ADDIS ABABA UNIVERSITY

ADDIS ABABA INSTITUTE OF TECHNOLOGY

SCHOOL OF CHEMICAL AND BIO ENGINEERING

PREPARATION OF COLLAGEN HYDROLYSATE SYNTAN FROM DELIMED PELT TRIMMINGS FOR POST TANNING APPLICATION AND FOR STABILZING COLLAGEN FIBERS

By

Mohammed Hussien

Advisors

Dr. Madhan Balaraman

Dr. Raghava Rao

A thesis submitted to the School of Graduate Studies of Addis Ababa University in partial fulfillment of the Degree of Master of Science in Leather Technology

September 2014

Addis Ababa, Ethiopia

ADDIS ABABA UNIVERSITY

ADDIS ABABA INSTITUTE OF TECHNOLOGY

SCHOOL OF CHEMICAL AND BIO ENGINEERING

PREPARATION OF COLLAGEN HYDROLYSATE SYNTAN FROM DELIMED PELT TRIMMINGS FOR POST TANNING APPLICATION AND FOR STABILZING COLLAGEN FIBERS

A thesis submitted to the School of Graduate Studies of Addis Ababa University in partial fulfillment of the Degree of Master of Science in Leather Technology

By

Mohammed Hussien

Approved by Board of Examiners

Dr. Ing. Berhanu Assefa (Head of School of Chemical and Bio engineering)	Signature	Date
Dr. Madhan Balaraman (Advisor)	Signature	Date
Dr. Raghava Rao (Advisor)	Signature	Date
Dr. Betelay Tekola (Internal Examiner)	Signature	Date
Dr. Yasmin Khambathy (External Examiner)	Signature	Date

Acknowledgements

I would firstly like to thank Allah, our creator, it's only and only because of Him and His help and His grace that this research study is possible.

Secondly I would like to thank my advisor, Dr. Madhan Balaraman, for his support and guidance without him this study would have been difficult. I would like to thank also Dr. Raghava Rao for his support and guidance. I would like to thank Mr. Wondu Legesse, Director General of LIDI, and his management staffs. I would like to thank also Prof. A. B. Mandal, Director of CLRI, Dr. Chandrasekaran and his team in the twinning project, I thank them from the bottom of my heart.

I also thank Satiesh Kumar and Sathiyamoorthy Perumal, PH D scholars in CLRI, for their help and support while conducting this research. I also thank my family for their support. It is not easy to name all others who supported me, I thank them all.

Table of contents

Page

List of Tables

Table 4.16:- Rates for organoleptic properties for leathers treated with CH that has been hydrolyzed for 2 hours 69

List of Figures

Abstract

Delimed pelt trimmings are trimmings that are obtained from limed pelt trimmings by removing lime. Limed pelt trimmings are one of the solid wastes that are generated in leather processing which is common in Ethiopia unlike other countries. These wastes are important resources for producing collagen hydrolysate which can be applied in leather processing operations. The aim of this study is to prepare collagen hydrolysate from delimed pelt trimmings and to apply it in wet blue goat skin in post tanning operation to determine the optimum hydrolysis conditions and molecular weight of the collagen hydrolysate. And also to determine its stabilizing effect on collagen fibers by treating rat tail tendon with the collagen hydrolysates by carrying out enzymatic and thermal stability experiments. The optimum conditions of hydrolysis for post tanning operation was determined by preparing six different types of collagen hydrolysates and apply it post tanning operation as a retanning agent. Based on the absorption of chemicals and organoleptic properties, the best collagen hydrolysate is selected and characterized further using FPLC and MALDI. The results showed that collagen hydrolysates play a role in stabilizing collagen fibers. And also the best collagen hydrolysate for post tanning is obtained when the proportion of high molecular weight to low molecular weight proportion of the fractions is 80% to 20%. And the range of the molecular weight should be in between 2000 to 6000 daltons. This can be achieved when the hydrolysis condition is carried out by using 0.8% trypsin for duration of 3 hours. It is concluded that by preparing collagen hydrolysates syntans from delimed pelt trimmings satisfactory results can be achieved by applying it in post tanning operation during leather processing and also they can be used for stabilization of collagen fibers.

1. Background

Ethiopia is one of the countries in the world who posses largest livestock population. Ethiopia stands eighth for cattle, twelfth for sheep and eighth for goat livestock populations [1]. 53.4 million cattle, 25.5 million sheep and 22.78 million goat livestock population are found in Ethiopia, which is the share of Ethiopia is 2.5% of the world livestock population [2].

At present there 27 tanneries operating in the country, employing over 5,000 people and having soaking capacity of 1.3 million pieces of hide and 32 million pieces of skins annually. The daily soaking capacity of tanneries is 145, 524 pieces of skins and 7,800 pieces of hides [3].

In order to process leather more than fifty structurally different types of chemicals are required although thousands of commercially different types of leather chemicals are available in the market. In Ethiopia more than 90% of leather chemicals are imported including syntans based on proteins. The only chemicals which are produced locally for leather processing applications are sulfuric acid, soda ash, wetting agents, lime and caustic soda.

Tanneries are one of highly polluting industries and have adverse impact on environment because of the generation of liquid, solid and gaseous wastes. Solid wastes generated in tanneries are raw hide waste, fleshing waste, limed hide waste, chrome shaving, trimming waste of wet blue, trimming waste of crust, buffing waste [4].

Solid wastes generated from tanning industries contain different chemicals which are used during leather manufacturing process. These tannery solid wastes have different characteristics. Some tannery solid wastes made of organic collagen protein will rot away after being placed for a period of time in nature environment. And the others contain toxic substances, such as chrome and aldehyde, which is harmful to environment and human beings.

However tannery solid wastes contain collagen protein, which is a valuable resource. Collagen proteins have application for making gelatin, additive component for cosmetics, biomaterial for medical products [5], for animal feed staff, nutrition component for health care products, and as raw material for protein based industrial products. Moreover some of tannery solid wastes contain chrome, which is a valuable material for tanning.

Thus, it is important to reuse this resource so as to reduce the pollution and to have better value addition to these wastes. This study was designed to prepare collagen hydrolysate from limed pelt trimmings for use as a syntan in tanning and post tanning operations, thereby enhancing the value of the tannery by-product. Additional the use of collagen hydrolysate would be ecologically more acceptable option than other commercially available syntans.

1.1 Statement of the problem

Protein based syntans are commonly used during post tanning operation of leather processing. In Ethiopia this type of syntans are employed for leathers which are meant for export. In 2006 E.C more than a total of 62 million square feet of leather have been exported from cow hide, sheep and goat skins. The input-output matrix for prepared for Ethiopian tanneries indicates that protein syntans have a coefficient of 7.2 on average, which means 7.2 g of proteins are consumed in order to produce unit square feet of leather.

Based on the value of input-output matrix, approximately 450 tons of protein based syntans are imported per year and consumed. The price of protein based syntan is two USD per kg. Thus protein based syntans having a value of about 900, 000 USD per year are imported to Ethiopia for the production of leather. However, this protein based syntans can be alternatively prepared from delimed pelt trimmings as collagen hydrolysate syntan, which are wastes of tannery and having lower value. The process for preparing collagen hydrolysate syntan from delimed pelt trimmings is not difficult and can be manufactured locally and can substitute the imported protein based syntans.

Trimming of limed pelt especially from hide is a common practice in Ethiopia which is not common in other countries. A survey was made in a typical Ethiopian tannery and the solid waste generated is presented in the following table.

Solid Wastes	*Kg/Ton of Wet salted hide*
Fleshing Waste	218.87
Limed Pelt trimming	**123.36**
Split	232.13
Shaving Dust	156.86
Wet blue trimming	31.84
Crust trimming	21.44
Buffing Dust	21.91
Finished trimming	13.1
Total	**819.51**

From the table above, about 15% of the solid waste generated in hide leather processing is contributed by limed pelt trimmings. Limed pelt trimmings are currently used in Ethiopia for making of glue, which is very low value application.

The value of limed pelt trimmings can be enhanced by converting them in to collagen hydrolysate syntan which can be used in tanning and post tanning operations of leather processing. Moreover the value of the tannery by-product by the use of collagen hydrolysate as a syntan would be ecologically more acceptable option than other commercially available syntans. For this reason this study has been proposed.

1.2 Objective of the study

1.2.1 General objective

The general objective of this study is to prepare collagen hydrolysate from delimed pelt trimmings for post tanning application in leather processing and to study the effect of collagen hydrolysate on the stability of collagen fibers.

1.2.2 Specific objectives

The specific objectives of this study are:-

- To prepare collagen hydrolysates from delimed pelt trimmings
- To characterize the chemical properties of delimed pelt trimmings
- To optimize protolytic conditions for preparing collagen hydrolysates from delimed pelt trimmings
- To characterize the collagen hydrolysate prepared
- To assess the effect of collagen hydrolysates on the stability of collagen fibers

1.3 Framework of the Study

The framework of this study is categorized in to three main groups. The first one is the assessment tanning ability of the collagen hydrolysate extracted from limed pelt trimmings. The second one is trials of the collagen hydrolysate as retanning agent for post tanning operation. And the third one is the characterization of the collagen hydrolysate which gives better results in post tanning operation.

1.4 Scope of the research

This thesis work concentrates on hide limed pelt trimmings of tannery solid wastes and their application for tanning and retanning of leather processing operations. It is also limited to optimization, characterization and assessing their tanning ability of the collagen hydrolysates extracted from these hide limed pelt trimmings. The extraction of the collagen hydrolysate from limed pelt trimmings is also limited to enzymatic hydrolysis. Experiments on tanning and post tanning are on rat tail tendon and wet blue goat skin respectively.

1.5 Significance of the research

Collagen hydrolysates extracted from limed pelt trimmings have both environmental and economical advantages. Foreign currency can be saved by import substitution of protein based syntans, if collagen hydrolysate syntan are manufactured locally.

2. Literature Review

2.1 Export performance of leather in Ethiopia

According to the report of leather industry development institute (LIDI), the export performance of tanneries from 2003-2006 E.C is presented in Table below.

	2003 E.C.		2004 E.C.		2005 E.C.		2006 E.C.	
Type	FOB value (million USD)	Quantity (million Sq. ft.)	FOB value (million USD)	Quantity (million Sq. ft.)	FOB value (million USD)	Quantity (million Sq. ft.)	FOB value (million USD)	Quantity (million Sq. ft.)
Sheep	67.91	53.58	72.28	42.33	65.30	32.83	63.91	35.16
Goat	21.99	23.62	22.74	20.43	32.28	24.18	27.66	20.88
Cow	4.59	4.77	4.71	4.31	2.87	2.49	6.12	6.36
Total	**94.50**	**81.97**	**99.72**	**67.07**	**100.45**	**59.50**	**97.69**	**62.40**

The volume of leather exported decreases from 2003 to 2006 E.C, but the value of export was increasing. The reason for this is due to transition from export of crust leather to a value addition of finished leather by discouraging export of crust with the imposition of higher tax, which has been implemented in 2005 E.C.

2.2 Import of protein based syntan in Ethiopia

The import of protein based syntan depends on the volume of leather produced for export. The input-output matrix for Ethiopian tanneries which has been prepared by LIDI indicates that protein based syntan have a coefficient value 0.0072 (i.e. 0.0072 kg of protein syntan are consumed for producing one square feet of leather). Based on this value the import of protein based syntan for the year 2006 E.C is estimated to be 450 tons of protein based syntans which is worth of 0.9 million USD.

2.3 Brief review on collagen

2.3.1 Collagen

Collagen is the most abundant protein in mammals. It is a predominant constituent in bone, cartilage, skin, tendon and tooth. The name ‘collagen’ comes from Greek meaning ‘glue

producer'. When collagen is heated in water, it gradually breaks down to produce soluble derived protein i.e. gelatin or animal glue [6].

2.3.2 Structure of collagen

Collagen is fascinating protein because of its unique chemical structure. All collagens are composed of 3 polypeptide alpha chains coiled around each other to form the tripe helix configuration [7]. The individual polypeptide chains of collagen each contain approximately 1000 amino acid residues [6]. The collagen molecule is approximately 300nm long and 1.5nm narrow [8]. The α chains are left handed helices that wrap around each other into a right handed rope like triple helical rod. The molecular weight of collagen is 300 KDa.

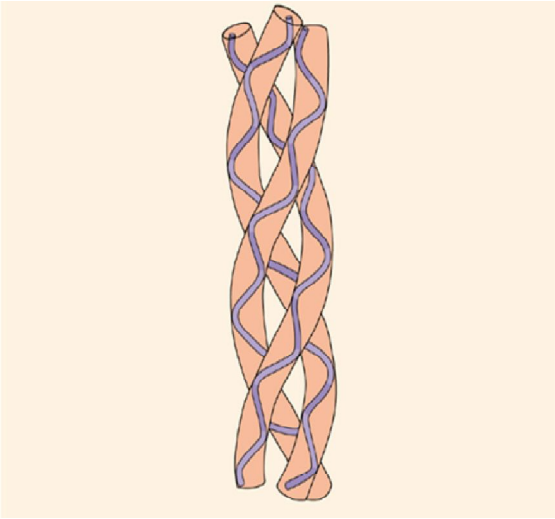


Figure 2.1:- Triple helical structure of collagen

Collagen has $(Gly\text{-}X\text{-}Y)_n$ repeating sequence where X and Y are side chains of any amino acid residues [9]. Thus glycine occupies every third position. Glycine is essential for the triple helical conformation because larger amino acids will not fit in the center of the triple helix. In α chain of type I collagen there are 338 Gly–X–Y triplets repeated in a sequence. The additional 32 amino acids flank the long triplet sequence at each end. They are known as telopeptides. The immino acids Proline in the X and Hydroxyproline in the Y positions are the most favored due to their highly stabilizing effect on the triple helix. The structure of collagen typically depends on high amounts of proline and hydroxyproline in α chains. Both these amino acids differ from other α-amino acids of proteins because they are amino acids with a rigid cyclical structure. This rigid structure prevents the rotation of the polypeptide chain backbone.

Collagen in tail tendon are indexed with a 10/3 symmetry (10 amino acids in 3 turns) [9]. Then it was reported that the first crystal structure of a collagen-like peptide, $(Pro\text{-}Pro\text{-}Gly)_{10}$, had a 7/2 symmetry (7 amino acids in 2 turns) [11]. The difference in triple-helix symmetry between the $(Pro\text{-}Pro\text{-}Gly)_{10}$ crystal and collagen fibers could have arisen from crystal packing effects, the unusually high imino acid content in the peptide compared with collagen, or from the absence of Hyp in the peptide [9].

Five of collagen molecules align longitudinally with an overlap of approximately one-quarter the molecular length to form a microfibril of diameter 3.6 nm. This so-called quarter stagger combined with the gap between successive macromolecules is responsible for the characteristic 64 nm banding pattern observed in the electron microscope and by x-ray diffraction [12].

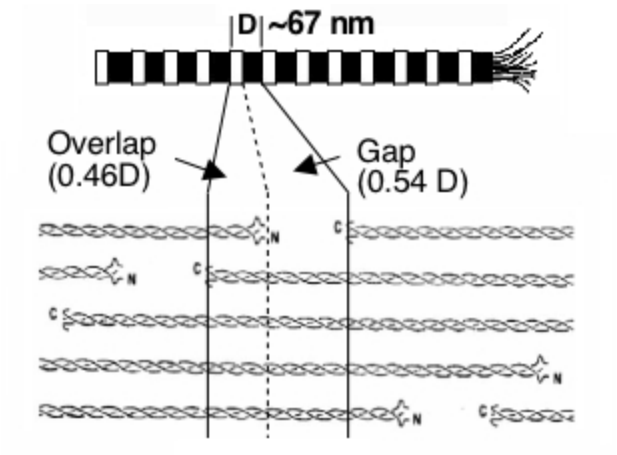


Figure 2.2:- Quarter staggered structure of collagen with D periodicity

2.3.3 Torsion angles and Ramachandran plot

In proteins the two torsion angles phi, Φ (bonds between N-Cα) and psi, ψ (Cα-C) describe the rotation of the polypeptide chain around the two bonds on both sides of the carbon atom. The Ramachandran plot provides an easy way to view the distribution of torsion angles of a protein structure [13]. The reason is that these angles provide the flexibility required for folding of the polypeptide backbone. It also provides an overview of allowed and disallowed regions of torsion angle values, serving as an important factor in the assessment of the quality of protein three-dimensional structures.

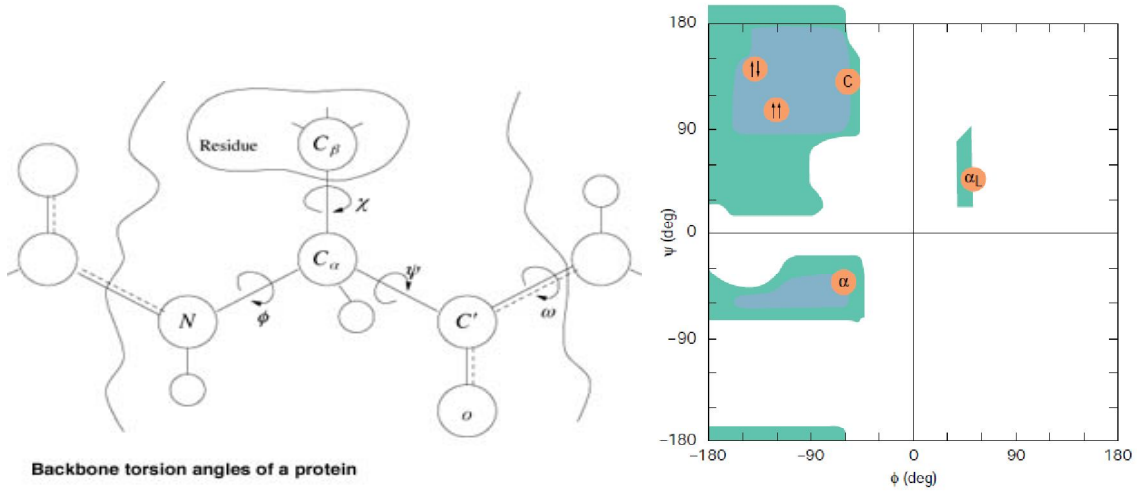


Figure 2.3:-torsion angles of peptide protein (left); Ramachandran plot showing allowed torsion angles for peptide protein (right)

The green-shaded regions indicate the sterically allowed Φ and ψ angles for all residues except Gly and Pro. For collagen the Φ and ψ angles in Gly-X-Y (Φ_G, ψ_G, ω_G, Φ_X, ψ_X, ω_X, Φ_Y, ψ_Y, ω_Y) equals (-74°, 170°, 180°, -75°, 168°, 180°, -75°, 153°, 180°)

2.3.4 Chemical composition of collagen

The most abundant constituent of collagen is glycine. Glycine makes up almost 1/3rd of the collagen molecule (i.e. 33%) [9]. Collagen contains two amino acids, hydroxy proline and hydroxy lysine, that are not found in other animal tissue proteins. Hydroxy proline is present as 1 in 10 residues (i.e. 10%) while Hydroxylysine is present as 1 in 200 residues (i.e. 0.5%). Proline and hydroxy proline together account for 2/9th of the residues (i.e. 22%) [14].

2.3.5 Role of hydrogen bonding in collagen

Hydrogen bonding is a critical part of triple-helix stabilization. The triple helix has repetitive backbone hydrogen bonding networks. It was clear from earliest models that one strong interchain peptide NH. . .OC bond could be formed per Gly-X-Y tripeptide unit (Hydrogen bond between the NH of Gly in one chain and the C=O of the residue in the X position of the neighboring chain). In addition, peptides with sequences where the X position is occupied by a residue other than Pro, show a second interchain hydrogen bond between the amide group of the X position residue and the C=O of the Gly residue, which is mediated by one

water molecule. The water molecules involved in the NH (X position). . .CO (Gly) hydrogen bond make additional hydrogen bonds with Hyp or side chains [9].

2.3.6 Types of collagen

Collagens represent a large family of proteins and at least 29 different collagen types have been described so far. Variations are brought by differences in the assembly of basic polypeptide chains, different lengths of the helix, various interruptions in the helix and differences in the terminations of the helical domains.

Collagens are divided roughly into 3 groups based on their abilities to form fibrils. The first one is fibril forming collagens. These are form banded fibrils. Type I, Type II, Type III, Type V, Type XI [15].

The second one is Fibril associated collagens with interrupted triple helices (FACIT); this group of collagens consists of proteins in which collagenous domains are interrupted by non collagenous sequences. These are associated with the surface of fibril forming collagens. Includes type IX, type XII, type XIV and perhaps type XVI also. The former three are unique in containing glycosaminoglycans components covalently linked to the protein molecule [15].

The third one is all other non fibrillar: they form the third group which includes Type IV, type VIII and type X (network forming collagens), Type VI (beaded fibril forming collagen), Type VII (anchoring fibrils and inter vertebrate cuticle collagen). In addition to the above collagen groups at least 10 non collagenous proteins incorporating short triple helical collagen domains have been described [16].

2.3.7 Degradation of collagen

Collagenases are enzymes that have specifically evolved to hydrolyze collagens because the triple helical collagen structure is resistant to most common proteinases. The collagenases belong to a family of enzymes called Matrix metalloproteinases (MMPs'). MMPs are a family of zinc-dependent proteolytic enzymes with the ability to catalyze the degradation of extracellular matrix components, including collagens [17].

Collagenases cleave α1(I) and α2(I) chains at the glycine 775 – isoleucine 776 and glycine 775 – leucine 776 bonds respectively. This results in release of cleavage fragments about three quarters and one quarter of the chain's original size. The released fragments have a lower T_m than the intact collagen molecule at the physiologic temperature, therefore become denatured and are subsequently denatured by other proteinases [18].

Collagen is an unusually stable protein and is susceptible to attack only by collagenase. Collagenases are present in different sources; predominantly in mammalian and bacteria.

The function of collagenase in microorganisms like *Clostridium histolyticum* is to digest the proteins for nutritional purposes or use to invade a collagen containing host. Contain seven different collagenases of 2 classes based on sequence homologies and work at hypersensitive regions of collagen. Bacterial collagenase cleaves collagen after Y residue in the sequence –Pro-Y-Gly-Pro-, where Y is mostly neutral amino acid and they can cleave collagen at more than six sites [19].

2.4 Review on collagen hydrolysate

Collagenous proteins in skin and hides, a meat production by-product, are an important raw material for the production of leather, gelatine and glues, foodstuffs, cosmetics, pharmaceuticals. Collagen hydrolysates are collagen fragments having an average molecular weight of 1000-30,000 g/mol (1-30 kDa) obtained by chemical or enzymatic hydrolysis [20].

Chi Yuanlong etal [21], extracted collagen hydrolysate from tannery skin shavings through enzymatic hydrolysis by using protease 1398, protease 2709, Alcalase, papain, pepsin, protease 537 and trypsin. There research provides a potential approach for molecular weight and molecular weight distribution control of collagen hydrolysates by enzymatic hydrolysis of tannery skin wastes. They evaluated the hydrolytic degree and molecular weight distribution (MWD) of the hydrolysates by the formaldehyde titration method, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), Tricine-SDS-PAGE and centrifugal ultrafiltration. They found that the low molecular weight (MW) collagen hydrolysates (i.e. <10 KDa), which has relatively high hydrolytic degree, were obtained by protease 1398, protease 2709, papain and Alcalase. The medium-MW collagen hydrolysates (i.e. 10-30 KDa) were obtained by using protease 537. And the high-MW collagen hydrolysates (i.e. >30 KDa), which has the lowest hydrolytic degree, were obtained by using pepsin.

Vera Kasparkova etal [22], used formic, phosphoric and nitric acids for the hydrolysis of chrome shavings. Their aim was to prepare low-molecular weight product (<5kda) to be used as plant bio-stimulator. The hydrolysis was monitored by Gel Permeation Chromatography (GPC). In their study they concluded that MW and MWD of hydrolysates are influenced by the choice of hydrolyzing acid, its concentration as well as by the time of acid treatment. Of all the three acids in the test, phosphoric acid is the most effective one. By combination of enzymatic and acid hydrolysis it is possible to prepare hydrolysates with a defined molecular weight for intended use.

Zhongkai Zhang etal [23], studied physicochemical properties of collagen, gelatin and collagen hydrolysate derived from bovine limed split wastes. Collagen was extracted in the acid solution containing pepsin, which only attacked the non-triple helical domain of native collagen, whereas gelatin and collagen hydrolysate were prepared under severe conditions above the

denaturation temperature. Their analysis using Sodium dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) analysis showed that the molecular weight distribution of collagen was very narrow (about 200 and 100 KDa for β and α chains respectively) compared with those of gelatin (less than 300 KDa and wide distribution) and collagen hydrolysate (less than 50 KDa and wide distribution). And the molecular weight of type I collagen was about 300 kDa. There results suggested that collagen derived from bovine limed split wastes has greater potential biomaterial utilization than gelatin and collagen hydrolysate due to its special physicochemical properties.

Wuyong etal [24], partially hydrolyze collagen from chromium containing leather waste, in which more than 50% of the fragments had molecular weights less than 21 kDa. They modified it with glutaraldehyde (0 to 18%). Gluteraldehyde formed intermolecular crosslink of the collagen hydrolysates as evidenced an increase in molecular weights. The 12% glutaraldehyde-hydrolysate protein (GHP) versus 0% gluteraldehyde-hydrolysate protein (HP) was used in a demonstration of leather filling. They observed that the GHP adhered better to chrome tanned calf leather than HP. The average increase in thickness of the leather (measured before neutralization and after filling) from butt area was 4.7% greater when 12% GHP was used as a filler than the control HP. To visualize the filling effect, they label glutaraldehyde hydrolysate protein and hydrolysate protein with fluorescent dye and the treated leather samples was evaluated by fluorescence microscopy. The result suggested potential filler for leather.

M.M. Taylor etal [25]**,** polymerize gelatins by using tansglutaminase as the cross-linking agent and they make the reaction to take place either in the leather itself or prepare it outside and then add it to the leather. Their results showed that enzymatically modified gelatin and casein could be used effectively as filler which are bound to the leather and would not come out easily.

F. Langmaier etal [26]**,** have prepared pure collagen hydrolysate with the use of commercially available enzymatic preparations from short cattle tendons (musculus extensor communis, musculus flexor digitorum, musculus flexor digitorum profundis) suitable for application in skin and hair care cosmetics. For the purpose of purification of short cattle tendons, they use specific lipases of microbial origin (Aspergillus. Concentrations of 1% (w/w) were applied to the starting collagenous materials which are grounded, at temperature of 38 ^{o}C,

with occasional stirring, for a period of 48 h. Then the purified collagen was dispersed in 500% (v/w) and denatured by heating the dispersion to 95 ^{o}C for 15 min. After cooling the dispersion to 45 ^{o}C 1.0% (w/w) of protease of bacterial origin (Bacillus subtilis) was added and hydrolysis was conducted under proper stirring for 3 h at that temperature. The protolytic enzyme was then inactivated by heating the reaction mixture for 5 min to 95 ^{o}C. The liquid phase was filtered off, vacuum thickened to half its volume and dried in a spray-dryer. Their hydrolysate contain neither lipoid nor amino saccharide components, content of primary amino groups reaches around 1.1mmol g-1 and the average molecular weight of the resulting collagenous hydrolysates does not exceed 2000 g mol-1 (2.0 kDa).

C.Cantera etal [27], hydrolyze chrome shavings by an alkaline-enzyme process, they develop besides collagen hydrolysate, a copolymer CH-acrylic acid: “acrylic-protein” retanning agent. They observed that CH enhances the leather grain properties and provides greater softness improving, masks preservation defects and damage marks on the skin and also intensifies colors and improves homogeneity of the dyeing. In their study they concluded that when CH is used along with an acrylic-retanning agent, it shows an interesting behavior in relation to the physical properties of leather - particularly with tear strength- increasing the resistance of the fibrous tissue

I. Bragança1 etal [28], produced fat and hydrolyzed protein from fleshing waste. The best result they found was a temperature of 60°C, 4 hours of hydrolysis, 2% of enzyme and 100% of water, with a fat extraction yield of 93%. The fat obtained through the hydrolysis process was used to produce sulphated oils. The protein hydrolysate the extracted was concentrated to about 40% of solids content and was used to prepare co-products of protein hydrolysate and glutaraldehyde.

A. Aslan etal [29], obtained Collagen hydrolysates from shavings of chrome tanned sheep skins and was used in a pretanning process for sheepskins and the effect on the efficiency of chrome tanning was evaluated. Six groups of half-skins were pretanned separately with 0, 1, 2, 3, 4 and 5% collagen hydrolysate before acidifying at the pickle stage. In the next stage, their PHs was adjusted to 3.2 and was tanned with basic chrome sulphate (33% basicity). In order to measure the efficiency of tanning with additions of collagen hydrolysates to pickle floats,

chromium oxide analyses were carried out for the wet-blue experiments and exhaust floats. It was stated that since hydrolysates contain high amounts of proteins and, have active groups of that can react with chromium, which leads that the leathers might take up more chromium. This property of hydrolysates resulted in increased chrome oxide contents of the leathers. They concluded that wet-blue leathers pretanned with 5% protein by-product (collagen hydrolysates) showed increased chromium take-up and had better physical characteristics.

F. Langmaier etal [30]**,** prepared hydrolysates of chrome-tanned leather waste from tanning manufacture and react or modify it with glutaraldehyde to produce thermo-reversible or thermo-irreversible gels. Solid leather waste from chrome-tanning was hydrolyzed by a commercially available endopeptidase of bacterial origin *(Bacillus licheniformis)* in an open reactor for 6 h at 70ºC. The required alkaline medium was produced by an addition of 1% vol. cyclohexylamine. The solid phase containing mainly chromic tanning salts in addition to a certain fraction of organic substances was filtered off and the liquid phase was vacuum-condensed to approx. 15% dry matter and drying was completed in a spray dryer. The tendency of reaction mixtures of collagen hydrolysates with glutaraldehyde to form gels were studied by reacting an aqueous solution of hydrolysate (25% w/v) with 1.25% glutaraldehyde (w/v, related to hydrolysate dry matter) at pH 5.5 and 40ºC. They suggested that thermo-reversible gels can be employed as glues, thermo-irreversible gels that are applicable in encapsulation techniques. This expands the possibilities of these products for other industrial applications.

2.5 Brief review on tannery solid wastes

2.5.1 Classification of tannery solid wastes

Tannery solid wastes can be categorized based on their contents. It can be classified as chrome free solid waste, chrome containing solid waste and dye stuff containing solid waste. Chrome free solid wastes are solid wastes that are generated before tanning. Raw hide/skin trimmings, fleshing, and limed pelt trimmings solid wastes fall under this category. The main impurities of this solid waste are salt, lime and sulphide.

Chrome containing solid wastes is solid wastes that are generated after tanning and before retanning, fatliquoring and dyeing operations. Wet blue shaving dusts and trimmings are the main solid wastes under this category. The main impurity is chrome.

Dyestuff containing solid waste is solid wastes that are generated after post tanning operations. Crust trimmings and buffing dust are the main wastes in this group. The main impurities are chrome, retanning agents, fatliquors and dyes.

2.5.2 Chrome free solid wastes

From chrome free solid wastes gelatin, collagen protein and regenerated hide can be prepared. Gelatin has the following application:- additive component for foods products, such as ice cream, jelly, canned meat, candy, and jam, raw material to produce capsule for medicine, raw material to produce photo film and raw material to produce binder for instrument or abrasive band.

Collagen hydrolysates has the following applications, additive component for cosmetics, nutrition component for health care products, raw material for medicinal products, protein material for animal feedstuff, raw material for protein-based industrial products.

Regenerated hide has the following applications: - pets products and collagen casing. Regenerated hide can replace split hide to make pets products, such as knotted bone-like product, and other shape products by machine. The thickness and shape of regenerated hide can be adjusted easily, which can avoid cut waste when hide is made into pet’s products. Regenerated

hide can replace small intestine to make sausage products. The length and diameter can be adjusted easily.

2.5.2.1 Preparation of gelatin

The raw material for preparing gelatin can be raw hide/skin trimmings and limed pelt (trimmings and splits). The main steps are liming (for raw hide/skin trimmings), washing to remove the unbound lime, deliming to remove the bound lime, degreasing to remove fats and oils. Then it will be followed by thermal hydrolysis. Hydrolysis is carried out at $60^{o}C$ and the hydrolysis liquor is recovered, filtered, concentrated and dried [31].

2.5.2.2 Preparation of collagen hydrolysates

The raw material for preparing collagen hydrolysates can be limed pelt trimmings and pelts. The main steps are washing to remove the unbound lime, neutralization with HCl to adjust the PH to 8. Followed by enzymatic hydrolysis with 0.5-1% enzyme and 2-4 hours hydrolysis duration time. Then filtering, concentrating and drying will be carried out in order to get the final product [31].

2.5.2.3 Preparation of regenerated hide

Principle of preparing regenerated hide is that there are abundant active groups on collagen fibers, such as collagen carboxyl, collagen amino and collagen hydroxyl. If the collagen fibers are close enough, the active groups can attract together and bind together after collagen fiber is dried. By this method small piece of hide can be produced into big piece of hide. It is edible because no binding agent is applied.

The main steps in preparing regenerated hide are alkali treatment, washing and neutralization, enzyme treatment, acid swelling, pulping and de-fibering, filtering, neutralization and shaping, drying and trimming [31].

2.5.3 Chrome containing solid wastes

From chrome containing solid wastes collagen hydrolysates, retanning agents, protein composite fiber and regenerated leather can be prepared. The reuse of wastes can be achieved by extracting collagen hydrolysate or wastes or by removing chrome from chrome (dechroming) containing solid wastes. In the isolation of collagen hydrolysate, the hydrolysate is dissolved in solution and chrome salt remains in the residue. Where as in the dechroming, chrome salt is dissolved in the solution and collagen fiber remains in the residue.

2.5.4 Method of hydrolysis for chrome containing solid wastes

There are different methods of hydrolysis to extract collagen hydrolysate from chrome containing solid wastes. The following are possible methods of hydrolysis: Alkali hydrolysis, acid hydrolysis, enzyme hydrolysis, combined hydrolysis (alkali-alkali, alkali-acid, and alkali-enzyme). The characteristics of each hydrolysis method are described below.

2.5.4.1 Alkaline hydrolysis

The acyclamide bond of collagen fiber is easy to be hydrolyzed at high temperature by alkali, and the alkali will be neutralized by the collagen carboxyl which is new-produced from the break of collagen acylamide. The hydrolyzed collagen component from leather waste is soluble in water, but the chrome in leather waste is insoluble in alkaline condition, and it can be realized to separate collagen protein and chrome salt [31].

The yield of collagen protein in alkaline hydrolysis is depended on the kind and the amount of alkali, hydrolyzing temperature and time. The chrome content, the ash content and the molecular weight are also depend on the kind and the amount of alkali, hydrolyzing temperature and time. Ash content of collagen protein is mainly depended on the solubility of alkali in water all the alkali solved in water will be mixed into collagen protein, which increase the ash content of collagen protein

The molecular weight of collagen protein is mainly depended on the pH of alkaline aqueous solution, if the pH arises, the hydrolyzing action on collagen protein becomes stronger, and the molecular weight of the isolated collagen protein becomes smaller accordingly

The chrome content of collagen protein is mainly depended on the pH of alkaline aqueous solution, the chrome content is increased with the pH value arises. For example, the chrome content of the collagen protein extracted by MgO is much less than NaOH, that is because MgO is a kind of weak alkali, and NaOH is a kind of strong alkali. There exists equilibrium of chrome salt in aqueous solution as following:

It can be found that chrome oxide is a kind of amphoteric oxide, it can be precipitated in alkaline solution, and it will be solved partly in strong alkaline solution, so it is important to adjust the pH value of the hydrolyzing solution to control the chrome content of collagen protein.

2.5.4.2 Acid hydrolysis

The acylamide bond of collagen fiber is easy to be hydrolyzed at high temperature by acid, and the acid will be neutralized by the collagen amino which is new-produced from the break of collagen acylamide. Sulfuric acid is used in acid hydrolysis, because it can be removed from the collagen protein by precipitating the SO_4^{2-} with Ca^{2+} to decrease the ash content. Furthermore, the acidity of sulfuric acid is very strong and it is much cheaper than other acid [31].

The collagen component is hydrolyzed from leather waste by acid and it is soluble in aqueous solution, at the same time the chrome salt in leather waste is also soluble in acidic condition, so additional precipitate is needed to remove chrome salt from collagen protein after acid hydrolysis. $Ca(OH)_2$ is preferable in the precipitate of acid hydrolyzing collagen protein solution, because the OH^- of $Ca(OH)_2$ can react with Cr^{3+} in the solution to form $Cr(OH)_3$ precipitate, and the Ca^{2+} of $Ca(OH)_2$ can react with SO_4^{2-} to form $CaSO_4$ precipitate

It can be found that the chrome content of collagen protein recovered by acid hydrolysis is several hundred times less of that of alkali hydrolysis. Collagen protein yield of acid hydrolysis from chrome shavings is much lower than alkali hydrolysis.

2.5.4.3 Enzyme hydrolysis

Chrome-containing leather waste is difficult to be hydrolyzed by enzyme. If the chrome-containing leather wastes is pretreated with alkali, the collagen protein yield will be improved

remarkably because the combination between chrome salt and collagen carboxyl is destroyed by alkali, and the leather wastes is easy to be hydrolyzed by enzyme. Thus, it is important for chrome-containing leather wastes to be pretreated with alkali before enzyme hydrolysis [31].

2.5.4.4 Combination of different hydrolysis method

Each hydrolysis method has its disadvantage; therefore the combination of different hydrolysis method is more practical in the isolation of collagen hydrolysate from chrome-containing leather wastes to overcome the disadvantages [31].

2.3.5 Dechroming of chrome containing solid wastes

There are three methods to remove chrome from chrome containing solid wastes, acid, alkali and oxidation methods. In acid method, the chrome in the wastes is dissolved into strong acid solution slowly under low temperature. After long time extraction, the structure of leather remains undestroyed on the whole [32].

In alkali method, the chrome in leather wastes can be precipitated by alkali slowly under low temperature. After long time extraction in alkali solution, the chrome residue can be removed by acid and the structure of leather remains undestroyed on the whole [32].

In oxidation method, Cr^{6+} is easy to be solved in water and does not combine with leather fiber, so oxidation agent can be applied to oxidize Cr^{3+} into Cr^{6+}, which can be removed from leather by washing [32].

2.6 Retanning agents

2.6.1 Retanning agents as a whole

The main objectives of the retanning are to improve the fullness and uniformity of substance, to fill the looser areas in order to get uniform substance and fibre compaction in all parts of the skins, to impart body and round feel, to avoid grain looseness and improve 'break' properties and grain tightness by filling the void spaces between grain and corium by selective filling and to improve the overall cutting value by bringing about uniformity of substance and fibre compaction [33].

The main retanning materials used for imparting fullness are vegetable tanning materials, phenolic syntans, acrylic and other resin tanning materials, protein based tanning agents, whitening syntans, polyurethane syntans, aldehydes and their derivatives, inorganic mineral tanning agents (chrome, aluminum, zirconium salts) and protein fillers and their derivatives [34].

Retanning agents can be broadly categorized as organic and inorganic retanning agents. Organic retanning agents can be classified as regular syntans, resins, polymeric acrylic resins and protein fillers. Regular syntans can be further classified into replacement and auxiliary syntans [33].

Replacement syntans are condensation products of aromatic compounds like phenol, naphthalene sulphonic acid with formaldehyde or urea. They are used as synthetic substitutes of vegetable tanning agents for retanning of chrome tanned wet blue leather or vegetable tanned leathers. They are favored for retanning chrome leather to improve fullness and level dyeing through uniform uptake and penetration of fatliquors and dyes which leads to improvement in buffing properties. They also reduce cationic charge of chrome leather [33].

The low molecular weight syntan of this group of the low pH value (1.2-1.6) are generally called bleaching syntans because these synthetic tanning agents can bleach vegetable tanned leathers and natural vegetable tannins by solubilizing the sludge or by lowering the pH value of the system etc. Chrome tanned leathers cannot be bleached with this bleaching syntans and therefore white retanning syntans are used for that purpose [33].

Resins are condensation products from formaldehyde with amino and amido compounds like urea, melamine and dicyandiamide. As the resin syntans are bigger molecules they are blended with dispersing agents for easier penetration into the skin or hide. Resin syntans are particularly used for filling those areas of the hide or skin where the fiber Structure is loose. Resin syntans penetrate the skin structure easily and get deposited in loose areas. Generally resin syntans based on melamine and dicyandiamide are preferred. Resin syntans are generally used in combination with auxiliary syntans. Auxiliary syntans help in penetration of resin syntans into the skin structure. Resin syntans give tighter fiber structure and finer grain break than vegetable tans [33].

Polymeric or acrylic syntans are mainly poly acrylics, i.e. polymerization products from acrylic acid derivatives. They are homo-polymers or co-polymers of acrylic acid, Meta acrylic acid and acrylonitrile in aqueous solutions. Acrylic syntans are useful in retanning of chrome leather for making suede leather. Acrylic syntans help in achieving round full firm handle and good grain characteristics with softness. Acrylic syntans are anionic in nature and can be used in combination with other anionic syntans. Acrylic tanning agents are light fast in nature and can be used in light fast leathers [33].

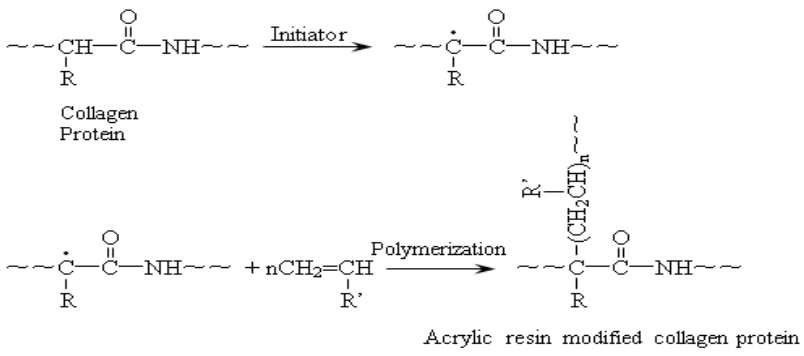
Inorganic retanning materials are mostly employed in rechroming stage for chrome tanned leathers or semi chroming in the case of vegetable tanned leathers. Basic chromium sulphate as such or higher basic chromium sulphate or self basifying chrome or chrome syntans may be employed in rechroming or semi-chroming. Sometimes aluminium based tanning salts or syntans are used in rechroming/semi-chroming to improve dyeability and get rich shades in dyeing. Zirconium tanning agents are used in certain types of leathers to get white leathers or to increase the dry heat resistance (pilot glove leather) or to reduce stretch in the leathers. In the case of semi-chroming of vegetable tanned leathers, inorganic tanning salts are used to build up cationic charge to increase affinity for dyes and fatliquors. Soft leathers with increased depth of shades in vegetable tanned leathers are possible only by increasing the contents of mineral tanning agents [33].

2.6.2 Retanning agent from collagen hydrolysate

Collagen hydrolysate can be graft copolymerized by aqueous acrylic monomer, such as acrylic acid, methyl acrylic acid, acryl amide and acrylonitrile, to produce protein-containing retanning agent of acrylic type. The retanning agent from leather wastes has good consistency with leather because of the collagen protein structure, and it can improve the whiten defect, which is very common to the leathers retanned with acrylic resin, because collagen protein is a kind of polyelectrolyte with ampholytic structure. By the same method, we can prepare other kinds of collagen protein based retanning agents, such as amine resin modified, PU modified, and phenol-formaldehyde resin modified retanning agents.

2.6.2.1 Acrylic resin modified collagen protein retanning agent

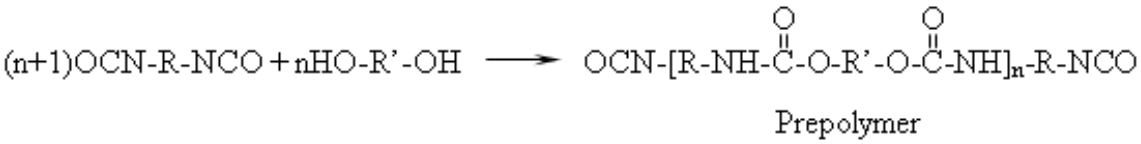
The active H-containing group can be initiated by radical initiator and copolymerized with aqueous acrylic monomer [34].



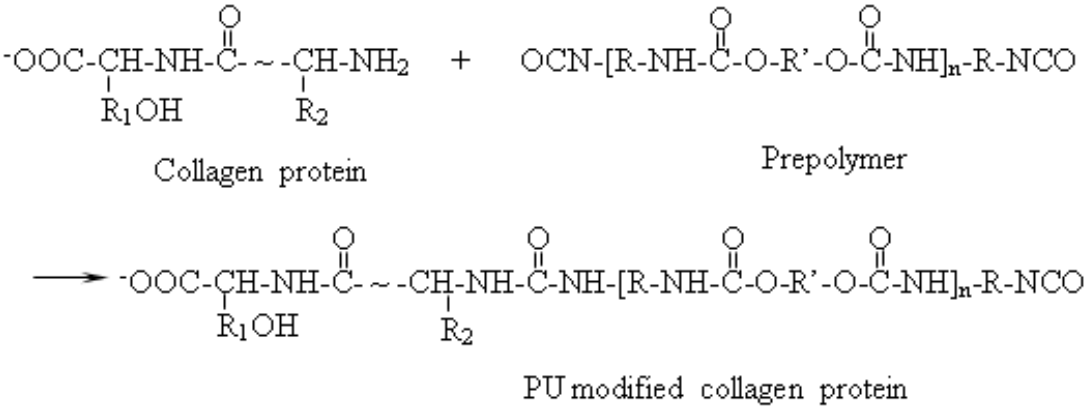
2.6.2.2 Polyurethane modified collagen retanning Agent

The amino group on collagen protein can react with isocyanate of polyurethane prepolymer to form PU modified collagen protein [35].

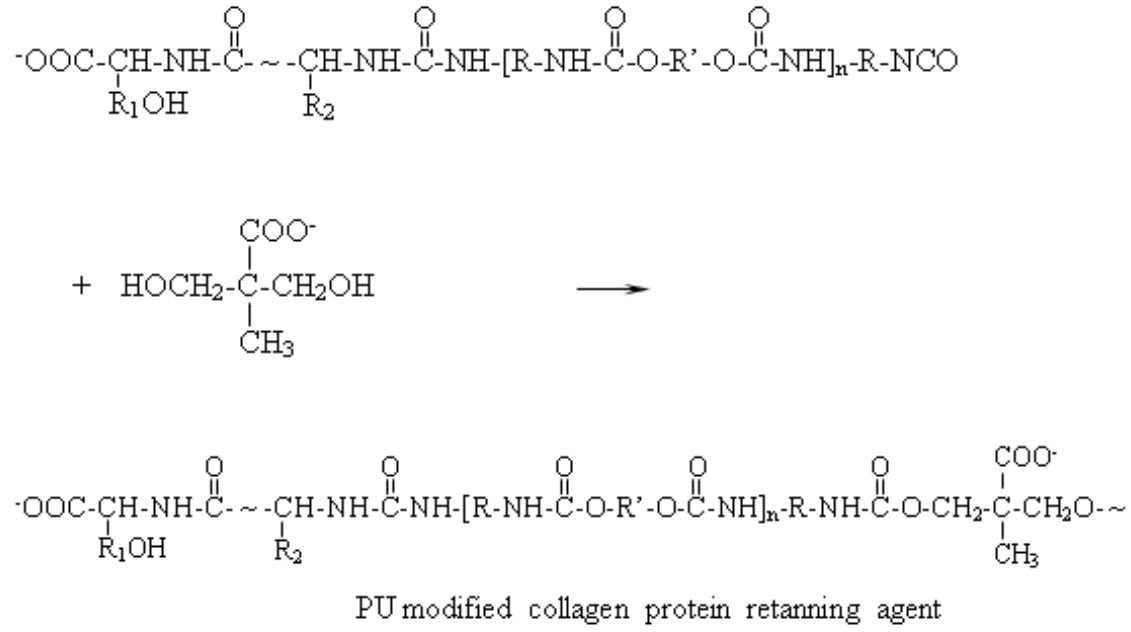
STEP 1: Synthesis of PU prepolymer



STEP 2: Synthesis of PU modified collagen protein



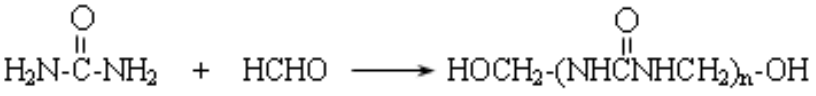
STEP 3: Synthesis of PU modified collagen protein retanning agent



2.6.2.3 Amine resin modified collagen protein filling agent

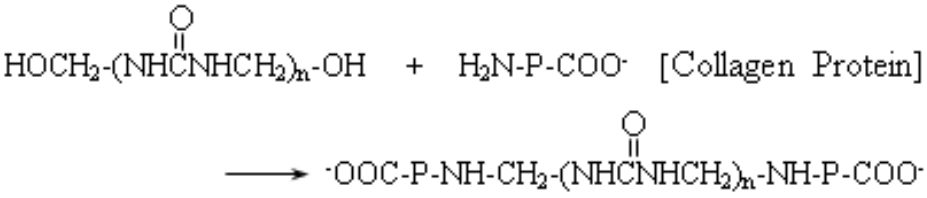
Amine resin modified collagen protein retanning agent can be prepared by reacting collagen protein with amine resin [36].

STEP 1:



Amine Resin Prepolymer

STEP 2:



Amine Resin Modified Collagen Protein Retanning Agent

2.7 Color

Color is the effect on the brain of an observer when the object is viewed in the presence of a light source. Colors are expressed in terms of hue (color), lightness (brightness) and saturation (vividness), in such a way also color can be quantified [37].

2.7.1 Scientific approach to color quantification

$$Object + Light\ Source + Observer \rightarrow Color$$

Computation of Tristimulus system [37]

$$X = \sum_{\lambda=400}^{\lambda=700} E_\lambda * \bar{x}_\lambda * R_\lambda$$

$$Y = \sum_{\lambda=400}^{\lambda=700} E_\lambda * \bar{y}_\lambda * R_\lambda$$

$$Z = \sum_{\lambda=400}^{\lambda=700} E_\lambda * \bar{z}_\lambda * R_\lambda$$

E_λ: CIE standard spectral power distribution data

$\bar{x}_\lambda$, $\bar{y}_\lambda$, $\bar{z}_\lambda$: - standard CIE observer data

R_λ: - spectral reflectance value of the sample

CIE $L * a * b$ color space

$$L^* = 116\left[\left(Y/Y_o\right)^{1/3}\right] - 16$$

$$a^* = 500\left[\left(X/X_o\right)^{1/3} - \left(Y/Y_o\right)^{1/3}\right]$$

$$b^* = 200\left[\left(Y/Y_o\right)^{1/3} - \left(Z/Z_o\right)^{1/3}\right] X_o$$

X_o, Y_o, Z_o are tri stimulus for the illuminant

L^* = Whether the sample is light or dark

a^* = If sample is red (+a^*) or green (-a^*)

b^* = If sample is yellow (+b^*) or blue (-b^*)

2.7.2 Colour difference value

Pass or Fail of a shade produced can be determined by measuring the L, a, b values for the swatch and the dyed sample and calculating the difference in terms of CIE DE (ΔE) value. Let the swatch has the colour values of L_s, a_s, b_s and for the dyed sample, L_D, a_D, b_D

The colour difference [37],

$$\Delta E = \sqrt{(L_D - L_S)^2 + (a_D - a_s)^2 + (b_D - b_s)^2}$$

For Good colour matching; DE value should be < 1.0. But for many shades, up to a value of 2.0 may be acceptable but depends on the spectral colors.

2.8 Enzyme inhibition

The inhibition is due to a substance, which binds with the enzyme and inhibits its function. The inhibitor may be organic or inorganic in nature. There are three categories of enzyme inhibition, reversible, irreversible and allosteric or non-covalently regulated enzyme inhibition [38].

2.8.1 Reversible inhibition

The inhibitor binds (non-covalently) with enzyme and inhibits its action. It can be removed by dialysis. Hence the name is reversible inhibition. Reversible inhibition is further subdivided into competitive, non competitive and uncompetitive inhibition [38].

In competitive inhibition, the inhibitor resembles the substrate (substrate analogue) and binds at the active site of the enzyme. So, there is no further catalysis. Competitive inhibition occurs between the substrate and inhibitor [38].

In non competitive inhibition, no competition occurs between substrate and inhibitor. The inhibitor binds at a site other than the active site on the enzyme surface and inhibits its action. The inhibitor has no structural resemblance with the substrate. It will not affect the binding of the substrate to the enzyme. The inhibitor can bind either to the enzyme or ES complex. The inhibitors, heavy metal ions (Ag^{+}, Pb^{2+}, Hg^{2+}) inhibit the enzyme by binding with functional group of amino acids like –SH group of cysteine or carbonyl group of histidine [38].

In uncompetitive inhibition, the inhibitor binds only to the ES complex and not to the free enzyme.

2.8.2 Irreversible inhibition

In irreversible inhibition, the inhibitors bind covalently with the enzyme and inactivate them [38].

2.8.3 Allosteric inhibition

In allosteric inhibition, the enzymes have additional site called allosteric site apart from the active site. The inhibitor binds to the allosteric site and inhibits its function. The inhibitor may be positive allosteric or negative allosteric effectors. Positive allosteric effectors increase the enzyme activity, where as negative allosteric effectors decrease the enzyme activity.

3. Materials and methods

3.1 Materials

Hide limed pelt trimmings, wet blue goat leather, rat tail tendon, enzyme (trypsin), sodium hydroxide, sodium chloride, chemicals for processing leather commercial grade (Sodium formate, Sodium bicarbonate, Lipoderm liquor SX-25, Dyestuff, Sellasol PR, Formic Acid), different reagent for analysis analytical grade (Chloramine T, Methyl cellosolve, citric acid monohydrate, glacial acetic acid, sodium acetate trihydrate, sodium hydroxide, Paradimethyl Amino Benzaldhyde, Perchloric Acid, standard hydroxyproline, Tris HCl, Calcium chloride, Collagenase, sodium dodecyl sulfate, Trinitro benzene sulfonic acid (TNBS), HCl, NaH_2PO_4, Na_2HPO_4, Formaldehyde, Phenolphthalein, Sodium carbonate, Dancyl chloride, Acetone, etc).

3.2 Equipments

Round bottom flask, conical flask, heating mantle, water bath, shaker with heating incubator, filter paper, funnel, beaker, centrifuge tube, hot air oven, deep freezer, lyophilizer, refrigerator, tannery machines (small testing glass drums, shaving machines, setting out machines, overhead drier and staking machines), analytical instruments (UV spectrophotometer, DSC, micro-shrinkage tester, FPLC, SEM, Microscope, MALDI, PH electrode, analytical weighing balance).

3.3 Methods

3.3.1 Raw material collection and pretreatment

Limed pelt trimmings from hide were collected. The weight of the pelt was determined and added in to small glass drums. 300% water based on its weight was added and washed for 10 minutes while the drum is running. Then the liquor is then drained and second washing also done like the above procedure and after that the liquor was drained. A fresh float of 100% water at 37^oC was then added and 1% ammonium chloride was added and the drum was closed and run for 45 minutes. The removal of lime was confirmed by checking the cross section of the pelt with phenolphthalein. If the cross section shows colorless it indicates that almost all the lime is

removed from the pelt. After that the liquor is drained and washed with water once to get clean delimed pelt trimmings.

3.3.2 Preparation of collagen hydrolysate

Then the delimed pelts were cut into small pieces. They are weighed and transferred in to 250 ml conical flask. Then 300% (v/w based on the wet weight) of water was added. The pelt is then denatured by heating at 80^{o}C for 1 hour in water bath. After that the hot melted dispersion is cooled to 37^{o}C. Then 0.8-1.2% trypsin 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 to 37^{o}C and the hydrolysis was carried out for 2-3 hours. After that the flask is removed from the incubator shaker and placed in water bath and heated at 80^{o}C for 30 minutes to deactivate trypsin enzyme or to stop the hydrolysis.

After cooling to room temperature, the extracted collagen hydrolysate was then filtered off with Whatman filter paper. The residue on the filter paper is discarded and the liquid phase was collected and placed in deep freezer at -40^{o}C to convert it into ice for overnight. Then it was dried with lyophilizer to get white collagen hydrolysate powder.

3.3.3 Characterization of delimed pelt trimmings

The delimed pelt trimmings were characterized for collagen, ash and fat content.

3.3.3.1 Collagen content

Known amount of delimed pelt was taken into hydrolysis tube and HCl was added to the sample in the hydrolysis tube and the final concentration of HCl is adjusted to 6N. The hydrolysis tube was then sealed and placed in hot air oven at 120^{o}C, 12 hours for complete hydrolysis into individual amino acids.

After incubation, the seal of the hydrolysis tube was cut open and the hydrolyzed sample poured in to porcelain crucible and evaporated in water bath. The evaporation was continued for 5 times by adding 2 ml of distilled water wash liquor from the hydrolysis tube to ensure the removal of HCl vapors as the presence of HCl will interfere in the assay on the hydroxyproline which is the unique amino present in collagen. Then finally the contents of the porcelain dish

were transferred into 50 ml volumetric flask by washing the crucibles and adding the wash solution into the flask. It was then made up to 50 ml. From this solution, 1 ml of solution was further made up to 10 ml with distilled water. Then 1 ml was taken for hydroxyproline estimation according to the procedure of J. F. Wossener which is attached as annex 1 [39]. Moisture content of the delimed pelt was measured at the same time, by taking 5 g of delimed pelt.

The calculation of the content of collagen is based on the following formula:-

$$\%\ \text{Hydroxyproline} = \left(\text{concentration in } \mu g \Big/ \text{weight of delimed pelt} \right) \times \text{dilution factor}$$

$$\%\ \text{Collagen} = \%\ \text{Hydroxyproline} \times 7.4$$

3.3.3.2 Ash content

Total ash content of the delimed pelt was determined according to SLC 6 (IUC 7; BS 1309:6). And it is attached as annex 2 [40].

3.3.3.3 Fat content

Fat content of delimed pelt was determined according to SLC 4 (IUC 4; BS 1309:4). And it is attached as annex 3 [41].

3.3.4 Characterization of the collagen hydrolysate

The collagen hydrolysate which gives better results in terms of fullness, dye absorbance and other organoleptic properties was selected and taken for characterization.

3.3.4.1 Collagen content

Collagen content was determined by estimating the hydroxyproline content in the collagen hydrolysate as indicated by procedure of Wossener which is attached as annex 1 [39].

3.3.4.2 Moisture content

Moisture content of the collagen hydrolysate powder was determined according to SLC 3 (IUC 5; BS 1309: 3) [45].

3.3.4.3 Ash content

Total ash content of the delimed pelt was determined according to SLC 6 (IUC 7; BS 1309:6). And it is attached as annex 3 [40].

3.3.4.4 Yield

The yield of collagen hydrolysate from delimed pelt of the trimmings was calculated. This is based on the content of solid content in delimed pelt before digestion and the solid content in the collagen hydrolysate after hydrolysis and filtration.

3.3.4.5 Determination of the total free amino groups by formol titration

Collagen hydrolysate solution

0.5±0.009 g (P) collagen hydrolysate powder was weighed and placed in 100 ml beaker. 20 ml demonized water was added. It was stirred until the collagen hydrolysate is completely dissolved. Then the pH was adjusted to 7.4±0.1 with 0.05N NaOH [46].

Preparation of formol reagent

500 ml of formaldehyde solution were diluted with 200 ml of de-ionized water and was then stirred. And the pH was adjusted to 7.4±0.1 with 0.05N NaOH just before use.

Titrations

35 ml of formol reagent was added to collagen hydrolysate solution and was stirred for 5 min. It was then titrated to pH 9.2±0.1 with 0.05 N NaOH (V) in the presence of phenolphthalein.

The total free amino groups (N_t) present in collagen hydrolysate is given as

$$N_t = 0.05 \times \frac{V}{P}\left(\frac{mmol}{g}\right)$$

3.3.4.6 Analyzing the collagen hydrolysate using Fast Protein Liquid Chromatography (FPLC)

Instruments dedicated to the separation of proteins have given rise to the technique of fast protein liquid chromatography (FPLC). There are no unique principles associated with FPLC; it is simply based on reversed-phase, affinity, exclusion, hydrophobic interaction and ion-exchange chromatography, and chromtofocusing. Mainly aqueous-based elution systems are used with special high capacity stationary phases of similar diameter to those used in conventional HPLC. However, the operating pressure (1-2MPa) is lower than conventional HPLC [47].

When it is known or suspected that the molecular weight exceeds approximately 2000 daltons for some or all of the sample components, then a separation using exclusion

chromatography is indicated. This method is based on the ability of controlled-porosity substrates to sort and separate sample mixtures according to the size and shape of the sample molecules [47].

Very large molecules cannot enter many of the pores, and they also penetrate less into the comparatively open regions of the packing. Thus exclude, they travel mostly around the exterior of the packing and elute at the bed void volume of the mobile phase. Very small molecules diffuse into all or many of the pores accessible to them. With a larger column volume at their disposal, small molecules exit the column last.

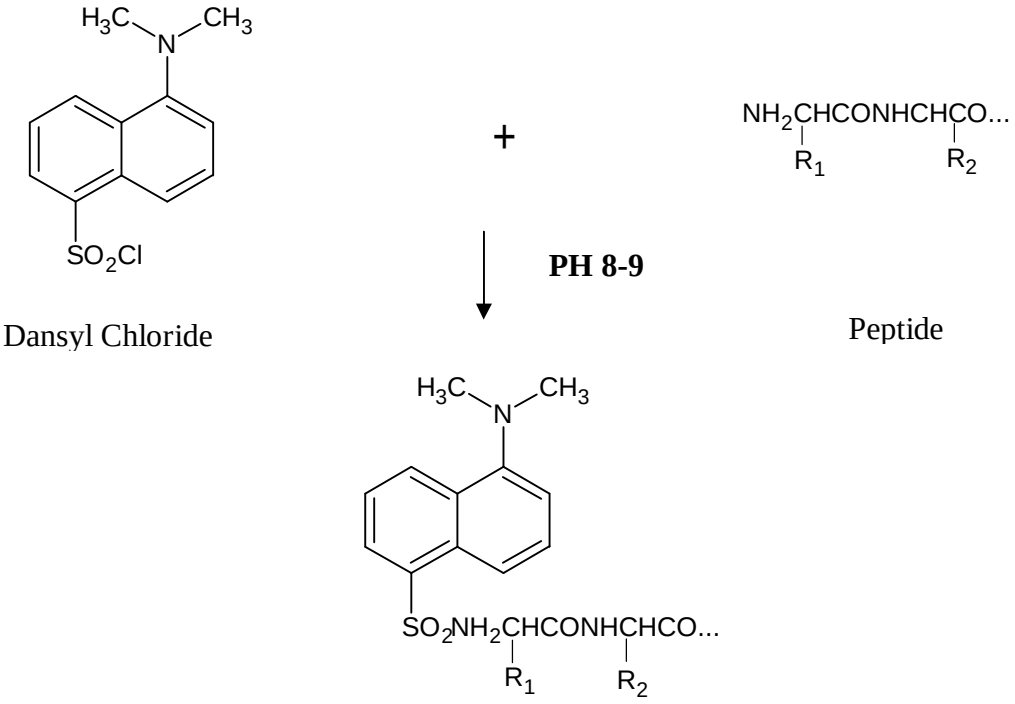
15 mg/ ml of collagen hydrolysate was prepared by dissolving collagen hydrolysate in distilled water; in this case distilled water was used as a mobile phase. Another solution of collagen hydrolysate was also prepared by dissolving it 25mM tris-HCl; in this case 25mM tris-HCl was used as a mobile phase. The samples were filtered with sterilized MCE membrane filter of pore size 0.22μm.

The column was packed by using 150ml of superdex 30 (prep grade) from GE Health care. 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 were injected in 0.5 ml loop. Each fractions of the sample were separated and collected separately, for this more than 25 ml of sample were injected. Each fractions separated are kept for further study.

From the chromatogram, it is possible to analyze how many fractions are formed by the enzymatic hydrolysis and the relative proportions of each fraction.

3.3.4.7 Derivitization of the collagen hydrolysate with dansyl chloride to determine the primary amines in the collagen hydrolysate

Dansyl Chloride (1-dimethyl aminonaphthalene-5-sulfonyl chloride) reacts with free primary amine groups of peptide and proteins as shown in below. The bond between the dansyl group and the N terminal amino acid are resistant to acid hydrolysis. The dansyl amino acid is fluorescent under UV light and is identified by thin layer chromatography on polyamide sheets [48]. By analyzing the UV spectrum of the dansyl chloride when it reacts with the peptide we can determine the amount of primary amines present in the collagen hydrolysate by using Beer-Lambert equation.



Dansyl Chloride

Peptide

The following reagents were prepared for dansyl experiment:-

- Dansyl chloride (1 mg/ml in Acetone)
- Sodium Hydroxide (0.05 N)
- Acetone
- Collagen Hydrolysate (5 mg/ml in Water)

The procedure of reaction is as follows:-

Four test tubes were prepared. In the first test tube (Experimental): 1 ml collagen hydrolysate, 1 ml water and 0.25 NaOH are mixed first and then 0.25 ml dansyl chloride was added finally. In the second test tube (Collagen Hydrolysate alone): 1 ml collagen hydrolysate, 1 ml water, 0.25 ml NaOH and 0.25 ml acetone was mixed. In third test tube (Dansyl alone): 2 ml water, 0.25 ml NaOH, 0.25 ml dansyl chloride was mixed. In fourth test tube (Base line correction or acetone alone) 6 ml Water, 0.75 ml NaOH, and 0.75 ml Acetone was mixed. All the test tubes were then heated in a water bath in dark condition at 60^{o}C for 1 hour. After this the test tube were taken for UV and scanned from 200 to 800 nm.

3.3.4.8 Analyzing the collagen hydrolysate with Matrix Assisted Laser Desorption/Ionization (MALDI-TOF)

MALDI is a soft ionization technique used in mass spectrometry, allowing the analysis of bio-molecules, bio-polymers (such as DNA, proteins, peptides and sugars) which tend to be fragile and fragment when ionized by more conventional ionization methods. The type of a mass spectrometer most widely used with MALDI is the TOF (time-of-flight mass spectrometer), mainly due to its large mass range [49].

The mechanism of MALDI involves two steps. The first step is desorption (i.e. release of molecules from the surface of the matrix) by UV laser beam. This leads to hot plume containing many species like neutral and ionized matrix molecules, protonated and deprotonated matrix molecules, matrix clusters and nanodroplets. The second step is ionization, in which the analyte molecules are ionized into protonated or deprotonated state in the hot plume [49].

Matrix is crystalline molecules which have relatively low molecular weight but they are not easily evaporating during sample preparation, they are a proton source for the ionization of the analyte and they typically contain chromophore [49].

In analyzing our collagen hydrolysate with MALDI-TOF, sinapinic matrix was used. Both positive and negative ions were collected and the spectrum of the hydrolysate m/z over a wide mass range versus intensity was plotted.

3.3.5 Application of the collagen hydrolysate in post tanning operation

In order to determine the applicability of collagen hydrolysates for post tanning; experimental trials using collagen hydrolysate for improving the properties of the leather were conducted using goat wet blue leather.

Six type of collagen hydrolysate were prepared based on the varying condition of the concentration of enzyme and duration of hydrolysis to get different molecular sizes of the collagen hydrolysate and is presented in table 2.1 to see the efficacy of the products for its effectiveness for the intended purpose.

Table 3.1:- Collagen hydrolysates prepared

Collagen Hydrolysate	*Amount of Enzyme trypsin (%)*	*Duration of digestion (hours)*
CH-1	0.8	3
CH-2	1.0	3
CH-3	1.2	3
CH-4	0.8	2
CH-5	1.0	2
CH-6	1.2	2

The prepared collagen hydrolysate products (six) prepared at different concentrations (0.8, 1.0, and 1.2%) of trypsin enzyme and different duration of hydrolysis (2 and 3 hours) were used for retanning trials Wet blue goat skins were sided in to four quadrants by cutting across the back bone line and along the belly horizontally as shown in figure 2.1below.

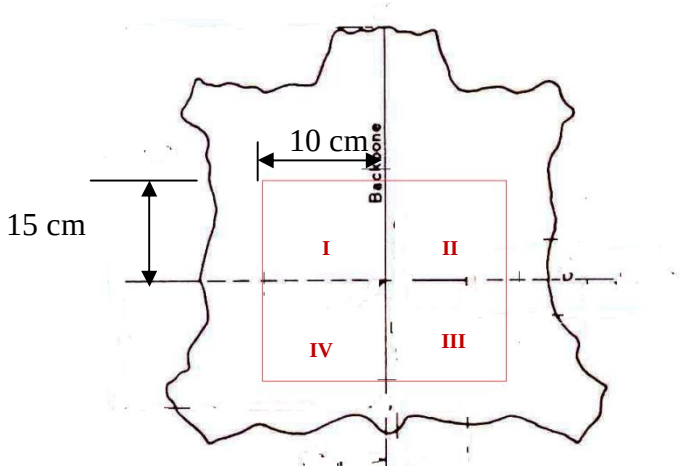


Figure 2.1 Allocation of the four quadrants of the wet blue goat leather for different types of collagen hydrolysate

Quadrant I was used as a control for trial with the commercial available syntan, Quadrant II was used for trial with the prepared collagen hydrolysate with 0.8% trypsin, Quadrant III for collagen hydrolysate with 1.0%, and Quadrant IV for collagen hydrolysate with 1.2%. The same procedure was repeated for 2 hrs and 3 hrs digestions.

Small glass drums of the same kind were used for processing the pieces of the quadrant leather, and same recipe except at the retanning stage were used. The recipe used is presented in table 2.2.

Table 3.2: Experimental/Control post tanning process for making upper Crust

Process	Percent	Chemicals	Time	Remark
Washing	200	Water, 40 ^{o}C	20’	
				Drain
Neutralization	150	Water, 40 ^{o}C		
	1	Sodium Formate	20’	
	0.7	Sodium bicarbonate	60'	pH 4.8/5.0
				Drain/Wash/Drain
	150	Water, 45 ^{o}C		
	2	Synthetic fatliquor	20’	
Retanning	*10*	*Collagen Hydrolysate (experimental)*	*30'*	
		Aromatic Sulphone-protein containing syntan (Control)		
Dyeing	3	Blue dyestuff	60'	
Fatliquoring	50	Water, 60 ^{o}C		
	8	Synthetic fatliquor	45'	
Fixation	0.5	Formic Acid (1:10 cold)	20'	
	0.5	Formic Acid (1:10 cold)	20'	pH 3.4/3.6
				Drain/Wash/Drain
		Pile and sam set and Hang over dry	L/O/N Next day	

Evaluation of the leathers treated with collagen hydrolysates was determined based on the percent absorption of collagen hydrolysate, dye and post tanning chemicals (fatliquors, retanning agents and dyes) by the leather. The more absorption value of collagen hydrolysates, dye, and post tanning chemicals, the more will be its fullness and dye exhaustion. Evaluation was also determined based on the percent change in thickness and weight of the leathers, quantification of colors, strength of leathers and organoleptic properties.

3.3.5.1 Percent absorption of collagen hydrolysate

Percent absorption of collagen hydrolysate (CH) was estimated in terms of hydroxyproline. The following equation was used for the balance:-

$$Amount\ of\ CH\ absorbed\ by\ leather = Amt\ CH\ offered - Amt\ CH\ in\ spent\ liquor$$

The amount of Hyp offered in the collagen hydrolysate can be easily estimated by following directly Hyp estimation procedure.

Determining the amount of collagen in the liquor

The discharged post tanning liquor was collected and its volume is determined. And then 3 ml of the post tanning liquor was taken and then it was evaporated in hot air oven for about 3 hours. The dried matters were then re-dissolved in 10 ml volumetric flask with distilled water. From this stock solution 200µl was pipette and placed in hydrolysis tube. 800µl of distilled water and 1000µl 12N HCl were then added into this hydrolysis tube. The tube was then sealed and digested for 8 hours in hot air oven at 120^{o}C. The tubes were de-sealed and poured in to porcelain crucible and washed with evaporated with distilled water up to five times to completely eliminate the acid. It was then dissolved and transferred to 50 ml volumetric flask and make up to the mark with distilled water. Then the normal procedure for Hyp estimation was followed.

$$Concentration\ of\ Hyp\ in\ spent\ liquor\ \left(^{\mu g}/_{ml}\right) = Concentration\ of\ Hyp\ as\ determined\ by\ UV\ \left(^{\mu g}/_{ml}\right) \times Dilution\ factor$$

$$Concentration\ of\ Collagen\ in\ spent\ liquor\left(\mu g/_{ml}\right) = Concentration\ of\ Hyp\ in\ spent\ liquor\left(\mu g/_{ml}\right) \times 7.4$$

$$Amount\ of\ collagen\ in\ spent\ liquor\ (\mu g\ or\ mg) = Concentration\ of\ Collagen\ in\ spent\ liquor\left(\mu g/_{ml}\right) \times Volume\ of\ spent\ lliquor\ collected$$

Calculation of collagen hydrolysate

$$\%\ Collagen\ \ Absorbed = \frac{Amount\ of\ Collagen\ Absorbed}{Amount\ of\ Collagen\ Offered} \times 100$$

Amount of collagen offered

The amount of collagen hydrolysate offered is calculated based on the weight shaved wet blue weight. According to table 3, 10% of collagen hydrolysate based on the weight of wet blue was offered.

The amount of collagen is calculated based on the collagen content of the collagen hydrolysate.

$$Amount\ of\ collagen = Collagen\ content\ (\%) \times Amountof\ collagen\ offered$$

3.3.5.2 Percent absorption of dye

$$Amount\ of\ dye\ absobed\ by\ the\ leather = Amount\ of\ dye\ offered - Amount\ of\ dye\ in\ the\ spent\ liquor$$

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

Determining the amount of dye in the liquor

Known concentration (5, 10, 20, 30, 40, 60, 80 and 100 ppm) of dye used was prepared. λ_{max} for the dye was determined by scanning one of the known concentration in UV-

Spectrophotometer. After getting λ_{max} the absorbance of the known concentration was read at λ_{max}.

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 up to the mark. Then the absorbance of this solution was read at the λ_{max} corresponding to the dye. From the standard calibration curve of the dye, the concentration of the dye in the prepared sample can be determined. And by calculating back we can determine the concentration of the dye in discharged liquor.

$$Concentration\ of\ dye\ in\ spent\ liquor\ (ppm) = Concentration\ of\ dye\ as\ determined\ by\ UV\ (ppm)\ \times Dilution\ factor$$

$$Amount\ of\ collagen\ in\ spent\ liquor\ (mg) = Concentration\ of\ Collagen\ in\ spent\ liquor\left({}^{mg}/_{ml}\right) \times Volume\ of\ spent\ lliquor\ collected\ (ml)$$

Dye offered

Amount of dye offered is straight forward, it is the amount of dye we put into the drum (i.e. 3% based on the weight of shaved wet blue).

3.3.5.3 Percent absorption of post tanning chemicals

Percent absorption of post tanning chemicals were calculated based on the total solid content of the fatliquors, dyes and syntans.

$$Total\ Solids\ absorbed\ in\ the\ leather = Total\ solid\ content\ offered - Total\ Solids\ in\ the\ spent\ liquor$$

Total Solids offered

Total solids offered for fatliquors, dyes and syntans was determined by evaporating known amount of them at 105^{o}C in hot air oven for 8 hours. And then total solid is calculated gravimetrically.

$$Total\ Solid\ offered = Total\ Solids\ of\ (fatliquors + dye + syntans)$$

Total Solids in the discharged liquor

The discharged post tanning liquor was collected and its volume is determined. Out of this 3 ml liquor was taken and poured in to porcelain crucible. It was then evaporated in hot air oven and the total solid content of the liquid was determined gravimetrically.

Thus,

$$\%\ Absorption\ of\ the\ Post\ tanning\ chemicals = \frac{Amount\ of\ Total\ Solids\ Absorbed}{Amount\ of\ Total\ Solid\ Offered} \times 100$$

3.3.5.4 Percent weight reduction of the processed leathers

Calculation of the percent weight reduction of the processed leather indicates the extent of filling of the post tanning chemicals. The weights of the wet blue pieces of leathers were taken and then after crusting the weight of dry crust leathers were measured.

$$\%\ Reduction\ in\ Weight = \frac{Wt.of\ Wet\ Blue - Wt.of\ Crust}{Wt.of\ Wet\ Blue} \times 100$$

3.3.5.5 Percent change in thickness

Thickness of the shaved wet blue at certain marked location was measured. Again the thickness of the crust leather at that certain marked location was read. The percent change in thickness of the leathers for treated with CH (2 and 3 hours of hydrolysis) and the control was calculated according to the following formula:-

$$\%\ Change\ in\ thickness = \frac{Thickess\ at\ wet\ blue\ stage - Thickness\ at\ crust\ stage}{Thickness\ at\ wet\ blue\ stage} \times 100$$

3.3.5.6 Strength characteristics

The four quadrant crust leathers were tested for physical strength properties. Sampling and conditioning were done according to the standards ISO 2418:2005 and ISO2419:2005. Physical strength properties such as tensile strength and elongation at break [44], and double edge tear strength were measured.

3.3.5.7 Quantification of colors of the leathers

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

3.3.5.8 Organoleptic evaluations

The organoleptic properties such as softness, fullness, roundness, smoothness of grain, grain tightness, intensity of the shade of the color and overall appearance of the crust leather for CH treated (2 and 3 hours hydrolysis) and for the control were evaluated by a group of experts. The values were rated from 1 to 10; higher value represents better functional property.

3.3.6 Effect of collagen hydrolysates on the stability of collagen fibers

In order to determine the effect of collagen hydrolysates on the stability of collagen fibers; enzymatic and thermal stability experiments were conducted using rat tail tendon collagen fibers. Also microscopic examination using compound and scanning electron scanning microscope were also carried out. It is known that rat tail tendons contain purest form of collagen.

Preparation of Rat Tail Tendon (RTT)

Collagen fibers were teased out from the tails of albino rats (Wistar strain). The rat tail tendons (RTT) were first washed with plain water at 4^0C. And then they were washed with 0.9% saline water at 4^0C. The RTT was then stored in the saline condition and preserved in the refrigerator at 4^oC and served as a stock of tendon for the whole experiment of tanning.

Preparation of buffers

Different phosphate buffers at PH 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 were prepared. The preparation of each buffer is mentioned as follows:-

- PH 4.0:- 5.04 g Na_2HPO_4, 3.01 g KH_2PO_4 in 1000ml distilled water
- PH 5.0:-1.76 g Na_2HPO4, 13.61 g KH_2PO4
- PH 6.0:- 6.15 ml, 0.2M Na_2HPO_4 and 43.85 ml, 0.2M $NaHPO_4$ diluted to 1000 ml with distilled water.
- PH 7.0:- 30.5 ml, 0.2M Na_2HPO_4 and 19.5 ml, 0.2M $NaHPO_4$ diluted to 1000 ml with distilled water.
- PH 8.0:- 47.35 ml, 0.2M Na_2HPO_4 and 2.65 ml, 0.2M $NaHPO_4$ diluted to 1000 ml with distilled water.
- PH 9.0:- 1.74 g KH_2PO_4 in 80 ml H_2O, adjust the pH with 1M KOH and dilute to 100 ml distilled water.

Treatment of RTT with collagen hydrolysate

2 mg rat tail tendon was weighed and placed in 2 ml eppendrof tube. To this, 0.7 ml of the buffers (section 3.3.2.3) were added and kept in refrigerator 4^oC for overnight to equilibrate

the pH environment of the tendons at respective pH's. Then 50% collagen hydrolysate based on the weight of tendons was added and made up with respective pH buffers to 1 ml. After the addition of collagen hydrolysate the solution was placed in orbital shaker for 24 hours at room temperature. After this the samples were taken for enzymatic and thermal stability experiments.

3.3.6.1 Enzymatic stability

The collagen hydrolysate treated RTT (section 3.3.2.4) were tested for resistance to collagenase. The resistance or enzymatic stability of native and collagen hydrolysate treated RTT by collagenase was analyzed by estimating the amount of hydroxyproline released in the solution after hydrolysis.

Collagenase (MMP1) preparation

Collagenase of conc. 1 mg/ml was prepared in Tris HCl buffer at PH 7.4.

Tris-HCl Buffer preparation for 100 ml

Tris (M.Wt. 121.14 g/mol) of 1.212 gm and 0.555 g $CaCl_2$ are added to 100 ml volumetric flask and make up it with water to the mark. Adjust the pH to 7.4 with HCl if necessary.

Procedure

The collagen hydrolysate treated tendons were washed with distilled water for three to five times to ensure the excess and unbound collagen hydrolysate are removed from the tendon. To this 700 µl Tris buffer and then 20µl collagenases (in the proportion of 100:1) was added and make up to 1ml with tris buffer. Then they are placed in orbital shaker at 37^oC in orbital shaker for 24 hours. Then they are placed in a centrifuge to settle down the degraded tendon. After this, 0.5 ml of supernatant solution was transferred into a hydrolysis tube for estimation of hydroxy proline content.

Estimation of Hydroxyproline

The determination of hydroxy proline in the supernatant solution was carried out according to the procedure of J. F. Wossener which is attached as annex.

Calculation of Collagen degradation

To convert amount of hydroxyproline into collagen, we use the following relationship:-

$$Collagen\ (mg) = Hydroxyproline(mg) \times 7.4$$

To calculate the degradation:

$$Percent\ degradation = \frac{Collagen\ (mg) of\ the\ supernatant\ solution}{Collagen\ in\ the\ tendon\ before\ degradation} \times 100$$

3.3.6.2 Thermal stability

After treating the tendons with collagen hydrolysates at pH 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0, the thermal stability was measured using micro-scale visual determination and differential scanning calorimetric (DSC) technique (for PH 5). As a control tendons are treated with only buffer at different PH without collagen hydrolysate.

Thermal stability at different concentration of collagen hydrolysate, (1:0.25, 1:0.5, 1:0.75, 1:1.0, 1:1.75 and 1:2) tendon to collagen hydrolysate concentrations, was determined by DSC.

Both methods are based on the principle that the thermal shrinkage of the collagenous matrices can be measured by monitoring the heat induced dimensional changes and phase transition. The dimensional change in the shrinkage process of the collagen fibre is detectable and the temperature at which the shrinkage takes place is a measure of hydrothermal stability of the fibers.

3.3.6.2.1 Microshrinkage analysis

The temperature at which the length of the collagen fibre shrinks approximately one-third of its original length is noted as the shrinkage temperature of the fibre (Borasky and Nutting 1949) [42].

Rat tail tendon treated with collagen hydrolysate at different pH and their respective controls were subjected to hydrothermal stability analysis. About 1 mm length of treated and untreated RTT was cut and placed in the groove of a microscopic slide. Few drops of water were added to the groove in such a way that the sample was completely immersed in water. The groove was covered with a cover slip. The slide with the sample was placed on a metallic heating stage carrying a thermometer. The metallic heating stage was mounted on a tripod. A vertically held microscope was mounted above the heating stage to view the dimensional changes of the sample. The rate of heating was maintained around 2^{o}C/min. The temperature at which the fibre shrinkage occurred was noted as the shrinkage temperature.

3.3.6.2.2 Differential scanning calorimeter measurements

Differential Scanning Calorimetry (DSC) is thermal analysis technique. In this technique, the sample and reference materials are subjected to a precisely programmed temperature change. When a thermal transition (a chemical or physical change that results in the emission or absorption of heat) occurs in the sample, thermal energy is added to either the sample or reference containers in order to maintain both the sample and reference at the same temperature. Because the energy transferred is exactly equivalent in magnitude to the energy absorbed or evolved in the transition, the balancing energy yields a direct calorimetric measurement of the transition energy. DSC can measure directly both the temperature and the enthalpy of a transition or the heat of a reaction [43].

Integration of the area under a DSC curve provides a direct measurement of ΔH for thermally induced transitions according to the equation

$$A = -k'm\Delta H$$

Where A is the area; k' the instrument constant, which is independent of the temperature; m the mass; and H the enthalpy of the reaction or transition [43].

The native and collagen hydrolysate treated RTT were blotted uniformly weighed and hermetically encapsulated in aluminium pans. The samples were fused in a differential scanning calorimetric cell of 910 differential scanning calorimeter, Dupont Instruments. The temperature was calibrated using indium as standard. The heating rate was maintained constant at 5^{o}C/min. The denaturation temperature, T_D (in ^{o}C) associated with the phase and enthalpy changes for native and fibres treated with collagen hydrolysates were determined.

3.3.6.3 Microscopic examination

The enzymatic degradation of native and collagen hydrolysate treated RTTby collagenase and native RTT which is not subjected to collagenase was analyzed by observing under compound and scanning electron microscopes.

3.3.6.3.1 Compound microscope

The samples (i.e. native RTT and collagen hydrolysate treated RTT which are subjected to collagenolytic hydrolysis, and native RTT which is not subjected to collagenolytic hydrolysis) were examined with compound microscope at 40X, 100X and 200X magnification level.

3.3.6.3.2 Scanning Electron Microscope (SEM) examination

The samples (i.e. native RTT and collagen hydrolysate treated RTT which are subjected to collagenolytic hydrolysis, and native RTT which is not subjected to collagenolytic hydrolysis) were examined with SEM at magnification level of 200X and 500kX.

4. Result and discussion

4.1 Characterization of delimed pelt trimmings

4.1.1 Collagen content

The collagen content based on the procedure mentioned in section 3.3.1.1 and according to Wossener (1961) was determined. It was found that, based on dry content; the delimed pelt contains 96.05% collagen. This implies that limed pelt is an ideal source of raw material for preparing collagen hydrolysates.

4.1.2 Ash content

Total ash content of the delimed pelt was determined according to SLC 6 (IUC 7; BS 1309:6) and it is found that the delimed pelt contains 1.73% ash based on dry delimed pelt. The ash content is primarily due to calcium salts which were not completely removed by deliming process.

4.1.3 Fat content

Fat content of delimed pelt was determined according to SLC 4 (IUC 4; BS 1309:4). Accordingly the fat content of the delimed pelt is 2.0 % based on dry delimed pelt weight.

4.2 Characterization of collagen hydrolysate

The collagen hydrolysate which gives better results in terms of fullness, dye absorbance and other organoleptic properties was selected and taken for characterization. In this case collagen hydrolysate which has been digested with a protolytic condition of 0.8% trypsin and 3 hour hydrolysis duration time was taken for characterization.

4.2.1 Collagen content

The collagen content of the collagen hydrolysate prepared is 96% based on dry weight.

4.2.2 Moisture content

The moisture content of the collagen hydrolysate powder was found to be 5%.

4.2.3 Ash content

The ash content of the collagen hydrolysate powder was found to 2.64% based on the weight of collagen hydrolysate powder (i.e. including moisture). The ash content is due to calcium ions which were removed completely during deliming of the pelt.

4.2.4 Yield

The yield in producing collagen hydrolysate from delimed pelt is presented in Table 4.1.

Table 4.1:- Yield of CH from delimed pelt trimmings

Sample weight for digestion (g)	*Total Solid (g)*		*Yield (%)*
	Before Hydrolysis	*After Hydrolysis and filtration*	
20	7.8	7.60	97.48

Thus, out of 100 g of delimed pelt, 97.48 g collagen hydrolysate will be produced.

4.2.5 Determination of the total free amino groups by formol titration method

As described in the methodology part, to reach end point of the titration, the hydrolysate consumed on average 6.4 ml of 0.05N NaOH.

Thus, the total free amino groups (N_t) present in collagen hydrolysate as given by$N_t = 0.05 \times \frac{V}{P}\left(\frac{mmol}{g}\right)$, will be:-

$$N_t = 0.05N \times \frac{6.4\ ml}{0.5\ g} = \mathbf{0.64}\ \left(\frac{\boldsymbol{mmol}}{\boldsymbol{g}}\right)$$

4.2.6 Analyzing the collagen hydrolysate using Fast Protein Liquid Chromatography (FPLC)

The chromatogram of the collagen hydrolysates that have separated by using distilled water as mobile phase is presented in Figure 4.1. The integration of the peak areas of the chromatogram is also given in Table 4.2.

It is observed that there are about 6 to 7 major fractions and about 4 minor fractions. The relative proportion of the first fraction is about 33%. The second and third fractions are also the major fractions accounting for about 30%.These fractions are eluted during initial period of running the chromatography. Then the next higher proportion of fraction is 14.88%. This fraction is coming relatively at a latter period.

Thus about 80% of the collagen hydrolysates have higher molecular weight (retention time 49 to 66.67 minutes) and 20% have lower molecular weight (retention time 71 to 126 minutes). Because, the higher molecular weights will elute first and the lower molecular weights will elute at last.

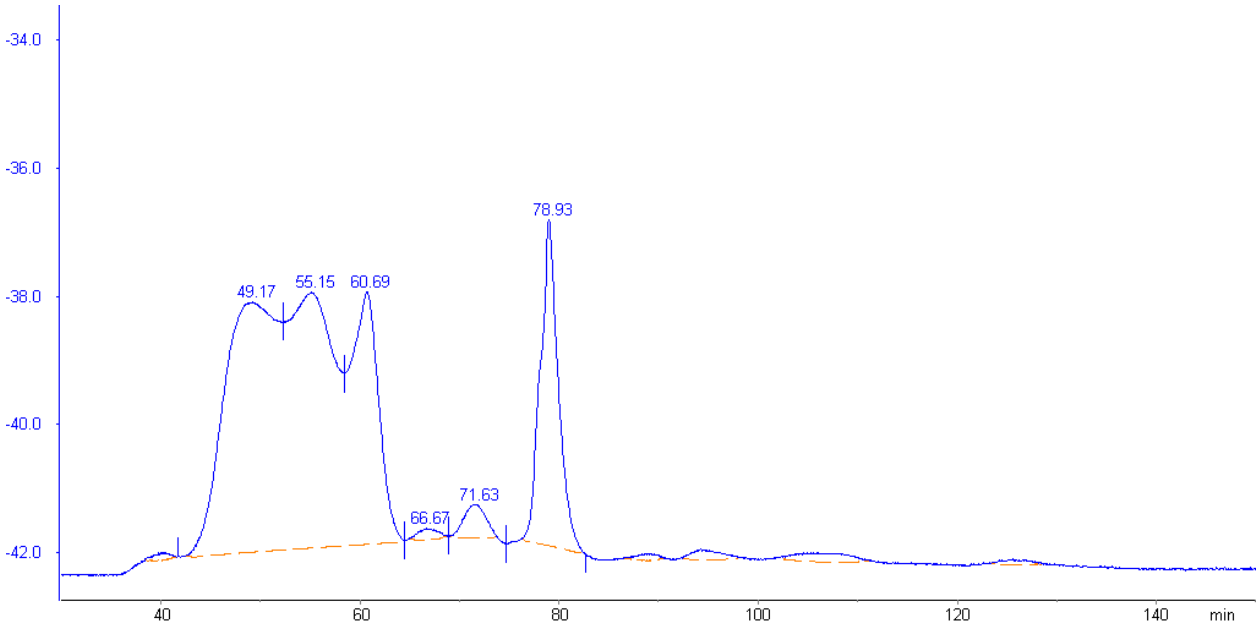


Figure 4.1:- Chromatogram of collagen hdyrolyzate with water as a mobile phase

Tale 4.2:- Relative proportions of each fraction of CH with tris-HCL as a mobile phase

No	*Retention (min)*	*Area (mAU*min)*	*Relative proportions of each fraction (%)*
1	49.17	25.67	33.49
2	55.15	22.08	28.80
3	60.69	13.75	17.94
4	66.67	1.23	1.62
5	71.63	2.50	3.27
6	78.93	11.41	14.88
7	88.91	0.25	0.32
8	94.19	0.55	0.71
9	105.49	0.42	0.53
10	107.89	0.48	0.62
11	125.60	0.15	0.20
12	126.83	0.11	0.14
Total		78.64	100.00

4.2.7 Derivitization of the collagen hydrolysate with dansyl chloride

The UV spectctrum of the derivitaized collagen hydrolysate with dansyl chloride along with the other controls and base line corrections (merged) are given in Figure 4.3. As expected the amine absorption for CH occured at λ_{max}215 nm. For dansyl chloride its λ_{max} occurred at 215 nm due to its amine group and 316 nm. λ_{max} of dansyl chloride shifted from 315 nm to 307 nm, more over its absorbance also decreased from 1.9 to 1.4 for the experimental part. This indicated that the amine group of the collagen hydrolysate reacted with the dansyl chloride.

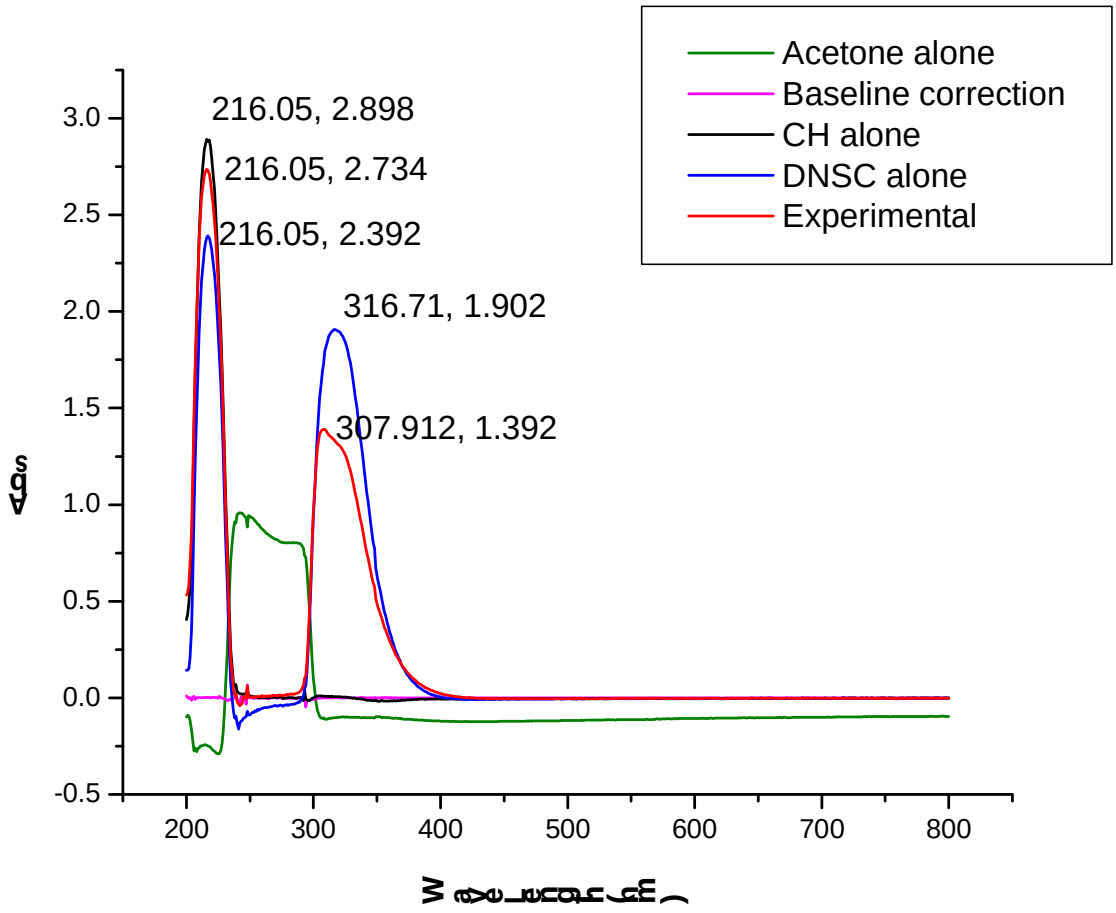


Figure 4.3:- UV-spectrum of derivitzed CH, DNSC alone, and CH alone

By using Beer-Lambert equation

$$A = \varepsilon c l$$

Where ε is the extinction coefficient in $M^{-1}Cm^{-1}$, c is the concentration in M, and l the path length in cm. The extinction coefficient of DNSC-Protein conjugate is 3300 $M^{-1}Cm^{-1}$. From the figure, the absorbance of the reacted Dansyl chloride is found to be 1.392. Thus the concentration of the reacted dancyl chloride will be:-

$$c = \frac{A}{\varepsilon l} = \frac{1.392}{3300\ M^{-1}cm^{-1} \times 1\ cm} = 0.000422M = 0.422mM$$

The number of moles of dansyl chloride reacted in 2.5 ml of solution will be:-

$$number\ of\ moles = 2.5\ ml \times 0.000422M = 0.001055\ mmol$$

According to the stochiometic relation between dansyl and primary amine (peptide), 1 mol of dansyl reacts with 1 mol of primary amine. Thus the number of moles of primary amines reacted with the dansyl chloride will be$0.001055\ mmol$.

In the reaction test tube we have added 1 ml of collagen hydrolysate at 5 mg/ml concentration. So the amount of collagen hydrolysate will be:-

$$Amount\ of\ collagen\ hydrolysate = 1\ ml \times 5\frac{mg}{ml} = 5\ mg$$

Thus, the amount of primary amine per gram of collagen hydrolysate will be:-

$$Amount\ of\ primary\ amine = \frac{0.001055\ mmol}{5\ mg} = \frac{1.055\ mmol}{5\ g} = \mathbf{0.211}\ \boldsymbol{mmol/g}$$

We have found that the total free amine groups to be 0.64 mmol/g. So out of the total free amine about 33% is in the form of primary amine.

4.2.8 Analyzing the collagen hydrolysate with Matrix Assisted Laser Desorption/Ionization (MALDI-TOF)

The spectrum of the collagen hydrolysate after analysing with MALDI-TOF is presented in Figure. It indicates that the molecular wight of the collagen hydrolysate ranges from 1746.96 to 5788.03 daltons. From the FPLC it was found that about ten fractions. From MALDI the molecuar weight of each fractions are indicated on each of specific peaks as it is indicated in the Figure 4.4.

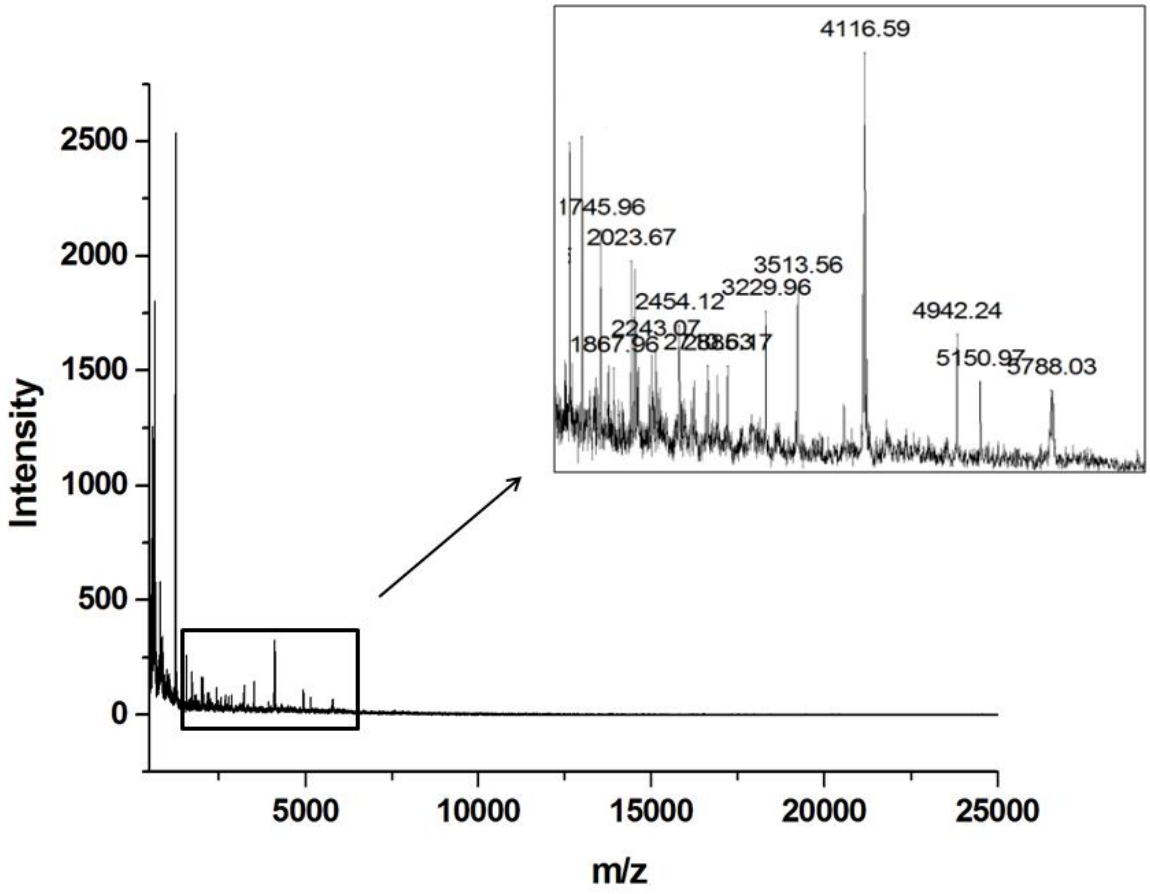


Figure 4.4:- Mass spectrum of CH using MALDI-TOF

4.3 Application of the collagen hydrolysate in post tanning operation

4.3.1 Leathers treated with CH (3 hours of hydrolysis)

4.3.1.1 Percent absorption of collagen hydrolysate

Determining the uptake of collagen hydrolysate

And the amount of collagen in the liquor as described in section 3.3.5.1 is presented in table 4.3.

Table 4.3:- Amount of collagen in spent liquor

Results	*Description*	*Collagen (g/ml)*	*Volume of spent liquor Collected (ml)*	*Total CH in spent liquor(g)*
II	CH-1 (0.8)	0.018	55.00	0.98
III	CH-2 (1.0)	0.018	58.75	1.04
IV	CH-3 (1.2)	0.019	67.50	1.31

Amount of collagen hydrolysate offered

The collagen hydrolysates offered are calculated based on the weight of shaved wet blue weight. The actual collagen content of the collagen hydrolysates was determined by hydroxyproline estimation method as described in the annex. It is determined that the content of collagen (based on hypro) in the collagen hydrolysates 91%. Thus the actual collagen content offered for the experimental trials are presented in table 4.4.

Table 4.4:-Amount of collagen offered

Results	*Description*	*Total solids of CH offered (g)*	*Actual CH offered (g)*
II	CH-1 (0.8)	8.03	7.30
III	CH-2 (1.0)	6.38	5.80
IV	CH-3 (1.2)	7.65	6.96

The percent of collagen absorption by the leather are presented in table 4.5.

Table 4.5:- Percent absorption of the collagen hydrolysate

Quadrant	*Description*	*CH offered (g)*	*CH in the spent liquor(g)*	*% CH absorbed by the leather*
II	CH-1 (0.8)	7.30	0.98	86.58
III	CH-2 (1.0)	5.80	1.04	82.09
IV	CH-3 (1.2)	6.96	1.31	81.21

From the table it can be observed that, quadrant II which has been treated with collagen hydrolysate that is digested with 0.8% trypsin for 3 hours has maximum absorption of collagen than other collagen hydrolysates (i.e. 1 and 1.2%). As the percent of trypsin used for hydrolysis increases the absorption of collagen hydrolysates by the leather decreases. This may be due to as the concentration of trypsin increases the extent of hydrolysis increases. This in turn will result lower size (molecular weight) of collagen hydrolysates. As the size of the collagen hydrolysates decreases the probability of them staying in the inter fibers of the leather matrix decreases and they will end up discharged in the spent liquor. Thus the percent absorption of the hydrolysates decreases.

4.3.1.2 Percent absorption of dye

Determining the amount of dye in the liquor

As described in section 3.3.5.2 amount of dye in the spent liquor is presented in table 4.6.

Table 4.6:- Amount of Dye in spent liquor

Quadrant	*Description*	*Dye conc. (mg/ml)*	*Volume of spent liquor collected (ml)*	*Dye(mg) in discharged liquor*
I	Control	0.37	65	23.88
II	CH-1 (0.8)	0.11	55	6.09
III	CH-2 (1.0)	0.04	58.75	2.62
IV	CH-3 (1.2)	0.04	67.5	2.40

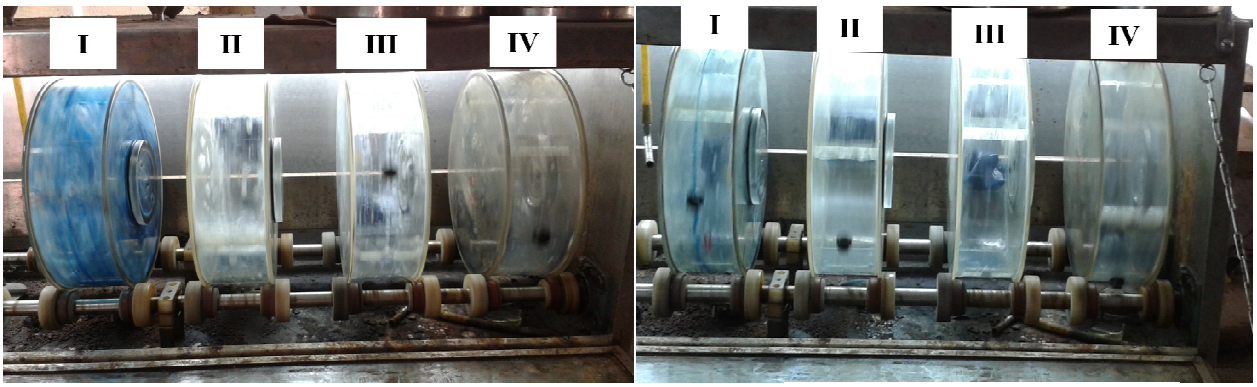
Dye offered and dye uptake

Amount of dye offered is calculated based on the shaved wet blue weight. Therefore the uptake of the dye by the leather is presented in table 4.7.

Table 4.7:- Percent Dye uptake of the CH treated leather and control

Quadrant	*Description*	*Dye (mg) offered*	*Dye (mg) in spent liquor*	*% Dye uptake*
I	Control	887.82	23.88	97.31
II	CH-1 (0.8)	95.40	6.09	99.36
III	CH-2 (1.0)	835.50	2.62	99.69
IV	CH-3 (1.2)	884.46	2.40	99.73

From table 8 it is observed that leathers treated with collagen hydrolysate (II, III and IV) have better dye uptake than the control which has been treated with the commercial syntan (I or Control). The differences in the dye uptake were also observed during processing i.e. before and after fixation with formic acid (Figure 4.5), and during sam setting of the leathers (Figure 4.6). The reason for high uptake of dye by the leathers which have been treated with collagen hydrolysates than the control is due to the collagen hydrolysates will create additional sites or functional groups where the dye will come and react either through electrostatic interaction or hydrogen bonding. Both of these interactions will enhance the fixation of the dye into the leather.



Before Fixation **After Fixation**

Figure 4.5:-Dye uptake of CH treated and control leather samples during processing (i.e. before and after fixation)

Experimental **Control**

Figure 4.6:-Leathers during sam setting

4.3.1.3 Percent absorption of post tanning chemicals

As described in section 3.3.5.3 the material balance of post tanning chemicals was calculated based on solid contents of collagen hydrolysates, fatliquors and dyes. The absorbance of post tanning chemicals are presented in table 4.8.

Table 4.8:- Percent absorption of post tanning chemicals for CH treated and control leather samples

Quadrant	*Description*	*Total solids offered (g)*	*Total solid in liquor (g)*	*% absorption to the leather*
I	Control	9.53	1.53	83.97
II	CH-1 (0.8)	11.20	1.79	83.99
III	CH-2 (1.0)	9.15	2.05	77.65
IV	CH-3 (1.2)	10.59	2.41	77.24

From the Table 4.8 it can be seen that quadrant I (control) and quadrant II(CH 0.8,) have better absorption of post tanning chemicals than others (quadrant II and III). The higher the absorption of the post tanning chemicals the better will be its fullness.

The higher absorption of post tanning chemicals in the case of quadrant II is due to the higher size of the collagen hydrolysates which have been hydrolyzed with low concentration of enzyme. The higher absorption of post tanning chemicals in the case of (quadrant I) is due to the higher molecular size of the commercial aromatic sulphone protein containing syntan used as a control.

4.3.2 Leathers treated with CH (2 hours of hydrolysis)

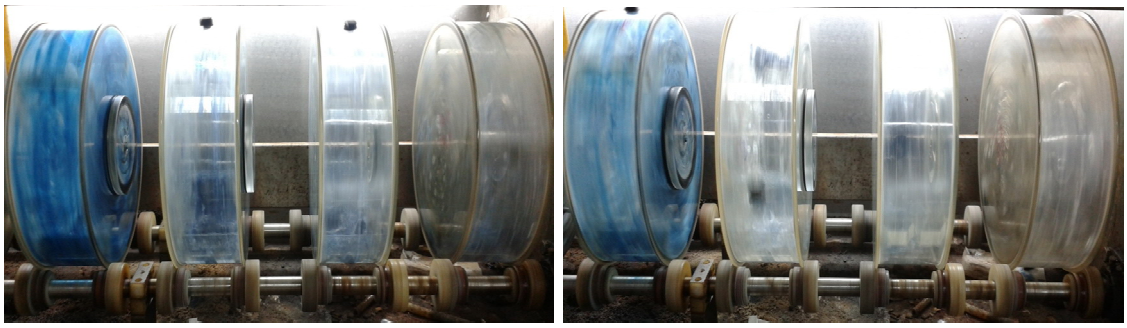
4.3.2.1 Percent absorption of dye

The dye concentration of the spent liquor which are determined according to the method mentioned in section 3.3.7.2 and dye uptake of leathers treated with collagen hydrolysates that has been digested for 2 hours versus the control is presented in table 10. The colors of the liquors

during processing i.e. before and after fixation are also shown in Figure 4.7 and collections of spent liquors after fixation are shown in Figure 4.8.

Table 4.9:- Percent Dye uptake of the CH treated leather and control (2 hours digested CH)

Quadrants	*Description*	*Dye concentration (mg/ml)*	*Volume of liquor collected (ml)*	*Dye offered (mg)*	*Dye in spent liquor (mg)*	*% Dye uptake*
I	Control	1.91	60.00	530.00	114.35	78.55
II	CH-4 (0.8)	0.06	59.50	560.00	3.35	99.41
III	CH-5 (1.0)	0.03	53.00	530.00	1.72	99.68
IV	CH-6 (1.2)	0.06	57.00	520.00	3.55	99.32



Before Fixation **After Fixation**

Figure 4.7:-Dye uptake of CH treated and control leather samples during processing (i.e. before and after fixation)(2 hours digested CH)

From the Table 4.9 and Figure 4.7 and 4.8 there is a significant difference in dye uptake between the leathers that have been treated with collagen hydrolysates (II, III and IV) and control (I). The dye uptakes of leathers treated with collagen hydrolysates are almost similar with sample III is marginally better. The reason for high uptake of dye for quadrant II, III and IV follows the same explanation as mentioned in section 4.3.1.2.

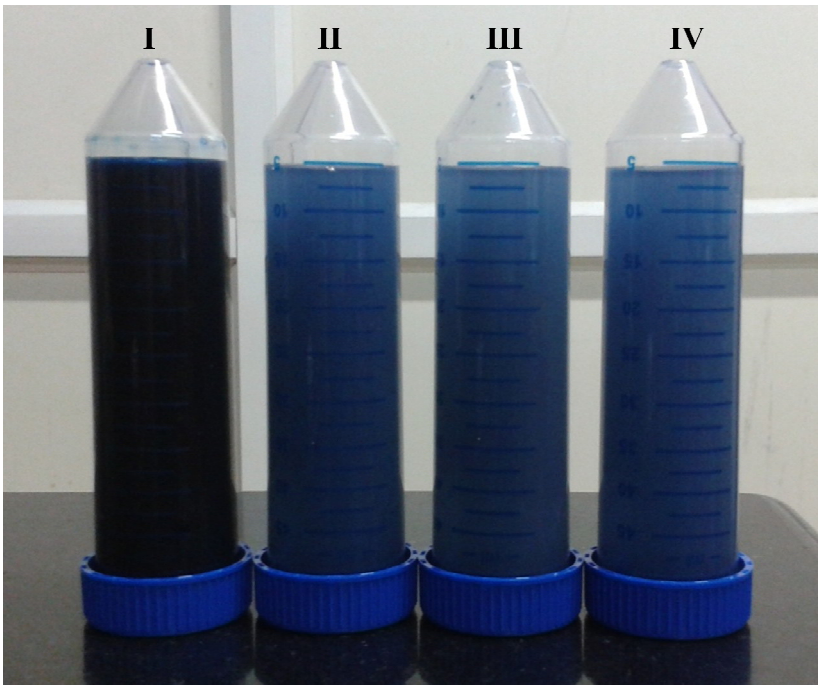


Figure 4.8:- Spent liquors collected after fixation

4.3.2.2 Percent absorption of post tanning chemicals

The percent absorbance of post tanning chemicals by the leathers that have been treated with hydrolysates which are digested for 2 hours versus the control are presented in Table 4.10.

Table 4.10 Percent absorption of post tanning chemicals for CH treated and control leather samples (2 hours digested CH)

Quadrant	*Description*	*Total solids offered (g)*	*Total solid in liquor (g)*	*% absorption to the leather*
I	Control	6.20	1.71	72.50
II	CH-4 (0.8)	6.05	2.13	64.82
III	CH-5 (1.0)	5.45	1.74	68.02
IV	CH-6 (1.2)	5.36	2.02	62.39

In such regard, the control is better than the hydrolysate. Among the hydrolysates quadrant III (CH-5 1.0) have high uptake of post tanning chemicals. This is due to the higher molecular size of the commercial syntan to be absorbed in the leather.

From the data of percent dye uptake (Table 4.9) and percent absorption of post tanning chemicals (Table 4.10) it is observed that quadrant III which has been treated with collagen hydrolysate using 1.0% trypsin for a duration 2 hours hydrolysis gave better results than others. In the 3 hours of hydrolysis CH-1 which has been digested with 0.8% trypsin gave good result in terms of percent absorption of post tanning chemicals. Where as in 2 hours of hydrolysis it gave lower result, the reason may be because the lower concentration of trypsin and the lower duration of hydrolysis may resulted a very high molecular size of collagen hydrolysates which are not capable of sitting or entering into pores of the collagen fibers of the leathers.

4.3.3 Percent weight reduction of the processed leathers

4.3.3.1 CH of 3 hours of hydrolysis

Percent weight reductions of leathers treated with hydrolysates 3 hours along with the controls are presented in Table 4.11. The lower the value of percent reduction of weight, the better fullness of the leathers will have. The reduction in the weight of the leathers is mainly due to the loss of moisture content. The moisture content of shaved wet blue is 30% where as that of dried crust leathers is 12%. However this factor is common to all quadrants. The difference in the percent of weight is coming from the difference in the uptake of post tanning chemicals namely the fatliquors, dyes and syntans. The higher the absorption of chemicals, the lower will be the percent reduction of weight and the higher will be the fullness of the leathers. From the Table 4.9 it can be observed that the control have better fullness than the CH treated leathers. This result is matching with the result mentioned in section 4.3.1.3 where the percent absorption of the post tanning chemicals is better for quadrant I and II. Among the CH treated leathers quadrant II is marginally better than others.

Table 4.11:- Percent weight reduction of CH treated and control leathers (3 hours digested CH)

Quadrant	*Description*	*Weight of wet blue (g)*	*Weight of crust leathers (g)*	*Percent reduction in weight*
I	Control	29.17	17.99	38.31
II	CH-1 (0.8)	32.56	18.71	42.54
III	CH-2 (1.0)	30.07	17.06	43.24
IV	CH-3 (1.2)	29.77	16.82	43.49

4.3.3.2 CH of 2 hours of hydrolysis

Percent weight reductions of leathers treated with hydrolysates 2 hours along with the controls are presented in table. From the Table 4.12 it can be observed that the control and sample III have better fullness than others. Here also it supports the result mentioned in section 4.3.2.2 where the percent absorption of post tanning chemicals is higher for quadrant I and III. As the percent absorption of post tanning chemicals increases the percent weight reduction decreases and the fullness of the leather will be improved.

Table 4.12:- Percent weight reduction of CH treated and control leathers (2 hours digested CH)

Samples	*Description*	*Weight of wet blue (g)*	*Weight of crust leathers (g)*	*Percent reduction in weight*
I	Control	19.27	12.31	36.00
II	CH-4 (0.8)	20.36	12.75	37.40
III	CH-5 (1.0)	19.21	12.35	35.69
IV	CH-6 (1.2)	18.91	12.04	36.35

4.3.4 Percent change in thickness

The percent reduction (change) in thickness measured at wet blue and crust stage are presented in Table 4.13. The percent change in thickness is also related with the percent reduction in weight and with the percent absorption of post tanning chemicals. The higher

absorption of post tanning chemicals will result a lower percent reduction of weight and inturn will result improved fullness of the leathers.

Table 4.13:- Percent Change in thickness of CH treated and control leathers

Samples	*Description*	*% Change in Thickness*	
		3 hours CH	*2 hours CH*
I	Control	13.00	8.50
II	CH (0.8)	13.30	10.97
III	CH (1.0)	20.11	8.03
IV	CH (1.2)	17.30	8.93

Here also the lower the value of % change in thickness in absolute value, the better will be its fullness. Thus from the table we can observe that the control (quadrant I) and quadrant II (CH 0.8) have better fullness than others for leathers treated with CH that has been hydrolyzed for 3 hours of duration. This result is in agreement with result mentioned in section 4.3.1.3 (percent absorption of post tanning chemicals) and section 4.3.3.1 (percent reduction in weight).

For leather treated with CH that has been hydrolyzed for 2 hours of duration sample III (CH 1.0) have better fullness than others. This result is also in agreement mentioned in section 4.3.2.2 (percent absorption of post tanning chemicals) and section 4.3.3.2 (percent reduction in weight).

4.3.5 Strength properties

The tensile strength and percent elongation are presented in the table and double edge tear strength is presented in Table 4.14.

Table 4.14:- Tensile and tear strength of CH treated and control leathers

Samples	*Description*	*Tensile Strength (N/mm^2)*	*Elongation (%)*	*Tear Load (N/mm)*
I	Control	19.37	63.13	49.21
II	CH (0.8)	28.38	64.10	58.92
III	CH (1.0)	33.29	68.07	64.98
IV	CH (1.2)	35.48	60.53	68.59

Table 4.14 suggests that the experimental leathers are better in tensile strength, tear strength and elongation than the control. Though the control (Quadrant I) and sample II are symmetrical there is a difference in strength properties due to collagen hydrolysate treatment. Sample II is relatively lower in strength properties when compared with sample III and IV. This may due the fact that sample III and IV are more closer to butt are region than sample II. And it is a known fact that butt regions of leather have better strength properties than other regions of leather.

The difference in tensile and tear strength between control and CH treated leathers is due to the effect of the syntans (commercial and CH syntans) on the fibers of the leathers. The commercial syntan as it can be seen from the organoleptic properties mentioned in section 4.3.7 is making the leather fibers very rough and hard. However CH syntans will make the leathers smooth and soft. As the fibers become very rough and hard they will not slide along each other or will have higher frictional forces among each other when external force or pressure is applied. So the fibers will break easily by the external force. This is similar to leathers which are treated with fatliquor will have higher lubrication (less frictional forces among the fibers) and the fibers will slide along each other due to external force. So the fibers will not break easily due to external pressure. This will result higher strength properties.

4.3.6 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 ΔE value by making the control leather as a reference are presented in Table 4.15.

Table 4.15:- L, a, b values of CH treated and control leathers

Samples	*Description*	*L*	*a*	*b*	*ΔE*
I	Control	39.96	-7.06	-20.1	-
II	CH (0.8)	29.62	-4.14	-20.64	10.76
III	CH (1.0)	26.94	-3.12	-19.03	13.65
IV	CH (1.2)	28.3	-3.43	-19.96	12.21

From the table it is observed that **ΔE** value is greater than 2. This suggests that there is significant difference in color shade between the control and the experimental leather samples. The color difference between the experimental leathers is more or less the same. This implies that the shade colors between the experimental samples (swatches) are matching.

The significant difference in the shade of the color between the control and CH treated leathers matches with the result mentioned in section 4.3.1.2 and 4.3.2.1 (percent absorption of dyes). Due to the additional sites created by the collagen hydrolysates more of dye will be fixed into the leather and will enhance the intensity of the shade of the leathers.

4.3.7 Organoleptic properties

The results of retanning experiments after analyzing the organoleptic properties of the experimental and corresponding control leathers by experts showed that the fullness and roundness properties of collagen hydrolysate syntan CH-2 0.8 (sample II) in table 4.14 and CH-5 1.0 (sample III) in Table 4.15 are comparable with that of the control. This result is also matching with the results mentioned in section 4.3.1.3 and 4.3.2.2 (percent absorption of post tanning chemicals), 4.3.3.1and 4.3.3.2 (percent reduction in weight), and 4.3.4 (percent change in thickness).

Whereas the grain tightness, grain smoothness, intensity of the shade/uniformity of the dyeing all the six collagen hydrolysate syntans (Table 4.14 and 4.15) are better than control leathers. In terms of the overall appearance sample II (CH-2 0.8) in the case of table 4.14 and sample III (CH-5 1.0) in the case of Table 4.15 are better than other samples.

Table 4.16:- Rates for organoleptic properties for leathers treated with CH that has been hydrolyzed for 3 hours

Properties	*I (Control)*	*II CH-1 (0.8)*	*III CH-2 (1.0)*	*IV CH-3 (1.2)*
Fullness	8	7	5.5	5.5
Softness	5	6	6	5
Grain smoothness	3	7	7	6
Color shade (intensity)	3	7	7	7
Roundness	8	7	5.5	5.5
Overall Appearance	7	7	6	6

Table 4.17:- Rates for organoleptic properties for leathers treated with CH that has been hydrolyzed for 2 hours

Properties	*I (Control)*	*II CH-1 (0.8)*	*III CH-2 (1.0)*	*IV CH-3 (1.2)*
Fullness	8	7	7	5.5
Softness	3	4	6	5
Grain smoothness	3	4	7	6
Color shade (intensity)	3	5	7	6.5
Roundness	8	7	7	5.5
Overall Appearance	4	5.5	7	6

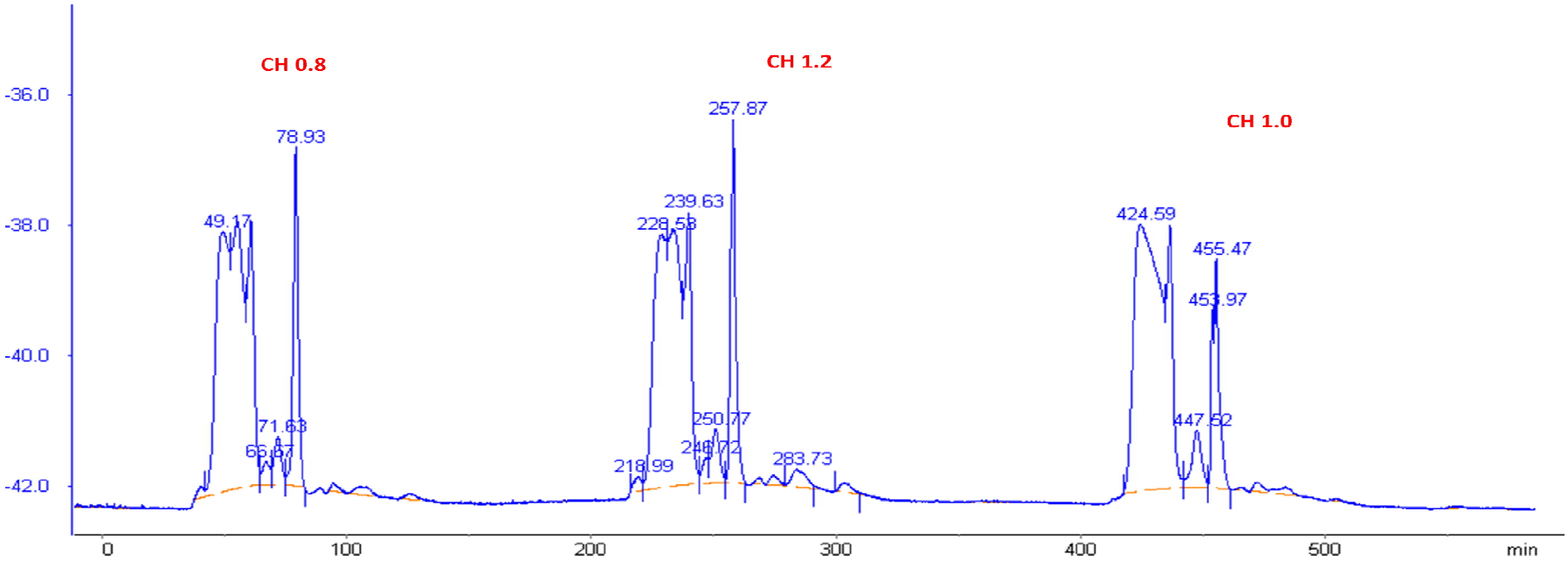
4.4 Comparison of the different types of collagen hydrolysates

Among the collagen hydrolysates that has been hydolysed for three hours, interms of collagen hydrolysate absorbtion by the leather (Table 4.3), CH-1 0.8 is the first, CH-2 1.0 is second and CH-3 1.2 is last. In terms of dye uptake all the three types of the collagen hydrolysates are almost equal and better than the control (Table 4.5). In terms of post tanning chemicals absorption by the leather (Table 4.6) the control and CH-1 0.8 are better than other hydrolysates. In terms of percent weight reduction (Table 4.9) the control is better than others, then follows CH-1 0.8. In terms of percent change in thickness (Table 4.11) the control and CH-1 0.8 are better than the others. And lastly in terms of orgaleptic properties (Table 4.14), control and CH-1 0.8 are better than the others. Overall CH-1 0.8 are giving better result than other types of collagen hydrolysates (hydrolysed for 3 hours)and giving comparable with the control.

Among the collagen hydrolysates that has been hydrolysed for three hours, interms of dye uptake (Table 4.7), all the three types of collagen hydrolysates are better than the control. In terms of post tanning chemical absorption (Table 4.8), the control is better than the hydrolysates followed by CH-5 (1.0). In terms of percent weight reduction (Table 4.10) the control and CH-5 (1.0) are better than the others. In terms of percent change in thickness (Tale 4.11) the control and CH 1.0 is better than others. In terms of organleptic properties (Table 4.15), CH-5 (1.0) is better than others. Overall CH-5 (1.0) is giving better result than other types of collagen hydrolysates hydrolysed for 2 hours.

This indicates that collagen hydrolysates which are prepared with 0.8% trypsin and 3 hours duration of hydrolysis is equivalent to those prepared with 1.0% trypsin and 2 hours duration of hydrolysis. This implies that with low concentration of enzyme and longer duration of hydrolysis gives same or equivalent result as that of higher concentration of enzyme and short duration of hydrolysis. So if the cost of enzyme is expensive, it is possible to use low concentration of enzyme and increase the duration of the hydrolysis. If the cost of enzyme is cheap it is possible to increase the concentration of enzyme and reduce the duration of hydrolysis.

The concentration of enzyme and duration of hydrolysis influences the characteristics of collagen hydrolysates prepared, which in turn affect the properties the leathers. Comparison of the three collagen hydrolysates (for the 3 hours of hydrolysis) using FPLC is presented in Figure



Calibrated Retention time (min)	CH 0.8			CH 1.0			CH 1.2		
	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 6	47.75	62.28		48.25	62.67		44.77	58.15	
7 to 12	13.76	17.94	80	12.32	16	78.67	13.99	18.17	76.31
13 to 23	3.75	4.89		4.11	5.33		3.01	3.91	
23 to 30	11.41	14.88	20	12.32	16	21.33	15.23	19.78	23.69
	76.67	100.00		77	100		77	100	

Though the pattern of the chromatogram for the three collagen hydrolysates seems to be similar, the relative proportions of each fractions are different between the hydrolysates. The high molecular weight fractions are those fractions which are eluting first (t_R: 49 to 61 for CH-1 (0.8), t_R: 228 to 240 for CH-2 (1.2), and t_R: 425 to 437 for CH-3 (1.0); and the low molecular weight frations are those fractions which are eluting last (t_R: 62 to 79 for CH-1 (0.8), t_R: 241 to 259 for CH-2 (1.2), t_R: 438 to 455 for CH-3 (1.0)).

The relative relative proportion of high to low molecular weight fractions is 80 to 20 for CH-1 (0.8), 78 to 22 for CH-2 (1.0) and 76 to 24 for CH-3 (1.2) hydrolysates. When the concentration of enzyme increases the percentage of higher molecuar weight fractions decreases and the lower molecuar weight fraction increases. So when the proportion of high to low molecular weight collagen hydrolysate fractions is 80 to 20, it is observed to give better result as a syntan for treatment of wet blue leathers with collagen hydrolysate. Further more, based on the results of MALDI in Figure 4.15, the molecular weight range of the CH-1 0.8 is observed between 2000 to 6000 daltons.

4.2 Effect of collagen hydrolysates on the stability of collagen fibers

To determine the applicability of the collagen hydrolysates for tanning, RTT were treated with CH according to procedure mentioned in section 3.3.2.4. The stability of tendons was assessed in terms of their resistance to degradation by enzymatic degradation. Further thermal stability of CH treated tendons was examined by measuring their shrinkage temperature using micro shrinkage and differential scanning calorimetric techniques.

4.2.1 Enzymatic stability

The enzymatic stability of tendons treated with collagen hydrolysates and their respective controls at different PH are presented in figure 4.9.

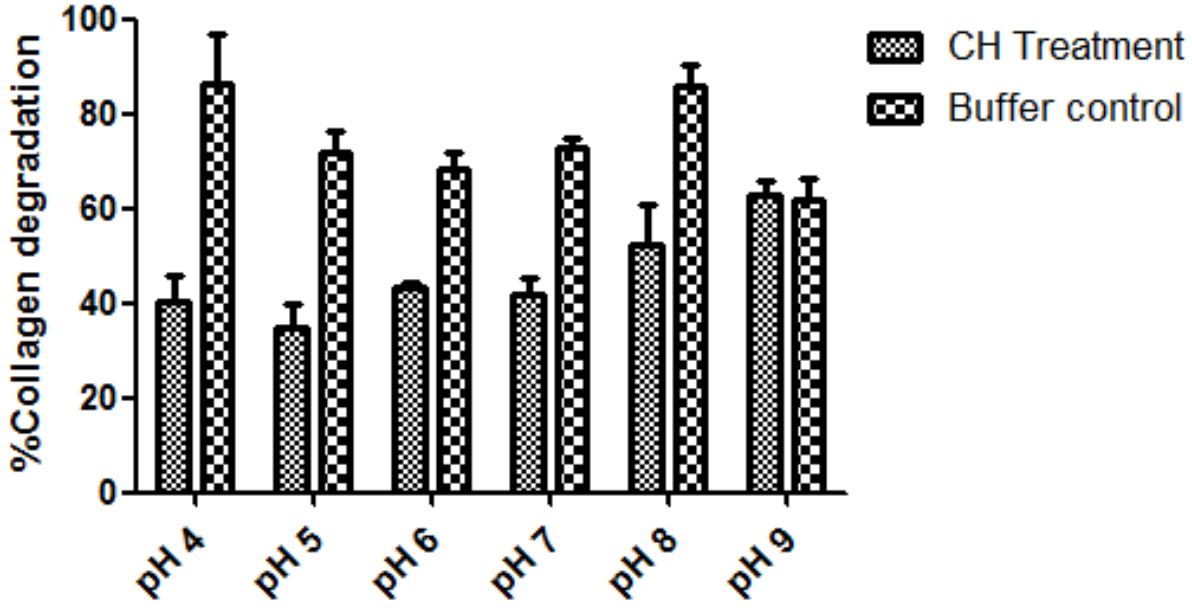


Figure 4.9:- Enzymatic stability of CH treated and Untreated RTT at different PH

The graph indicates that those tendons treated with collagen hydrolysates have better enzymatic stability than the controls. It can be also seen that at PH 4 and 5, the difference between the experimental and the control is significant indicating that there is better interaction between collagen hydrolysate and tendon. Whereas at PH 9 the degradation is almost equal for both the experimental and control. The higher enzymatic stability of the CH treated tendon as compared to native tendon may be due to three reasons. The first is collagen hydrolysates contain

different functional groups like carboxyl, hydroxyl and amine groups which are capable of forming hydrogen bonding with that of collagen in the tendon. This will stabilize the collagen matrix due to the extra hydrogen bond networking. The second is the collagen hydrolysates are charged fragments having both positive and negative ends depending on pH environment. This charged species can form electrostatic interaction with the collagen molecules also containing both positive and negative charges. This electrostatic interaction though it is weak can play its own role in stabilizing the collagen in the tendon. The third reason is collagen hydrolysates can act as inhibitor and can bind with the enzyme and inhibits its function. Competitive inhibition is likely to occur here, because both collagen hydrolysate (inhibitor) and the substrate (tendon) have structural resemblance. So collagen hydrolysate binds with the active site of enzyme and stops further catalysis.

4.2.2 Thermal stability

4.2.2.1 Microshrinkage analysis

The thermal stability as determined by micro shrinkage tester is presented in figure 4.10. From the figure it can be seen that the tendons which are treated with collagen hydrolysates have a shrinkage temperature of about 54^{o}C, whereas those not treated have a shrinkage temperature of about 50^{o}C. This shows that tendons treated with collagen hydrolysate have better thermal stability than the control. The reason for higher shrinkage temperature can be due to hydrogen bond networking and electrostatic interaction as mentioned in section 4.2.1

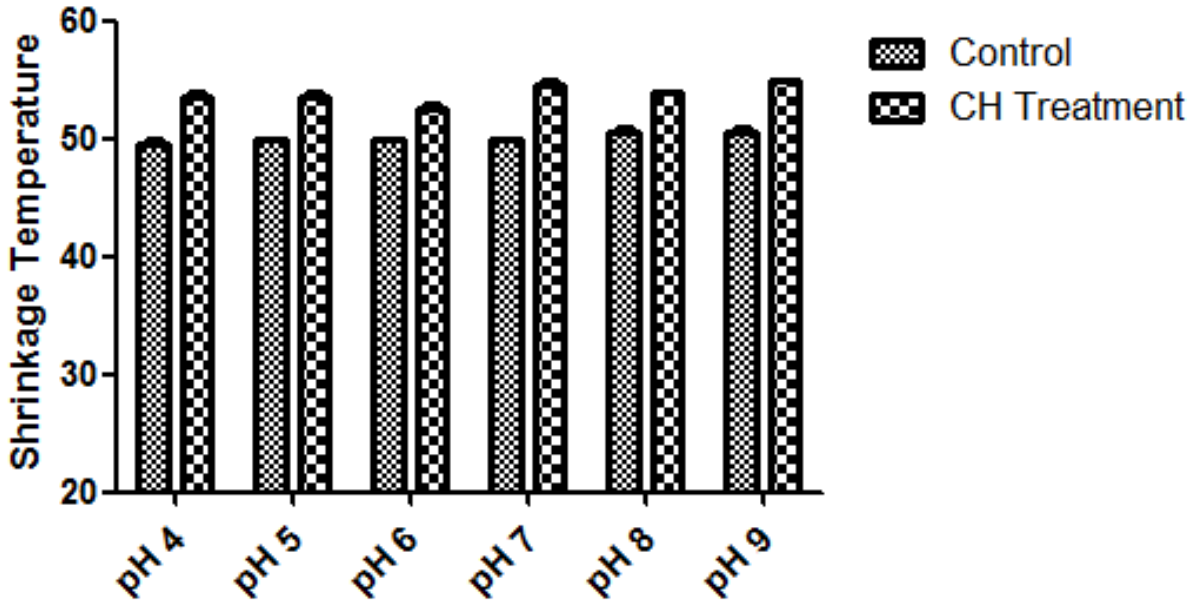


Figure 4.10:- Thermal stability of CH treated and Untreated RTT at different PH

4.2.2.2 Differential scanning calorimetry measurements

The tendons treated with collagen hydrolysate at PH 5 and its respective control are scanned with DSC and presented in figure 4.11.

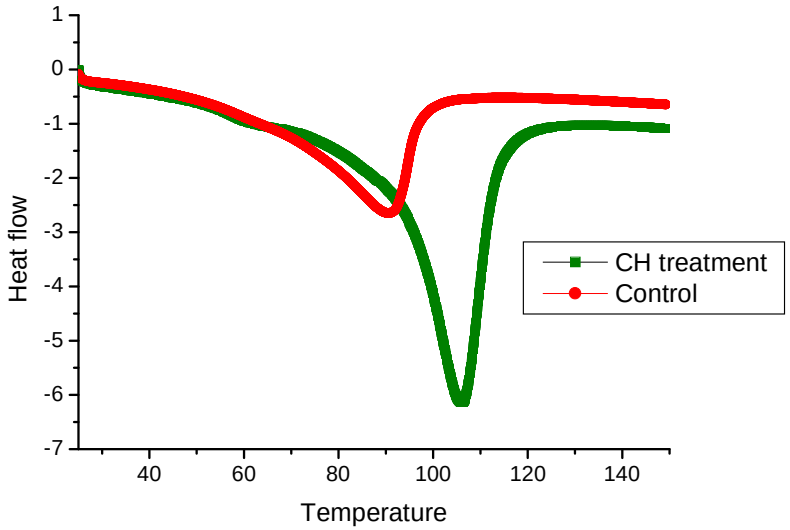


Figure 4.11:- DSC thermograph for CH treated and untreated RTT at PH 5

From the figure 4.3, the phase transition as can be seen by deep depression, occurred at around 90°C for the control and at around 108°C for the tendon treated with collagen hydrolysate. This curve like shrinkage shows that the tendons treated with collagen hydrolysate have better thermal stability than the control indicating there is interaction between the hydrolysates and tendons which leads to stabilizing of collagens in the tendon.

Thermal stability with DSC for different concentration

The thermal stability (by using DSC) of tendons which are treated at different concentration of collagen hydrolysate is presented in figure 4.12. It indicates that those tendons which are treated with collagen hydrolysate have higher thermal stability than the control which is not treated with collagen hydrolysate. The maximum thermal stability was found when the proportion of tendon to collagen hydrolysate is 1:1.5 based on its weight.

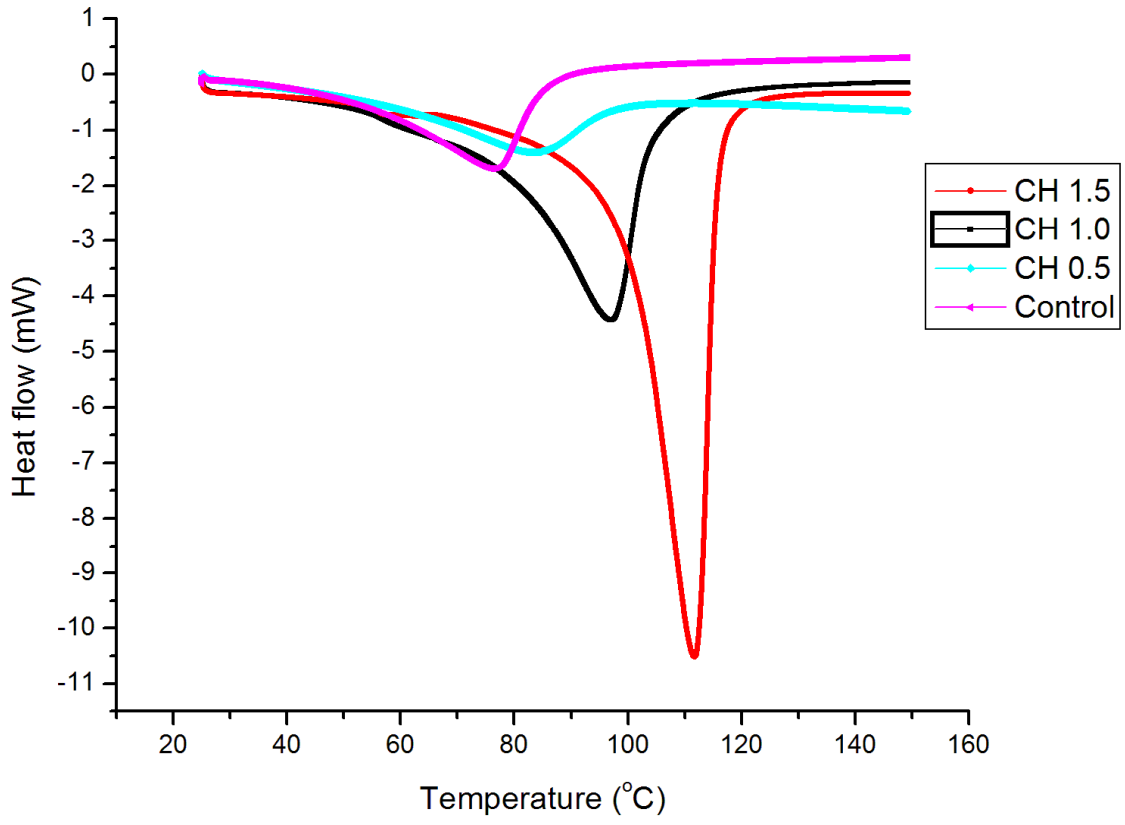


Figure 4.12:- DSC thermograph of treated RTT at different concentration of RTT and native RTT

4.2.3 Microscopic examination

4.2.3.1 Compound microscope

Microscopic examinations of collagen hydrolysate treated and untreated tendons after exposing to collagenase are shown in figure 4.13. Also as a control a native tendon which is not subjected to collagenase are shown in the figure. They were examined at 40X, 100X and 200X magnification level.

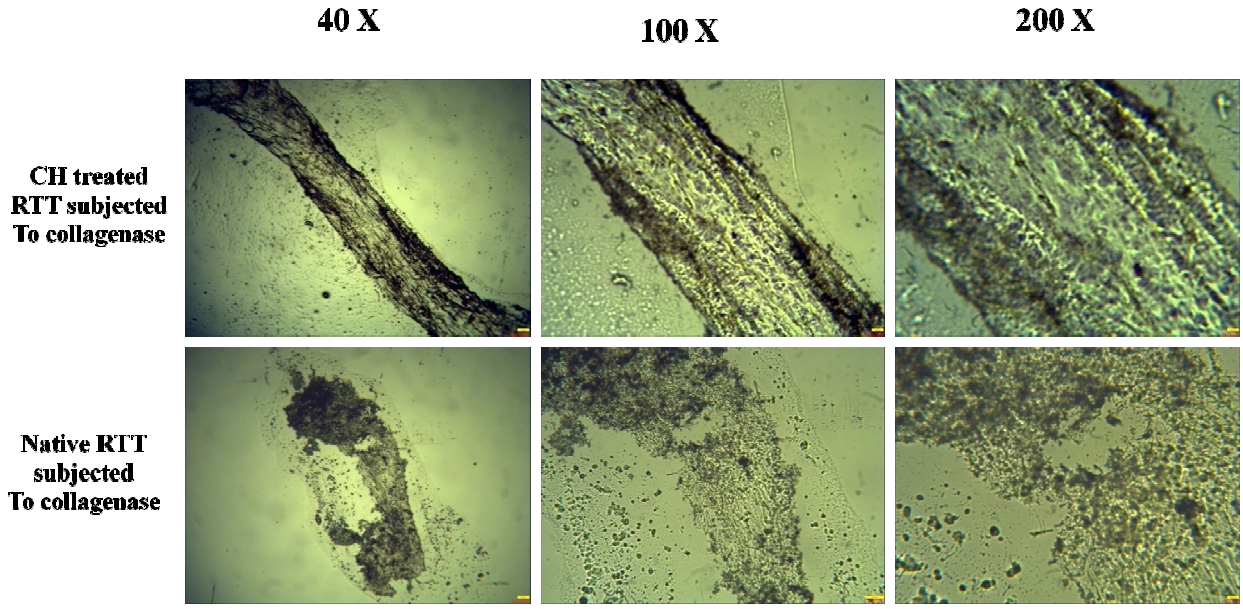


Figure 4.13:- Micrograph of CH treated and untreated RTT which are subjected to collagenase and native RTT (no collagenase treatment)

As it can be from the figure, the tendons which are not treated with collagen hydrolysate (second row) are highly degraded. Whereas tendons which are treated with collagen hydrolysate (first row), are resistant to collagenase and show less degradation. The treated tendons did not lose their intact structure.

4.2.3.2 Scanning Electron Microscope (SEM)

Microscopic examinations of collagen hydrolysate treated and untreated tendons after exposing to collagenase with SEM are shown in figure 4.14. Also as a control a native tendon which is not subjected to collagenase are shown in the figure 4.14.

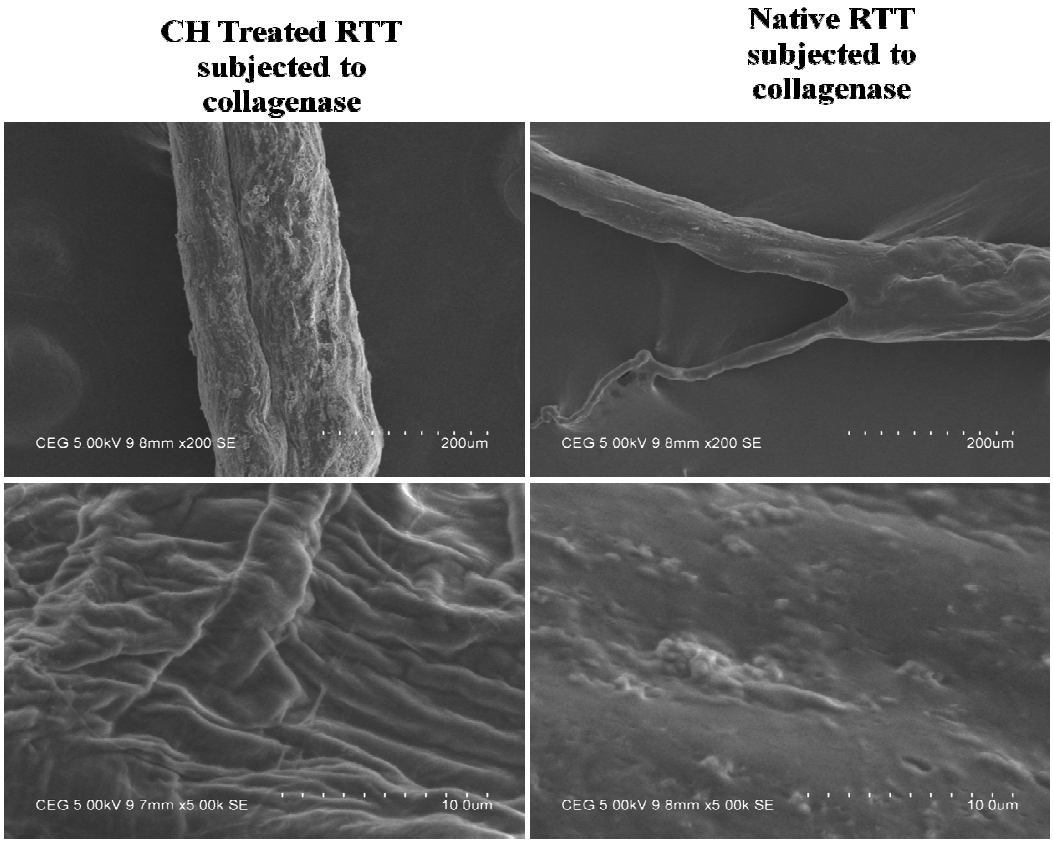


Figure 4.14:- Micrograph of CH treated and untreated RTT which are subjected to collagenase and native RTT (no collagenase treatment)

Here also, it can be clearly seen that the untreated tendons (second column) are highly degraded when compared to treated tendons (first column). The degradation is observed from the point of fiber splitting and reduction of the thickness of tendons. The treated tendons though they are intact like the native, there are surface degradation. Another important observation is with high magnification (second row), the treated tendons show different surface structure which are not observed in the native and untreated tendons. This may be due to the deposition of collagen hydrolysates on the surface of the tendon.

5. Conclusions and recommendations

5.1 Conclusions

From limed pelt trimmings, through enzymatic route it is possible to produce collagen hydrolysate that can be applied in tanning and post tanning operations of leather processing.

Results of enzymatic stability, shrinkage temperature and microscopic examination indicate that collagen hydrolysate plays a role in stabilizing collagen fibers.

In post tanning operations, results of the uptake of dye indicate that collagen hydrolysates significantly improve the exhaustion of the dyes. About 99% of dye uptake can be achieved by treating the leathers with collagen hydrolysate. The data of tensile and tear strength indicates that collagen hydrolysate will increase 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.

The concentration of enzyme and duration of hydrolysis affects the collagen hydrolysate prepared. This in turn affects the properties of the leathers. Results indicate that when the proportion of high molecular weight to low molecular weight is 80% to 20% in the collagen hydrolysate with better properties of the leathers are achieved. Moreover the molecular weight fractions of CH resulted in better leather characteristics were observed in the range of 2000 to 6000 daltons. In the collagen hydrolysate about 10 fractions collagen hydrolysate peptides were found.

The above mentioned proportion mentioned can be achieved by using 0.8% trypsin and for 3 hours hydrolysis or 1% trypsin and 2 hours hydrolysis. Compromise between the two factors is dependent on the cost of enzyme and value of time. As the concentration of enzyme increases, the proportion of high molecular weight decreases and the lower molecular weight increases.

Thus, limed pelt trimmings are important resource for producing collagen hydrolysates which can be applied for tanning and post tanning operations. So they have both economical and environmental benefits.

5.2 Recommendations

In Ethiopia, at present limed pelt trimmings are used to prepare glue, which has a low value. Better value addition of limed pelt trimmings can be achieved by converting them into collagen hydrolysate syntan that can be applied in tanning and post tanning operations.

Further study in collagen hydrolysates can be done by separating the fractions into separate peptides and treating the tendons with each of fractions and to observe the enzyme and thermal stability of tendons. And also, collagen hydrolysates can be modified by converting the carboxylic group of the peptides into aldehydes. Aldehydes have better reactivity with collagen fibers. The present study has used enzymatic route to prepare collagen hydrolysates from limed pelt trimmings. Further works can be done using alkaline hydrolysis and optimizing the conditions for better post tanning operation.

References

1. FAO World Statistical Compendium For Raw Hides and Skins, Leather And Leather Footwear, 2011
2. Central Statistic Agency (CSA), Agricultural Sample Survey, Addis Ababa, February 2011
3. http://ethiopianleather.org/sector.asp
4. J Kanagaraj, K C Velappan, N K Chandra Babu and S Sadulla, Solid wastes generation in the leather industry and its utilization for cleaner environment- A review, Journal of Scientific and Industrial Research, Vol 65, July 2006, pp 541-548
5. Ramshaw, J.A.M., Werkmeister, J.A. and Gattaner, V. (1995), Collagen-based biomaterials, Biotechnology Genetic Eng Rev., 13, 335-382
6. Arnab Bhattacharjee and Manju Bansal, Collagen Structure: The Madras Triple Helix and the Current Scenario, Life, 57(3): 161 – 172, March 2005
7. Ramachandran, G. N. (1967), Structure of Collagen at the Molecular Level. In ‘‘Treatise on Collagen,’’ (G. N. Ramachandran, Ed.), pp. 103–183. Academic Press, New York.
8. Karl E. Kadler, David F. Holmes, John A. Trotter. and John A. Chapman, Collagen fibril formation- review article, Biochem. J. (1996) 316, 1±11
9. Barbara Brodsky and Anton V. Persikov, Molecular structure of the collagen triple helix
10. Fraser, R.D.B., MacRae, T.P., and Suzuki, E. (1979), Chain conformation in the collagen molecule. J. Mol. Biol. 129, 463–481.
11. Okuyama, K., Arnott, S., Takayanagi, M., and Kakudo, M. (1981). Crystal and molecular structure of a collagen-like polypeptide (Pro-Pro-Gly)10. J. Mol. Biol. 152, 427–443.
12. Eric Baer, James J. Cassidy, and Anne Hiltner, Hierarchical Structure of Collagen Composite Systems, http://pubs.acs.org, May 8, 1992
13. C. Ramakrishnan, Ramachandran and his Map, Resonance October 2001, pp 48-56
14. Ramshaw JAM, Shah NK, Brodsky B. Gly-X-Y tripeptide frequencies in collagen: a context for host guest triple-helical peptides. J. Struct. Biol 1998;122:86–91. [PubMed: 9724608]
15. David R. Eyre, Jiann-Jiu Wu, Collagen Cross-Links, Top Curr Chem (2005) 247: 207–229

16. Michel Van Der Rest and Robert Garrone, Collagen family of proteins, The FASEB Journal, Vol. 5 October 1991, pp 2814-2823
17. Ivano Bertini, Marco Fragai, Claudio Luchinat, Maxime Melikian, Mirco Toccafondi, Janelle L. Lauer, and Gregg B. Fields, Structural Basis for Matrix Metalloproteinase 1-Catalyzed Collagenolysis
18. Gross J., Harper E., Harris E., McCroskery P., Highberger J., Corbett C. and Kang A. (1974), 'Animal collagenases: Specificity of action and structures of the substrate cleavage site', Biochem. Biophys. Res. Commun., Vol. 61, pp.605-612.
19. Oshima G., Shimabukuro H. and Nagasawa K. (1979), 'Mode of actionof bacterial collagenase on a synthetic substrate (Pro-Pro-Gly)5', Biochim. Biophys. Acta, Vol. 567, pp.492-400.
20. http://www.pbgelatins.com/binaries/Hydrolysates%20UK_tcm11-12473.pdf
21. Chi Yuanlong1, Cui min, Cui xiaoju, Zhangwenhua, LiaO Xuepin and Shi Bi, Enzymatic Hydrolysis of Skin Shavings for Preparation Of Collagen Hydrolysates with Specified Molecular Weight Distribution, JSLTC, vol 96, pp16-20
22. Vera Kasparkova, Karel Kolomaznik, Lenka Burketova, Vladimir Sasek, Lubomir Simek, Characterization of Low-Molecular Weight Collagen Hydrolysates Prepared by Combination of Enzymatic and Acid Hydrolysis, JALCA, Vol. 104, No. 2, pp 46-51, 2009
23. Zhongkai zhang, Guoying Li* and Bi Shi, Physicochemical Properties Of Collagen, Gelatin And Collagen Hydrolysate Derived From Bovine Limed Split Wastes, JSLTC, Vol. 90 p.23-28
24. Wuyong Chen, Peter H. Cooke, Gary DiaMaio, Maryann M. Taylor and Elanor M. Brown, Modified Collagen Hydrolysate, Potential for use as filler for leather, JALCA, Vol. 96, No:7, pp 262-267, 2001
25. M. M. Taylor, L. Bumanlag, W.N. Marmer and M. Brown, Use of Enzymatically Modified Gelatin and Casein As Fillers in Leather Processing, JALCA, Vol. 101, No. 5, Page 169-178, 2006
26. F. Langmaier, M. Mladek, K. Kolomaznik and S. Sukop, Collagenous hydrolysates from untraditional sources of proteins, International Journal of Cosmetic Science, 2001, 23, 193-199

27. C. Cantera, J. Martegani, G. Esterelles And J. Vergara, Collagen Hydrolysate: 'Soluble Skin' Applied In Post-Tanning Processes Part 2: Interaction With Acrylic Retanning Agents, JSLTC, Vol 86, p195-202
28. I. Bragança1, A. Crispim, A. Sampaio, E. Ramalho, F. Crispim, N. S. Caetano,and P. C. Silva, Adding Value to Tannery Fleshings: Part I – Oils and Protein Hydrolysates – Production and Application, JSLTC, Volume 97 Page 62-67
29. A. Aslan G. Gülümser and B. Ocak, Increased Chromium Tanning Efficiency with Collagen Hydrolysates, JSLTC, Vol. 90 p.201-204
30. F. Langmaier, K. Kolomazník And M. Mládek, Modifying Products Of Enzymatic Breakdown Of Chrome-Tanned Leather Wastes With Glutaraldehyde, JSLTC, Vol. 87 p.55.-61
31. Ding Zhiwen, Jia Guangyu, Ma Zhaoguo, Pang Xiaoyan, System of Reusing Technologies for Different Leather Wastes, China Leather, 2009/19
32. Han Jiao-jiao, Zhu Jian, Dan Wei-hua, Zeng Rui, Mi Zeng-jian, The Methods of Dechroming of Contained Chrome Leather Waste, Leather Science and Engineering, 2010/01
33. Tony Covington, Tanning Chemistry the science of leather, Published by The Royal Society of Chemistry, 2009
34. Miao Yin, Yang Xiao-feng, LI Man-ni, Study on Retanning Property of the Modified Protein Tanning Agent L-4, Acta Scientiarum Naturalium Universitatis NeiMongol, 2006
35. Xu Yanlin, Sun Jing, Pang Xiaoyan, Ding Zhiwen, Mechanism of Polyurethane Modified Collagen and its Application (III): retanning and filling agent of polyurethane modified collagen protein, China leather, 2010
36. Ding Zhi-wen; Yu Shu-xian; Zhang Wei; Pang Xiao-yan, Synthesis and Applying of Collagen Protein Retanning Agent Modified by Amine Resin, China Leather, 2007/09
37. A Guide to understanding color communication, X-Rite, Incorporated 2007
38. http://en.wikibooks.org/wiki/Structural_Biochemistry/Enzyme/Reversible_Inhibitors
39. J. F. Woessner, The determination of hydroxyproline in tissue and protein samples containing small proportions of this immino acid, Archives of Biochemistry and Biophysics 93, pp 440-447, 1961
40. SLC 6 (IUC 7; BS 1309:6), Determination of sulphated total ash

41. SLC 4 (IUC 4; BS 1309:4), Determination of Substances (Fats and Other Solubles) Soluble in Dichloromethane
42. Borasky, R., and Nutting, G. C. (1949). J. Amer. Leather Chemists Ass., 44, 830.
43. Willard H., Merritt L, Dean J., Settle F., Instrumental Methods of Analysis, 7th edition, pp 762-765
44. ISO 2418:2005 and ISO2419:2005 tensile strength and elongation at break
45. SLC 3 (IUC 5; BS 1309: 3), Determination of volatile matter
46. Alain Denis, Noe lle Brambati, Bernadette Dessauvages, Sylviane Guedj, Claude Ridoux, Nicole Meffre, Claude Autier, Molecular weight determination of hydrolyzed collagens, Food hydrocolloids, pp 989-994, 22 (2008)
47. Keith Wilson and John Walker, Principles and techniques of biochemistry and molecular biology, 6th edition pp 513-514
48. John M. Walker, The Dansyl Method for Identifying N-Terminal Amino Acids, Methods in Molecular Biology, Vol. 32. pp 321-328
49. Richard B. Cole, Electrospray and MALDI mass spectrometry, 2nd edition, pp 149-183

Annex

Determination of Hydroxy proline

Reagents

Hydroxy proline standard

A stock solution is prepared by dissolving 25 mg of vacuum-dried L-hydroxy proline in 250 ml of 0.001 N HCl standards are prepared daily by diluting the stock with water to obtain concentrations of 1-5 μg/2 ml.

Buffer

Fifty grams of citric acid monohydrate, 12 ml of glacial acetic acid, 120 g sodium acetate trihydrate, and 34 g sodium hydroxide are made to a final volume of 1 liter in distilled water. The PH is carefully adjusted to 6.0 and the buffer is stored in refrigerator under toluene.

Chloramine T (Sodium P-Toluene sulfon chloramide)

A 0.05 M solution is prepared fresh daily by dissolving 1.41 g Chloramine T in 20 ml Water. 30 ml of methyl cellosolve and 50 ml buffer are added. The solution is kept in a glass-stoppered flask.

Methyl Cellosolve (Ethylene Glycol Mono-methyl Ether)

Preparations free of interfering substances.

P-Dimethyl amino benzaldehyde

A 20% solution is prepared shortly before use by adding methyl cellosolve to 20 g of Pp-dimethyl amino benzaldehyde to give a final volume of 100 ml. This may be warmed in 60^{o}C bath to facilitate solubilization. If the solutions are deep blue or purple, recrystallization of this reagent by the method of Adams and Coleman may be necessary.

Perchloric Acid

A 3.15 M solution is prepared by diluting 27 ml of 70% Perchloric acid to 100 ml with water.

Procedure

Sample preparation

Samples (proteins, tissues, fluids etc) are hydrolyzed by adding HCl to a final concentration of 6 N. The samples are sealed in small Pyrex test tubes and hydrolyzed for 3 hour at 130^{o}C. The tubes are then opened. The contents are decanted into a graduate cylinder or volumetric flask. The tubes are washed thoroughly with water and the washings are combined with the hdyrolyzate. Several drops of 0.02% methyl red indicator are added, followed by 2.5N NaOH for neutralization. Final adjustments are made with dilute HCL and NaOH until the indicator turns slightly yellow corresponding to PH 6-7.

Reaction procedure

Samples are prepared as above. 2 ml portions containing 1-5 µg hydroxy prolines are placed in 16x150 mm test tubes. A series of standards is prepared containing 0-5 µg hydroxy proline in 2 ml. Hydroxy proline oxidation is initiated by adding 1 ml Chloramine T to each tube. The tube contents are mixed by shaking a few times and allowed to stand 20 min at room temperature. Chloramine T is destroyed by adding 1 ml Perchloric acid to each tube in the same order as before. The contents are mixed and allowed to stand for 5 minutes. Finally, 1 ml p-dimethyl amino benzaldehyde solution added. The mixture is shaken until on schliren can be seen. The tubes are placed in a 60^{o}C water bath for 20 minutes. Then cooled in tap water for 5 minutes (The developed color is stable for at least 1 hour). The absorbance of the solutions is determined spectrophotometrically at 557 nm.

Notes

- Absorbency is linearly related to the amount of hydroxy proline over the range 0-5 µg.
- In the region 5-10 µg there is a slight negative deviation of about 2% hence it is recommended that samples be diluted to contain 1-5 µg Hyp per 2 ml.
- The millimolar extinction coefficient of the chromogen is found to be 56.3 at 557 nm.
- Samples (purified calfskin gelatin) had an average Hyp content of 14.34±0.16%.

Annex

Determination of Sulphated Total Ash (SLC 6)

1. Scope

   This method is applicable to all types of leather. The method may be inaccurate by the extent to which the leather contains silicone or Organo-metallic compounds.

   The amount of mineral substances found by ashing can differ from the actual content owing to decomposition, reduction, or the escape by volatilization of certain salts. By treating the ash with sulphuric acid the salts and oxides are converted into sulphates, but some salts will again be transformed into oxides at the selected temperature of ignition. To determine the total mineral content, e.g., within the framework of a complete leather analysis, the water-soluble and water-insoluble inorganic substances can be ascertained by calculation or determined separately.

   Ammonium salts are not determined by this method (compare with SLC 5), but can be determined as described in SLC 8.

2. Definition

   For purpose of this method the following definitions apply.

   Sulphated total ash: - Residue obtained from burning leather in an open crucible after sulphating, as described in this method.

   Sulphated water insoluble ash: - Residue obtained when leather, previously extracted with water as described in SLC 5, is burnt in an open crucible after sulphating, as described in this method.

3. Reagents

   The following reagents are required.

   Sulphuric acid reagent solution, approximately 2N

4. Apparatus

   Usual laboratory apparatus is required and, in particular, the following

   (a) *Crucibles and dishes,* of glazed porcelain, platinum, or quartz.

   (b) *Muffle furnace,* capable of being maintained at temperature close to, but not exceeding, 750^{o}C.

5. Procedure

   Sample and grind in accordance with SLC 1 and 2. Weigh 2.5 g of the sample to the nearest 0.001 g, and carefully carbonize it over a low flame in a crucible which has been previously heated to 750°C, cooled, and weighed, so that the leather burns with a small flame. Carbonize fatliquored leather particularly carefully so that the grease burns very slowly. Then thoroughly moisten with the sulphuric acid solution and heat over a low flame until sulphur trioxide fumes are no longer visible. Heat more vigorously, and then ignite in the furnace at 750°C until completely ashed. Cool in the desiccators and weigh. Repeat the addition of acid, heating, cooling and weighing until the mass of the residue is constant.

6. Notes on the procedure

   a. It is advisable to extract silicone-impregnated leather with dichloromethane before determining the sulphated total ash.

   b. For the determination of the sulphated total ash, the dry leather obtained from the determination of volatile matter, in accordance with SLC 3, can be used in all cases.

   c. If a carbon-free residue cannot be obtained in spite of heating at 750°C, it should be moistened with a little ammonium nitrate solution and the heating repeated until the ash is free from carbon.

   d. At temperature above 750°C some loss of mass from the residue is possible due to volatilization of certain inorganic salts. For this reason, close control is essential to prevent the maximum furnace temperature from exceeding 750°C.

7. Expression of results

   Calculate the following percentages.

$$Sulphated\ total\ ash, percentage\ by\ mass = \frac{100M_1}{M_o}$$

   Where M_1 is the mass of sulphated total ash

   And M_o is the mass of the original sample of leather

Annex

Determination of Substances (Fats and Other Solubles) Soluble in Dichloromethane

SLC 4 (IUC 4; BS 1309:4)

1. Scope

   This method is applicable to all types of leather.

   Not all fatty and similar substances can be extracted from leather with organic solvents; they may be in part soluble and partly bound to the leather. On the other hand, the solvent can dissolve non-fatty substances, e.g. sulphur and impregnants, both of which cause difficulty in the determination of the acid value and saponification value of the fat.

   As the extraction is frequently done in conjunction with determination of free fatty acid content of the leather, a suitable procedure for determination of the free fatt acids extracted by this method si included.

   NOTE: - The apparatus and technique described in this method are also suitable for the extraction of leather by solvents other than dichloromethane. If, for any purpose, other solvents are used, the solvent or solvents used should be stated in the test report.

2. Definition

   For the purposes of this method the following definition applies.

   Extractable substances: - Fats and other soluble matter which can be extracted from leather with dichloromethane.

3. Principle

   The prepared leather is extracted continuously with dichloromethane. Solvent is evaporated from the extract which is then dried at 102 ^{o}C.

4. Reagents

   The following reagent is required

   Dichloromethane, boiling point 38^{o}C to 40^{o}C, freshly distilled and kept in a dark flask over calcium oxide.

   NOTE 1:- Dichloromethane that has stood for a long time should be tested for the presence of any hydrochloric acid which may have formed, as follows.

Shake 10 ml of dichloromethane with 1 ml of 0.1N silver nitrate solution. If the silver nitrate solution becomes turbid the dichloromethane should be redistilled and kept in a dark flask over calcium oxide.

NOTE 2:- Dichloromethane which has been used for this method can be recovered and re-used after distillation.

NOTE 3:- Dichloromethane has toxic properties and should be used with caution.

5. Apparatus

Usual laboratory apparatus is required and, in particular, the following

(a) *Soxhlet apparatus*

(b) *Filter paper thimbles* of suitable size and manufacture, or suitable glass filter bells. Schleicher and Schull Thimbles No 603 or Whatman Extraction Thimbles 33 mm x 80 mm are known to be satisfactory.

(c) *Oven,* capable of being maintained at 102±2 ^{o}C, complying with the requirements of BS 2648.

6. Procedure

Sample and grind in accordance with SLC 1 and 2. Weigh 10 ± 0.1 g of the prepared sample and press evenly into the filter paper thimble, or into the filter paper thimble, or into the glass bell. Cover the leather with a thin layer of cotton wool. Dry the extraction flask with two glass beads in it by heating for half an hour at 102±2 ^{o}C. Weigh after cooling in a desiccator.

Begin the continuous extraction with dichloromethane, then, after at least 30 changes of solvent, distil the dichloromethane from the flask containing the extract. Dry the extract in the oven for four hours at 102±2 ^{o}C (if drops of water are visible before drying, add 1-2 ml of ethanol). Weigh after cooling for 30 min in the desiccator. Repeat the drying, cooling and weighing, at least twice more, but with drying periods of 1 hour until either the further loss in mass does not exceed 10 mg, or the total drying time equals 8 hours.

7. Notes on the procedure

a. Dichloromethane can also dissolve non-fatty materials from the leather, e.g. sulphur (the presence of sulphur is recognizable by yellow precipitate in the flask). As sulphur causes difficulty it can be removed in the following way. Dissolve the extract in the smallest possible quantity of diethyl ether and filter

through a little cotton wool into a previously weighed flask. After thoroughly washing out the cotton wool filter with ether, remove the ether from the extract in the flask by distillation over a bath of hot water from which any flame has previously been removed. If sulphur is again precipitated, repeat the procedure. After the diethyl ether has been distilled off, dry the flask and residue and weigh.

b. The extract can be used for analysis, e.g. to determine acid and saponification values of the fats, or to determine the free fatty acid content of the leather.

c. After removal of the solvent the extracted leather may be required for determination of water soluble matter in accordance with SLC 5.

8. Expression of results

Calculate the following percentage

$$Extractable\ substances, percentage\ by\ mass = \frac{100M_1}{M_o}$$

Where M_1 is the mass of the extract

And M_o is the mass of sample used

Declaration

I, the undersigned, declare that this thesis is my original work and has not been presented for a degree in any University, and that all the source of materials used for the thesis has been duly acknowledged.

Declared by:

Name: - _____

Signature: - _____

Date: - _____

Advisor

Name: - _____

Signature: - _____

Date: - _____