NOVEL ENZYMATIC APPLICATIONS TO THE LEATHER INDUSTRY WITH SPECIFIC REFERENCE TO MICROBIAL TRANSGLUTAMINASE

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To my father (26-10-1943* 15-08-2005†)
who made
most of my dreams to come true

ABSTRACT

This project has investigated the potential of using the protein cross-linking enzyme, microbial transglutaminase (mTG), as a tanning agent, using native bovine hide as substrate. Treatment of bovine hide with mTG led to the covalent cross-linking of collagen molecules with approximately 4 nmol of cross-link per mg of collagen. However, mTG treatment did not affect the denaturation temperature of native bovine hide when used alone or together with other proteins or bifunctional diamines as cross-linking facilitators. In addition, mTG crosslinking of either chrome or glutaraldehyde tanned bovine hide led to a decrease in tensile strength. However, the resistance of cross-linked bated bovine hide towards either acid hydrolysis or proteolytic digestion with collagenase was significantly increased. Despite these beneficial changes, the use of mTG as an alternative tanning agent seems unlikely given that the most important aspects of a tanned hide are increased hydrothermal stability coupled with good tensile strength properties.

As a result, the focus of the research was amended to evaluate the potential of applying mTG during the dyeing operations, by cross-linking of dye-binding carrier peptides into tanned hides to facilitate an increase in potential dye-binding sites. Initial results at laboratory scale using both freeze dried bated bovine hide and chrome tanned crust leather with keratin hydrolysate as the carrier protein and mTG as the cross-linker indicated an increase of depth of shade of –1.5 DL units (grain side) when using acid dyes, the colour fastness was also improved. A full-scale industrial trial of this novel process was undertaken using a commercial process in LINK-project partner tanneries with wool on sheepskin. The outcome of the industrial trials indicated that treatment with mTG and keratin hydrolysate at full scale production resulted in leathers with a significantly deeper shade (approximately -1 DL unit) and the leathers were also faster to treatment with artificial perspiration solution. The treatment also improved the wool resistance to abrasion.

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Publications

Papers

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Patents

- **Addy V.L., Clara S., Collighan R. and Griffin M.** Enzymatic Treatment of Hides and Leather. PCT/GB2004/003076

List of Abbreviations

$(NH4)_2SO_4$	Ammonium sulphate
ANOVA	Analysis of variance
BSA	Bovine serum albumin
BTC	Biotin cadaverine
BUT	Butanol
$Ca(OH)_2$	Calcium hydroxide
Ca^{2+}	Calcium cation
$CaCl_2$	Calcium chloride
CH_3COOH	Acetic acid
cm	Centimetres
Cr^{3+}	Chromium (III)
DCC	Dicyclohexylcarbodiimide
DL	Differences in lightness of the leather
DMAP	Dimethyl amine pyridine
DMF	Dimethyl-formamide
DMSO	Dimethylsulphoxide
DSC	Differential scanning calorimetry
EDTA	Ethylene di-amine tetraacetic acid
GC	Gas chromatography
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
Hyp	Hydroxyproline
K^+	Potassium cation
KCl	Potassium chloride
KDa	Kilodaltons
KH_2PO_4	Potassium di-hydrogen phosphate
KOH	Potassium hydroxide
KPa	Kilopascals
M	Molar
ml	Millilitres
mM	Milimolar
mS	Milisiemens
mTG	Microbial transglutaminase
Na_2HPO_4	Di-sodium hydrogen phosphate
Na_2S	Sodium sulphide
$NaCH_3COO$	Sodium acetate
NaOH	Sodium hydroxide
NH_3	Ammonia
NH_4^+	Ammonium
NH_4HCO_3	Ammonium hydrogen carbonate
NMR	Nuclear magnetic resonance
PBS	Phosphate buffered saline
PD	Purified dye
pH	Negative decimal logarithm of hydrogen ion concentration
S^{2-}	Sulphide
SDS	Sodium dodecyl sulphate
SE	Standard error
SP	Subproduct of the coupling reaction

t-BOC	Tert-butyl pyrocarbonate
TCA	Trichloroacetic acid
TG	Transglutaminase
TLC	Thin Layer Chromatography
TOC	Total Organic Carbon
Tris	Tris(hydroxymethyl)-aminoethane
Uv-vis	Ultraviolet visible

TABLE OF CONTENTS

1	Chapter 1. Introduction	1
1.1	**Leather technology**	1
1.2	**The concept of tanning**	3
1.3	**Leather dyeing**	8
1.4	**Current enzymatic applications in leather processing**	14
1.4.1	Enzymes in soaking	14
1.4.2	Enzymes in liming	14
1.4.3	Enzymes in bating	15
1.4.4	Enzymes in degreasing	16
1.5	**Transglutaminases**	17
1.6	**The aims of the project**	27
2	Chapter 2. Materials and Methods	28
2.1	**Materials**	28
2.2	**Degree of deamidation during liming**	30
2.3	**Design of an alternative unhairing process**	32
2.4	**SDS-page**	34
2.5	**Microbial transglutaminase activity assay**	34
2.6	**Determination of mTG-available glutamine content**	34
2.7	**Determination of collagen/leather denaturation temperatures**	35
2.8	**ε-(γ-glutamyl) lysine crosslink analysis**	35
2.9	**Hydroxyproline quantification to determine the resistance towards collagenase attack and acid hydrolysis**	37
2.10	**Analysis of mTG penetration into bated hide**	37
2.10.1	Uptake of mTG by bated hide	38
2.10.2	Detection of biotin in hide sections	38
2.11	**Isometric tension measurements**	39
2.12	**Tensile strength testing of leather**	39
2.13	**Dye modification for incorporation into leather by mTG**	42

2.13.1	Amino-acid protection	**42**
2.13.2	Coupling reaction	**42**
2.13.3	Deprotection step	**43**
2.13.4	Dye incorporation into N-N' dimethylcasein by mTG	**43**
2.14	**Uptake of mTG by crust leather**	**45**
2.15	**Microbial transglutaminase mediated incorporation of keratin hydrolysate into freeze dried bovine hide and crust bovine leather**	**47**
2.16	**Dyeing**	**47**
2.17	**Colour measurements**	**49**
2.18	**Fastness to perspiration solution measurements**	**49**
2.19	**Wash fastness measurements**	**49**
2.20	**Wool resistance to abrasion**	**49**
2.21	**Statistical analysis**	**50**
3	**Chapter 3. Microbial Transglutaminase as tanning agent**	**51**
3.1	**Introduction**	**51**
3.2	**Results**	**53**
3.2.1	Investigation of glutamine and asparigine hydrolysis during liming	**53**
3.2.2	Design of an alternative unharing process	**59**
3.2.3	Effect of the chemicals applied in the early stages of leather processing and[^{14}C] putrescine incorporation into bated hide	**66**
3.2.4	Effect of crosslinking of bated bovine hide collagen by mTG on denaturation temperature	**69**
3.2.5	Effect of cross-linking of bated bovine hide collagen by mTG on resistance towards microbial collagenase	**70**
3.2.6	Effect of cross-linking of bated bovine hide collagen by mTG on resistance towards acid hydrolysis	**70**
3.2.7	mTG mediated crosslinking of bated hide on the physical properties of leather	**75**
3.3	**Discussion**	**84**

4	**Chapter 4. Dye modification for mTG mediated incorporation into leather**	95
4.1	**Introduction**	95
4.2	**Results**	97
4.2.1	Amino acid protection	97
4.2.2	Coupling reaction and deprotection step	97
4.2.3	Microbial transglutaminase mediated incorporation of the modified dye into N,N'-dimethyl casein as a model system	110
4.3	**Conclusion**	114
5	**Chapter 5. Microbial transglutaminase mediated incorporation of keratin hydrolysate and its effect on dyeing**	115
5.1	**Introduction**	115
5.2	**Microbial transglutaminase uptake by crust leather and potential for crosslinking**	116
5.3	**Microbial transglutaminase mediated incorporation of keratin hydrolysate into crust leather**	116
5.4	**Initial investigations of mTG mediated incorporation of keratin hydrolysate into leather and its effect on dyeing**	121
5.5	**Microbial transglutaminase mediated incorporation of keratin hydrolysate in crust leather and its effect on dyeing process on a larger scale**	132
5.6	**Microbial transglutaminase mediated incorporation of keratin hydrolysate in wool on sheepskin and its effect on dyeing**	136
5.7	**Discussion**	144
6	**Chapter 6. General discussion and future work**	148
	References	156

LIST OF TABLES

Table I	Typical shrinkage temperature values for varying tanning chemistries	5
Table II	Amount of dye in percentage held by varying hide powder substrates with dipole interactions, ionic interactions and other interactions	12
Table III	Reactivity of microbial transglutaminase (mTG) towards various food proteins	22
Table IV	Protocol of the Dispase/Urea unhairing process	32
Table V	Tanning protocol for the Dispase/Urea unhairing method	33
Table VI	Beamhouse protocol	40
Table VII	Glutaraldehyde tanning protocol	41
Table VIII	Tanning protocol for the production of crust leather	46
Table IX	Dyeing protocol for bovine crust leather	47
Table X	Dyeing protocol for crust wool on slink leathers	48
Table XI	pH effect on amide hydrolysis	57
Table XII	Results on Dispase/Urea Unhairing	61
Table XIII	Crosslinking effect on the denaturation temperature measured by DSC	72
Table XIV	Dye incorporation by mTG into N,N'-dimethylcasein	112
Table XV	Amino acid composition of wool keratin	145

LIST OF FIGURES

Figure 1	Glutaraldehyde mediated crosslinking via formation aldimine bond	7
Figure 2	Scheme of the TG-catalysed transfer reactions	20
Figure 3	Effect of pH and temperature of *Bacillus Circulans* TG	21
Figure 4	Number of TG related patents filed since 1975 to 2001	21
Figure 5	Chromatogram of 1mM ammonium chloride solution	55
Figure 6	Calibration curve of Ammonium Chloride solutions	55
Figure 7	Relationship between conductivity an [KCl]	56
Figure 8	Ammonium released by collagen I due to liming conditions	56
Figure 9	Ammonium released by rawhide due to liming conditions	57
Figure 10	Hydrolysis of glutamine due to alkaline conditions at pH 12.5 and conductivity 37.5 mS/cm adjusted with Kcl or $CaCl_2$	58
Figure 11	SDS page of Dispase	61
Figure 12	Appearance of the grain of the samples that underwent dispase/urea and pickling with swelling	63
Figure 13	Protein removal assessment during the urea treatment	64
Figure 14	Protein removal assessment during pickling with swelling	65
Figure 15	^{14}C Putrescine incorporation into bated hide by mTG	68
Figure 16	Microbial transglutaminase mediated crosslinking effect on resistance towards microbial collagenase	73
Figure 17	Microbial transglutaminase mediated crosslinking effect on resistance towards acid hydrolysis	74
Figure 18	Assessment of the effect of freeze-drying on the penetration of mTG into bated bovine hide by confocal microscopy	77
Figure 19	Hydrothermal stability of leather, treated with mTG, before and after subsequent chrome tanning	78
Figure 20	Results of crosslinking analysis for mTG treated bated samples	79
Figure 21	Hydrothermal stability of leather, treated with mTG, before and after subsequent glutaraldehyde tanning	80
Figure 22	Isometric Tension of mTG mediated crosslinked bated hide	81
Figure 23	Effect of mTG mediated crosslinking on tensile strength of chrome tanned leather	82
Figure 24	Effect of mTG mediated crosslinking on the tensile strength of	83

	glutaraldehyde tanned leather	
Figure 25	Reactions involved in glutaraldehyde mediated crosslinking of proteins	94
Figure 26	(A) Example of an acid dye. (B) Example of a reactive dye with the competing reaction between water and the amines	96
Figure 27	Chemical structures of the products involved in the protection step	101
Figure 28	^{13}C NMR spectra of the t-BOC 6 amino hexanoic acid in DMSO	102
Figure 29	Chemical structures of the products involved within the coupling reaction	103
Figure 30	^{13}C NMR of PD in methanol	104
Figure 31	^{13}C NMR of Acid Blue 92 in DMSO	105
Figure 32	^{13}C NMR of DMF in DMSO	106
Figure 33	^{13}C NMR of eluent mixture (Butanol/Methanol 70:30 in DMSO)	107
Figure 34	^{13}C NMR of SP in DMSO	108
Figure 35	Chemical structures of the activated t-BOC 6 amino hexanoic acid (*O*-acylisourea) and the *N*-acylurea	109
Figure 36	Dye effect on [^{14}C] putrescine incorporation by mTG into N,N'-dimethylcasein	112
Figure 37	Dye effect on fluorescein cadaverine incorporation by mTG into N,N'-dimethylcasein	113
Figure 38	Uptake of biotinylated mTG into bovine crust leather	118
Figure 39	Uptake of biotinylated mTG into dry bovine leather	119
Figure 40	Uptake of biotinylated keratin hydrolysate into crust leather	120
Figure 41	Dye release from freeze-dried bated leather samples treated with mTG and/or biopolymers (putrescine and/or keratin) by perspiration solution treatment vs differences in lightness of the leather grain surface	123
Figure 42	Dye release from freeze-dried bated leather samples treated with mTG and/or biopolymer (putrescine and/or keratin) by perspiration solution treatment vs differences in lightness of the leather flesh surface	124
Figure 43	Dye release from freeze-dried bated leather samples treated with mTG and/or biopolymer (putrescine and/or keratin hydrolysate) by perspiration solution treatment vs differences in lightness of the leather grain + flesh surface	125
Figure 44	Dye remaining in solution at the end of the dyeing process, bated leather	126

	samples treated with mTG and/or biopolymer (putrescine and/or keratin hydrolysate) vs differences in lightness of the leather grain + fresh surface	
Figure 45	Dye release from crust leather samples treated with mTG and/or biopolymer (putrescine and/or keratin) by perspiration solution treatment vs differences in lightness of the leather grain surface	127
Figure 46	Dye release from crust leather samples treated with mTG and/or biopolymer (putrescine and/or keratin) by perspiration solution treatment vs lightness of the leather flesh surface (n=4)	128
Figure 47	Dye release from crust leather samples treated with mTG and/or biopolymer (putrescine and/or keratin) by perspiration solution treatment versus differences in lightness of the leather grain + flesh surface	129
Figure 48	Dye remaining in solution at the end of the dyeing process crust leather samples treated with mTG and/or biopolymer (putrescine and/or keratin hydrolysate) vs lightness of the leather grain + flesh surface	130
Figure 49	Effect of the keratin offer on the increase in depth of shade of leather and fastness to perspiration solution	131
Figure 50	Loss of colour due to wash fastness treatment solution versus lightness of the leather grain side	133
Figure 51	Loss of colour due to wash fastness treatment solution versus lightness of the leather flesh side	134
Figure 52	Dye release from crust leather samples treated with mTG and biopolymer by perspiration solution treatment vs differences in lightness of the leather grain + flesh surface	135
Figure 53	Loss of colour due to wash fastness treatment solution versus differences in lightness of the leather (n=12 wool on slink skin crust leather)	138
Figure 54	Dye release from crust leather samples treated with mTG and biopolymer by perspiration solution treatment vs lightness of the leather (n=12 wool on slink skin crust leather)	139
Figure 55	Wool resistance to abrasion after 1000 rubbing cycles	140
Figure 56	Dye release from crust leather samples treated with mTG and biopolymer by perspiration solution treatment vs differences in lightness	141

of the leather. (n=12 wool on slink skin crust leather). Results from the industrial trials

Figure 57 Loss of colour due to wash fastness treatment solution vs differences in lightness of the leather (n=12 wool on slink skin crust leather). Results from the industrial trials 142

Figure 58 Wool resistance to abrasion after 1000 rubbing cycles (n=12). Results from the industrial trials 143

Chapter 1. Introduction

1.1. Leather technology

The British Standard definition of leather as given in BS 2780:1972 is hide or skin which still retains its original fibrous structure more or less intact, and which has been treated so as to be resistant to putrefaction even after exposure to water. The hair or wool may or may not have been removed (Sykes, 1981)

Tanning, defined as the chemical stabilisation of collagen derived from this raw hide (Heidemann, 1993 (a)), is the core operation in leather processing. Beside tanning, other chemical and physical processes are involved in leather production. This can mainly be divided into; beamhouse operations which aims at removing non structural proteins and hair providing a substrate suitable to be tanned, tanning and finishing. Beamhouse includes soaking, unhairing/liming, deliming, bating and pickling. Tanning which, as previously described, chemically stabilises collagen. Finally, finishing gives leather the properties required for its subsequent use. (Addy, 1998)
These processes are described in further detail below:

- **Soaking:** This step is required to clean and rehydrate the skin or hide after salting (the traditional method for preservation). Glycosaminoglycans such as hyaluronic acid and dermatan sulphate, and non collagenic proteins, e.g. globulins and albumins, are partially removed. Water and biocides are the chemicals employed during soaking. (Addy, 1998)

- **Unhairing/liming:** The aim of unhairing/liming process is removal of hair, epidermis hyaluronic acid, dermatan sulphate and non-collagenous proteins such as albumins and globulins; and a partial loosening of the collagenous structure. Calcium hydroxide and sodium sulphide are generally applied. The solution raises the pH to around 12.5 resulting in swelling of structure. Sodium sulphide is a reducing reagent that attacks the disulphide bonds of keratin facilitating its removal, the main component of hair. Calcium has lyotropic properties which results in

hydrogen bonding disruption within the collagen structure with the consequent opening up of the fibres. (Addy, 1998)

- **Bating:** At this stage, the hide is treated with enzymatic based products, e.g. trypsin and proteases from bacterial origin (Green, 1957; Christner 1992), to complete the removal of non structural proteins. The softness of the final product will partially rely on the degree of bating. (Addy, 1998)

- **Pickling:** The pH is lowered to 2.5-3 adding mixtures of formic/sulphuric acid with salt (to prevent swelling) to achieve the necessary conditions for chrome tanning operations. At this pH chrome salts easily penetrate into hide. (Addy, 1998)

- **Tannage:** Different kind of tannage chemistries; e.g. aldehydes, hydrolysable tannins, condensed tannins, aluminium salts or synthetic resins containing either hydroxyls or amine groups, may be applied as tanning chemicals. However, the most effective tannage is achieved with chrome (III) basic sulphate salts. The reaction between chrome salts and collagen is pH dependent. The fundamental chemistry involved in chrome tannage is the formation of a complex between Cr(III), glutamic acid and aspartic acid, hydroxyl groups and water. The reaction starts at pH 2.5. At this pH carboxylic acids are protonated and the Cr(III) complex can not be formed, allowing for full penetration of the chrome into the hide. Slow basification up to pH 3.8-4.2 leads to the formation of the above mentioned complex producing the desired tannage effect. Chrome tanned hides have high hydrothermal stability, with shrinkage temperatures above 100 °C. (Addy, 1998)

- **Finishing operations:** Leather undergoes further treatments, e.g. retannage, addition of oils and dyeing, to impart the softness and handle properties required for the end product. (Addy, 1998)

1.2 The concept of tanning

Tanning may be defined as the chemical stabilisation of the collagen derived from an animal source such as hide or skin. Due to this chemical stabilisation some of the physical properties of the collagen become changed (Heidemann, 1993(a)). Examples of these physical properties include increase of the resistance towards proteolytic attack (Ishii, 1992), increase of the resistance to deterioration by moist heat and perspiration (Bowes and Raistrick, 1965), increase of the resistance to hot water (Gerngross and Horasan, 1965), increase of the resistance to acid and alkali hydrolysis and increase of the hydrothermal stability (Bowes *et al.*, 1965).

Collagen hydrothermal stability can be defined as the stability of collagen tertiary structure in the presence of water within a range of temperatures (Engel, 1987). When collagen is heated to a certain temperature, a partial loosening of the structure takes place and the fibres shrink. This temperature is known as the shrinkage temperature. The shrinkage temperature of untanned bovine hide is 65 °C (Balakrishnan and Selvarangan, 1986). Since shrinkage is an endothermic event, it can be measured by differential scanning calorimetry (DSC). Only chemicals which enhance the hydrothermal stability of hide are considered as tanning agents for the leather industry. Chemicals that modify other properties of the hide, but do not satisfy such requirement are disregarded as tanning agents from a practical perspective (Collighan *et al.*, 2004)

The literature also reports that the introduction of more crosslinks in native collagen by both crosslinking reagents and due to the collagen ageing process results in modification of its isometric tension (Heidemann, 1993 (a)). This is defined as the tension generated in a sample of collagen throughout the shrinking transition when the length is maintained by clamping the two ends of the sample (Trinick *et al.*, 1977). Peters *et al.* (1990) applied this method to investigate glutaraldehyde tanning. The curves obtained when plotting the developed tension versus the temperature showed a change in the slope at the temperature that the shrinking transition occurs. If the sample has been crosslinked, both natural crosslinking due to the ageing process of collagen and by crosslinking reagents, the pattern of the curve changes.

Historically, the tanning properties of a chemical, has been related to its ability to cross-link the collagen molecule with the consequent increase of its hydrothermal stability (Covington *et al.*, 1998). However, the literature also reports that the shrinkage temperature does not only depend upon the cross-linking density, other factors such as the position of the cross-link and the type of cross-link (e.g. covalent versus hydrogen bonding or versus metal complex bond) have also to be considered (Heidemann *et al.*, 1981). Furthermore, work carried out in collagen chemistry, and not only from a leather science perspective, has also provided evidence that solvent displacement of the water surrounding the collagen triple helices modifies the denaturation temperature of collagen. The displacement of water by a solvent such as 1,2-propanediol in collagen like model peptides largely increase the denaturation temperature (Engel J., 2004). Examples of current tanning technologies include metal, organic and combination of metal-organic tannages (Kochta *et al.*, 1990).

Covington *et al.* (2001) have recently investigated the kinetics and thermodynamics of the shrinking transition and their implications to the varying tanning chemistries. They cluster the tanning chemistries, in two groups. The first group includes the tanning agents that increase the shrinkage temperature up to 80 °C. The second group consist of tanning agents that brings about a hydrothermal stability in excess of 100 °C. Their work conclude that is incorrect to explain tanning only upon the degree of cross-linking. They state that:"Thermodynamic studies of the events that take place during the shrinking transition have suggested that the change in the shrinkage temperature depends on the amount of supramolecular water that surrounds the collagen triple helices. Tanning agents with the ability to displace the supramolecular water within the collagen matrix, with the consequent change of this environment should provide an increase of the shrinkage temperature in the order of 40 °C. In contrast to that, if the tanning reaction only results in an increase of the degree of crosslinking without changing further the amount of supramolecular water, the increase in shrinkage temperature will be only about 20 °C". Examples of these two populations are shown in Table I.

Table I. Typical shrinkage temperature values for varying tanning chemistries*

Tannage	Ts (°C)
Chromium (III)	100-120
Semi metal (hydrolysable vegetable tannin and Aluminium)	100-120
Zirconium	80-85
Aldehyde	75-85
Phenolic syntan	70-85
Aluminium (III)	65-75
Melamine/formaldehyde resins	80-100

* source Covington *et al.* (2001)

Tanning with mineral salts dates from the ancient times (Chakravorty *et al.*, 1958). However, the interest of the leather industry for mineral tanning was rather dormant until Knapp in 1858 started to investigate the rationale behind mineral tanning (Anon, 1996).

Some metal salts can be applied as a tanning reagents due to their ability to form complexes with the side chains of collagen with the consequent increase of its hydrothermal stability (Gustavson (a1) 1956; Heidemann, 1993(a)). Although many metal salts have been shown to have the ability to form complexes with collagen, the industry have only exploited the tanning ability of chromium, aluminium, zirconium, titanium and iron salts for tanning purposes. Furthermore, unlike these metal salts other metal salts with complex formation properties do not possess tanning ability (Bowes, 1946).

The basic chromium sulphate salts are the preferred choice amongst the other inorganic salts since chrome tanning results in leather with an improved overall performance (Heidemann, 1993 (a)) The mechanism of chrome tanning involves the formation of a complex between the Cr^{3+} as the electron acceptor, the carboxylic acids of collagen (aspartic and glutamic acid), the sulphate and the hydroxyl as the electron donors (Gustavson, 1956 (a1)). Chromium (III) tanning confers a hydrothermal stability above

100 °C and this is unique between the single tannages. The reason why chromium (III) tannages produces such a high shrinkage temperature remains unclear. It is postulated that the introduction of a rigid structure making crosslinks brings about high shrinkage temperature. (Covington *et al.*, 1998).

The tanning industry would ideally like to continue to utilise chromium as a tanning agent. However, the drive for more environmentally friendly production techniques is driving the industry to find alternative tanning agents (Covington *et al.*, 1987).
Existing mineral-free tannages tend to yield leathers with lower shrinkage temperatures compared to chrome tanned leather. The commercial mineral-free tanning materials are mostly aldehydic in nature, often based on glutaraldehyde or modified glutaraldehyde. Many giving positive results with current formaldehyde legislation (Lampard, 2001).

The first reference found in the literature about glutaraldehyde mediated cross-linking of proteins dates back to 1957 (Seligsberger *et al.*, 1957). Their work provided evidence of the ability of glutaraldehyde to make leather and the advantages in respect with formaldehyde. It was initially believed that glutaraldehyde reacts with the amino groups of the protein leading to the formation of a Shiff base or aldimine as shown in Figure 1. However, Blass *et al.* (1976) suggested that the type of cross-linking provided by glutaraldehyde was somehow more complex, since the aldimine bond should be reversible to acid hydrolysis. They used monomeric glutaraldehyde and demonstrated that the type of cross-linking was not reversible to acid hydrolysis which consequently suggest that an aldimine bond is not involved in the type of cross-linking. However, the literature stills reports that glutaraldehyde mediated cross-linking of proteins involves the formation of aldimine bonds (Paul *et al.*, 2004). Chrome and aldehyde tannages are the preferred choice by the industry to provide tanning effect.

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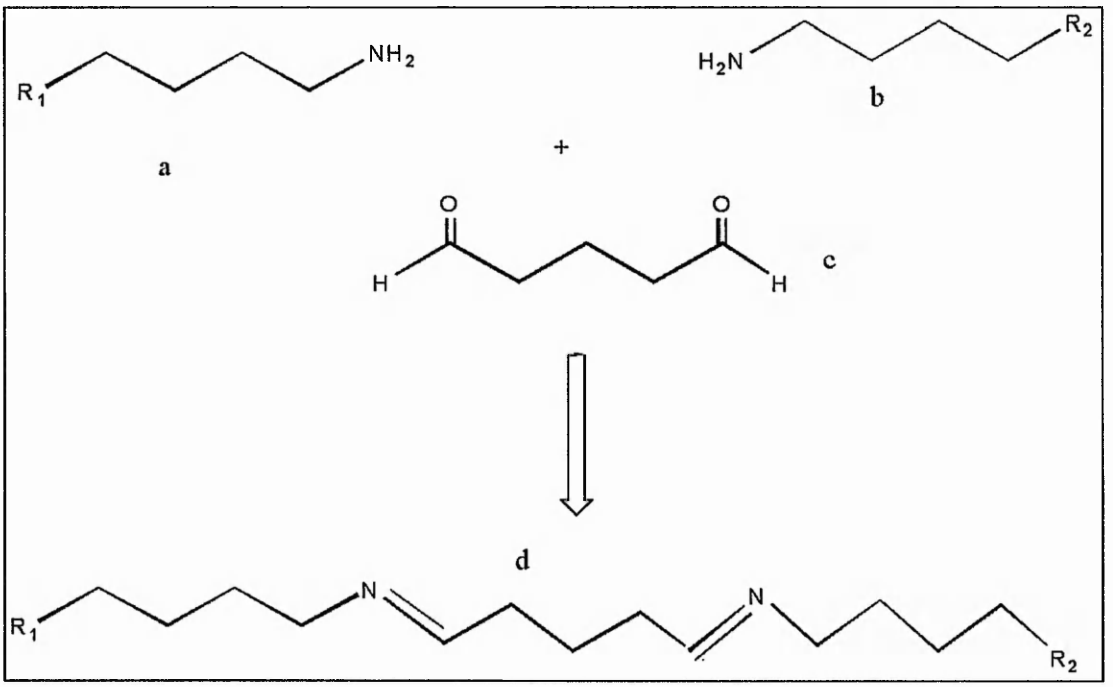


Figure 1. Glutaraldehyde mediated crosslinking via the formation of aldimine bond. The primary amines are depicted in a and b, the glutaraldehyde is labelled as c. The aldimine bond is depicted in d.
(Source Heidemman, 1993 (a))

1.3 Leather dyeing

Improvement of the dyeing properties of the leather has tradionally been an area of research in the leather industry, since dyes are some of the most expensive chemicals in leather production and there is a huge scope for fastness improvement (Dyson, 1990 and Hudson, 2000). Three types of dyes are mostly applied in leather production

- Acid and direct dyes. Acid dyestuffs have a high charge density and binding to collagen fibers is pH dependent. Direct dyes are in contrast little influenced by pH. They have only sufficient charged groups on the molecule to allow them to be water soluble (Heidemann, 1993 (d)). They can have a variety of chemical structures. They are general larger in molecular size than acid dyes and have lower water solubility. The affinity for the fiber depends upon ionic interactions, Van der Waals' forces, aromatic effects and hydrophobic interactions. Satisfactory shades are achieved with these dyes although problems with the fastness properties of the leather are usually seen (Heidemann, 1993(d); Hudson, 2000).

- Reactive dyes. These have a functional group in the molecule that enables the dye to be covalently fixed in the leather fiber. Covalent bonds are formed between the dye and the nucleophilic groups of the substrate. In contrast to acid dyes, reactive dyes ensure good fastness properties at the expense of the shade. Unsatisfactory shades are caused by the undesired side reaction between water and the reactive group of the dye, as well as the lack of nucleophilic groups in the leather (Heidemann, 1993(d)).

The chemical behaviour of collagen, the raw material for leather production, is modified by the physical and chemical operations carried out during the pre-tanning and tanning operations of leather processing. Although the dyeing mechanism of textiles such as wool, cellulose, nylon, cotton or polyesters has been extensively studied, the mechanisms involved in leather dyeing has not yet been fully elucidated (Muralidharan *et al.*, 1990).

The different tanning and post-tanning chemistries applied bring about such variability in the dyeing substrate, that a comprehensive study about leather dyeing has yet to be published. However, if the substrate modifications due to the tanning process are not considered, the principles of dyeing from textiles can be applied to the leather.

The most simplistic approach to explain the interaction between proteins, e.g. tanned collagen, and an anionic dye is that the coloured molecule is bound to cationic groups of proteins, in particular to the protonated ε-amino groups of lysine (Haurowitz, 1963). Thus, on the basis of this ionic bond, the dyeing process is improved by adding to the leather matrix a substrate which is rich in amines. Many applications that make use of this technology can be found in the textile and leather industries (Schaffer *et al.* 1986; Heller et *al.* 1987; Burkinshaw *et al.* 1993; Hudson 2000). Examples of such approach include the use of amino silanes and chitosan in leather manufacture. The first technology improves the dyeing properties of the leather by pretreatment of the leather with the unpolymerised aminosilane, the product subsequently polymerises within the leather matrix as it reacts with water, resulting in leather fast polymer rich in sites for dye fixation. Consequently, the leather has better dyeing properties (deeper shade and better dyes fastness) (Hudson, 2000).The second makes use of the cationic character of chitosan. Treatment of chrome grain leather with chitosan enhances the depth of shade (Cogen, 1987). Despite this deeper shade, comparable wash fastnesses are observed (e.g. chitosan treated samples against control).

Although it is well accepted that ionic bonding is one of the mechanism for dye fixation, many authors have provided evidence that dye and protein interaction is somewhat more complex .

Work carried out by Haurowitz with bovine serum albumin and methyl orange as a model system concluded that although binding of the dye to the protein is initiated by the long range electrostatic forces (e.g. ionic bonding), the short-range Van der Waals' forces are more efficient after binding has been established. Thus, these are responsible

for the apparent irreversible binding of the dye to the protein. The text also suggests that hydrophobic interactions between dye and protein take place (Haurowitz, 1963).

Gustavson introduces into leather the outcome of the textile studies about fibre and dye interactions. Although he observes a certain disparity between different authors, he concludes that dyeing of polypeptides may involve sites located on the backbone of the polymers, their side chains and amino groups (Gustavson (a2), 1956).
Nakamura *et al.* (1991) studied how the forces of interaction between the dyes and leather fibres are related to the colour fastness. They reviewed the role of the hydrophilic-hydrophobic characters of the dyes and leather. Their findings enabled them to conclude that the interaction between the dyes and leather were hydrophobic in nature increasing with hydrophobicity of alkyl side-chains on the dye anion and collagen amino acid.

Knight *et al.* (1980) showed that the use of acrylate resins in the retannage intensified the shade and improved fastness. They found that greatest shade intensification occurred when applying the polymer prior to dyeing with anionic dyestuffs. The presence of polar groups in the polymer resulted in a lower dye fixation.

Muralidharan *et al.* (1990) studied the chemical and physical interactions involved in leather dyeing using hide powder as a model compound. They used C.I Acid Yellow 36 because it has a simple structure, having only one reactive sulphonic acid group in addition to the azo group. Stripping studies of the dyed samples indicated the nature of the dye-fibre bonds.. As shown in Table II, the outcome of their work demonstrated that mechanism of dye fixation is not only underpinned by a salt linkage between the sulphonate group of the dye and the cationic residues in the leather matrix.

Britten (1994) stated that increasing hydrophobicity of anionic dyestuffs results in leather with deeper shades and better fastness due to the partially hydrophobic character of the leather. He advocates for dyeing systems to combine hydrophilic and hydrophobic dyes. Hydrophilic dyes have to be applied due to their low affinity

towards leather. Hence, facilitating dye penetration with the consequent uniform distribution across the cross-section of the leather. Build up of the shade is achieved by applying hydrophobic dyes which react with the surface of the leather.

Whilst the use of cationic fixatives to improve dyeing in leather has proved to be a valid option, the mechanism of leather dyeing has shown to be so complex that the use of other dyeing auxiliaries should not be overlooked (Schaffer *et al.*, 1986; GB 2 184739, 1987; Burkinshaw *et al.*, 1993; Hudson, 2000). The interest in production of collagen hydrolysates from chrome shavings has increased in the past few years[i]. Many ways have been explored to utilise the available hydrolysate. One of the interesting routes for the leather industry is to use the hydrolysate from leather processing as retanning reagents. Two differents routes can be undertaken. The first route utilises the protein hydrolysate in combination with acrylic resins to produce graft polymers for retanning. Alternatively, the collagen hydrolysate is applied alone (Ma, 1999). Amongst other benefits, application of collagen hydrolysate alone or combined with acrylic as retanning reagents may improve the dyeing properties of the subsequent leather (Cantera, 2000; Ma, 2004).

[i] Chrome shavings: After the chrome tanning process, hides are shaved to required thickness, the solid waste generated by this operation is called chrome shavings

Table II. Amount of dye in percentage held by varying hide powder substrates with dipole interactions, ionic interactions and other interactions*

Substrate	Dipole interactions (%)	Ionic interactions (%)	Other interactions (%)
Native hide powder	1.00	13.35	85.65
Esterified hide powder	0.89	8.95	90.16
Formaldehyde tanned hide powder	5.41	18.40	76.19
Acetylated hide powder	1.67	16.09	82.24
Chrome tanned hide powder	1.81	20.70	77.49
Dinitrophenylated hide powder	0.51	1.75	97.74
Deguanidated hide powder	5.30	31.50	63.20

* Original data from (Muralidharan *et al.*, 1990)

Keratin hydrolysate, formed during the liming process, is one of the major pollutants in the tannery effluent since is responsible to a great extent for the high chemical oxygen demand (COD) of the effluent (Cantera, 1998). Current beamhouse technologies have addressed this issue by applying hair save techniques. The use of enzymes (Germann, 1997) or application of hair immunisation chemistries (Cantera, 2001(a); Cantera 2001(b)) brings about unhairing methods that loosen rather than dissolve the hair, with the consequent COD reduction. Although hair immunisation is technically satisfactory, its full implementation has yet to be accomplished and the high COD of liming effluents is still an issue to be addressed (Mischitz, 1988; Cantera, 1998; Marsal, 2002).

Since there is evidence that other mechanisms, alongside ionic interaction, are involved in dye fixation (Britten, 1994; Muralidharan *et al.*, 1990), the use of other compounds to improve dyeing performance, beside cationic fixatives has to be considered. Amongst these compounds collagen hydrolysate and keratin hydrolysate in conjunction with transglutaminases have shown their ability to improve dyeing performance in textiles such as wool (Winkler, 1999; Cortez, 2002; Cortez 2004)

1.4 Current enzymatic applications in leather processing

Enzymes are currently considered an integral part of leather processing. Although traditionally enzymes were only used for the bating stage of the beamhouse operations, current processing also utilises enzymes in the soaking, liming and to some extent degreasing stages of leather making. The use of pancreatic enzymes has been superseded by microbial proteases, and enzymes are available which cover the pH extremes encountered during leather processing (Addy, 1998).

1.4.1 Enzymes in soaking

The enzymes used in soaking are carbohydrases and proteases. These target a broad range of components which include solidified fats, carbohydrates and non-collagenous proteins which sometimes covers the external surface of the hide, making contact between collagen fibres and water difficult. The advantages of an enzymatic assisted soaking include shorter wetting times, loosening of the scud[ii], initiation of fiber opening, and production of a product with less-wrinkled grain (Pfleiderer, 1985). However, a major disadvantage of their use is the added costs involved. Recent work carried out by Tozan *et al.* (2002) has shown that enzymes such as xylanases, cellulases and ligninases can successfully be applied to breakdown the dung carried by the hides from the abattoir. Application of such enzymes facilitates the soaking operations.

1.4.2 Enzymes in liming

The aim of unhairing/liming process is removal of hair, epidermis hyaluronic acid, dermatan sulphate and non-collagenous proteins such as albumins and globulins, and a partial loosening of the collagen structure. A solution of calcium hydroxide and sodium sulphide is applied to raise the pH to around 12.5. At such high pH value the hide fibres combine with an appreciable amount of alkali. During the reaction the carboxyl groups are ionised and the amino groups are in the free base form. To compensate the charge balance water enters the hide by osmosis and the collagen protein swells. Sodium sulphide is a reducing reagent that attacks the disulphide bonds of keratin, the main

[ii] Scud: A film or deposit of waste matter appearing on the surface of leather in process after certain operations

component of hair, facilitating its removal. Calcium has a lyotropic effect within the hide structure providing a partial loosening of the structure (Addy, 1998).

Proteolytic enzymes are used in the liming stage. They can be used to target the proteoglycans still present within the skin structure (Addy, 1998). The degradation of keratin protein is difficult due to the presence of cystine residues within the molecule structure which give stability to the protein, and enzyme systems based on the keratinases applications have not been totally successful for this application (Cantera, 2001(a)). Also the epidermal layer presents a barrier to the penetration of chemicals and enzyme will therefore need to migrate from the flesh surface of the skin through to the grain layer to reach the hair follicle (Frendrup, 2000). It is now possible to reduce sulphide at its source using enzyme-assisted processes. The enzymes origin can be animal, bacterial, fungal or plant (Palanisamy *et al.*, 2004). Enzymatic unhairing generally uses proteolytic enzymes in combination with small amounts of sulphide and lime (Palanisamy *et al.*, 2004). The advantage of enzyme assisted unhairing is a reduction in the COD load of the effluent, as the hair is loosened by selective break-down of the cementing substances in the hair follicle, thereby keeping the hair shaft intact (Green, 1952).

However, because commercially are often general proteases, there is a risk associated with enzyme assisted unhairing such as damage of the dermal collagen. Attack of the dermal collagen may result in a leather with grain damage which is not suitable to manufacture high quality products (Paul *et al.*, 2001).

1.4.3 Enzymes in bating

The function of the bating step is to clean the skin of any remaining protein debris from the liming stage and to continue with the degradation of interfibrillary non collagenous proteins. The choice of protease will depend on the deliming method used. As the preferred method of deliming is using ammonium salts, the proteases used for bating will need to be active at pH 8-9, and pancreatic (e.g. trypsin) and bacterial (e.g. *Bacillus Subtilis* protease) derived proteases fulfil this criteria (Christner, 1992).

1.4.4 Enzymes in degreasing

Some types of skins, specially some types of sheepskins, present a high content of fat which needs to be removed during the tanning operations. The leather industry currently has two technologies to ensure cost effective grease removal adequate with the required product quality, these are solvent extraction and aqueous emulsification using detergents (Addy, 1994).

Conventional wet solvent degreasing is done through the use of non-polar alkane based solvents (usually paraffin and white spirit) (Innes *et al.*, 1952) which function to solubilise the cutaneous lipid. However the leather industry is driven towards solvent free degreasing systems due to environmental problems associated with their use. The degreasing mechanism follows three stages: breakdown of the proteic membrane of the fat containing cell, surfactant assisted emulsification of the fat in water or solvent solubilisation and removal of the fat. Therefore, the ideal enzymatic preparation should provide the three effects (proteolysis, lipolysis and emulsification) (Palinasamy *et al.*, 2004). Zhang *et al* (1982) used an alkaline lipase in combination with proteinase and pancreatin to improve the degreasing effect in softening pigskin. Work carried out by Addy *et al* (2002) suggest that lipid hydrolases can be used to target and hydrolyse ovine storage lipid and that such information could be built upon to develop effective-enzyme based degreasing systems.

The leather industry is being driven to apply new biocatalyst in a view to reducing the effluent load (Palanisamy *et al*, 2004). Transglutaminases, the protein crosslinking enzymes (Li 2002). might be suitable option to be implemented either in tanning to increase the hydrothermal stability or during post tanning to modify the physical properties of the leather.

1.5 Transglutaminases

The term "transglutaminase" was introduced by Heinrich Waelsch and co-workers to describe an enzyme that was present in the liver and some other tissues of guinea pig. The enzyme was reported to have transamidating activity. In contrast to other enzymes that modify the amides of free glutamines such as glutaminases, γ glutamyltransferase and glutamine synthetases, the novel enzyme had the ability to modify peptide bound glutamines (Collighan and Griffin 2003; Griffin *et al.* 2004 and Verderio *et. al*, 2004).

Transglutaminases (TG, referred to as the R-glutaminyl-peptide, amine-γ-glutamyl transferase. EC 2.3.213) catalyse post translational modification of proteins, which results in crosslinking of proteins and or incorporation of primary amines. Due to such modification, the physical, chemical and biological properties of the proteins become changed (Aeschliman *et al.*, 1994). The post translational modification takes place via an acyl-transfer reaction between a peptide bound glutamine and a suitable acyl aceptor. This include the ε-NH_2 side chain amino group of a protein bound lysine residue, primary amino groups of a variety of compounds such as polyamines, and even water in the absence of other acyl aceptor (Li, 2002; Cortez *et al.*, 2004)

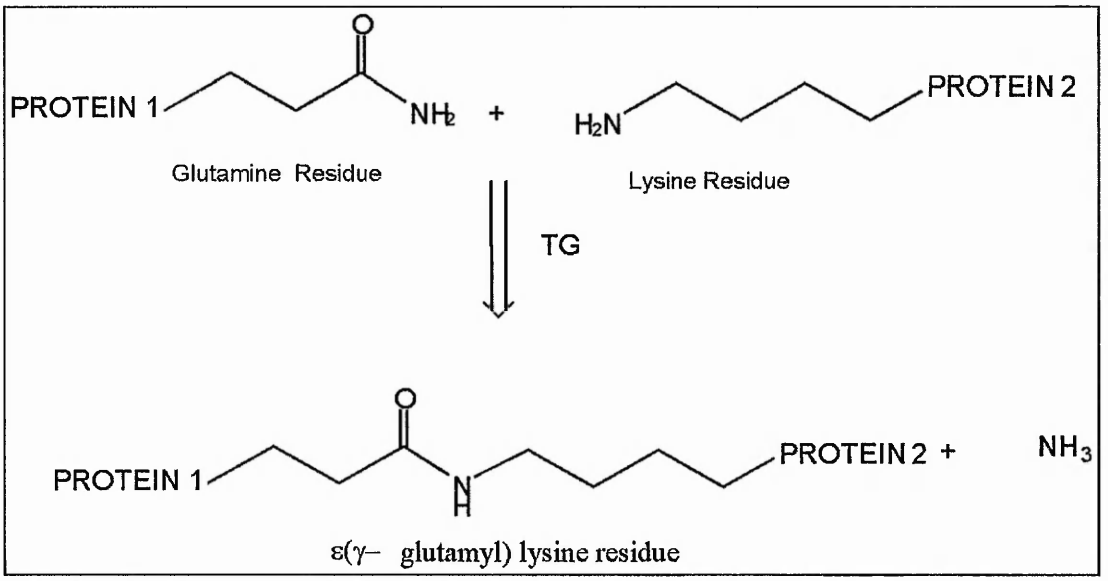
The covalent cross-linked iso-peptide is stable and resistant to proteolysis, and thereby enhancing the resistance of the protein to chemical, enzymatic and mechanical disruption (Aeschliman *et al.*, 1994). The reactions catalysed by TG are shown in Figure 2.

Transglutaminases occur in a broad variety of living systems. Sources of TG include microorganisms such as those that belong to the *genus Streptoverticillium*, to *Bacillus Subtilis*, various *Actinomycetes* and *Myxomycetes*, fish species and other marine sources, from plants and mammals (Collighan *et al.*, 2002). Mammals provide the blood clotting protein activated Factor XIII. Liver TG can be obtained from guinea pigs. In general, TG from animal sources require calcium ions for activity (Aeschliman *et al.*, 2000). Recombinant forms of TG may be produced by genetic engineerins

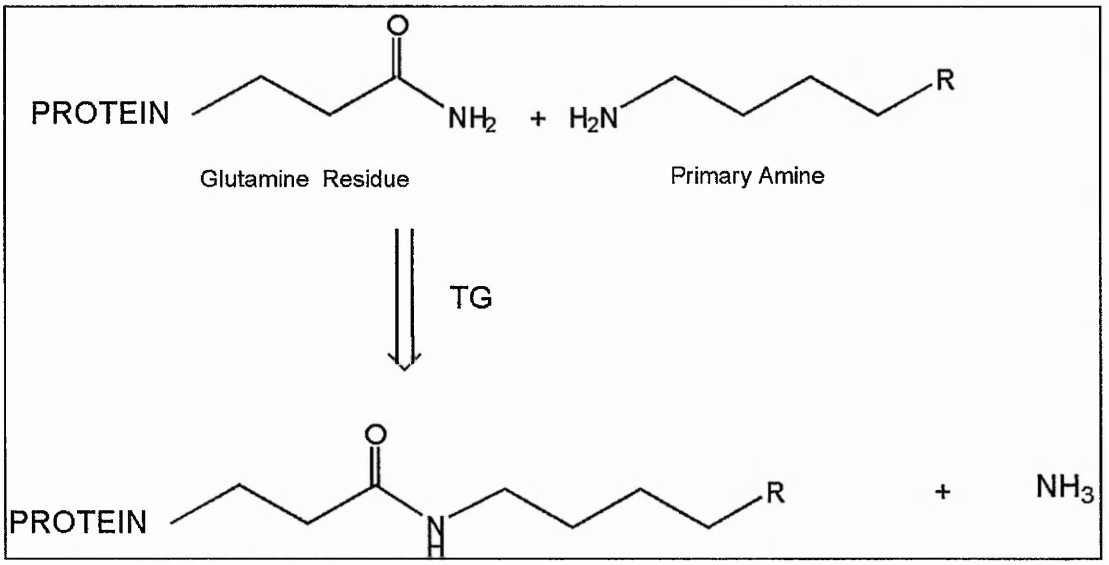
methods as heterologous proteins produced in bacterial, yeast, and insect or mammalian cell culture (Collighan *et al.*, 2002).

These different groups of enzymes each have their own characteristics with respect to substrate specificity, mode of activation, temperature stability and pH dependence (Colligan *et al.*, 2002) For example, as shown in Figure 3, the much widely used microbial TG from *Streptoverticillium* is Ca^{2+} independent, has a broader substrate range, higher temperature tolerance and broader pH range compared to the well characterized mammalian Type 2 tissue TG, thus suiting its use in a wide range of high throughput, large yield commercial applications (Collighan *et al.*, 2002)

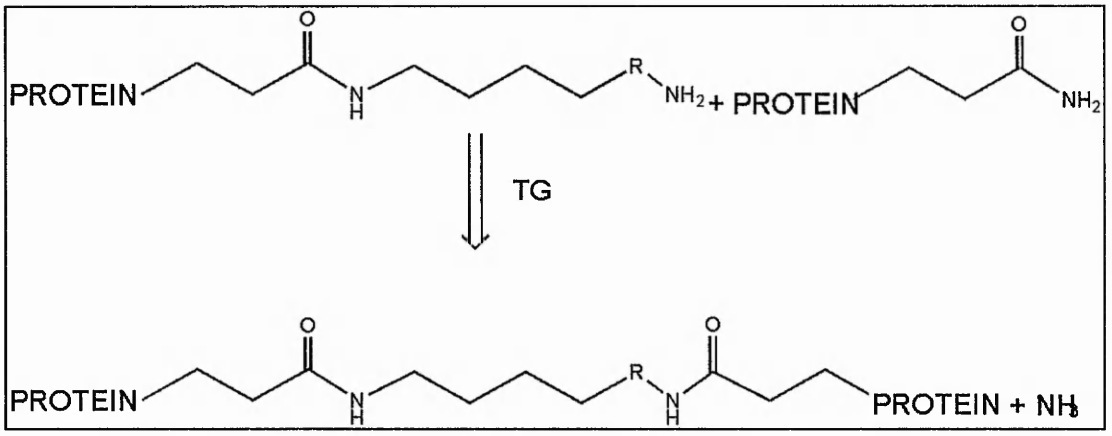
(i) Formation of the ε(γ-glutamyl) lysine cross-link between proteins



(ii) Incorporation of a primary amine into a peptide bound glutamine residue



(iii) Formation of N,N'–bis(γ-glutamyl) polyamine lin



(iv) Formation of peptide-bound glutamic acid

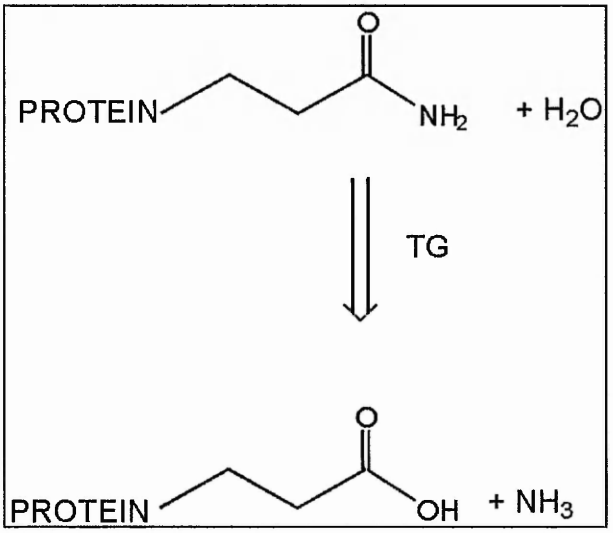


Figure 2. Scheme of the TG-catalysed transfer reactions
Source Li, (2002)

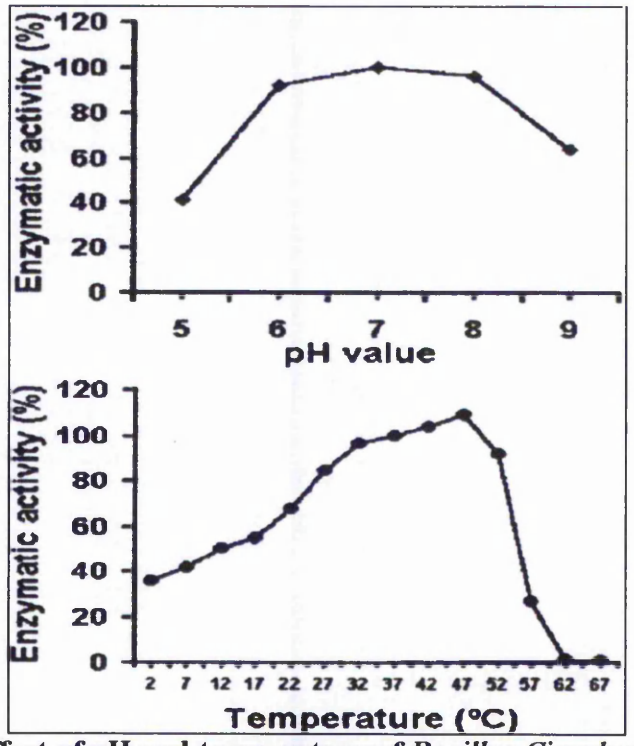


Figure 3. Effect of pH and temperature of *Bacillus Circulans* TG.

Source Barros Soares *et al.*, (2003)

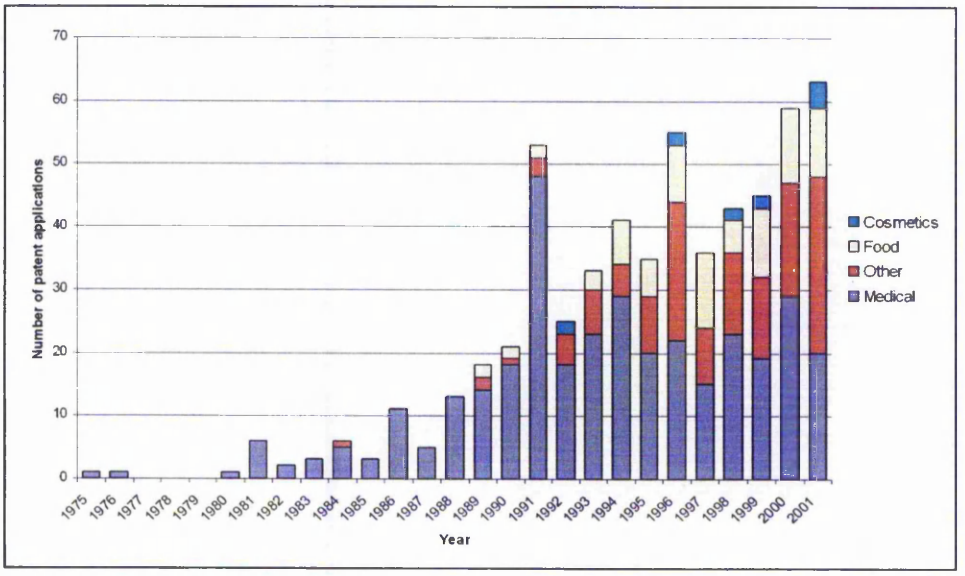


Figure 4. Number of TG related patents filed since 1975 to 2001.

Source Collighan *et al.* (2002)

Table III. Reactivity of microbial transglutaminase (mTG) towards various food proteins

SOURCE	PROTEIN	REACTIVITY
Milk	Casein	Very good
	Sodium caseinate	Very good
	α-lactalbumin	Depending on conditions
	β-lactoglobulin	Depending on conditions
Eggs	Egg white protein (ovalbumin)	Depending on conditions
	Egg yolk protein	Good
Meats	Myoglobin	Depending on conditions
	Collagen	Good
	Gelatin	Very good
	Myofibril:myosin	Very good
	Myofibril:actin	Poor
Soybean	11S globulin	Very good
	7S globulin	Very good
Wheat	Gliadin	Good
	Glutenin	Good

Source: Yokoyama *et. al* 2004

The first reference found in the literature about TG studies in a view to assess the potential of industrial application, particularly about modification of proteinaceous food, dates back to 1980 (Matheis *et al.*, 1987). At these early stages the tissue TG (Type II) purified from guinea pig liver and bovine plasma TG (factor XIII) were the enzymes most commonly investigated (Matheis *et al.*, 1987). However, in 1989 these were superseded with the discovery of the microbial enzyme from *Streptoverticillium sp.* The enzyme derived from the microbial source, in contrast to the mammalian enzyme, featured some advantages such as Ca^{2+} independence, same levels of activity at lower concentrations, lower substrate specificity and easier bulk production by recombinant expression technology with the consequent price reduction (Collighan *et al.*, 2002). This price reduction resulted in a steady rise of TG applications in the industry as shown in Figure 4. Examples of TG in food processing include paste, meat, diary and bakery applications (Collighan *et al.*, 2002; Parsons 2004).

The addition of TG during the setting process of Surimi (Japanese fish paste) was the first TG application in food processing. This application results in higher breaking stress and better elasticity. There is evidence to relate these changes in the physical properties of the fish paste with TG mediated crosslinking, as the fish proteins presented a degree of TG mediated crosslinking up to 3μmol of ε-(γ-glutamyl)lysine/100g gel (Seguro *et al.*, 1995). Similar changes in the physical properties of processed meat have also been reported. This improvement in the physical properties prevents fragmentation and breakage during the process with the consequent product maximisation which ultimately results in savings for the food manufacturer (Kuraishi *et al.*, 1997). Benefits to the consumer are health based, as TG can replace other binders such as salts and phosphates. The enzyme is added in a mixture with the meat to be glued and a solution of sodium caseinate, as a consequence the meat is held in a crosslinked matrix of protein (Collighan *et al.*, 2002).

Since wheat glutens are rich in glutamine proteins TG have also been successfully applied in the production of noodles to increase their strength, even after cooking and

reduce the starch released after cooking producing pasta less sticky and with a higher content of nutrients (Kuraishi *et al.*, 2001)

It is well documented that casein is a good substrate for TG and the main protein in milk (Coussons *et al.*, 1992; Liu and Damodaran, 1999; Vasbinder *et al.*, 2003). Therefore, it is not then surprising that the enzyme is broadly applied in dairy products such as yoghurt, cheese and ice-cream. TG treatment of milk prior to fermentation in the production of yoghurt results in a finished product with increased firmness, higher viscosity, and reduced water separation properties (Ishii *et al.*, 1994). The reduced whey separation observed after TG treatment of milk is also a beneficial property in the manufacture of cheese products, where the yield of curd is also significantly increased. A further benefit of the application of TG in the dairy industry is in the manufacture of low calorie ice cream which is softer, smoother and easier to scoop (Okada *et al.*, 1993).

Films can be casted with gelatins treated with TG and mixed with glycerol as a plasticizer. Increasing the amount of enzyme the films have higher tensile strength, are less soluble in water and have improved water absorption properties. These products showed potential to be used as edible films and sausage casings. Furthermore they might be used in the packaging material market, a market not previously utilised because of the poor mechanical properties of the gelatin films (Taylor *et al.*, 2002)
As shown in Figure 2 (ii) TG has the ability to incorporate a suitable primary amine into a peptide bound glutamine residue. This has been utilised to modify the skin by covalent incorporation of primary amine compounds. Examples of this application include incorporation of antimicrobials, UV-absorbers, skin and hair conditioning agents, anti-inflammatories, antioxidants, colouring agents, perfumes and insect repellants into human skin, hair and nails (Bailey *et al.*, 1996).

Other TG applications are found in the leather and textile industries. Leather can be supplied with a finish of casein which is hardened by means of a hardening agent, such as an aldehyde, an aziridine or an isocyanate. However, this hardening agents suffer

from the disadvantage that they are toxic or even very toxic with the consequent health related issues for the operators in the leather finishing plants. Transglutaminases can substitute this hardening agents with the advantage that they are a non toxic material (Collighan *et al.*, 2002). Transglutaminases hardened finishes did exhibit the same quality in regard to rubbing fastness in comparison to the traditional hardener agents (Rasmussen *et al.*, 1996). Transglutaminases alone or in combination of a suitable protease treatment of wool improves some of the properties of the finished fabric. Examples of improved fabric properties include shrink resistance, handle, appearance, wettability, reduction of felting tendency, increased whiteness, reduction of pilling, improved softness, tensile strength retention, improved stretch, improved burst strength, and improved dye uptake and dye washfastness (Cortez *et al.*, 2004). As in the cosmetics industry, the enzyme can also be utilised to incorporate primary amine containing compounds such as perfumes, insect repellants, dyes, softeners, water repellants, antimicrobials, sunscreens, and other proteins or hydrolysed proteins such as keratin, silk, casein, fibronectin or collagen (Cortez *et al.*, 2004)

Since there is evidence that the tanning effect is attained by the introduction of cross-links within the collagen matrices (Covington *et al.*, 1998) and mTG has been showed to have this ability (Li, 2002). This project has investigated the potential of using mTG as a tanning agent.

As already discussed, other mechanisms, alongside ionic interaction, are involved in dye fixation (Britten, 1994 and Muralidharan *et al.*, 1990), the use of other compounds to improve dyeing performance, beside cationic fixatives has been investigated. Amongst these compounds collagen hydrolysate and keratin hydrolysate in conjunction with TG have shown their ability to improve dyeing performance in textiles such as wool (Cortez, 2002 and Winkler, 1999). Furthermore, keratin hydrolysate is a waste product in the leather industry which highly contributes of the total COD generated in leather processing, and this is an issue that needs to be adressed (Landmann, 1990). Therefore, it appears plausible to investigate the potential of using mTG mediated

incorporation of keratin hydrolysate in the retanning operations and its consequent effect on the dyeing properties of the leather.

1.6 The aims of the project

This work has been carried out as a part of the DTI funded project Novel Enzymatic Applications to the Leather Industry with Specific Reference to Transglutaminases. The project was carried out under the Link (Biocatalyst) program in collaboration between Nottingham Trent University, BLC Leather Technology Centre Ltd., Stirling Leather Ltd., Eagle Ottawa Warrington Ltd and Pittards PLC. Most of the work discussed in the thesis has been carried out by the author. However, some of the data presented along the text were provided by Nottingham Trent University. This has been stated appropriately.

The aims of this project were:

- To determine the effect of the protein crosslinking enzyme microbial transglutaminase (mTG) alone or with crosslinking facilitators on the physical properties of the leather. Work has particularly focussed on the ability of mTG to increase the denaturation temperature of collagen and the mechanical properties of the subsequent leather.

- To evaluate the potential of applying transglutaminases during the dyeing operations. Two routes have been undertaken to improve the dyeing properties of leather. The first involved the chemical modification of azo dyes. (p-(p-aminophenylazo) benzene sulphonic acid 2-naphtol) via addition of an alkylamine side chain such that it becomes a substrate of mTG, thus facilitating its incorporation into protein bound γ-glutamyl residues of hide or crust leather. The second has evaluated the ability of mTG to incorporate proteins (carrier proteins) into dried bated hide or crust leather which increases the reactivity of the modified hide/leather to dyestuffs. A number of carrier proteins are potentially available in bulk including casein or its hydrolysate and hydrolysates of keratin. The latter has the advantage in that it is a waste product from tanneries and can therefore be reused.

Chapter 2. MATERIALS AND METHODS

2.1 Materials

All the materials were purchased from Sigma-Aldrich,with the exception of the listed below:
Ajinomoto:
Microbial Transglutaminase

Basf:
Chromium Sulphate 33%
Luganil Black NT
Luganil Braun NT
Myacide As
Tan Base from Basf

Bayer:
Tanigan RFS

Dr. Boehme:
Cutapol TIS

Clariant:
Pancreol Bate 10K

Fisher Chemicals:
Acetic Acid
Ammonia
Ammonium Chloride
Ammonium Sulphate
Butanol
Calcium Chloride
Calcium Chloride
Calcium Hydroxide
Dimethyl-formamide
Dioxane
Ethyl acetate
Formic Acid
Hexane
Methanol
Oxalic Acid
Potassium Chloride
Potassium Chloride

Potassium Hydrogen Sulphate
Potassium Hydroxide from Fisher
Potassium Hydroxide from Fisher
Sodium Chloride from Fisher
Sodium hydrogen Carbonate
Sodium Hydroxide
Sodium Sulphide
Sulphuric Acid
Trichloroacetic Acid
Urea

Packard:
Soluene 350
Ultima Gold XR

Room&Haas:
Leukatan 1084

Schill + Seilacher
Lipsol
Derugan 3080

Tanac:
Mimosa

TFL:
Neutrasol IW
Sellaset Brown H
Sellaset Red H
Sellaset Yellow H
Sellasol NG
Sellasol TD
Tannesco HN

Trumpler:
Trupotan MT

2.2 Degree of deamidation during liming.

The degree of deamidation during liming was determined by measuring the ammonia released in solution as ammonium by ionic chromatography using a Dionex DX-100 column (Nickel *et al.*, 1999).

Five thousand three hundred forty-nine mg of ammonium chloride were weighed and the volume made up to 1l with double distilled water, with a resistance of 18.2 MΩ and trace of Total Organic Carbon (TOC), to give a ammonium stock solution 100 mM. Aliquots were taken and the volumes made up to assay two different ranges of molarities: 10, 1, 0.1 and 0.01 mM and 1, 0.5, 0.2, 0.1 and 0.05 mM. The sensitivity of the detector was set up at 30 μS. Each standard was run in triplicate.

One mg collagen samples were placed into 1.5 ml vials which contained 1ml of 100 mM, 10 mM and 1mM potassium hydroxide at pH 13.0, 12.0 and 11.0. The vials were sealed and incubated for sixteen hours at 25 °C. One hundred μl of trichloroacetic acid (TCA) were injected into each vial to convert the possible ammonia to ammonium. The samples were transferred to eppendorf tubes and centrifuged for 5 min at 12000 rpm. Five hundred μl of each sample were pipetted into the chromatographic vials and analysed by ionic chromatography. Ammonium was measured in all incubation solutions as a control. All the experiments were run in triplicate.

Further work was undertaken with 50 mg of collagen in 1.5 ml of solutions pH 12.5 and a ionic strength such that the conductivity of the solutions were 37 $mS.cm^{-1}$. The pH and the ionic strength were adjusted with KOH and/or $CaCl_2$, KCl.

Finally, the liming process was reproduced at the laboratory scale. Raw hide was soaked for four hours, freeze dried, and mechanically dehaired. Twenty-five mg of powdered raw hide underwent liming (2.4%w/w Na_2S 3%w/w $Ca(OH)_2$ in 1ml of distilled water) for 16 hours.

Investigation of degree of deamidation during liming using glutamine as a model system, was carried out as follows. Glutamine was dissolved in solutions at pH 12.5 and ionic strength with a conductivity 37 mS/cm adjusted with KCl or $CaCl_2$. The glutamine content was measured at varying time intervals using a commercial method for amino acid analysis by GC (Gas Chromatography). (EZ:faast phenomenex). Five, ten, twenty-five and fifty µl of standard solution 1 and 2 plus 100 µl of the internal standard were prepared in each case for amino acid analysis by SPE (Sorbent Phase Extraction) using sorbent cartridges provided in the kit. The standards were eluted in aqueous phase, and derivatized in one step with the reagent provided by the kit, at room temperature. The derivatization reagent were added in organic phase, and the reaction products (alkylated amino acid derivatives) were extracted into the same phase. Fifty µl of sample were analysed in each case following the standards protocol. Samples were analysed on a Hewlett Packard 5890 Gas Chromatograph. The GC column was the Zebron ZB-PAAC-MS, 10M X 0.25mm ID column supplied with the kit (1µl injected splitless, 50 kPa column head pressure, 280 °C injector temperature, 280 °C detector temperature, 75 °C(2minutes)-25 °C /min-320°C (1.20min). MS conditions: 2600 EM volts, acquisition 3.60-13.00 min, scan 45-450 amu, threshold 150, sampling 2(1.98 scans. s^{-1})

2.3 Design of an alternative unhairing process

The Dispase urea treatment was carried as shown in Table IV.

Table IV. Protocol of the Dispase/Urea unhairing process

Operation	Chemical	Quantity (w/w-hide)	T (^{0}C)	Time (h)	Observations
A. Proteolytic Treatment	Dispase Myacide As Tap water	0-1 units/g-hide 0.1 % 200 %	37	6	
B. Drain					
C. Wash x2	Tap water	200 %	20	0.5	2 washes of 15 minutes
D. Urea treatment	Urea Tap water	0-50-100-150-200 mM 200 %	20	16	
E. Drain					
F. Wash	Tap water	200 %	20	0.5	2 washes of 15 minutes
G. Drain					
H. Pickling	Sulphuric Acid Sodium Chloride Tap water	1 % 0-10 % 200 %	20	16	2 additions of sulphuric acid diluted 1:10 over the first two hours, check final pH 2.5

Float samples were taken at different intervals of time to determine the degree of protein removal due to urea. This was achieved by measuring the total protein concentration in the float by DC Protein Assay (Biorad). Five dilutions of a protein standard containing 0.2 mg.ml^{-1} to 1.5 mg.ml^{-1} protein (BSA) were prepared in the same buffer as the sample. One hundred µl of standards/samples were added into test tubes plus 500µl of working reagent (20µl of reagent S, SDS per 1.0 ml of reagent A, alkaline copper tartrate solution) and vortexed. Four ml of reagent B (dilute Folin reagent) were added and vortexed. The standards/samples were left to stand for 15 minutes and the absorbance was read at 750 nm with a Pye Unicam SP8-100 ultraviolet spectrophotometer from Phillips. The same procedure was carried out when samples

underwent the pickling treatment.The samples were subsequently processed to the dyed crust stage, following the protocol shown in Table V.

Table V. Tanning protocol for the Dispase/Urea unhairing method

Operation	Chemical	Quantity (%w/w)	T (^{0}C)	Time (h)	Observations
A. Chrome Tanning	Chrome Sulphate (33% basicity) Sodium chloride Tap water	8 6 100	20	4	After four hours check complete chrome penetration
A. Chrome Tanning	Tan Base	0.4	20-40	4	4 x 0.1 additions/ increasing temperature 5 ^{0}C every hour
Adjusting final pH to fix chrome	Sodium Hydrogen Carbonate	4x0.05 additions	40	1	Check pH 3.8-4.2 shrinkage temperature above 100 ^{0}C
B. Drain					
C.Wash	Tap water	200	20	0.5	2 washes of 15 minutes
D.Drain			20	48	Left on the horse for 48 hours and split to 2 mm thickness, weigh samples
E. Neutralising	Sodium Hydrogen Carbonate Tap water	0.7 100	20	1	All the percentages based on split samples weight Check pH 5.5-6
F. Dyeing	Luganil Braun NT	5	20-50	3	Increase temperature 5 ^{0}C every half an hour.
G. Fatliquoring	Lipsol 59	5	50	3	Emulsified in water at 50 ^{0}C (1:10)
H .Fixing	Formic Acid	1	50	2	2 additions of 1:10 diluted formic acid over the first hour. Check pH 3.5-3.8
I. Drain					
J. Wash	Tap water	200	20	0.5	2 washes of 15 minutes
K. Drain					

2.4 SDS page

Dispase purity was determined by SDS-PAGE under reducing conditions according to Laemmli (1970). All the reagents for SDS-PAGE were from Bio-Rad Laboratories. The electrophoresis were performed in a Mini-Protean electrophoresis unit (Bio-Rad Laboratories) using a 15% acrylamide separating gel and a 4% acrylamide stacking gel. Staining was carried out with Coomassie Brilliant Blue. All the reagents used were supplied by the kit.

2.5 Microbial transglutaminase activity assay

Microbial transglutaminase activity was determined by the method of Lorand *et al.* (1972), utilising the incorporation of [^{14}C] putrescine into N-N'-dimethyl casein. A reaction mixture containing a specified amount of mTGs in 28 mM Tris HCl pH 7.4, 3.85 mM dithiothreitol, 5mg/ml N-N'-dimethyl casein, 1.2 mM 3.97mCi mmol-^{1} [^{14}C]-putrescine, was incubated at 37 °C. The experiments were undertaken at varying concentrations of the biocide Myacide As, $CaCl_2$, S^{2-}, $(NH_4)_2SO_4$ and the bating preparation Pancreol Bate 10K. At specified time intervals, 10 µl aliquots were removed and spotted onto 1 cm^2 square of filter paper, which were placed into ice cold 10 per cent trichloroacetic acid (TCA). Unincorporated [^{14}C] putrescine was removed by washing three times for 10 minutes with ice cold 5% (w/v) TCA, 5 minutes with acetone/ethanol (1:1), 5 minutes with acetone, and the filters were air dried. Radioactivity associated with each filter was counted using a scintillation counter. mTG activity was calculated as nmol of [^{14}C]-putrescine incorporation per hour per mg of enzyme after subtraction of the control without the enzyme.

2.6 Determination of mTG-available glutamine content

Freeze dried bated bovine hide powder (2.5 mg) was placed into 1.5 ml tubes and 500 µl of a reaction mix consisting of 50 mM Tris HCl, 3.5 mM dithiothretiol, 1.2 mM [^{14}C]-putrescine, 100 µgml^{-1} mTG at pH 7.5 was added. The tubes were vortexed well and incubated at 37 °C for 2 hours, with intermittent mixing. The hide was pelleted by

centrifugation at 13000 g for 5 minutes and the supernatant carefully decanted. The pellet was resuspended in 1 ml of ice cold 10% (w/v) TCA as before. The wash was repeated five times and one further was with distilled water was performed. The pellet was resuspended in 500 µl of Soluene 350 and dissolved by incubation at 60 °C for 2 hours, vortexing occasionally. Aliquots were transferred to scintillation vials, mixed with 2ml of liquid scintillation cocktail (Ultima Gold XR) and counted in a scintillation counter.

2.7 Determination of collagen/leather denaturation temperatures

The denaturation temperatures were measured by differential scanning calorimetry (DSC) using a Mettler TA30 system with a low or medium pressure cell and a scan rate of 5 °C min^{-1} throughout the range 25 °C to 110 °C. The onset of denaturation was calculated automatically using a custom analysis program. Excess water was removed from treated collagen samples by blotting with tissue in order to reduce the water content to a level which allowed a sufficient amount of bated hide to be placed into a DSC sample pan.

2.8 ε-(γ-glutamyl) lysine crosslink analysis

Samples of dried powdered bated hide were suspended in 1 ml of 0.1M ammonium bicarbonate pH 8.0 and 1 crystal of thymol to prevent bacterial growth during subsequent incubations. Proteolytic treatments were then performed in the following way: 10µl of 0.5M $CaCl_2$ and 10µl of collagenase (Clostridiopeptidase A) (10mg.ml^{-1} in 0.1M NH_4HCO_3 were added and incubated at 37 °C for 16hr with shaking.

Ten µl of subtilisin carlsberg (10mg.ml^{-1} in 0.1M NH_4HCO_3) was added and incubated at 32°C for 16hr with shaking. The subtilisin digestion was repeated twice more. A

protein assay was perfomed at this point to give an indication of protein concentration for subsequent calculations. 10µl of pronase (15mg.ml^{-1} in 0.1M NH_4HCO_3) was added and incubated at 32°C for 16hr with shaking. This digestion was repeated once. Proteases were inactivated by heating to 100°C for 15min. Magnesium chloride was added to a final concentration of 5mM. Leucine aminopeptidase was activated by mixing the following components and incubating for 3hr at 37°C: 10µl manganese chloride (50mM), 90µl Tris HCl pH8.0 (10mM), 100µl leucine aminopeptidase (22.75u). Prolidase was activated by mixing the following and incubating at 37°C for 3hr: 20µl of manganese chloride (50mM), 80µl of Tris HCl pH8.0 (10mM), 80µl of distilled water, 20µl of prolidase (38.6U). 90µl of the activated leucine aminopeptidase and 75µl of activated prolidase was added and incubated at 37°C for 16hr with shaking. The leucine aminopeptidase and prolidase digestions were repeated once more. The pH of the sample was adjusted to between 6.75 and 7.0 with HCl and 10µl of carboxypeptidase Y (20mgml^{-1}) was added and incubated at 30°C for 16hr with shaking. Samples were freeze dried and stored at -20°C.

Freeze dried samples of enzymatically digested hide samples were resuspended in 0.1M HCl and sonicated for 2min to aid dispersion. An aliquot (10-90µl) was loaded onto a Dionex DC-4A resin column 0.5cm x 20cm using a Pharmacia Alpha Plus amino acid analyser. The column was eluted with lithium citrate buffers (Pico buffer system IV) at a flow rate of 25ml per hour. The column was eluted with buffer A pH 2.9 for 30min at 40°C, buffer B pH 3.04 for 22min at 40°C, buffer C pH 2.95 for 23min at 40°C and 20min at 65°C, buffer D pH 3.39 for 30min at 65°C and buffer E pH 3.44 for 60min at 65°C. Derivatisation was performed post column using o-phtalaldehyde and absorbance was measured at 450nm. Dipeptide was determined by addition of known amounts of ε–(γ-glutamyl)-lysine to the sample and comparing peak areas.

2.9 Hydroxyproline quantification to determine the resistance towards collagenase attack and acid hydrolysis.

Raw hide was processed following sodium hydroxide/sodium sulphide liming with subsequent acid sulfuric deliming. After three washes with water, the samples were frozen in liquid nitrogen, freeze dried for 72 h and finely grounded. 0.1 g of the hide powder were treated in 1ml 50 mM Tris HCl pH 7.4 0.5 $mg.ml^{-1}$ mTG for 2 hours at 37 °C in a Tris HCl buffer solution pH 7.4 (controls without mTG were also carried out for each sample). After the treatment, the samples were frozen in liquid nitrogen and freeze dried overnight. 50 mg of the samples were treated with 100 μg of collagenase from *Clostridium histolyticum* in 10 ml of Tris HCl buffer solution pH 7.4 5 mM $CaCl_2$. The collagenase digestion was carried out overnight at 37 °C. 5ml of the solution were hydrolysed overnight in 5ml of 6M HCl at 100 °C and Hyp content analysed by the Chloramine T method (Balint, 1977). The experiments that investigated the effect of mTG mediated crosslinking on the resistance towards acid hydrolysis were performed as previously. The only variation introduced with respect to the previous experiments was that the hydroxyproline content was determined by a commercial method for aminoacid analysis by GC (Gas Chromatography Mass Spectrometry). (EZ:faast kit from Phenomenex.)

2.10 Analysis of mTG penetration into bated hide

Microbial transglutaminase ($1mg.ml^{-1}$) was dialysed against 0.1M $NaHCO_3$ pH 9 overnight at 4°C. After dialysis, a solution of $1mgml^{-1}$ biotinamidocaproate N-hydroxysuccinimide ester in dimethylsulphoxide (DMSO) was added to each enzyme solution in the ratio 1:8. Labelling reactions were incubated overnight at 4°C with gentle agitation. The reactions were then dialysed against 50mM Tris HCl, 1mM EDTA pH7.5 overnight at 4°C to remove unincorporated label and DMSO. Labelled proteins were analysed by SDS-PAGE and a blotted gel was used to assess the biotin

incorporation by reacting with an avidin-peroxidase conjugate and staining with 3,3' diaminobenzidine (SigmaFast DAB peroxidase substrate).

2.10.1 Uptake of mTG by bated hide

Reaction mixes for the rehydration of the freeze-dried samples were prepared on ice as follows. 50mM Tris HCl pH 7.5, 0.5mg.ml^{-1} biotinylated mTG. Reaction mixes (2ml per hide section) were added to samples of freeze-dried hide and shaken vigorously on a platform orbital shaker at room temperature for 2 hour to allow uptake. The samples were then incubated with shaking for a further two hours at 37°C. A companion experiment was set up identically except that native enzymes (i.e. not biotinylated) were used and 0.5mM biotin cadaverine was added to the reaction mixtures prior to soaking. Incorporation of BTC by mTGs would then occur at the limits of penetration of the enzymes and would also confirm that enzyme activity was maintained within the hide.

The biotinylated enzyme treated hide samples were fixed with 4% (w/v) neutral buffered formalin to ensure that the biotinylated mTG could not diffuse during subsequent processing. Samples treated with enzyme and biotin cadaverine were washed overnight at 4°C in 50mM Tris HCl pH 7.5, 2mM EDTA, with shaking, to ensure removal of unincorporated biotin cadaverine. Samples were then frozen and 20μm sections were cut using a cryostat.

2.10.2 Detection of biotin in hide sections

Sections were washed in phosphate buffered saline (PBS) to remove cryostat mounting medium and salts. The sections were incubated in blocking solution (1% bovine serum albumin in PBS) for 60 min at 37°C and washed again with PBS. Cy5-streptavidin conjugate (2μg.ml^{-1} in blocking solution) was added to each section and incubated overnight at 4°C. Sections were washed 3 times with PBS and mounted using a fluorescence mounting medium. The samples were examined by fluorescent confocal

microscopy using laser excitation at 633nm and detection of emitted light through a 665nm long pass filter.

2.11 Isometric tension measurements

Samples of freeze dried bated hide were cut in 40x3 mm^2 square strips. The samples were hold under constant tension in the isometric tension apparatus, equilibrated in a water bath at 35 °C for 5 minutes, the samples were heated up to 70 °C at a heating rate of 1 $°C.min^{-1}$ and the tension generated recorded.

2.12 Tensile strength testing of leather

Pieces of hide (10x10 cm), processed to the bated stage (as shown in Table VI) were treated with 200 ml of a solution 0.5 $mg.ml^{-1}$ mTG in 50 mM Tris HCl pH 7.4. The sample were left to stand for 16 hours at 4 °C in static conditions and 2 hours at 37 °C with constant agitation in a rotating drum. Pieces of hide were then further processed to finished leather by washing with water, pickling, chrome tannage (as shown in Table V) or glutaraldehyde tanned (as shown in Table VII), dyeing and fatliquoring. Samples were then toggled, air dried, cutted from the butt area (backbone direction and perpendicular to the backbone direction) conditioned for 48 hours at 20 °C and 60% relative humidity and the tensile strength recorded automatically using the Instron 4301 apparatus with a custom analysis program.

Table VI: Beamhouse[i] protocol

Operation	Chemical	Quantity (% w/w)	T (^{0}C)	Time (h)	Observations
A. Soak	Tap water	200	20	0.2	
B. Drain					
C. Main soak	Tap water	300	20	3	Hide was fleshed after soaking
D. Drain					
E. Unhairing	Na_2S	1	20	0.5	
	NaOH	1.14		2	
	Na_2S	0.5		16	
F. Drain					
G. Wash	Tap water	200	20	0.5	2 washes of 15 minutes
H. Deliming	H_2SO_4	1	20	5	In 4 additions diluted 10% w/w over the first two hours. pH 8-8.5 cross-section clear to phenolphtalein
I. Drain					
J. Wash	Tap water	200	20	0.5	2 washes of 15 minutes
K. Bating	Tap water Pancreol Bate 10K	100 0.1	37	1 hour	
L. Drain					
M. Wash	Tap water	200	20	0.5	2 washes of 15 minutes

[i] The beamhouse protocol includes all the operations carried out prior to tanning. Further details of the different steps have been described in the Introduction

Table VII. Glutaraldehyde tanning protocol

Procedure	(w/w%)	Temp(° C)	Product	time	Notes
A. Wash	200	30	water	15	
	0.20	30	oxalic acid		to remove iron stains
B. Drain					
C. Wash	100	30	water		
D. Drain					
E. Tannage	100	30	water	60	
	2.5		derugan 3080 (33%)		
	2		sodium formate		
	1		Tanigan PAKN	15	check pH 4.8
F. Fatliquoring	2		cutapol TIS	30	
G. Wash	200	30	water	10	
H. Drain					
I. Wash	200	20	water	10	
J. Drain					
K. Retannage	80	35	water		
	6		leukotan 1084	20	
	8		tanigan RFS	30	
	6		trupotan MT	30	
L.Dyeing	3		luganil black NT	60	
	6		cutapol TIS	60	
	1	45	lipsol SQ/ LQ	120	
	100		water		
	1.5		formic acid (10%)	40	2x10 +20 pH 3.8
M. Drain					
N. Wash	200	45	water	30	
O. Drain					
P. Fatliquoring	200		water		
	6		cutapol TIS		
	2		lipsol SQ LQ		
	2		leukatan 1084		
Q. Top dyeing	1		luganil black NT	20	
	1		formic acid (10%)	40	2x10 + 20 pH3.4
R. Drain					
S. Wash	200	25	water	10	samm[ii] and toggling[iii]

[ii] Samm: removal of the excess of water by mechanical pressure
[iii] Toggling the leathers are stretched prior to air drying to maximise the area

2.13 Dye modification for incorporation into leather by mTG

2.13.1 Amino-acid protection

A solution of 6 amino hexanoic acid (4.792 g) in a mixture of dioxane (80 ml), water (40 ml) and 1 M sodium hydroxide (40 ml) was stirred and cooled in an ice-water bath. Di-t-butyl pyrocarbonate (9.037 g) was added and stirred at room temperature for 2 hours. The solution was concentrated in high vacuo to about 50 ml, cooled in an ice-water bath, covered with a layer of ethyl acetate (120 ml) and acidified with 1 M potassium hydrogen sulphate solution to pH 2.5. The aqueous phase was extracted with ethyl acetate (3x60 ml). The ethyl acetate extracts were pooled, washed with water (2 x 120 ml), dried overnight with anhydrous sodium sulphate and evaporated to dryness in vacuo. The residue was recrystallised with hexane (Vogel, 1989 (a)). A solution of the recrystallised product was analysed by ^{13}C NMR.

2.13.2 Coupling reaction

The reaction was carried out by dissolving 10 mmol of t-BOC amino acid and 10 mmol of dicyclohexyl carbodiimide (DCC) in 15 ml of dry dimethyl-formamide (DMF). The solution was allowed to stand under a N_2 atmosphere for 30 minutes (solution 1). 2.5 mmol of dye (Acid Blue 92) and 1 mmol of dimethyl amino-pyridine (DMAP) were dissolved in 20 ml of dry DMF, the solution stirred for 30 minutes and added slowly to solution 1. Stirring under a N_2 atmosphere was continued overnight at 80°C (Sakamoto *et al.*, 1994). The mixture gave rise to a precipitate (B), which was filtered and DMF from the liquid phase evaporated to dryness under high vacuum at 40°C (D). Thin Layer Chromatography (TLC) (70% butanol: 30% methanol was carried out on the starting material (A), the precipitate (B) and the dry residue (D).

D was dissolved in 50 ml of methanol, 30 g of silica added and the suspension dried under vacuum and loaded onto a chromatography column with silica gel. Column

chromatography was performed with 70% butanol: 30 % methanol (v/v) as eluent. Separation was assessed by TLC with silica gel (70% butanol: 30% methanol) and the plate sprayed with ninhydrin, the desired fractions pooled and chromatography repeated in order to increase the degree of purification. The purified fractions from the second column were evaporated under vacuum (in the text this will be subsequently referred as PD). ^{13}C NMR on PD was also carried out. The fractions from a subproduct of the reaction were also collected, pooled and dried under vacuum (in the text this will be subsequently referred as SP) and analysed by ^{13}C NMR.

2.13.3 Deprotection step

Five hundred mg of PD were dissolved in 20 mL of a mixture of trifluoroacetic acid/acetonitrile (30:70) at 0 °C and stirred continuously for 60 minutes. The solution was allowed to stand at room temperature for a further 60 minutes (Vogel, 1989). The reaction was monitored by TLC with silica gel 70% butanol: 30 % methanol (v/v). Once the tert-butyl group was removed, an excess of acetonitrile was added in order to precipitate the dye. The solid was washed with acetonitrile (2x10mL) and dried under vacuum at room temperature.

2.13.4 Dye incorporation into N-N' dimethylcasein by mTG

Dye incorporation was attempted at varying pH and temperature. The assay conditions were pH 5.5, 6.5 and 7.4 at 40, 50 and 60 °C respectively.

1mL stock solutions of PD (12 mM), N-N' dimethylcasein (25 $mg.ml^{-1}$) and mTG (1 $mg.ml^{-1}$) were made up in CH_3COOH/ $NaCH_3COO$ (85:15, 200 mM) pH 5.5, Na_2HPO_4/KH_2PO_4 (69:31, 100 mM) pH 6.5 and 50 mM Tris HCl pH 7.4. Ten µl of PD stock solution , 20 µL of N-N' dimethylcasein stock solutions and 10 µL of mTG stock solutions were transferred into 0.5 mL eppendorf tubes and the volume made up to 100 µL with the appropriate. Triplicates at each pH were made up as incorporation was attempted at 40, 50 and 60 °C. 1.2 mM, 5 $mg.ml^{-1}$ and 0.1 $mg.ml^{-1}$ were the final concentrations for PD, N-N' dimethylcasein and mTG respectively. The control

solutions were made up as described above but in the absence of mTG. Incorporation of PD into N,N' dimethylcasein was assessed qualitatively by TLC with silica gel 70% butanol: 30 % methanol (v/v)

Two inhibition studies with PD were carried out by assessing the effect of PD on mTG mediated [^{14}C] putrescine or fluorescein-cadaverine incorporation into N-N' dimethylcasein using a range of PD concentrations (0, 0.5, 1, 5 and 10 mM).
The effect of PD at varying concentrations (0, 0.5, 1, 5 and 10 mM) on mTG activity was carried out utilising the incorporation of [^{14}C]- putrescine into N-N'-Dimethyl casein based upon the method of Slaughter *et al.* (1992)

For the fluorescein cadaverine inhibition assay, a 96 well plate was coated with N,N'-dimethylcasein (250 µl of 10 mg/mL of N,N'-dimethylcasein in 100 mM Tris HCl pH 8.5) for 16 hours at 4 °C. Wells were then washed twice with PBS containing 0.05 % v/v Tween 80, and twice with distilled water. Wells were blocked with 250 µl of 100 mM Tris HCl pH 8.5, 3% w/v BSA and shaken for 30 minutes at room temperature. Plates were washed as described previously with an additional final wash with 100 mM Tris HCl pH 8.5,100 mM PD, 0.1 mg.ml^{-1} fluorescein-cadaverine and 1 mg.ml^{-1} mTG stock solutions were made up in 100 mM Tris HCl pH 8.5. To each plate was added 150 µl of fluorescein-cadaverine stock solution, 20 µl of mTG stock solution, x µl PD stock solution (depending on the PD concentration assayed) and the volume made up to 200 µl with Tris HCl pH 8.5. Controls with no mTG were also carried out. The wells were incubated at 37 °C for 15 minutes and the incorporation reaction was terminated by washing as described previously. To each well was added 200 µl of 100 mM Tris HCl pH 8.5 containing 1% (w/v) BSA and a 1:2000 dilution of monoclonal antifluorescein antibody. The plates were incubated for 2 hours at 37 °C and washed as above. To each well was added 200 µl of 100 mM Tris HCl pH 8.5 containing 1% BSA and a 1:3000 dilution of goat anti-mouse IgG horseradish peroxidase conjugate. The

plates were incubated for 2 hours at 37 °C and washed as above. Peroxidase activity was detected by the addition of 100 µl of 0.05 M phosphate citrate buffer, 0.014% (v/v) H_2O_2 pH 5.0 and 0.075 mg/mL 3,3',5,5'- tetramethylbenzidine. Colour development was terminated by the addition of 50 µl of 2.5 M H_2SO_4 and the absorbance read at 450 nm.

2.14 Uptake of mTG by crust leather

Raw hide was processed to the crust leather following the modified beamhouse protocol (shown in Table VI), pickled, chrome tanned and fatliquored as shown in Table VIII. Biotin incorporation in mTG and uptake of the enzyme, and assessment of uptake was carried out as described in 2.10.

Table VIII. Tanning protocol for the production of crust leather

Operation	Chemical	Quantity (%w/w) (respect soaked and fleshed hide)	T (^{0}C)	Time (h)	Observations
A.Pickling	Formic Acid (90% w/w)	1		1	Added diluted 1:10 with water (w/w)
	Sulphuric Acid (98 %w/w)	1		16	Added diluted 1:10 with water (w/w)
B.Chrome Tanning	Chrome Sulphate (33% basicity) Sodium chloride Tap water	8 6 100	20	4	After four hours check complete chrome penetration
Chrome Tanning	Tan Base	0.4	20-40	4	4 x 0.1 additions/ increasing temperature 5 ^{0}C every hour
C. Adjusting final pH to fix chrome	Sodium Hydrogen Carbonate			1	Check pH 3.8-4.2 shrinkage temperature above 100 ^{0}C
D. Drain					
E.Wash	Tap water	200	20	0.5	2 washes of 15 minutes
F. Drain			20	48	Left on the horse for 48 hours and split to 2 mm thickness, weigh samples
G. Neutralising	Sodium Hydrogen Carbonate Tap water	0.7 100	20	1	All the percentages based on split samples weight Check pH 5.5-6
H. Fatliquoring	Cutapol Tis	5	50	3	Emulsified in water at 50 ^{0}C (1:10)

2.15 Microbial transglutaminase mediated incorporation of keratin hydrolysate into freeze dried bovine bated hide and crust bovine leather

Freeze dried bated samples of approximately 1/4 of A4 (50 g) size were treated in a range of enzyme reaction 250 ml 50 mM Tris HCl pH 7.4 solutions, containing one or more of mTG, keratin or putrescine, at 4 °C for 16 hours. (0.1 $mgml^{-1}$ mTG, 2 mM putrescine and 20 $mgml^{-1}$ keratin hydrolysate). After wetting back, the treatment was continued for a further 2 hours at 37 °C to allow for enzyme incorporation of the keratin and/or putrescine into the leather matrix. The samples were then pickled, chrome tanned and fatliquored as shown in Table VIII. The dyeing protocol was carried out as shown in Table IX. The experiments with crust leather were undertaken identically but with the substrate produced as in Table VIII.

2.16 Dyeing

All the dyeing protocols with bovine crust leather were undertaken as shown in Table IX.

Table IX. Dyeing protocol for bovine crust leather

Operation	Chemical	Quantity (% w/w) (respect dry weight crust leather)	T (°C)	Time (h)	Observations
A.Retanning	Tanigan RFS	3	25	1	
	Mimosa	10	25	2	
B.Fatliquoring	Cutapol TIS	3	50	1	Emulsified in water 1:10
C. Dyeing	Acid Blue 92	5	50	2	Check dye penetration
D. Fixing	Formic Acid (90%)	2	50	Every 15 minutes	Added diluted 1:10 in water in 3x0.66% additions until pH 3.5-4
E. Washing	Water	3x200	25	Every 15	Samm and toggle

The dyeing protocols for wool on slink skins were carried out as shown in Table IX (both laboratory scale tannery trials and full scale).

Table X. Dyeing protocol for crust wool on slink leathers

Operation	Chemical	Quantity (% w/w) (respect dry weight crust leather)	T (°C)	Time (h)	Observations
A.Degreasing	ESM	5	50	1	
B. Wash	Water	1000	50	0.25	Cold refloat
C.Retanning	Tannesco HN	10	25	1	Emulsified in water 1:10
D.Drain and wash	Water	1000	25	0.25	
E.Neutralisation	Water Ammonia (35%)	1000 1	25	1	Added diluted 1:10 in water until pH 8-8.5
F.Auxiliaries	Sellasol NG Sellasol TD	1	25	0.5	
Auxiliaries	Neutrasol IW	3	25	0.2	
G. Dyeing	Sellaset Yellow H	1.194	25	1.5	Check penetration and colour
	Sellaset Red H	0.512			
	Sellaset Brown H	1.416			
H.Fixing	Formic Acid (90%)	1	25	20 minutes apart additions until pH 3.5-4 diluted 1:10 in water	
I. Wash	Water	1000	25	Several washes until clear, samm and toggle	

2.17 Colour measurements

2x3 cm^2 samples of dyed leather were cut and the depth of shade of the grain and or flesh side was measured as lightness of the leather (L) using the spectrophotometer MS 2020 from Instrumental Colour Systems with a custom analysis program

2.18 Fastness to perspiration solution measurements

2x3 cm^2 samples of dyed leather were left to stand for two hours at 37 °C in 3ml perspiration solution (pH 8 5mg.ml^{-1} sodium chloride, 5mg.ml^{-1}tris (hydroxymethyl) aminomethane, 0.5 mg.ml^{-1} urea and 0.5mg.ml^{-1} nitrilotriacetic acid). The dye released due to the treatment with perspiration solution was measured at the wavelength of the maximum of the spectra with a Pye Unicam SP8-100 ultraviolet spectrophotometer from Phillips.

2.19 Wash fastness measurements

2x3 cm^2 samples of dyed leather were left to stand for 30 minutes at 40 °C in 30 ml wash fastness solution (5mg.ml^{-1} SDS). The samples were air dried, and the lightness of the leather was measured prior to and after testing with the spectrophotometer MS 2020 from Instrumental Colour Systems with a custom analysis program. The resistance to wash fastness was expressed as the reduction in lightness due to treatment with the wash fastness solutions.

2.20 Wool resistance to abrasion

A circular test specimen was taken from the sheep leather sample, weighed and placed in the specimen holder with the wool facing downwards. The test conditions were 12 K Pa and 1000 cycles. Once the test was completed, the weight of the samples was measured again. The resistance of wool to abrasion was expressed as the relative lost in weight

2.21 Statistical Analysis

Statistical Analysis, ANOVA and t-test, were performed using Microsoft Excel from Windows 2000.

Chapter 3. Microbial Transglutaminase as tanning agent

3.1 Introduction

The objective of the work was to investigate the effect of microbial transglutaminase (mTG) mediated crosslinking on the physical properties of the hide. These include the hide hydrothermal stability measured by means of DSC, resistance towards collagenase attack, resistance to acid hydrolysis, tensile strength and isometric tension. Some of the data are compared with glutaraldehyde type crosslinking, as this is similar to that provided by mTG.

Initial work investigated the optimum conditions to apply mTG as tanning agent. To achieve this end four different routes were undertaken. Since the literature has reported that some of these residues undergo hydrolysis, e.g. asparagine, glutamine and arginine, due to the alkaline conditions encountered during liming (Higberger *et al.*, 1941; Warner, 1942; Bowes, 1949; Menderes *et al.*, 1999), the first route involved simulation of liming using insoluble Type I Collagen and middle splits of raw hide as a model substrate and real substrate, respectively, with subsequent measurement of the ammonia released. In order to overcome any possible issue of glutamine hydrolysis during liming operations, the second route was the design of an unhairing process which was functional at a neutral pH. This substrate should be more suitable for mTG mediated crosslinking. Although washing is commonly practised between the different steps of the process, the literature provides evidence that complete removal is not always attained. An example of this would be the emission of hydrogen sulphide from the deliming and pickling floats (Kendle, 1983). Therefore some of these chemicals may be carried over to a certain extent to subsequent steps of the process. Consequently, the third route investigated the effect caused by commonly applied chemicals during beamhouse on mTG activity (e.g. sodium sulphide, calcium chloride and ammonium sulphate and enzymatic preparations for bating). To ensure optimal cross-linking of collagen only, the ideal stage for mTG treatment should be after bating, since most of the non-collagenous proteins are removed at this stage (Heidemann, 1993 (c)). The fourth route quantified the amount of available glutamines by measuring the amount of [^{14}C] putrescine incorporated into bated hide by means of mTG .

With the process conditions optimised, work investigated the effect of mTG mediated crosslinking on the physical properties of the hide. Initial work used bated hide powder to circumvent any issue of enzyme penetration. The effect of mTG mediated crosslinking of bated hide on its shrinkage temperature as measured by DSC, resistance towards collagenase attack and acid hydrolysis were investigated.

Further work was undertaken with full substance bated hide and the effect of mTG mediated crosslinking on its physical properties was also evaluated. These included the shrinkage temperature and isometric tension. The samples were further processed to the dye crust stage following two different tannages (chromium and aldehyde) and the shrinkage temperature and the mechanical strength of the subsequent leathers were also evaluated.

3.2 Results

3.2.1 Investigation of glutamine and asparagine hydrolysis during liming

The degree of deamidation during liming was determined by incubating insoluble bovine Type I Collagen as model substrate at pH 11, 12 and 13 at 25°C for sixteen hours with subsequent measurements of ammonia released in solution as ammonium by cationic chromatography. Further work was undertaken with middle splits of mechanically unhaired soaked hide which underwent traditional liming and the concentration of ammonia liberated measured as previously.

Initial experiments determined the retention time for ammonium (see Figure 5) and the concentration range where the technique would give a linear response. Linearity was observed up to 1mM concentrations as shown in Figure 6.

The pH of the solution was adjusted with potassium hydroxide instead of sodium hydroxide, because the sodium is eluted before ammonium which may bring about overlapping of peaks between these two cations. Given the glutamine and asparagine content of bovine type I collagen, 3.57 and 3.80 % (w/w) respectively (Gustavson, 1993 (b)), it was expected that, if hydrolysis had taken place, 1 mg of collagen would give an ammonium concentration in solution within the range of the calibration curve. However, hydrolysis was not observed in any of the experiments (Table XI).

In order to determine if deamidation had taken place, or the levels of ammonia released were lower than the detector sensitivity, a second set of trials was carried out under the same pH conditions but using 50 mg of collagen. The ionic strength and the effect of Ca^{2+} and K^{+} was also considered. To achieve this end, a 100 mM KOH solution with the ionic strength adjusted to 0.5 with $CaCl_2$ was prepared. Since $Ca(OH)_2$ is insoluble the concentration of OH^- and Ca^{2+} should not be the one which had been theoretically adjusted. Measurement of the pH and the conductivity gave rise to pH 12.5 (against the theoretical pH 13) and 37 mS/cm (a neutral solution with the ionic strength 0.5 adjusted with $CaCl_2$ brings about a conductivity of 119mS/cm) indicative of $Ca(OH)_2$ precipitation. Consequently, the experiments with the ionic strength adjusted with KCl were performed by adjusting the pH to 12.5 with KOH and the conductivity to 37

mS/cm with KCl by interpolating this value in the graph shown in Figure 7. Incubation at pH 12.5 with the conductivity 37 mS/cm in the presence of Ca^{2+} over sixteen hours of insoluble Type I Collagen resulted in hydrolysis of the amides to such an extent that the concentration of the ammonium released in solution was 2.8 mM. Complete hydrolysis of asparagine and glutamine should bring about an ammonium concentration equal to 26.6 mM as calculated below.

$$\frac{50\,\text{mg of collagen} \times \frac{3.57\,\text{mg glutamine}}{100\,\text{mg collagen}} \times \frac{1\,\text{mmol glutamine}}{146\,\text{mg}} \times \frac{1\,\text{mmol } NH_4^+}{1\,\text{mmol glutamine}}}{0.001\,\text{L}} = 12.2$$

mM

$$\frac{50\,\text{mg of collagen} \times \frac{3.80\,\text{mg glutamine}}{100\,\text{mg collagen}} \times \frac{1\,\text{mmol glutamine}}{132\,\text{mg}} \times \frac{1\,\text{mmol } NH_4^+}{1\,\text{mmol glutamine}}}{0.001\,\text{L}} = 14.4$$

mM

Therefore, only 10.6% (100x(2.8/26.6)) of the amides appeared to undergo hydrolysis. Surprisingly, hydrolysis did not occur at the same pH and ionic strength conditions but in the presence of K^+ instead of Ca^{2+}, which would suggest that the reaction is favoured by Ca^{2+}. Assuming that freeze dried soaked raw hide has a 95% content of collagen (Bugby *et al.*, 1988) the complete hydrolysis of the collagen would result in a $[NH_4^+]$= 12.6 mM (25 mg in 1 ml). Thus, investigation of hydrolysis in liming medium splits of soaked raw hide as a real substrate concluded that 27% of the amides were hydrolysed. To ensure that the effect of Ca^{2+} was not an artefact of the measurements, the amino acid glutamine was incubated in the same pH and ionic strength conditions (e.g. pH 12.5 and a ionic strength that would bring about 37 mS/cm conductivity) but adjusted with K^+ or Ca^{2+} as a model system. As shown in Figure 10, a significant reduction ($p<0.005$) of glutamine was observed in both cases. However, the reduction took place to a higher extent in the presence of Ca^{2+}. After 48 hours in the presence of Ca^{2+} nearly all the glutamine was hydrolysed, whilst with K^+ 50% of glutamine still remained intact.

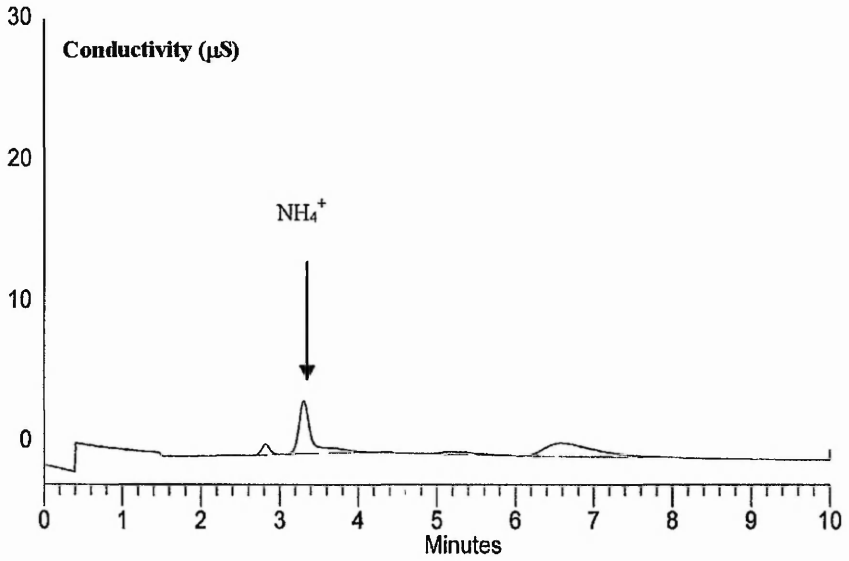


Figure 5. Chromatogram of 1 mM ammonium chloride solution.

The x axis represents the time and y the conductivity in µS. The retention time of ammonium was found to be 3.3 minutes.

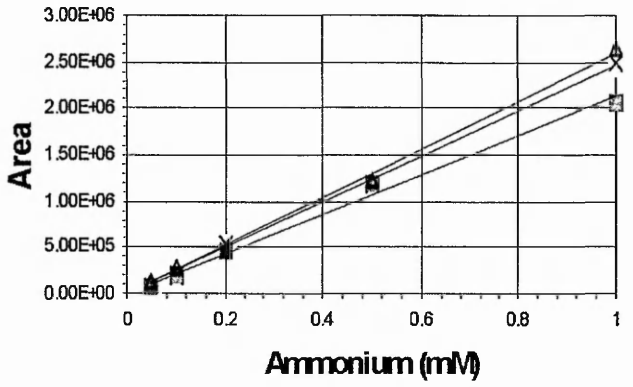


Figure 6. Calibration curve of ammonium chloride solutions.

The concentrations are displayed in the x-axis. The y-axis shows the area of the integrated peak. The chart displays three replicates of each standard (each standard was prepared in triplicate with one injection)

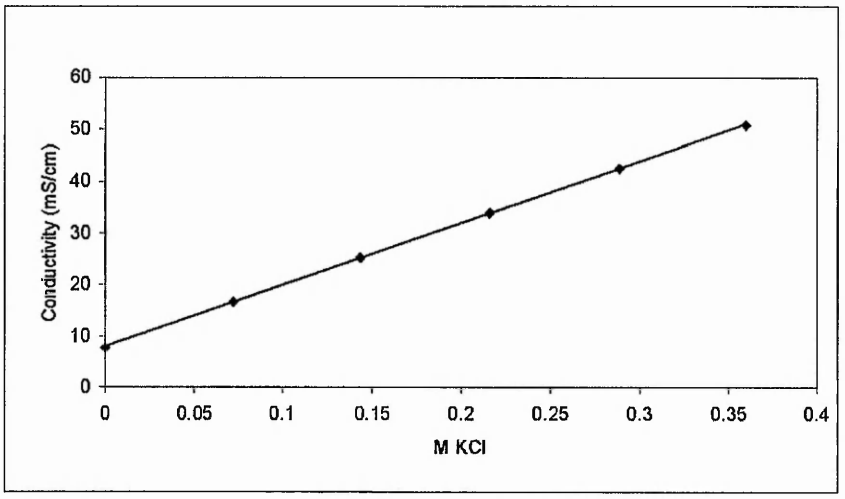


Figure 7. Relationship between conductivity and [KCl]

The x axis represents the concentration of calcium chloride in molarity, the y axis represents the conductivity in mS/cm. This experiment was undertaken to work in the pH and ionic strenght conditions achieved in liming.

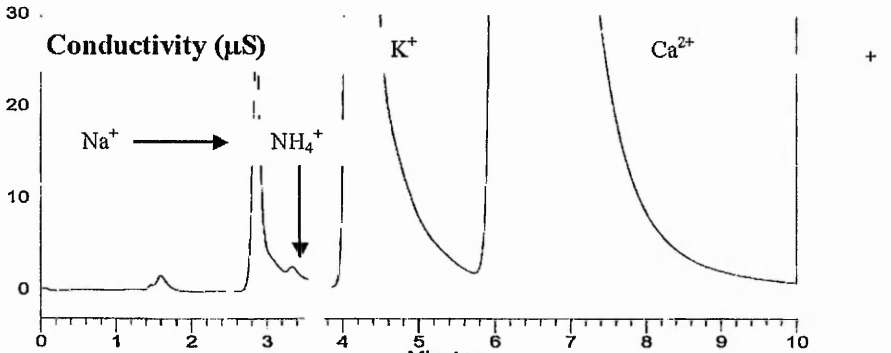


Figure 8. Ammonium released by collagen I due to liming conditions.

50 mg collagen incubated in 1ml of a solution at pH 12.5 with a conductivity of 37 mS/cm with Ca^{2+}. Retention times in minutes are displayed in the x axis. The y axis shows the conductivity in µS. As in the chromatogram from the standard solution (Figure 10) a peak can observed with the retention time of ammonium (3.3 min.). Standards were used to identify the other peaks.

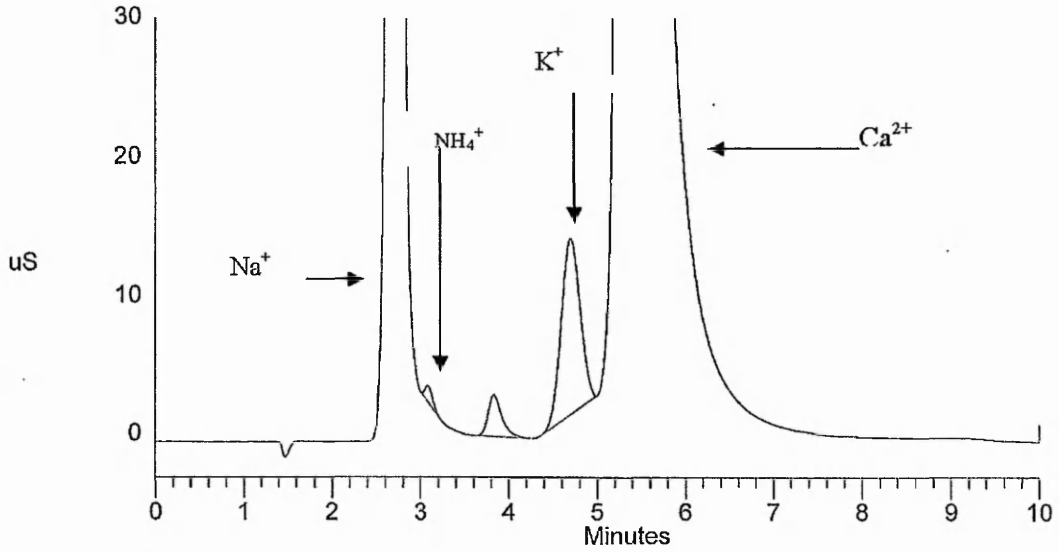


Figure 9. Ammonium released by raw hide due to liming conditions.

25 mg freeze dried soaked hide incubated in 2 mL of liming solution (600 mM Na_2S and 200 mM $Ca(OH)_2$). The y axis shows the conductivity in µS. As in the chromatogram from the standard solution (Figure 5) a peak can be observed with the retention time of ammonium (3.3 min.). Standards were used to identify the other peaks

Table XI. pH effect on amide hydrolysis. The results are the average of three replicates of the same experiment. The table shows the ammonia released in solution expressed as ammonium in the varying experiments carried out

Substrate	Concentration (mg/ml)	[KOH] mM	pH	Ionic Strength	$[NH_4^+]$ mM released
Insoluble Collagen Type I	1	1	11	Not adjusted	Undetected
Insoluble Collagen Type I	1	10	12	Not adjusted	Undetected
Insoluble Collagen Type I	1	100	13	Not adjusted	Undetected
Insoluble Collagen Type I	50	76 mM	12.5	To a conductivity 37 mS/cm with KCl	Undetected
Insoluble Collagen Type I	50	100 mM	12.5	To a conductivity of 37 mS/cm with $CaCl_2$	2.8 SE± 0.1
Middle splits of raw hide	25	600 mM Na_2S 200 mM $Ca(OH)_2$	12.5	Not adjusted	3.4 SE±0.3

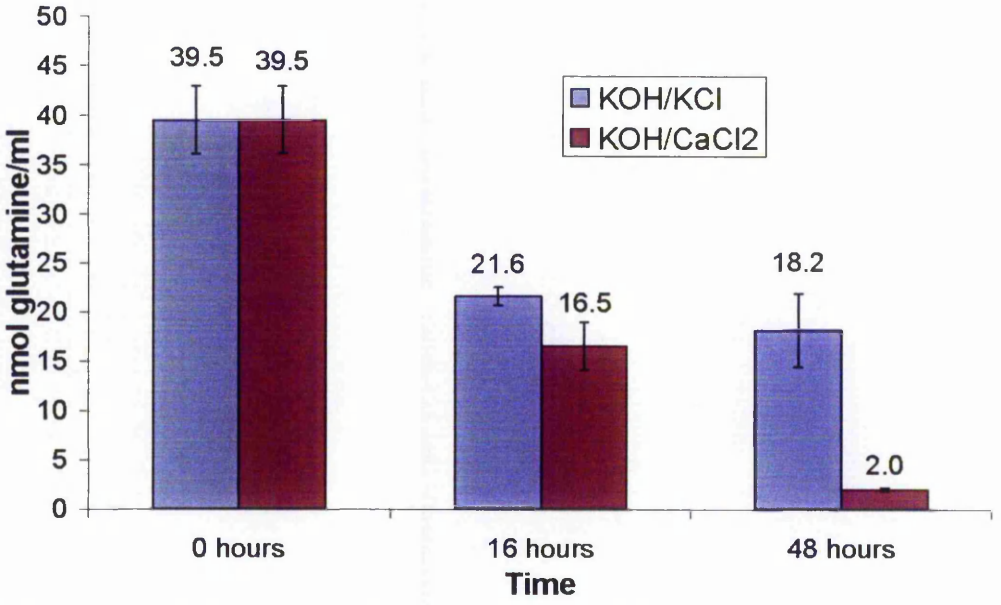


Figure 10. Hydrolysis of glutamine due to alkaline conditions at pH 12.5 and conductivity 37.5 mS/cm adjusted with KCl or $CaCl_2$ (n=3). The results are the average of three replicates of the same experiment. The error bars show the standard error. The presence of calcium resulted in a significant reduction of the amount of glutamine ($p<0.005$), e.g. 21. vs 16.5 nmol glutamine/ml at 16 hours and 18.2 vs 2.0 nmol glutamine/ml at 48 hours

3.2.2 Design of an alternative unhairing process

The first experiments were designed with a view to determining the minimum concentration of Dispase which would enable complete hair removal as well as the effect of swelling on unhairing. As already discussed in the introduction, the osmotic swelling that takes place during liming facilitates the opening-up of the hide structure. However, apart from the osmotic swelling the literature also refers to the lyotropic swelling. This is defined as the break down of the hydrogen bonding network within the collagen structure by lyotropic reagents (Braybrooks *et al.*, 1939). The literature also reports the lyotropic properties of varying ions. Ions that have the greatest such effect (exhibiting weaker interactions with water than water itself) are known as structure-breakers or lyotropes, whereas ions having the opposite effect are known as structure-makers or kosmotropes (exhibiting strong interactions with water molecules) (Lo Nostro, 2002). The cation calcium has good lyotropic properties and play an important role on the structural disruption that occurs during liming (e.g. Ca^{2+} is more lyotropic than Na^{+} which results in a higher disruption of the hide structure) (Mellon *et al.*, 1960). The literature also reports unhairing technologies based on combination of proteolytic enzymes and lyotropic reagents (Frendrup, 2000). Urea was the choice of lyotropic reagents with an initial concentration 200 mM (which corresponds to theoretical amount of Ca^{2+} in a liming float). The results shown in Table XII demonstrate that 0.5 unit/ml is the minimum concentration of Dispase. Experiments A,B,C investigated varying enzyme concentration whilst maintaining the urea and swelling constant. Experiment A, was rated 3, whereas B (0.5 unit/mL) and C (1 unit/mL) marked with 5. Swelling improved hair removal, since hair removal efficiency of samples that underwent swelling was rated in all of the cases as one degree higher than not swollen samples (Experiment A rated 3 versus D rated 2, Experiment B rated 5 versus E rated 4 and Experiment C rated 5 versus F rated 4). The outcome of the first trials demonstrated that the combination of Dispase/Urea and salt free pickling enabled for complete hair removal. However, the grain of the samples was damaged to such an extent that the process was not viable for full grain leather production. The subsequent set of trials investigated the effect of urea on the process. The results shown in Table

XII suggest that urea does not affect the unhairing efficiency of the process, as the same degree of unhairing can be seen in reduced urea concentrations, e.g. sample K [urea] 200 mM rated 5, sample J [urea] 150 rated 4, sample I [urea] 100 rated 5. Furthermore, the scanning electronic microscopy (SEM) micro-graphs showed in Figure 11 provide evidence that the extent of grain damage correlates well with increasing urea concentrations, as more severe damage is observed with higher urea concentrations, e.g little damage observed in the sample G which did not undergo urea treatment, to extensive damage in sample I where the urea concentration was 100 mM, to complete grain removal in sample K where urea concentration was 200 mM. Although little damage was observed in the sample that was only treated with Dispase, the purity of the preparation used was assessed by SDS- PAGE, as damage might be associated with other proteases present in the preparation applied. As shown in Figure 12, SDS page confirmed the presence of only a protein with a molecular weight of 65KDa, the molecular weight of Dispase (Takekawa *et al.*, 1991)

Since removal of non collagen proteins is one of the objectives of liming, this was investigated during these trials. Protein removal was assessed by measuring the total protein concentration in the float at different time intervals during both the urea and pickling stage of samples that had been previously treated with 0.5 unit/g-hide of Dispase. The results are displayed in Figure 13 and Figure 14.

Figure 13 shows that higher urea concentrations enables for more efficient protein removal. Although such levels of urea alone should not result in solubilisation of proteins (Gustavson, 1960) it appears plausible to suggest that a synergistic effect between Dispase and urea is occurring, thus the mechanism of solubilisation would be via urea mediated disruption of proteins which had been previously attacked by Dispase as reported in the literature (Campbell *et al.*, 1973). From Figure 14 it can mainly be concluded that protein is still removed during the pickling step and some residual urea in the samples or the urea mediated disruption in the previous step is governing the process.

Table XII. Results on Dispase/Urea Unhairing. The results are a subjective measurement in scale 1-5 units, where 5 is equal to complete hair removal

Trial	Dispase (units/mL)	Urea (mM)	Swelling	Rate
A	0.1	200	✔	3
B	0.5	200	✔	5
C	1	200	✔	5
D	0.1	200	✘	2
E	0.5	200	✘	4
F	1	200	✘	4
G	0.5	0	✔	4
H	0.5	50	✔	5
I	0.5	100	✔	5
J	0.5	150	✔	5
K	0.5	200	✔	5

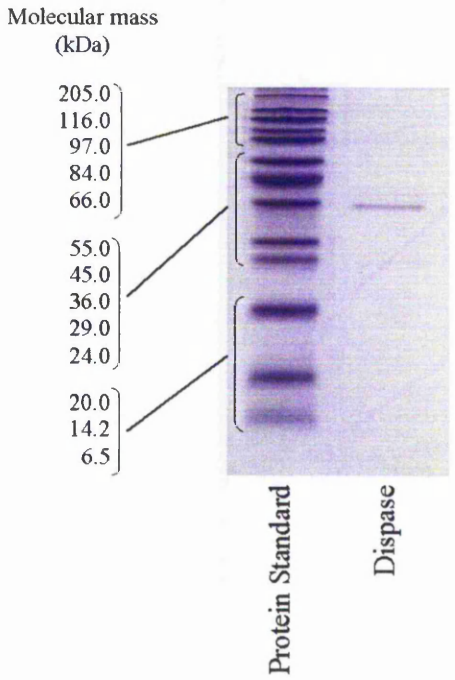
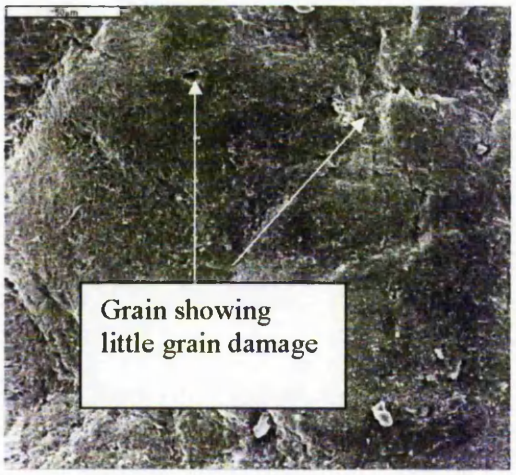
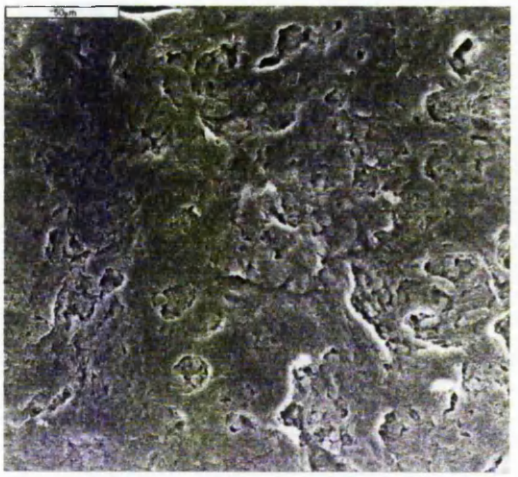


Figure 11. SDS page of Dispase

The panel shows the molecular weight of Dispase. On the left side of the SDS page displays the molecular weight of the varying standards. On the ride side, it appears a single band with a molecular weight of approximately 65 kDa which corresponds to the molecular weight of Dispase. Other bands are not observed confirming the purity of the enzyme and therefore eliminating the possibility that the grain damage was not associated with other proteases that might be present the enzyme preparation applied.

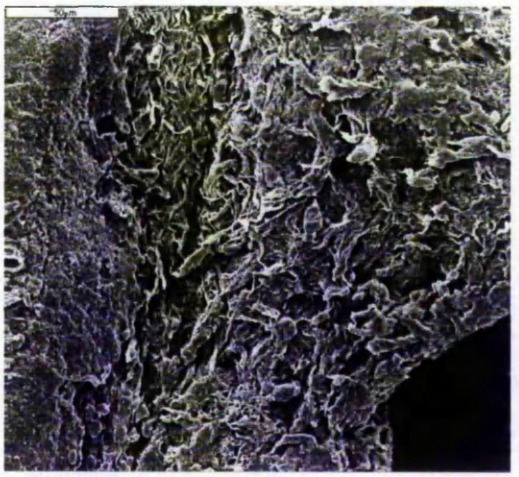


G Grain of sample G x600

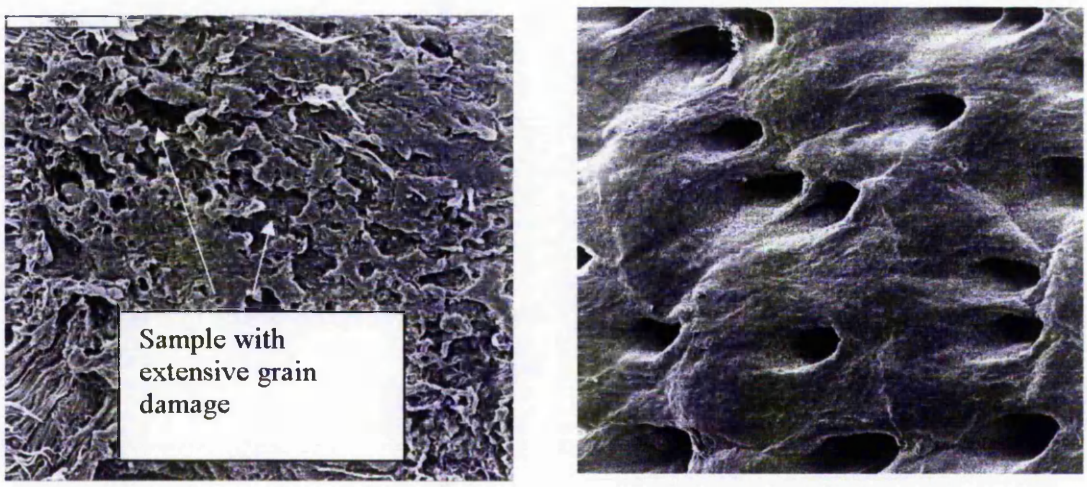


H Grain of sample Hx600

I Grain of sample Ix250



J Grain of sample Jx600



K Grain of sample K x600 **L Example of sample without grain damage**

Figure 12. Appearance of the grain of the samples that underwent dispase/urea and pickling with swelling.

The panel illustrates the grain damage observed in the samples with varying urea concentrations. The micrograph labelled as L is an example of the appearance of a sample of leather without grain damage which had been limed using the traditional treatment with sodium sulphide and lime. As shown in the micrographs, increasing the urea concentration resulted in more severe damage (G 0 M urea, H 0.05 M urea, I 0.1 M urea, J 0.15 M urea and K 0.2 M urea)

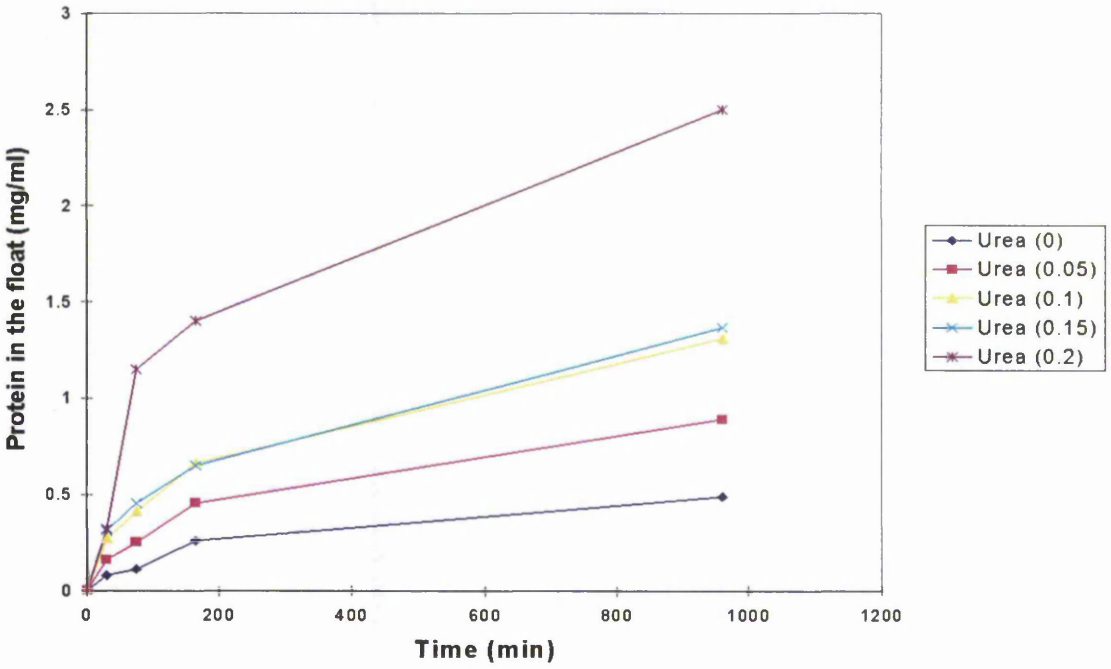


Figure 13. Protein removal assessment during the urea treatment.

The variable of the x axis shows the intervals of time in which protein analysis of the float were performed. The variable of the ordinate axis shows the protein concentration in the float expressed as mg protein/ml-float. at those times. The experiments were carried out at varying urea concentrations. The urea concentration is expressed as molarity

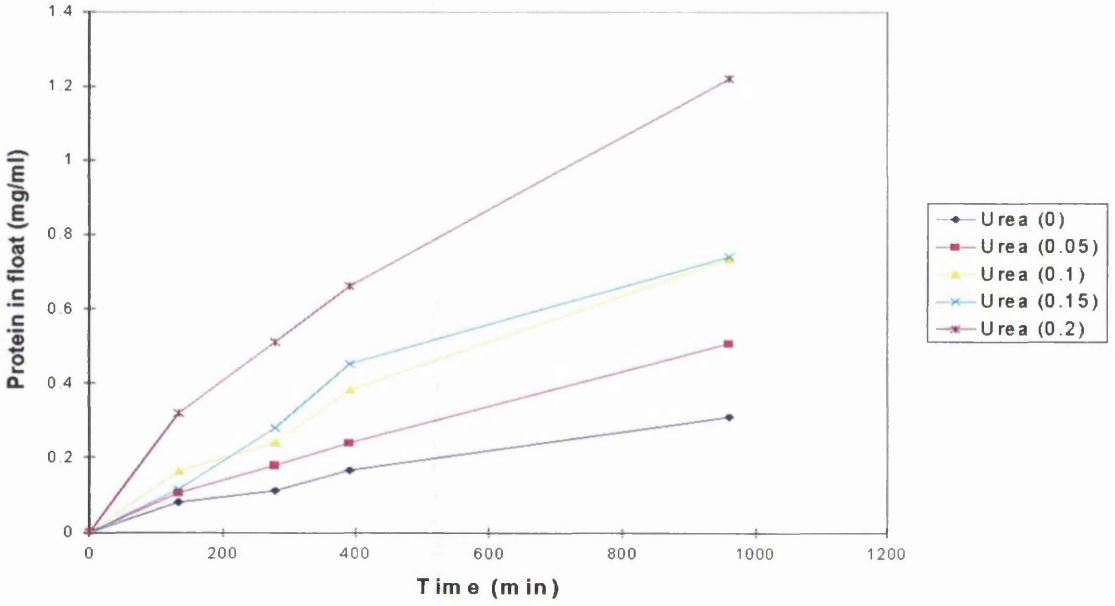


Figure 14. Protein removal assessment during pickling with swelling.

The variable of the x axis shows the intervals of time in which protein analysis of the float were performed. The y axis shows the protein concentration in the float expressed as mg protein/ml-float.at those times. Each curve relates to the previous urea treatment. The urea concentration is expressed as molarity.

3.2.3 Effect of the chemicals applied in the early stages of leather processing and [^{14}C] putrescine incorporation into bated hide

Previous to the crosslinking of leather, the effect of the chemicals applied in the early stages of leather processing on the activity of mTG was investigated. To achieve this end, varying mTG activity assays were performed in the presence of the biocide Myacide As, sodium sulphide, calcium chloride, ammonium sulphate and Pancreol 10K (a enzyme preparation used for bating). The concentrations applied were up to the standard concentrations utilised during traditional beamhouse processing., 0.003% (w/w) Myacide As did not inhibit the activity of mTG. Na_2S reduced the specific activity of mTG to 24% at 2.4% (w/w) Na_2S, the concentration used for the liming process. $CaCl_2$ reduced the specific activity of mTG by 57% at concentrations higher than 50mM. Ammonium ions inhibited mTG, removing all activity at concentrations higher than 1% (w/w) $(NH_4)_2SO_4$. Pancreol Bate 10K produced a very slight reduction in specific activity of mTG at 1mgml^{-1}, the concentration used during bating. This work was carried out by Nottingham Trent University.

Since calcium chloride and ammonium sulphate used during liming and deliming inhibited mTG activity, the processing of hide to the bated stage was modified. Lime was substituted by sodium hydroxide, and deliming was done with sulphuric acid instead of ammonium sulphate.

The quantitative measurement of available glutamines for mTG mediated crosslinking in bovine hide was undertaken via the incorporation of [^{14}C]-putrescine into bated hide (modified liming process, traditional liming and after Dispase unhairing) powder. The incorporation of [^{14}-C] was done by Nottingham Trent University.

Hide powder was the substrate in order to eliminate any variables resulting from access of the enzyme to the hide substrate. As shown in Figure 15, the maximum amount of [^{14}C]-putrescine that could be incorporated into bated bovine hide by 100μgml^{-1} mTG in 4 h was 3.4 nmol putrescine/mg, which represents 0.34 glutamine residues per collagen monomer (100kDa) with hide which had undergone the modified liming process (e.g. NaOH instead of $Ca(OH)_2$ in liming plus a deliming with H_2SO_4).

However, bated hide which had undergone traditional liming, could only incorporate 2.7 nmol putrescine mg^{-1} in 4 h at a concentration of 100μgml^{-1}, which equates to 0.27 glutamine residues per collagen monomer (100kDa) (Collighan *et al.*, 2004). This would match with what has been reported on the effect of chemicals applied in the early stages of the process on the activity of microbial mTG, since Ca^{2+} and $(NH_4)_2SO_4$ showed the ability to inhibit mTG activity. The other possible explanation why traditional liming resulted in lower [^{14}C]-putrescine incorporation might be that there would be less available glutamines since higher amide hydrolysis was observed in traditional liming as discussed previously. In fact the differences between the degree of hydrolysis with KOH/KCl or KOH/$CaCl_2$ liming after 16 hours is 5.1 nmol/ml as shown in Figure 10 which is about 24% very close to 22% reduction observed in the experiment where [^{14}C]-putrescine was incorporated in NaOH/Na_2S and $Ca(OH)_2$/Na_2S limed bated hide. Surprisingly, [^{14}C]-putrescine incorporation was further reduced 5 fold in bated hide which had undergone Dispase unhairing which would suggest that residual Dispase inhibits mTG or Dispase cleaves peptide fragment which are glutamine rich with the consequent reduction of available sites for putrescine to be incorporated.

Since lower [^{14}C]- putrescine incorporation was observed for bated hide powder which had been processed by means of Dispase or $Ca(OH)_2$/Na_2S liming with subsequent $(NH_4)_2SO_4$ deliming their use in the project was discontinued.

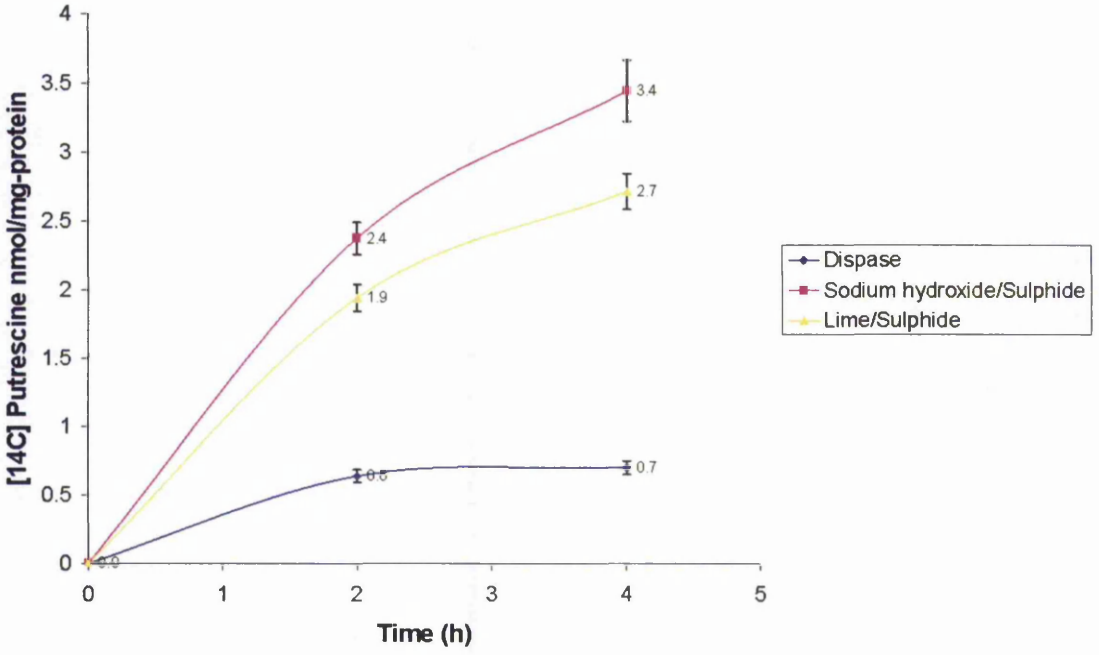


Figure 15. ^{14}C Putrescine incorporation into bated hide by mTG.

The x-axis represents the time intervals where putrescine incorporation was assessed. The y-axis the amount of ^{14}C putrescine incorporated in nmol per mg of protein, it was assumed that all the hide was protein. The results are the average of three replicates of the same experiments. The error bars show the standard error.

3.2.4 Effect of crosslinking of bated bovine hide collagen by mTG on denaturation temperature

Having confirmed that hide collagen was a substrate for mTG in terms of glutamine, the ability of mTG to incorporate ε(γ-glutamyl)lysine crosslinks was investigated. Using powdered bated bovine hide as the substrate, mTG mediated crosslinks were quantified by exhaustive proteolytic digestion followed by cation exchange chromatography. The results showed that mTG, when using bovine bated hide powder prepared using the modified beamhouse process (Na_2S/NaOH liming and H_2SO_4 deliming), large amounts of ε(γ-glutamyl)lysine crosslink were incorporated. The maximum number of ε(γ-glutamyl)lysine crosslinks that could be incorporated into bated bovine hide collagen by 0.5mgml^{-1} of mTG was 4 crosslinks per mg of hide. This was increased to 9 crosslinks per mg of hide when casein was applied as a crosslinking facilitator (Table XIII). The cross-linking analysis was undertaken by Nottingham Trent University. However, introduction of such levels of crosslinking did not bring about any change in the denaturation temperature of collagen as measured by DSC. To ascertain why such level of crosslinking did not modify the hide shrinkage temperature, this was compared with that provided by glutaraldehyde. The amount of glutaraldehyde crosslinking was determined by measuring the free lysines after 6M HCl hydrolysis by Gas Chromatography Flame Ionisation Detection (GC FID). It was assumed that the glutaraldehyde crosslinking was stable to acid hydrolysis and that the loss of two free lysines was equivalent to one glutaraldehyde crosslink. Table XIV demonstrates that mTG treatment results in a much lower degree of crosslinking than glutaraldehyde (10 fold). Although introduction of casein as crosslinking facilitator increases the degree of crosslinking up to 9 nmol of crosslink per mg of protein this is still 5 fold lower than glutaraldehyde mediated crosslinking and still not sufficient to attain the tanning effect. In the light of this result, it was decided to assess the use of alternative treatments in conjunction with mTG to facilitate crosslinking. Bated hide was sectioned (20 μm) and treated to remove all non-collagenous material by washing with Triton X-100, reduction with sodium sulphite, and chondrotinase ABC and hyaluronidase digestion. Linkers were used to determine whether crosslinking could be enhanced in this way. These were poly-L-lysine, gelatin, and the aliphatic diaminoalkanes 1,6-

diaminohexane, 1,7-diaminoheptane, 1,8-diaminooctane and 1,12-diaminododecane. Again no differences in the denaturation temperatures were observed as shown in Table XIV.

3.2.5 Effect of crosslinking of bated bovine hide collagen by mTG on resistance towards microbial collagenase

The results displayed in Figure 16 provide evidence that mTG mediated crosslinking results in a hide more resistant towards microbial collagenase. However, the increase of resistance towards microbial collagenase is much higher in the samples that were crosslinked with glutaraldehyde (6.8 fold,) which matches with the fact that glutaraldehyde brings about a higher degree of crosslinking.

3.2.6 Effect of crosslinking of bated bovine hide collagen by mTG on resistance towards acid hydrolysis

The effect of mTG mediated crosslinking on the resistance to acid hydrolysis was the last parameter investigated. Raw hide was processed following the modified beamhouse process ($NaOH/Na_2S$ with subsequent H_2SO_4 deliming) freeze dried and treated with mTG. The resistance to acid hydrolysis was determined as described in material and methods.

Samples which had undergone glutaraldehyde mediated crosslinking were treated in the same manner. Whilst acid treatment did not bring about significant swelling in the glutaraldehyde treated samples, this was observed in the untreated controls and mTG mediated crosslinked samples. Since, other than the denaturation temperature of hide as measured by DSC, any chemical that brings about a decrease of swelling under acidic conditions will be considered to have some tanning ability (Heidemann, a 1993). This would be again indicative that the degree or the type of crosslinking provided by mTG was not sufficient to attain the tanning effect. The swelling observed also made it difficult to take the aliquot for Hyp measurements. Consequently, the experiments were repeated but in 0.075 M HCl and 5% w/w NaCl which would eliminate swelling facilitating thus the Hyp measurements. As shown in Figure 17, although a significant increase ($p<0.005$) to acid hydrolysis was observed for the mTG sample (the [Hyp] due

to acid hydrolysis was 680 mmol/ml in the untreated control against 508 mmol/ml in the mTG treated sample), this was much lower than that observed in the glutaraldehyde sample (hydroxyproline was not detected).

Table XIII. Crosslinking effect on the denaturation temperature measured by DSC (the results are the value provided by a single measurement)

Sample	**Crosslink (nmol/mg-protein)**	**Denaturation Temperature (°C)**
Bated hide	0.25	60.3
Glutaraldehyde	44	78
mTG	4.7	59.2
casein	0.21	60.1
mTG + casein	9	59
poly L-lysine	Not determined	57.3
mTG + poly L-lysine	Not determined	58.9
gelatin	Not determined	59.9
mTG + gelatin	Not determined	58.7
diaminohexane	Not determined	59.0
mTG + diaminohexane	Not determined	59.6
diaminoheptane	Not determined	58.0
mTG + diaminoheptane	Not determined	59.4
diamino-octane	Not determined	59.1
mTG + diaminooctane	Not determined	59.2
diamino-dodecane	Not determined	58.8
mTG + diaminododecane	Not determined	59.6

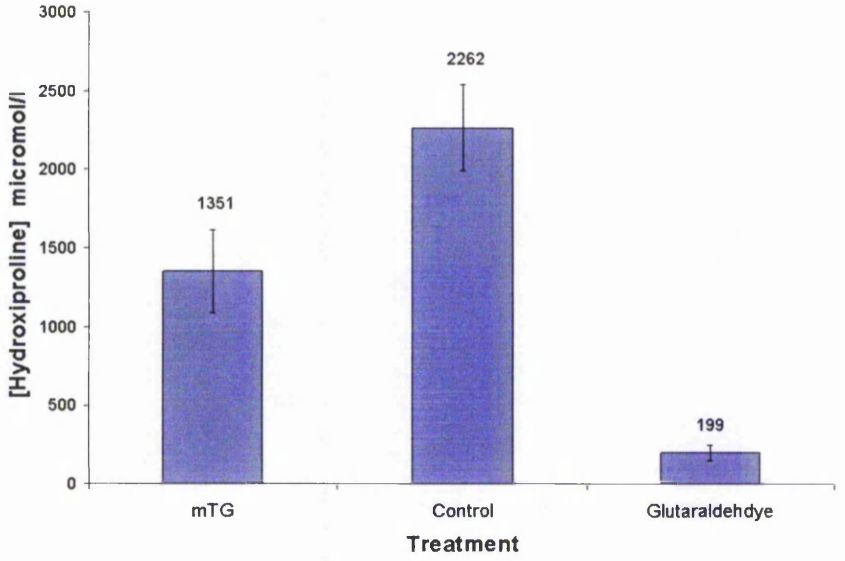


Figure 16. Microbial trasnglutaminase mediated crosslinking effect on resistance towards microbial collagenase. Higher values of hydroxyproline are indicative of less resistance towards microbial collagenase. The results show the average of three replicates of the same experiment, the error bars show the standard error. The results show a significant increase ($p<0.005$) to the resistance to microbial collagenase for both mTG and glutaraldehyde mediated crosslinked bated hide. However, the increase of resistance towards microbial collagenase is much higher in the samples that were crosslinked with glutaraldehyde (6.8 fold,) which matches with the fact that glutaraldehyde brings about a higher degree of crosslinking.

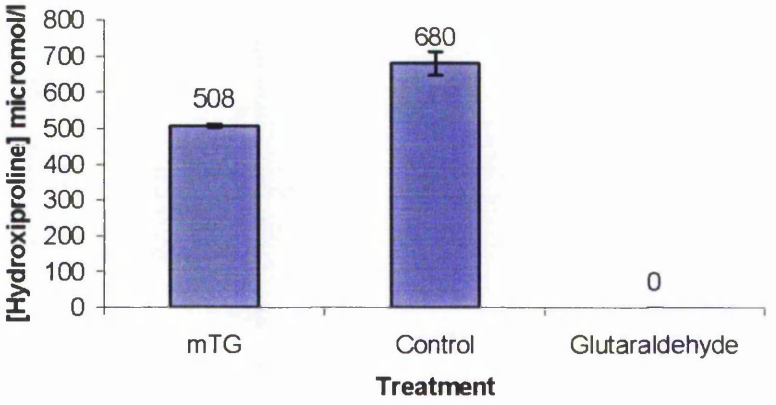


Figure 17. Microbial transglutaminase mediated crosslinking effect on resistance towards acid hydrolysis. Higher values of hydroxyproline indicate less resistance towards acid hydrolysis. The results show the average of three replicate of the same experiment. The error bars show the standard error. The results show a significant increase ($p<0.005$) to the resistance to acid hydrolysis for both mTG and glutaraldehyde mediated crosslinked bated hide. Although a significant increase ($p<0.005$) to acid hydrolysis was observed for the mTG sample (the [Hyp] due to acid hydrolysis was 680 mmol/ml in the untreated control against 508 mmol/ml in the mTG treated sample), this was much lower than that observed in the glutaraldehyde sample (hydroxyproline was not detected).

3.2.7 Effect of mTG mediated crosslinking of bated hide on the physical properties of the leather

Although mTG mediated crosslinking did not enhance the hydrothermal stability of collagen, further experiments were undertaken on a larger scale to determine if TG mediated crosslinking could bring about changes in other physical characteristics of the leather, such as the tensile strength.

It was predicted that problems of enzyme penetration into full substance bated hide could arise due to the size of the enzyme (35 kDa mTG) (Kashiwagi *et al.*, 2002). Penetration of the enzyme may be enhanced by freeze drying the hide prior to any enzyme treatment. The dried hide would behave as a sponge when rehydrated in an enzyme solution, and the enzyme up-take would be increased. The samples were freeze dried to allow for complete re-hydration of the sample when it is wet back. Penetration of the enzyme after freeze drying was assessed. Controls with wet bated hide were also carried out. As shown in Figure 18, mTG penetrated well throughout freeze dried hide (Figure 18 C)). However, in wet bated hide, mTG only penetrated to a depth of approximately 1mm through both surfaces of the section (Figure 18 B). This work was carried out exclusively by Nottingham Trent University.

After optimisation of mTG treatment, by substitution in the beamhouse process of $Ca(OH)_2$ and $(NH_4)_2SO_4$ with NaOH and H_2SO_4, respectively, to avoid mTG inhibition by Ca^{2+} and NH_4^+, and freeze drying of bated hide prior to treatment with mTG, the experiments were scaled up to 1/6 of A4 size samples of full substance, bated hide. It was possible that the distance between glutamine and lysine residues in the collagen quaternary structure was too long for the formation of intermolecular ε-(γ-glutamyl) lysine crosslinks. To overcome this possibility, the use of a crosslinking facilitator, such as the mTG substrate casein, to increase the degree of crosslinking between adjacent collagen triple helixes was investigated.

In order to investigate whether mTG crosslinking could affect the tensile strength and the shrinkage temperature of finished leather, bated bovine hide, prepared using the modified beamhouse process, was treated with mTG and further processed to finished

leather (chrome and glutaraldehyde tanned leather). As shown in Figure 19 and Figure 21 mTG mediated crosslinking did not modify the denaturation temperature of the subsequent leather. This was true for the two tanning reagents investigated. As previously found with powdered bated hide powder, although mTG shows the ability to incorporate 4 nmol-crosslink/mg-protein and this can be further increased to 9 nmol-crosslink/mg-protein in the presence of a crosslinking enhancer, the type or the degree of crosslinking incorporated is not sufficient to attain the tanning effect (Figure 20).

Figure 23 shows that treatment with mTG reduced the tensile strength by 34 per cent. A similar but slightly less pronounced effect was seen after crosslinking in the presence of 2 per cent casein where the tensile strength reduction with mTG was 27 per cent. The data shown in Figure 24, also demonstrates that treatment with mTG or mTG and casein also reduces the tensile strength by 13% and 14% respectively when the samples are subsequently tanned with glutaraldehyde.

As shown in Figure 22, a preliminary investigation on the effect of mTG mediated crosslinking of bated hide on the isometric tension generated during the shrinking transition of bated hide suggests that mTG mediated crosslinking changes how the tension is generated during the transition. Again indicative that crosslinking is occurring and modifies to a certain extent the physical properties of the hide. However, it would be too simplistic a further detailed analysis of data provided by duplicates of a single experiment.

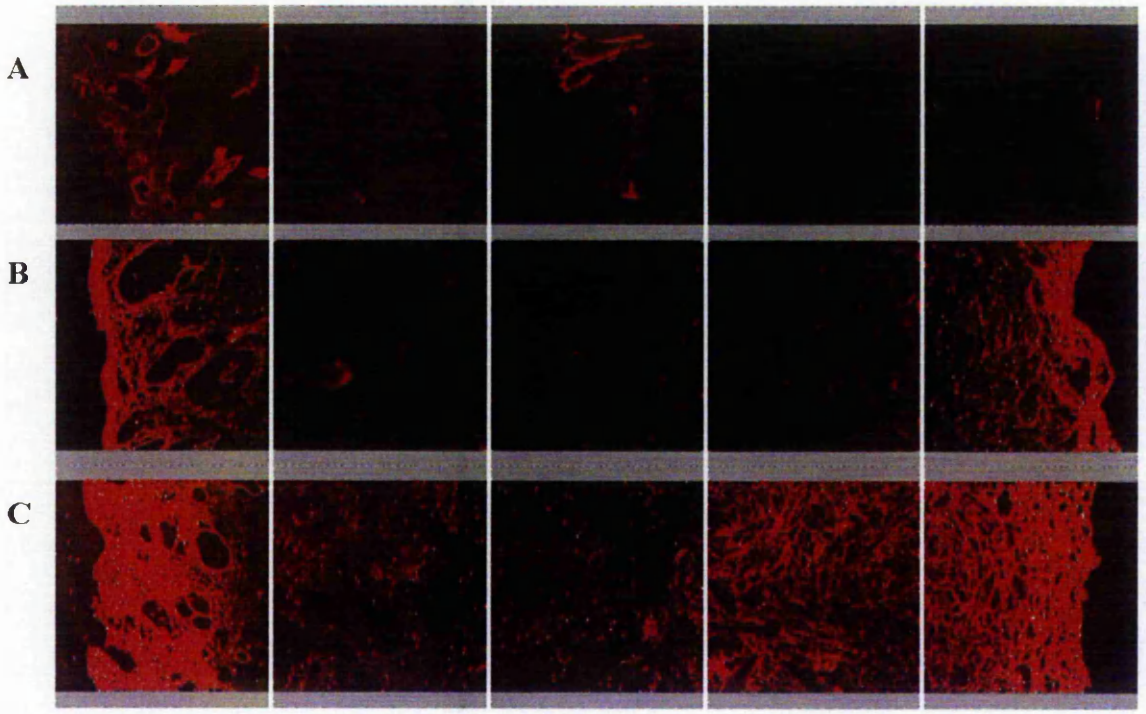


Figure 18. Assessment of the effect of freeze-drying on the penetration of mTG into bated bovine hide by confocal microscopy.

A) freeze dried bated control, B) wet bated + biotinylated mTG, 60 min soak, C) freeze dried bated hide + biotinylated mTG, 15 min soak. Confocal microscopy was done by Nottingham Trent University. The areas of the micrograph in red correspond to the part of the cross-section of the hide that was accessible to mTG. This is much more extensive in C (sample that previously freezed dried) when compared with B (longer time but withot freezed drying) and A (untreated control)

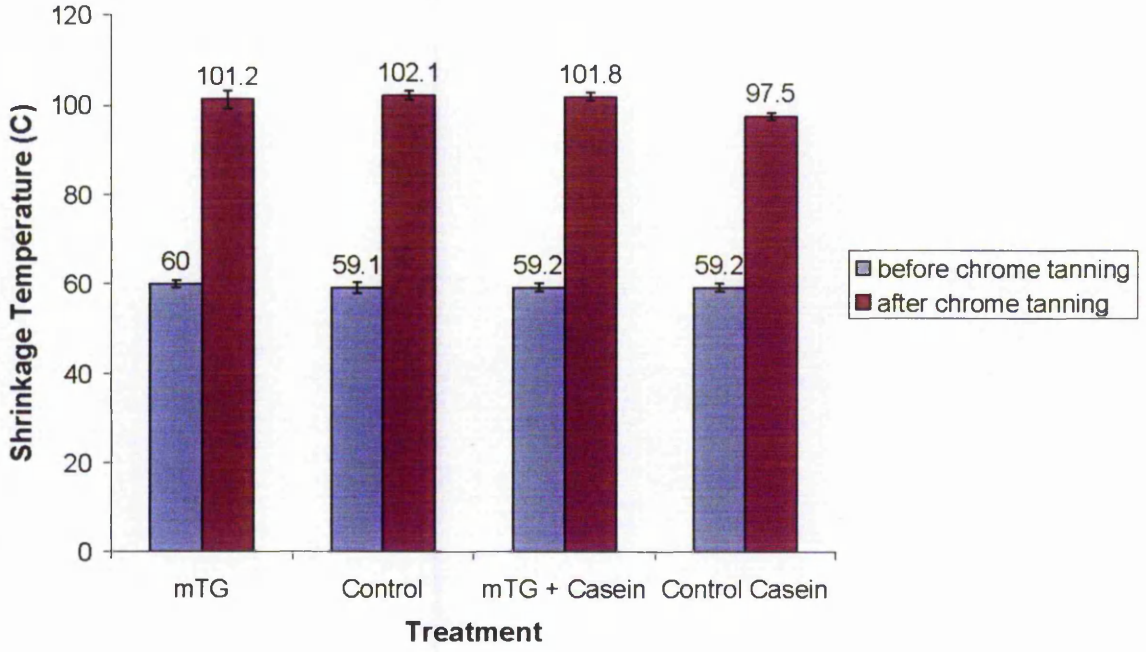


Figure 19. Hydrothermal stability of leather, treated with mTG, before and after subsequent chrome tanning.

The x-axis displays the varying treatments the y-axis the shrinkage temperature as measured by the onset value of DSC. The results are the average of three replicate of the same experiment. The y error bars show the standard error. Microbial transglutaminase alone or in conjunction with casein did not modify the shrinkage temeprature before tanning and after chrome tanning.

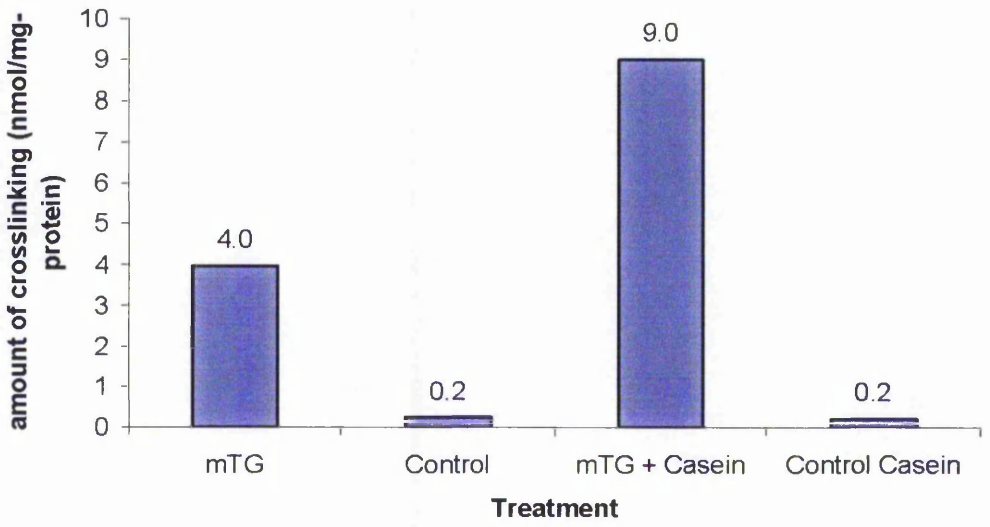


Figure 20. Results of crosslinking analysis for mTG treated bated samples n=1

The x-axis displays the varying TG treatments. In the y-axis number of crosslinks(nmol/mg-protein). mTG's treatments were carried out after the bating step. The results are the value from a single experiments. These results were done with samples of 2 mm thickness of freeze dried bated hide, which are different to the results displayed in Table XIII (those were done with powdered bated hide). The protocol of quantification of cross-links was carried out by Nottingham Trent University.

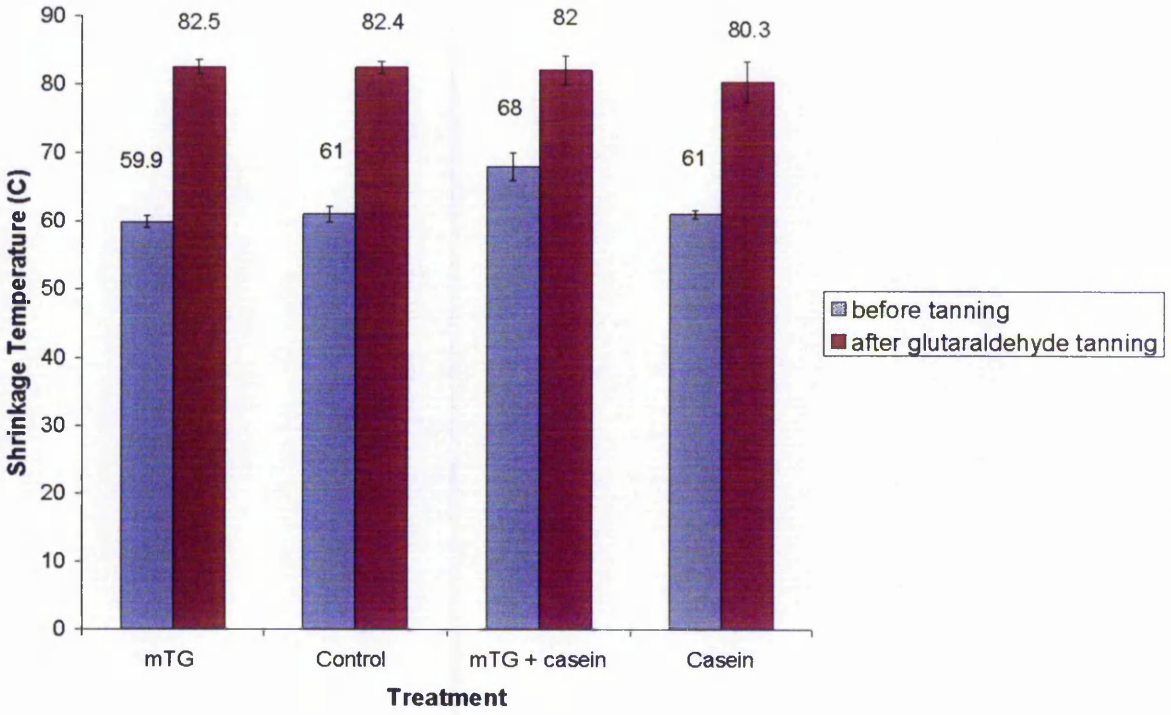


Figure 21. Hydrothermal stability of leather, treated with mTG, before and after subsequent glutaraldehyde tanning.

The x-axis displays the varying treatments the y-axis the shrinkage temperature as measured by the onset value of DSC. The results are the average of three replicate of the same experiment. The error bars show the standard error. Microbial transglutaminase alone or in conjunction with casein did not modify the shrinkage temeprature before tanning and after glutaraldehyde.

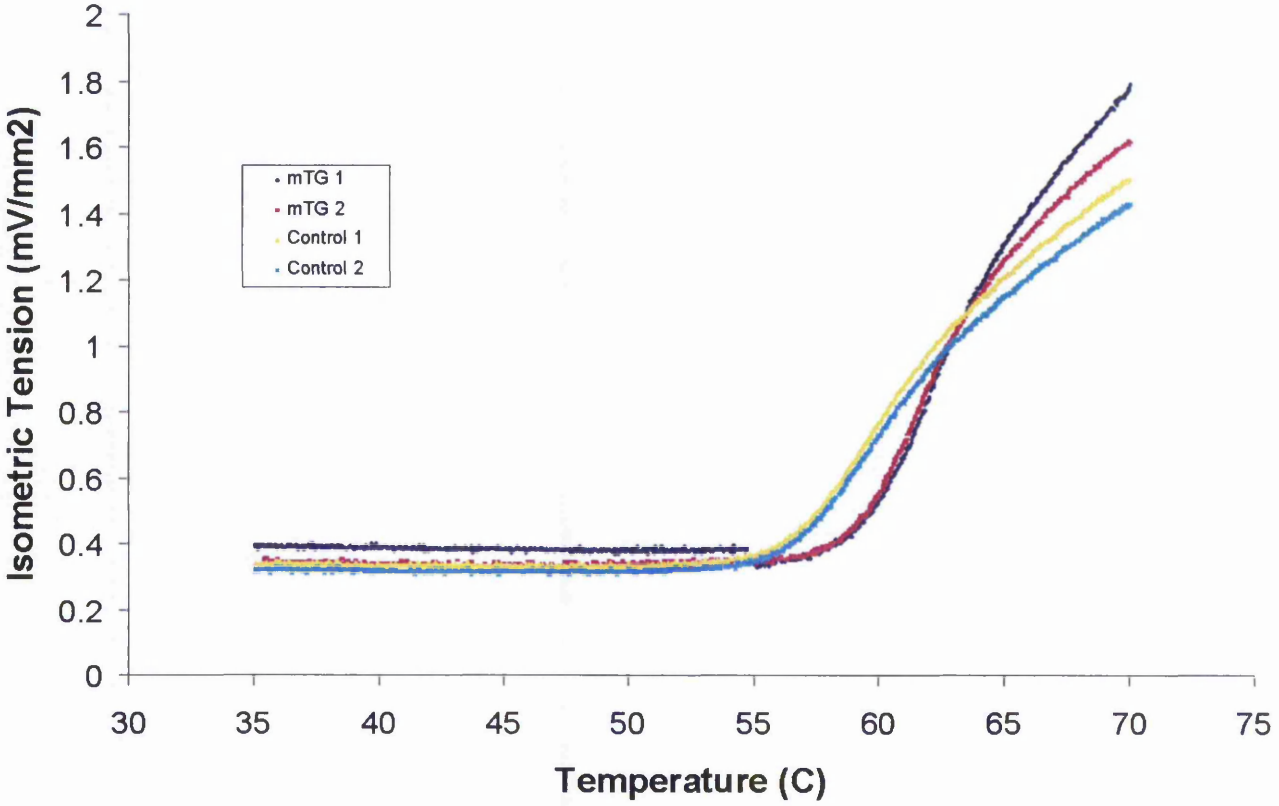


Figure 22. Isometric Tension of mTG mediated crosslinked bated hide.
The x-axis displays the temperature and the y-axis the tension generated by the sample. The pattern of the curve suggests that mTG mediated cross-linking has an effect on the force generated by the shrinking transition.

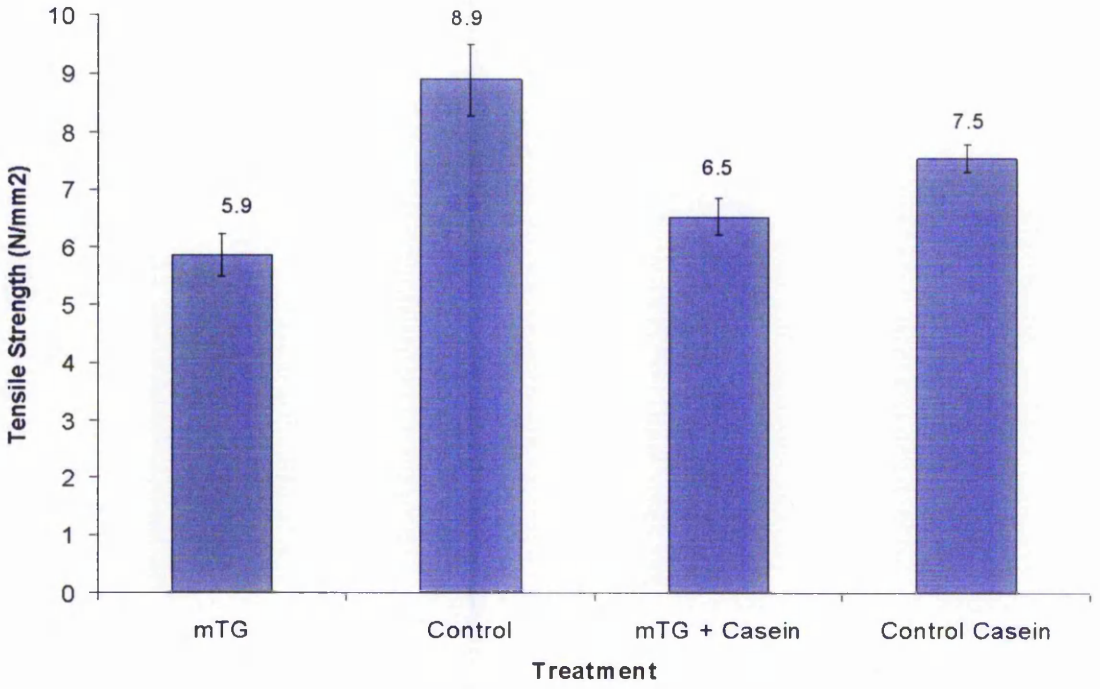


Figure 23. Effect of mTG mediated crosslinking on tensile strength of chrome tanned leather

The treatments were carried out after bating and the hides were subsequently chrome tanned and processed to dyed crust. The results are the average of 8 measurements. 2 tensile strength measurements per sample x 4 samples. The error bars show the standard error. Surprisingly, mTG alone or in conjunction with casein resulted in a significant reduction of tensile strength ($p<0.005$)

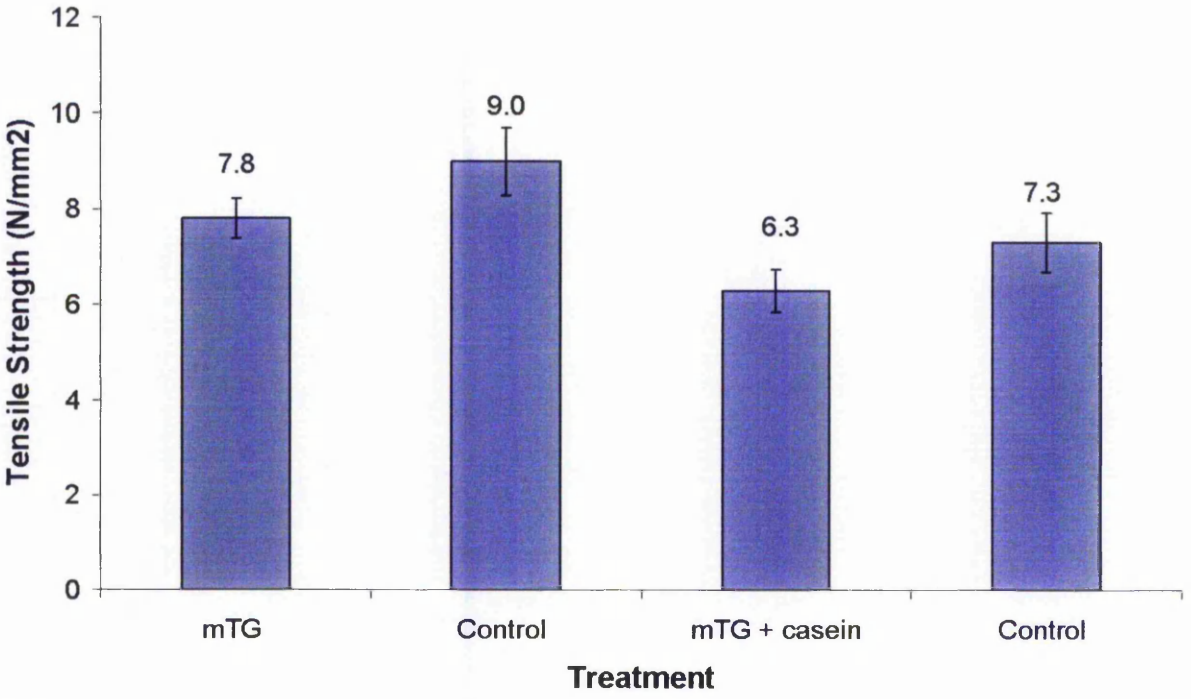


Figure 24. Effect of mTG mediated crosslinking on the tensile strength of glutaraldehyde tanned leather.

The treatments were carried out after bating and the hides were subsequently glutaraldehyde tanned and processed to dyed crust. The results are the average of 8 measurements. 2 tensile strength measurements per sample x 4 samples. The error bars show the standard error. As with chrome tanned leather, mTG alone or in conjunction with casein resulted in a significant reduction of tensile strength ($p<0.005$)

3.3 Discussion

The literature has reported that the amide side chains of glutamine and asparagine chains undergo alkaline hydrolysis due to conditions encountered during liming. The amide sites are converted to the carboxylic acids (glutamic and aspartic acid) and NH_3 is released in solution. As a consequence of this hydrolysis the isoelectric point of collagen becomes changed (Highberger *et al.*, 1941; Bowes, 1946; Bowes, 1949; Menderes *et al.*, 1999). However, the literature provides a disparity of isoelectric point values for limed and unlimed collagen . Heidemann (1993 (c)) states that the isoelectric point of unlimed collagen is 9 which drops to the region of 6 during liming. Other authors, e.g. Gustavson (1956 (b)) affirms that the isoelectric point of unlimed collagen is between 7 and 8, by liming it is shifted to 5.3. Beek *et al.* (1939) observed that liming lowered the isoelectric point of collagen from the neutral or slightly alkaline range to approximately pH 5. Menderes *et al.* (1999) agree with the latter author as they report that the liming shifts the isoelectric point from pH 7.9 to pH 4.5-5

Collagen, as other natural occurring macromolecules and proteins, acquires a charge when dispersed or solubilised in water, the overall charge depends on the pH of the medium. For instance, in acidic environments protons attach to basic groups, and the net charge of the macromolecule is positive; in basic media the net charge is negative as a result of proton loss. At the isoelectric point there is no net charge on the macromolecule. The most common technique applied to measure the isoelectric point of a protein is by monitoring the electrophoretic mobility of the protein within a pH range. The pH of no electrophoretic mobility is the isoelectric point (Gustavson b, 1956). This technique should be the most accurate technique to measure the change of isoelectric point in collagen, which would be indicative of glutamine hydrolysis that apparently takes place during liming operations. The use of this technique would only be possible using soluble collagen as a model substrate. This model system was considered not sufficiently close to real tannery conditions. Consequently, the investigation of glutamine hydrolysis during liming did not follow this route. A second alternative to be considered was the method proposed by Korn A.H *et al* (1971) who advocated development of a quick method to estimate the isoelectric point of insoluble

collagen. To achieve this end, samples of hide powder were equilibrated with dilute buffer solutions and the change in the pH of the buffer measured. The buffer shifts gave smooth curves when plotted; the point of zero shift was taken as the isoelectric point. Work carried out by BLC (Clara, 2003) provided evidence that the method appeared to be valid for an estimation of the isoelectric point which had undergone liming for the same period of time. It was apparent that different buffer concentrations and different ionic strength brought about approximately the same isoelectric point value (5.5). However, comparison between sixteen hours and forty eight hours liming gave rise to inconsistent results. Although longer liming resulted in an increase of the reactivity of hide powder towards chrome salts, which was supportive to the theory that the glutamine and asparagine residues undergo hydrolysis during liming, isolectric point measurement by the buffer shift method did not match with what is found in the literature. Menderes et *al* (1999) stated that an indication that de-amidation occurs is the presence of ammonia in liming floats. Wet-salted cow hide was washed with cold water for 45 minutes, soaked with 3% (w/w) brine for 22 hours at 20 °C to remove the hyaluronic acid, then split at the grain corium junction to remove hair, epidermis and elastin. The middle splits were limed with 3% (w/w) calcium hydroxyide in 100% (w/w) float at 25 °C for up to hundred and twenty hours. The ammonia contents of process liquors were determined spectrophotometrically, using Nessler's reagent. According to their work there are two mechanisms by which the reaction can proceed: direct hydrolysis, with hydroxyl catalysis, or intramolecular catalysed hydrolysis. A consequence of the intramolecular mechanism is that the naturally occurring L-asparagine is racemised to D-aspartic acid. They concluded that there was no initial rise in the amount of D-aspartic acid during the first twenty-four hours of liming. Therefore, the hydrolysis should be dominated by the direct mechanism. The amounts of L-aspartic acid and D-aspartic acid was measured by Gas Chromatography after hydrolysis with hydrochloric acid. The present study considered that the formation of the aspartic acid or glutamic acid due to liming should not be measured after acid hydrolysis, since this may give rise to false positives as the hydrolysis of the amides to the acids may be taking place whilst preparing the sample with hydrochloric acid prior to amino acid analysis, e.g. the amide-N content of collagen is determined by

measuring the ammonium released in solution after 10N HCl hydrolysis at 37 ºC for 9 days (Loeven, 1954) . Released ammonia measurements were not carried out by the Nessler method since a distillation step is required prior to the analysis. Although the work carried out by Menderes et *al.* (1999) did not perform distillation, this step should not be omitted since calcium which is present in high concentration in liming floats, interferes with the nesslerization (Fishman, M.J.; Friedman, L.C, 1989). Consequently, it was considered more appropriate to determine the degree of deamidation by measuring the ammonia released in solution as ammonium by ionic chromatography (Vogel, 1989). In this way, possible interferences should be circumvented.

The results reported in this thesis match to a certain extent with what is found in the literature, since a certain degree of hydrolysis was observed. However, amide hydrolysis has not often been measured directly, most of the occasions being reported as an indirect measurement of the change in the isoelectric point of collagen and comparison of the data provided by this thesis with what is found in the literature appears to be difficult. The most recent paper found in the literature that discusses the amide hydrolysis during liming (Menderes *et al.*, 1999) states that after 16 hours 42.5 % of the amides have been hydrolysed a figure higher than the 27% reported in this thesis (as shown in Table XI). The literature has reported that hydrolysis of amides in proteins is catalysed by base, heat and ionic strength, dictated by the pH and the adjacent amino acids (Fessenden *et al.*, 1990). However, other authors consider that amides easily undergo hydrolysis under basic conditions (Finar, 1983).

It is surprising, that hydrolysis was not observed in the samples incubated at pH 12.5 with a conductivity 37 mS/cm adjusted with KCl whereas the same pH and ionic strength conditions but in the presence of Ca^{2+} brought about hydrolysis (as shown in Table XI). This would suggest that Ca^{2+} is involved in the reaction mechanism of the alkaline hydrolysis of amides. One mechanism for dissolved metal ion catalysis is via coordination with the hydrolysable functional group on the substrate, increasing its electrophilic nature (metals are strong Lewis Acid or electrophiles). This is similar to acid catalysis. This is more important with substrates that have auxiliary donor group,

as in asparagine and glutamine hydrolysis, that can participate in bi-dentate chelation of the metal which may be favouring the hydrolysis (Buckingham, 1977, Houghton, 1979 and Ketalaar et *al.* 1956). The factors favouring complex formation are small highly charged ions with suitable energy orbitals of low energy which can be used for bonding (Lee, 1991). All the elements in the alkaline earth group e.g. Ca^{2+} (ionic radius 114 pm) form divalent ions, and these are smaller than the corresponding alkali metals e.g. K^{+}(ionic radius 152 pm), hence alkaline earth elements are better at forming complexes than alkali metals (Cotton *et al.*, 1988). Therefore Ca^{2+} might be participating in a bi-dentate chelation with the consequent catalysis of the hydrolysis. Incubation of glutamine at pH 12.5 and 37mS/cm conductivity adjusted with KCl or Ca_2Cl further suggested that Ca^{2+} is involved in the hydrolysis reaction (results shown in Figure 10).
It was attempted to develop an alternative unhairing process that was functional at neutral pH and would therefore eliminate the possible deamidation that could occur during liming.

This alternative method would also be more amenable for further enzymatic modification of the hide, e.g. by mTG. The new method relied on the combination of dispase a neutral protease of bacterial origin (Paul *et al.*, 2001), urea a lyotropic reagent to disrupt hydrogen bonding (Gustavson, 1960) and a pickling system without salt to bring about the swelling effect which is usually introduced during liming (Manzo *et al.*, 2004).

The possibility of chemical solubilisation of keratin at neutral pH by means of thio-compounds with subsequent thiol alkylation was also considered. However, initial literature search demonstrated that full unhairing effect is only achieved when the process is carried out in the presence of a strong alkali such as sodium hydroxide (Heidemann, c 1993, Kamal *et al.* and Landmann 1992). Thiourea which cleaves the cystine to a sulfoxilat, has been shown to have unhairing ability in weak alkaline conditions (Heidemmann, 1993). However, thiourea is classified as a carcinogen (IARC, 1974). Consequently, chemical unhairing at neutral pH was not considered viable.

The literature has reported that proteolytic attack of the hair roots and the epidermis is favoured by pre-treatment of the hide with alkali (Heidemann, 1993 (c)). Alkali swells which results in disruption of the epidermis structure facilitating the penetration of enzymes into the follicles and into the papillary layer (Germann, 1997; Cantera, 2001). It also reports that specific enzymes which only target the epidermis and the hair root have to be yet discovered. Extensive degradation of the root sheet and epidermal proteins is not possible without hydrolysis of the surrounding molecular sheets of the collagenous corium. Since the necessary hydrolysis takes places very slowly, the enzyme may be simultaneously attacking the superficial layers of the hide with the consequent grain damage (Burton, 1958).

Stenn et *al.* (1989) reported that Dispase was a rapid, effective, but gentle agent for separating intact epidermis from the dermis and intact epithelial sheets in culture from the substratum. In both cases it effects separation by cleaving the basement membrane zone region while preserving the viability of the epithelial cells. They investigated its substrate specificity using purified basement membrane components as substrate. SDS –PAGE of the peptides released by Dispase digestion provided evidence that the enzyme cleaves fibronectin and type IV collagen, but not laminin, type V collagen, serum albumin, or transferrin. The action of Dispase on collagen appeared be selective for type IV collagen in that several stable degradation products were formed, whereas the enzyme degraded type I collagen only minimally (Stenn *et al.*, 1989). Paul et *al.* (2001) have reported that Simmons et *al.* have also used Dispase to produce dermal plugs from human skin, although, successful in terms of removal of the epidermis and associated hair, it was not clear from their description if the underlying dermis had been damaged. Brady *et al.* (1990) proposed a model for proteolytic depilation of skins. In terms of their model, depilation is caused by disruption of the basement membrane at the dermal-epidermal junction through the degradation of its constituent molecular components by combinations of proteases with completely specificities, resulting in the removal of both the epidermis and associated wool or hair.

Paul et *al.*(2001) followed this approach and investigated the potential of Dispase as a depilatory reagent, as Dispase had been reported to be specific for collagen type IV and other components present in the basal membrane. Their results demonstrated that Dispase had potential for use in beamhouse processing. They found that the enzyme facilitates intact hair removal and efficient solubilisation of the epidermis without evidence of grain damage. However the enzyme did not bring about complete hair removal. In the light of what has been discussed above, Dispase appeared to be the ideal candidate for unhairing at neutral pH.

If the osmotic swelling needed to open up the hide structure would not occur during Dispase unhairing, This had to be induced subsequently. Since alkaline swelling was not an option (Beek *et al.*, 1939 and Menderes *et al.*, 1999) swelling was introduced by carrying out a salt free pickling.

The literature also reports that unhairing can be achieved by means of lyotropic reagents (Burton, 1956). Normally this type of unhairing takes some days, but in combination with enzymes, which in some cases are derived are from the hide itself, complete unhairing is possible for corrected grain leather (Frendrup, 2000). It can be concluded that the novel method was successful in terms of hair removal. Its efficiency can be attributed to Dispase, urea and the swelling achieved during pickling. However, the process conditions required to complete successful hair removal were detrimental to the quality of the final product. Even the samples processed following milder conditions presented a degree of damage unacceptable to the production of full grain leather.

The results reported in this thesis correlate well with what has been reported in the literature. Firstly, because complete hair removal was achieved by combination of a proteolytic enzyme and a lyotropic reagent (as shown in Table XII). Unfortunately, as also reported in the literature, that has always being observed in conjunction with grain damage, which does not allow the implementation of the novel technology for full grain leather production (as shown in Figure 12). Secondly, although a experiment with only

Dispase has not been carried out, the closest experiment to those carried out by Paul *et al.* (2001), (i.e. Dispase, without urea treatment and acid swelling) did not bring about complete hair removal (as shown in Table XII). That is not in complete agreement with what they found, as they concluded that no damage was observed. Although this can not be confirmed with only the current data. It would be worth considering as an hypothesis that the damage observed might have been introduced by the salt free pickling. The possibility that damage is caused by Dispase should not be ruled out, since the enzyme according to the literature has little activity on Collagen Type I. Besides the grain damage observed in the samples unhaired by means of the novel method, investigation of [^{14}C] putrescine incorporation into bated hide which had undergone Dispase unhairing provided evidence that little putrescine was incorporated (as shown in Figure 15) indicative that the novel method was not more amenable for mTG application. In the light of this results the use of Dispase to bring about unhairing at neutral conditions was abandoned.

Work carried out in the same project investigated the effect of mTGs mediated crosslinking on the denaturation temperature of soluble rat tail collagen. Colligan *et al.*, (2004) stated that "the crosslinking of soluble rat tail collagen by mTGs prior to, and during fibril formation should theoretically provide a true measure of the maximum amount of ε(γ-glutamyl)lysine that can be incorporated, since the enzyme has relatively free access to their amino acid side chain. mTG alone incorporated 8.9 nmol/mg-protein and in conjunction with casein as a crosslinking facilitator this was increased further to 15.2 nmol/mg. However such level of crosslinking did not modify the denaturation temperature. Although much lower levels than this were sufficient to alter the melting point of the soluble rat tail collagen gels, resulting in stability to boiling, no change in the hydrothermal stability of the collagen as measured by DSC was observed, suggesting that mTGs would be also unlikely to affect the hydrothermal stability of bovine skin collagen"

The question that arises from the results of the work carried out in this thesis is if tanning, as this measured by the denaturation temperature of collagen by DSC, only

implies crosslinking of collagen or whether further changes need to be introduced in the collagen structure to bring about a change in the temperature of the shrinking transition. Although a direct comparison with glutaraldehyde mediated crosslinking can not be done, since the degree of crosslinking achieved by mTG alone or with a crosslinking enhancer such as casein is always lower than glutaraldehyde (as shown in Table XIII), the results would suggest that to modify the shrinkage temperature the tanning agent should provide further changes in the collagen structure other than only an increase in the degree of crosslinking. The fact that mTG mediated crosslinking significantly modified other physical properties of the hide such as the resistance towards collagenase attack (see Figure 16), to acid hydrolysis (see Figure 17), the isometric tension generated during the shrinking transition (see Figure 22), the melting point of collagen gels and the tensile strength of chrome (as shown in Figure 23) and glutaraldehyde leather (see Figure 24) would support that modification of the shrinkage temperature is not as simple as only incorporation of inter or intra crosslinks in the collagen triple helices. If that was the case, although only little, some change in the denaturation temperature should be expected. In the same manner only moderate changes in the resistance towards collagenase attack, acid hydrolysis, isometric tension, the melting point of collagen gels or the tensile strength of the finished leather have been measured.

In a recent paper published by Covington *et al.* (2001) a "new theory of the origin of hydrohermal stability is defined, based upon the relative contributions of entropic and enthalpic changes to collagen by interactive chemical processes. These changes bring about stabilisation by the formation of matrices, that are capable of producing high hydrothermal stability". Although it was not the purpose of this thesis to fully discuss the kinetics and thermodynamics of the shrinking transition to postulate a tanning theory that might predict what is the nature of the collagen and tanning agent interaction that results in an increase in the shrinkage temperature, an intuitive review of the chemistry that brings about changes in the denaturation temperature might explain why mTG mediated crosslinking does not tan. Taking glutaraldehyde mediated crosslinking as an example, as already discussed that in the best case scenario the

amount of crosslinking provided by glutaraldehyde would be at least 5 fold compared with the amount incorporated by mTG. Beside of higher degree inter or intra molecular collagen crosslinking, recent work carried out by Roy *et al* (2004) in crosslinking of enzyme crystals has discussed the ability of glutaraldehyde to form a mixture of oligomers of different lengths and structures in aqueous solutions which makes its use as protein crosslinkers unpredictable to a certain extent. According to this work, glutaraldehyde can form long chain adducts in solution. Cross-linking with glutaraldehyde forms strong covalent bonds between the ε-amino groups of lysine residues within and between the enzyme molecules. As a consequence, the crystals become cross-linked throughout tri-dimensional matrix as shown in Figure 32, and it can not only be explained by simple imine formation between collagen ε-amino lysines. If this concept is applied to collagen and tanning, to a certain extent glutaraldehyde mediated crosslinking should be providing a matrix surrounding the collagen structure with the consequent change in the denaturation temperature. It is very unlikely that mTG mediated crosslinking even in the presence of a crosslinking facilitator such as casein would provide such a change in the environment that surrounds collagen which would explain why the tanning effect as this measured by DSC is not attained.

Investigation of the effect of mTG mediated crosslinking on the physical properties of chrome tanned and glutaraldehyde tanned leather showed a decrease in tensile strength after mTG treatment (as shown in Figures 23 and 24), the contrary effect that may be expected by covalently crosslink the collagen fibres. However, the strength of leather might be determined in part by the capability of collagen bundles to deform under external forces. mTG crosslinking may covalently fix the bundles into a conformation that is not necessarily the most optimal for increased tensile strength. The closest model to compare what is reported in this thesis with what has been previously found would be investigation of the ageing effect of bovine hide on the tensile strength of the subsequent leather. Russell *et al.,* (1977) investigated the tensile strength of two breeds of bulls at various ages. They observed a progressive decrease in tensile strength of chrome tanned leathers with increasing age. They state that the tensile strength is determined by "the intrinsec strength of the individual fibres as well as the degree of

fibre realignment in the direction of the applied force. Leather response under these conditions could be expected to depend on the degree of crosslinking within fibres. In the case of tensile strength, an increase in fibre crosslinking (as occurs due to collagen ageing) can be expected to lead to fibre embrittlement and earlier failure due to restricted fibre realignment". This would explain why mTG mediated crosslinking of bated hide results in tanned leather with lower tensile strength

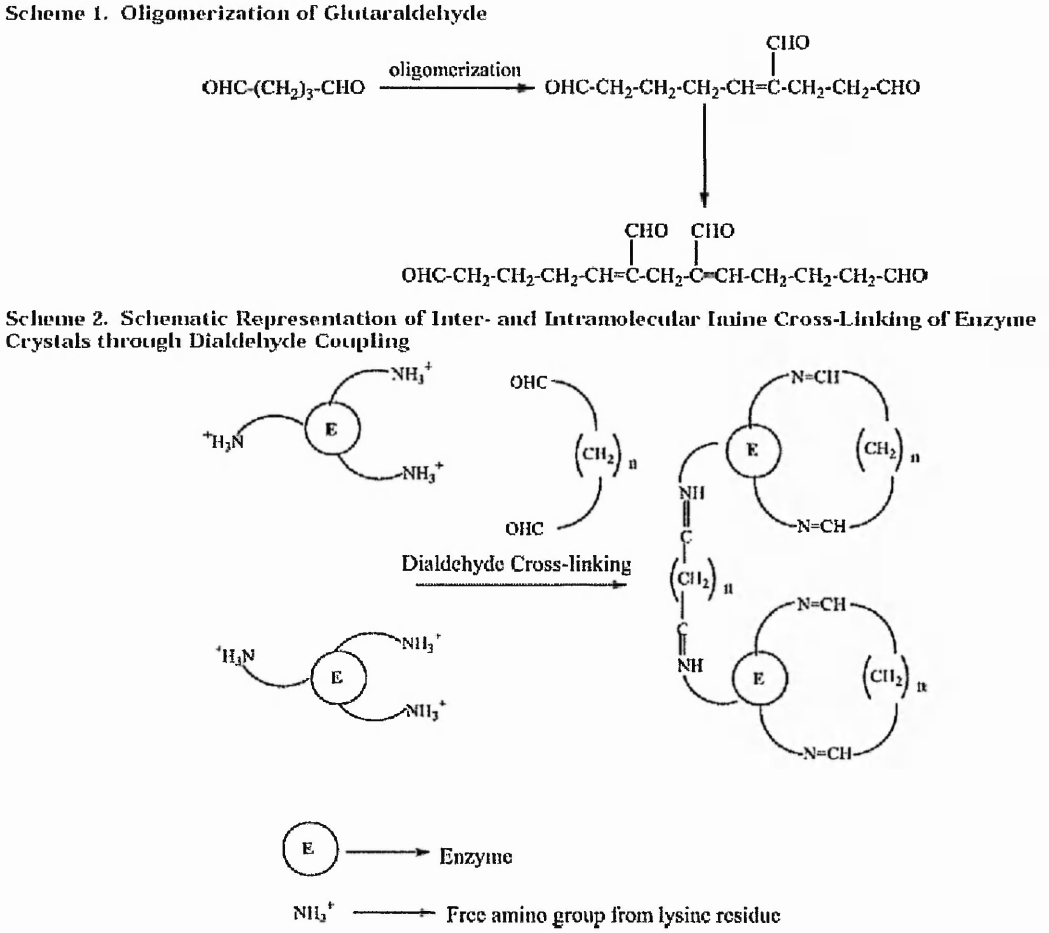


Figure 25. Reactions involved in glutaraldehyde mediated crosslinking of proteins.

Source from Roy *et al.* (2004)

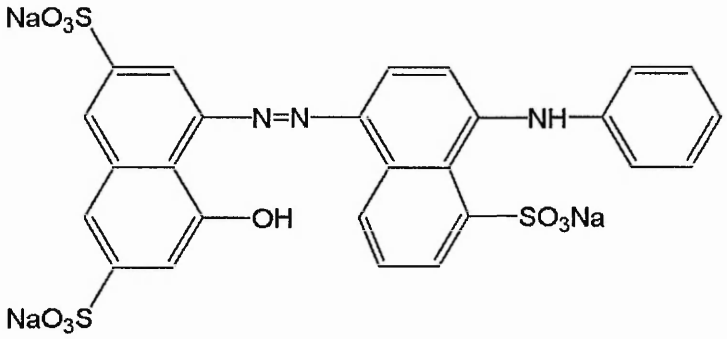
Chapter. 4 Dye modification for mTG mediated incorporation into leather.

4.1 Introduction

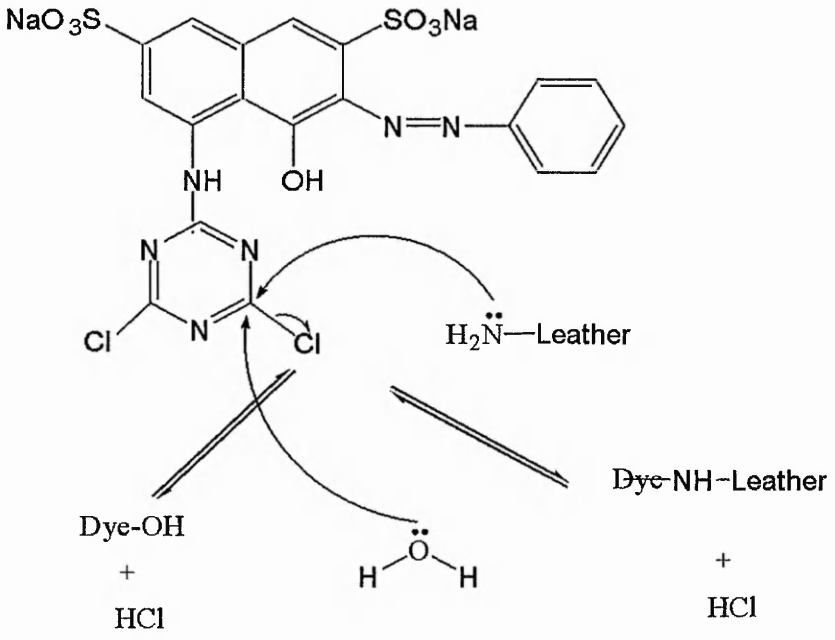
Since transglutaminases failed to increase the shrinkage temperature of bated hide, further work investigated the potential of applying transglutaminases during the dyeing operations of leather processing. Further work investigated the chemical modification of an acid dye via addition of a side chain with a primary alkyl amine. Thus, this could be incorporated into the leather by mTG (Cortez *et al.*, 2002).

The benefit of using a dye capable of being crosslinked by mTG into the leather is clear. This new dye would not undergo hydrolysis during the dyeing process with the consequent improvement of dye uptake and shade of the dyed leather (Figure 26).

The dye modification involved a coupling reaction between the phenolic hydroxyl group of an azo dye and the carboxylic acid of 6-aminohexanoic acid to obtain the desired compound. Three reactions were involved in the dye modification. Protection of the amine of the amino acid to prevent self-polymerisation, a dicyclohexylcarbodiimide (DCC) and dimethyl amine pyridine (DMAP) catalysed coupling reaction between the phenolic hydroxyl from the dye and the carboxylic acid from the amino acid, and deprotection of the amine incorporated into the dye. The reactions were monitored by Thin Layer Chromatography and ^{13}C nuclear magnetic resonance (NMR). With the incorporation of the primary amine confirmed, further work ascertained whether the modified dye was an effective substrate for mTG. This involved mTG mediated incorporation of the modified dye into N-N' dimethyl casein as a model system.



(A)



(B)

Figure 26. (A) Example of an acid dye. (B) Example of a reactive dye with the competing reaction between water and the amines

4.2 Results

4.2.1 Amino acid protection

^{13}C NMR provided evidence that the amino group had been protected. Figure 27 shows the chemical structure of the protected 6-amino hexanoic acid, the peaks labelled are subsequently identified in the ^{13}C NMR spectra showed in Figure 28.

Starting from the highest chemical shift value (1), the first signal to appear is from the carbonyl of the carboxylic acid. Continuing to lower chemical shift values, (3) is due to the carbonyl of the protecting group. At δ=77.9 appears the signal derived from the quaternary carbon of the tert-butyl group (4), which is followed by the DMSO peaks. Finally, the alkyl carbons give rise to five peaks on the right hand end of the spectrum. The three magnetically equivalent carbons of the protecting group (5) give rise to the highest peak. This gives a total of 8 identified carbons. The ninth peak (2) has not been identified because is probably overlapped by the DMSO peaks.

4.2.2 Coupling reaction and deprotection step

Acid Blue 92 was the choice of dye for modification because it has only one functional group capable of coupling with the carboxylic acid of the amino acid. Dry conditions were required as water might compete with the carboxylic acid for DCC (diciclohexyl carbodiimide), with a consequent reduction of the reaction yield. The chemical structures of the reagents involved in the reaction are shown in Figure 29.

The reaction was carried out by dissolving 10 mmol of t-BOC amino acid and 10 mmol of DCC in 15 ml of dry dimethyl-formamide (DMF). The solution was allowed to stand under a N_2 atmosphere for 30 minutes (solution 1). 2.5 mmol of dye (Acid Blue 92) and 1 mmol of DMAP were dissolved in 20 ml of dry DMF, the solution stirred for 30 minutes and added slowly to solution 1. Stirring under a N_2 atmosphere was continued overnight at 80°C. The mixture gave rise to a precipitate (B), which was filtered, and DMF from the liquid phase evaporated to dryness under high vacuum at 40°C (D). TLC (70% butanol: 30% methanol was carried out on the starting material (A), the precipitate (B) and the dry residue (D).

D was dissolved in 50 ml of methanol, 30 g of silica added and the suspension dried under vacuum and loaded onto a chromatography column. Column chromatography was performed with 70% butanol: 30% methanol as eluent. Separation was assessed by TLC (70% butanol: 30% methanol) and the plate sprayed with ninhydrin, the desired fractions pooled and chromatography repeated in order to increase the degree of purification. The purified fractions from the second column were evaporated under vacuum (PD). ^{13}C NMR on PD was also carried out. The fractions from a subproduct of the reaction were also collected, pooled and dried under vacuum (SP) and analysed by ^{13}C NMR.

As shown in Figure 29 an esterification between the carboxylic acid of t-BOC 6-amino hexanoic acid (3) and the phenolic hydroxyl of the dye (4) was the reaction that was expected to occur. DCC was used to activate the carboxylic acid, allowing for the formation of the isourea (6), a good leaving group which promotes the nucleophilic attack of the carbonyl by the dye. DMAP was likely to act only as a base, although it may play a more specific role (Vogel, 1989).

Preliminary assessment of the reaction by TLC indicated that it took place (data not shown), as the precipitate (B) and the liquid phase of the reaction mixture (D) gave rise to an additional spot. No t-BOC 6-amino hexanoic acid was detected by TLC although there was a second additional spot (SP) in D. That could be the activated t-BOC 6-aminohexanoic acid or a sub product from a side reaction. The new coloured compound (PD) was separated from SP by column chromatography. The yield of the reaction was only 24.3 %. ^{13}C NMR confirmed that PD was the modified dye.

Examination of the ^{13}C NMR of PD (Figure 30) starting from the more deshielded carbons, 1 (δ=174.1577) arises from the carbonyl of the ester formed. Moving to more shielded regions of the spectra, DMF 1 (δ=158.4805) can be assigned to the carbonyl from DMF, a solvent difficult to remove due to its high boiling point, as it also appears in the ^{13}C NMR spectra of pure DMF (Figure 32 DMF 1 δ=162.2738). The aromatic carbons of the dye give rise to the groups the peaks with chemical shifts between 150

and 110 as demonstrated in Figure 31. Therefore the group of peaks that arise in the same region have been assigned to the same atoms in the PD ^{13}C NMR spectra (Figure 30). Peak 4 in Figure 30 (δ=79.7432) can be assigned to the carbon 4 in Figure 29 (3) as give rise to the same peak in Figure 28. The carbon bonded to the alcohol of butanol has been assigned to the peak labelled as BUT 1 in Figure 33 with δ=60.5044. Consequently, the peak with the same chemical shift in Figure 30 comes from butanol. Peak 2 (δ=41.1767) arises from the aliphatic carbon attached to the amine (also labelled as 2 in Figure 27). In contrast to the NMR of t-BOC-6amino hexanoic acid (Figure 28) (analysis done in DMSO) the analysis was done with the sample dissolved in methanol and overlapping of peaks did not happen. The peaks labelled as DMF 2, DMF 3, BUT 2, BUT 3 and BUT 3 can be assigned to both solvents as shown in Figure 32 and Figure 33. The 5 peaks at the right end of the spectra are the peaks derived from the protecting group, as seen also in Figure 28. Only one peak remains unidentified. Together, these ^{13}C NMRs provided sufficient evidence that PD was the targeted compound.

SP also underwent ^{13}C NMR , as shown in Figure 34, the spectra would suggest that SP was the activated t-BOC 6-aminohexanoic acid (O-Acylisourea). The literature reports that the acylisourea is highly reactive and side reactions occur if the nucleophilic attack is slow. The collapse of the O-Acylisourea is by an intramolecular acyl-transfer mechanism which sometimes competes significantly with the desired attack by an external nucleophile. When this happens, the much less reactive N-acylurea is formed. Its formation not only reduces the yield, but may give rise to purification problems (Waring, 1990).

Examination of the ^{13}C NMR of SP (Figure 34), the peaks labelled as 1, 2, 3, 4, 5, 6 and 7 can be assigned to carbons 1, 2, 3, 4, 5, 6 and 7 (Figure 33). The eleven aliphatic carbons from the acylisourea (8 in Figure 34) give rise to the eleven peaks labelled as alkyl in Figure 34. Only two peaks remain unassigned. Consequently the product is the acylurea either in its O-acylisourea or N-acylurea form. Since O-acylisourea is very reactive and unstable, it is very likely that the activated O-acylisourea acid underwent an intramolecular acyl-transfer rearrangement with the consequent reduction of the

yield. The t-Boc group was removed from the modified dye as described in Chapter 2, section 2.11.3. Thin Layer Chromatography (TLC) indicated that removal of the protecting group was carried out successfully. At this stage, mTG mediated incorporation of the modified dye into N-N' dimethylcasein was investigated.

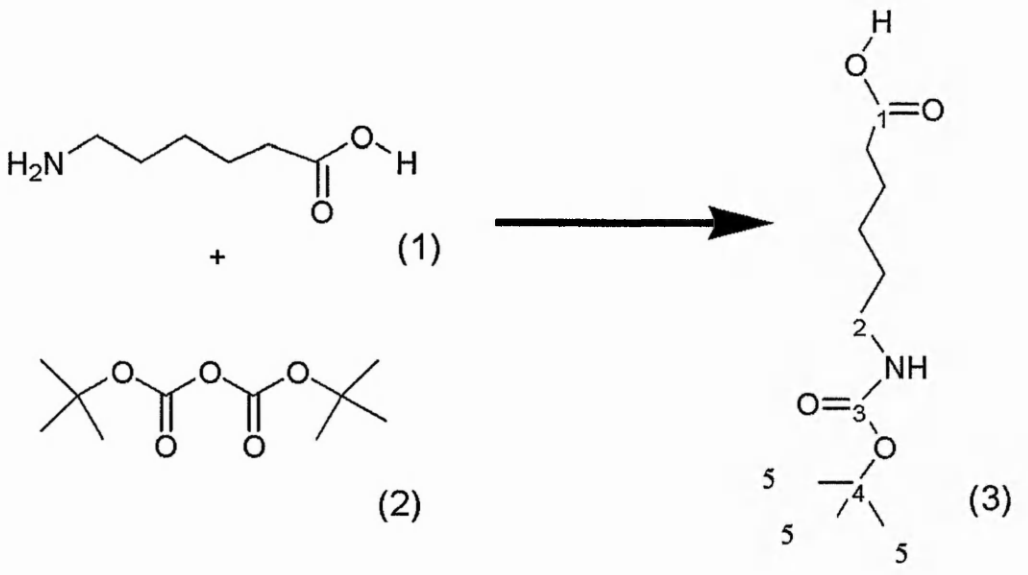


Figure 27. Chemical structures of the products involved within the protection step. Starting from the left of the figure, structures of the 6 amino-hexanoic acid (1), di-tertbutyl pyrocarbonate (2) and the t-BOC 6-aminohexanoic acid (3) are depicted. The varying carbons in the 5-BOC 6-amino hexanoic acid are labelled as 1, 2, 3, 4 and 5 and identified as such in the text and in Figure 28.

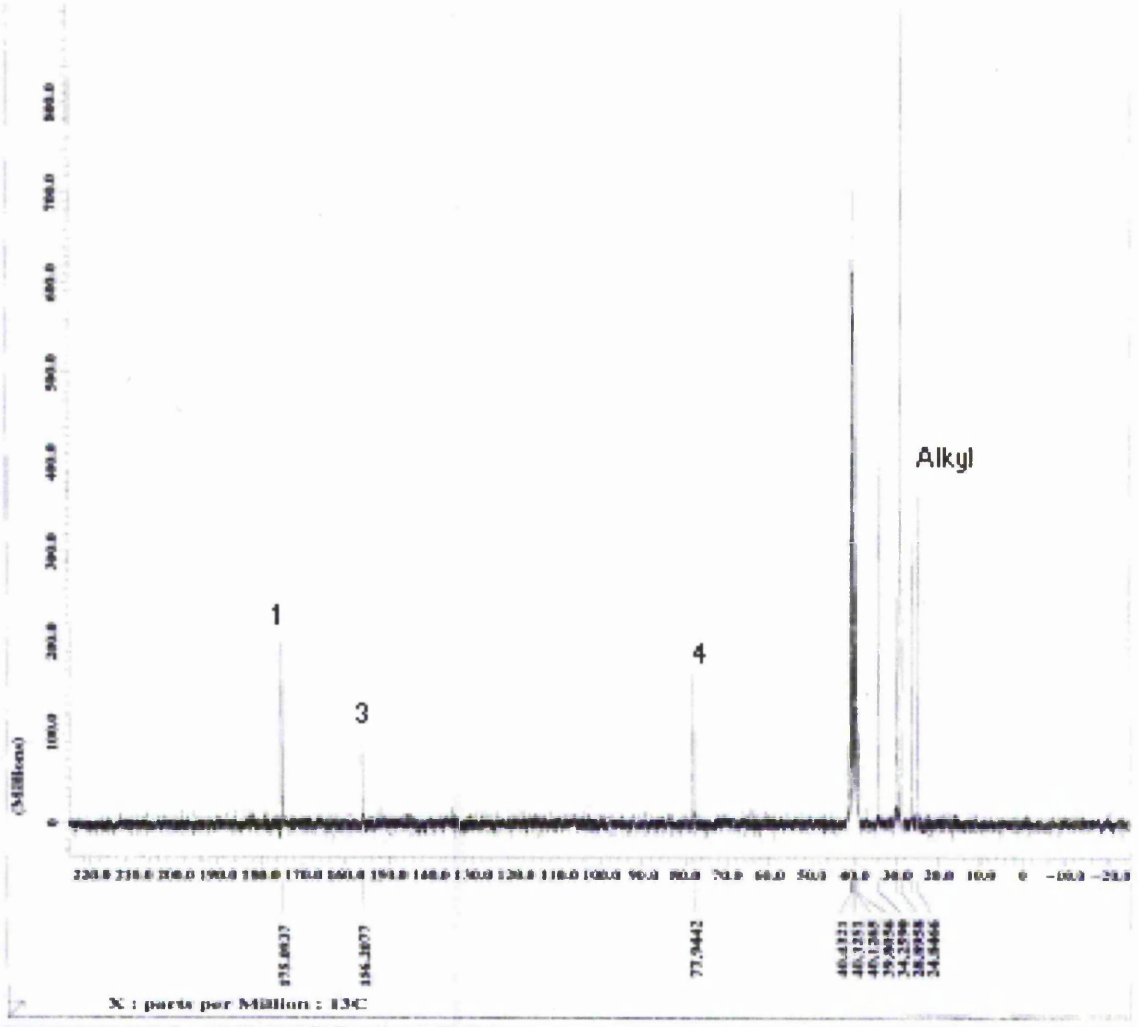


Figure 28. ^{13}C NMR spectra of the t-BOC 6 amino hexanoic acid in DMSO. The x-axis displays the carbon chemical shifts (δ in ppm) of the t-BOC 6-aminohexanoic acid. The y-axis shows the abundance of each signal. Some peaks are identified according to the labels of Figure 27.

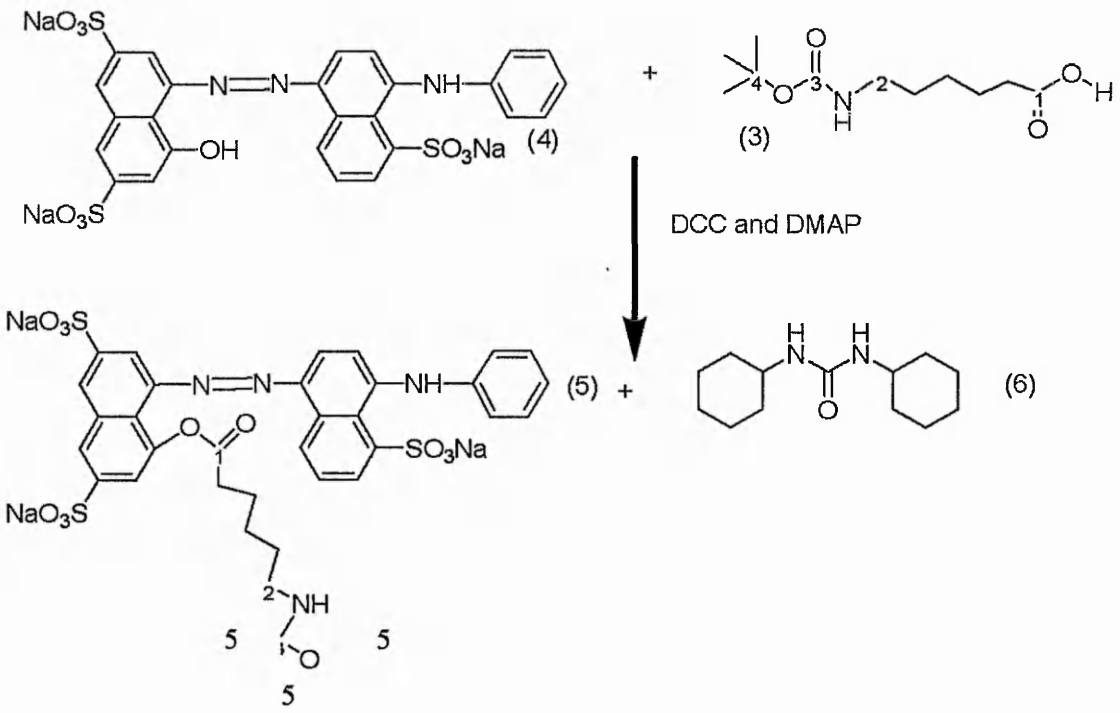


Figure 29. Chemical structures of the products involved within the coupling reaction. Starting from up to down Acid Blue 92 (4), t-BOC 6-aminohexanoic acid(3), modified dye (5) and the isourea (6) are depicted. The protecting group was removed prior to mTG mediated incorporation into N-N' dimethylcasein as described in material and methods.

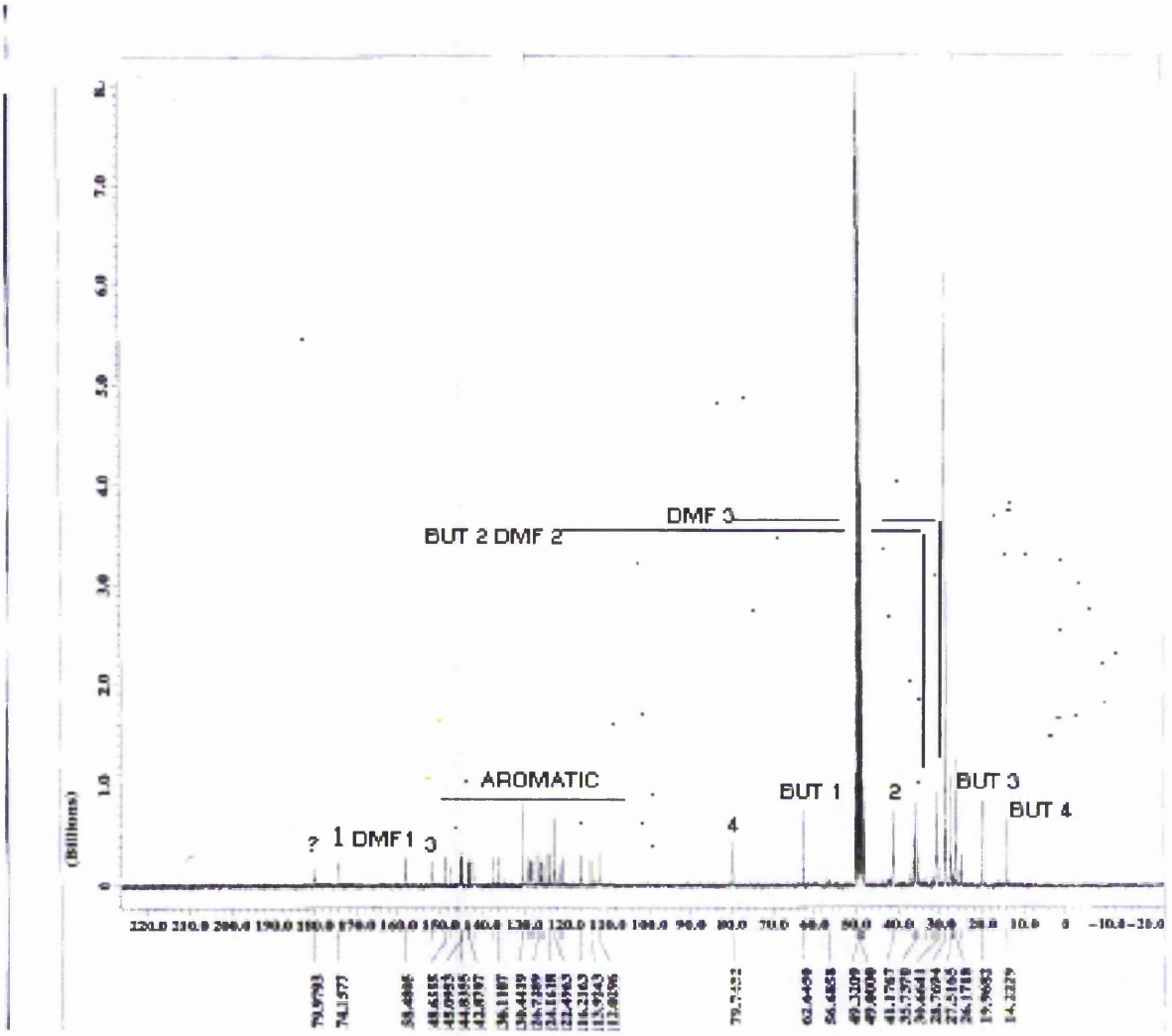


Figure 30. ^{13}C NMR of PD in methanol.

The x-axis displays the carbon chemical shifts (δ in ppm) of PD, the y-axis the abundance of each signal. Some peaks are identified according to the labels of Figure 28, additional peaks that arise from some impurities are also identified

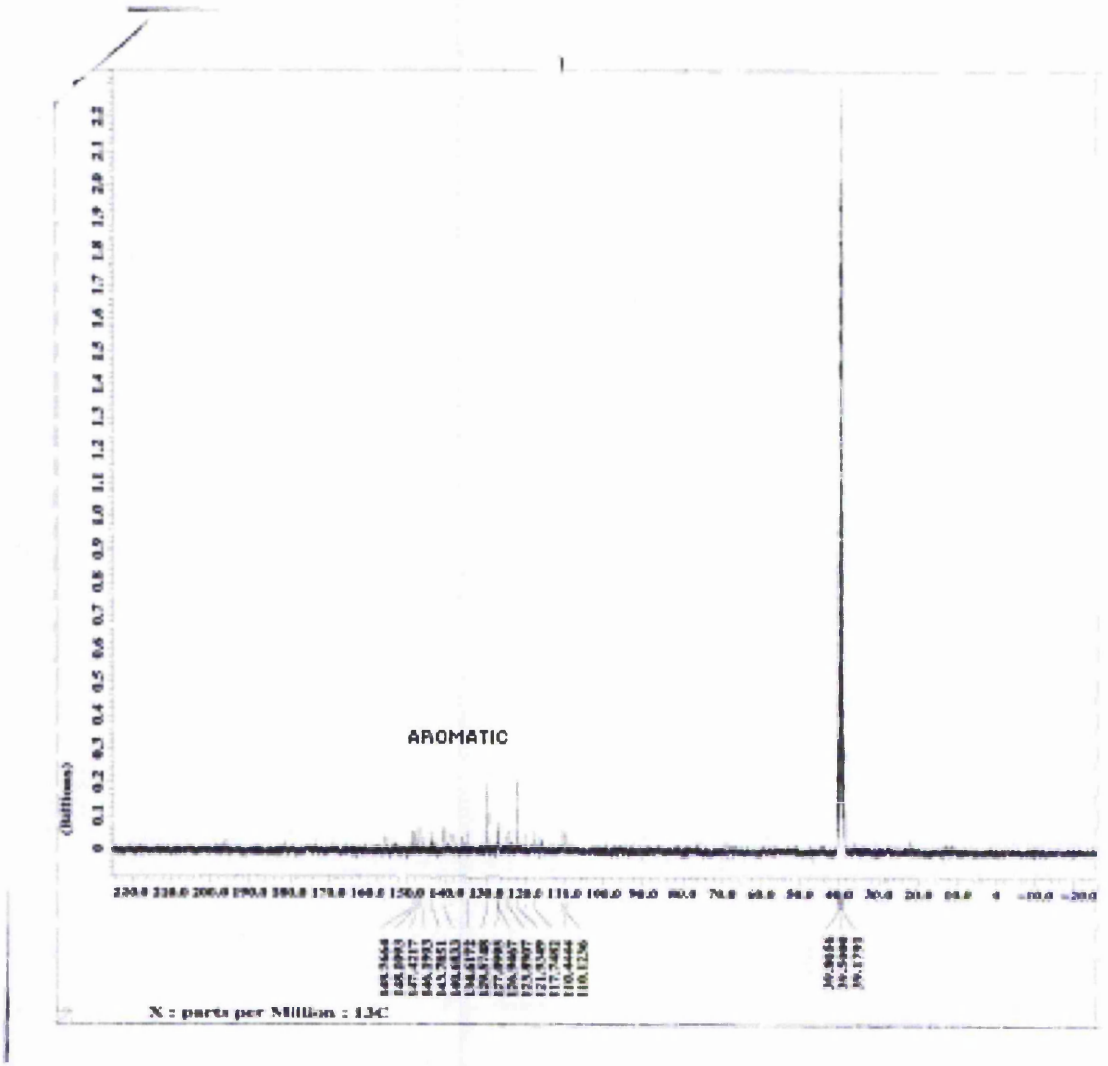


Figure 31. ^{13}C NMR of Acid Blue 92 in DMSO.

The x-axis displays the carbon chemical shifts (δ in ppm) of PD. The y- axis the abundance of each signal.

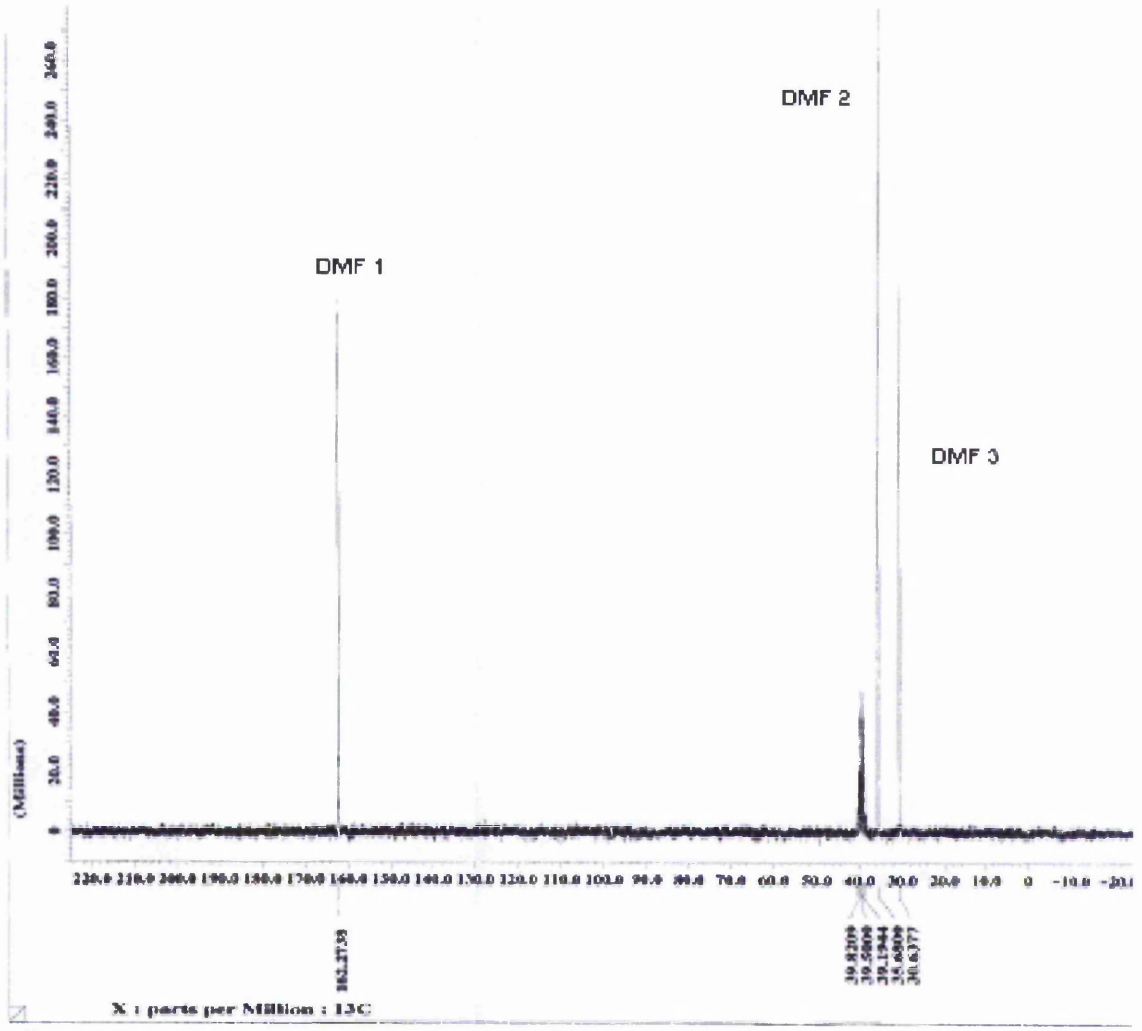


Figure 32. ^{13}C NMR of DMF in DMSO

The x-axis displays the carbon chemical shifts (δ in ppm) of PD. The y-axis the abundance of each signal. The three peaks that arise from DMF are labelled.

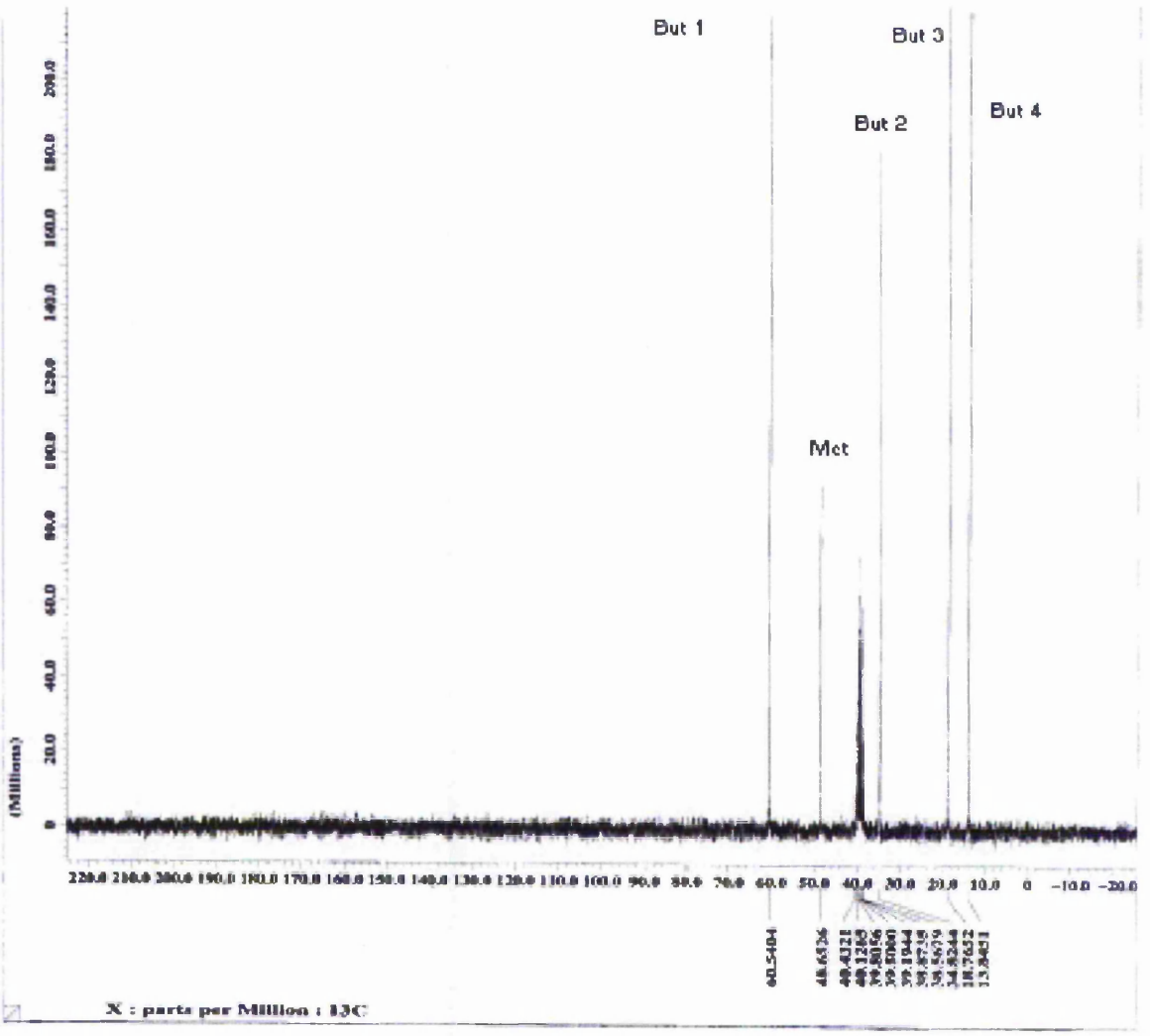


Figure 33. ^{13}C NMR of eluent mixture (Butanol/Methanol 70:30 in DMSO).

The x-axis displays the carbon chemical shifts (δ in ppm) of PD. The y-axis the abundance of each signal. The four peaks that arise from butanol and methanol are labelled.

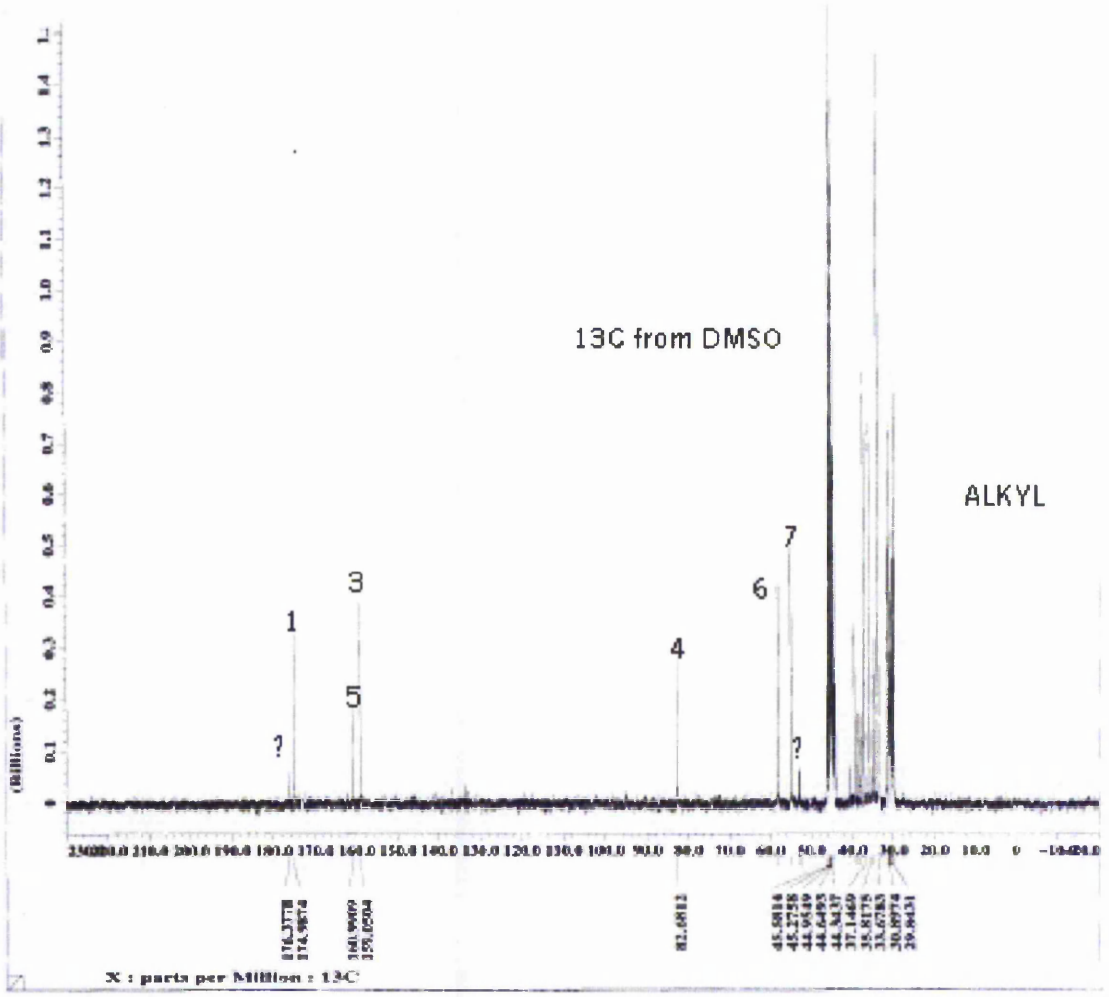


Figure 34. ^{13}C NMR of SP in DMSO.

The x-axis displays the carbon chemical shifts (δ in ppm) of SP. The y-axis the abundance of each signal. Some peaks are identified according to the labels of Figure 34.

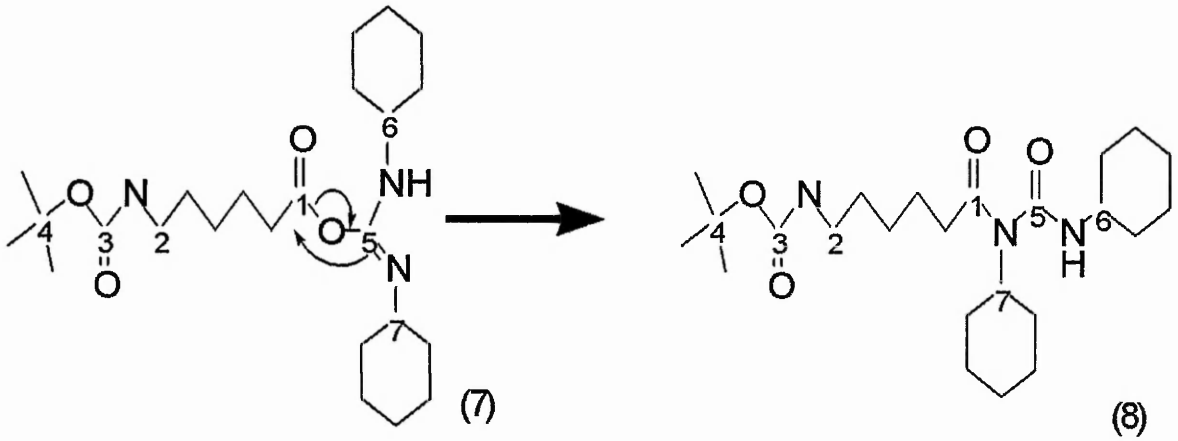


Figure 35. Chemical structures of the activated t-BOC 6 amino hexanoic acid (*O*-acylisourea) and the *N*-acylurea .

Starting from the left of the figure, structures of the *O*-acylisourea (7) and *N*-acylurea (8) are depicted.

4.2.3 Microbial transglutaminase mediated incorporation of the modified dye into N,N'-dimethyl casein as a model system

Microbial transglutaminase mediated incorporation of the modified dye into N,N'-dimethylcasein as a model system was assessed by means of TLC. If the dye was a mTG substrate this would be covalently linked into the protein with the consequent change of mobility in the silica gel between the unattached dye and the dye incorporated into the protein. Consequently, dye incorporation would bring about two spots, one spot from the dye incorporated and a second spot from the unreacted dye. The ability of mTG to crosslink the dye was investigated at varying pH and temperature conditions. Surprisingly, as shown in Table XV, incorporation was not observed, indicative that the dye was not a substrate for mTG.

In the light of these results, further work was undertaken in a view to understand why the dye was not a suitable substrate for mTG mediated incorporation. Two inhibition experiments were undertaken. Such experiments would provide information about the accessibility of the amine moiety of the dye to the active centre of the enzyme. To achieve this end, the inhibitory effect of the dye on [^{14}C] putrescine and fluorescein-cadaverine incorporation into N,N'-dimethylcasein was investigated.

The literature has reported the best amine substrates carry a large hydrophobic substituent attached to the alkylamine side chain of about 7.2 A in length. This hydrophobic substituent interacts with the hydrophobic binding region of the enzyme from where the alkyl side chain reaches into a narrow crevice towards the active center and positions the primary amine of the substrate to attack the carbonyl group of the acyl enzyme intermediate (Folk, 1980).

The first experiment would determine whether the alkyl amine of the dye could reach the narrow crevice. If that was the case, it would compete with [^{14}C] putrescine for such a site subsequently inhibiting its incorporation. No inhibition would indicate that the primary amine was not accessible to the active centre of the enzyme. This would explain why the modified dye was not a suitable substrate for mTG.

As shown in Figure 36, the modified dye did not inhibit [^{14}C] putrescine as higher dye concentrations did not lower the amount of radioisotope incorporated. The second experiment aimed at investigating whether the substrate cold be positioned in the enzyme binding site. This was achieved by measuring mTG-mediated incorporation of a bulky substrate, such as fluourescein-cadaverine.into N-N' dimethylcasein at varying concentrations.

As shown in Figure 37, reduction of fluorescein-cadaverine incorporation was observed with increasing concentrations of dye. The stereochemistry of the dye allowed for two hypotheses: the dye was either too bulky or the polarity of the sulphonic groups of the naphthalene ring attached to the side chain (Figure 29 (5)) did not allow the dye to sit in the enzyme binding site. This preliminary result suggested that the modified dye was inhibiting fluorescein-cadaverine incorporation into N,N'-dimethylcasein.

Consequently, it could be concluded that the sulphonated naphthalene ring carried by the alkyl amine could sit in the enzyme binding site and, therefore, that the stereochemistry of the dye was correct in terms of size and polarity. However, the primary amine with the five carbon aliphatic chain did not have the right geometry and so could not reach the active site centre. This is the most likely explanation of why the dye was not a mTG substrate.

Table XIV. Dye incorporation by mTG into N,N'-dimethylcasein

	pH 5.5			pH 6.5			pH 7.4		
T (°C)	40	50	60	40	50	60	40	50	60
Incorporation	None	None	None	None	None	None	None	None	None

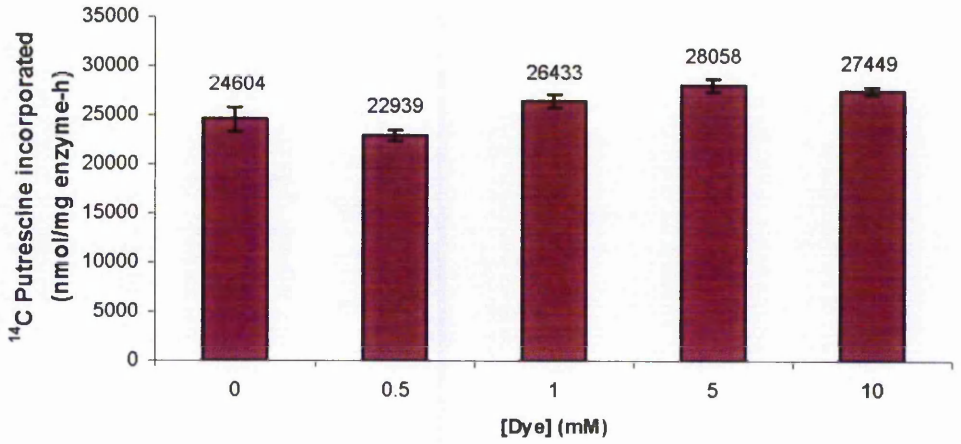


Figure 36. Dye effect on ^{14}C putrescine incorporation by mTG into N,N'-dimethylcasein.

The x-axis represents the concentrations of dye assayed. The y-axis ^{14}C Putrescine incorporated in nmol/mg enzyme/h. The results are the average of 2 replicates of the same experiment. The error bars show the standard error.

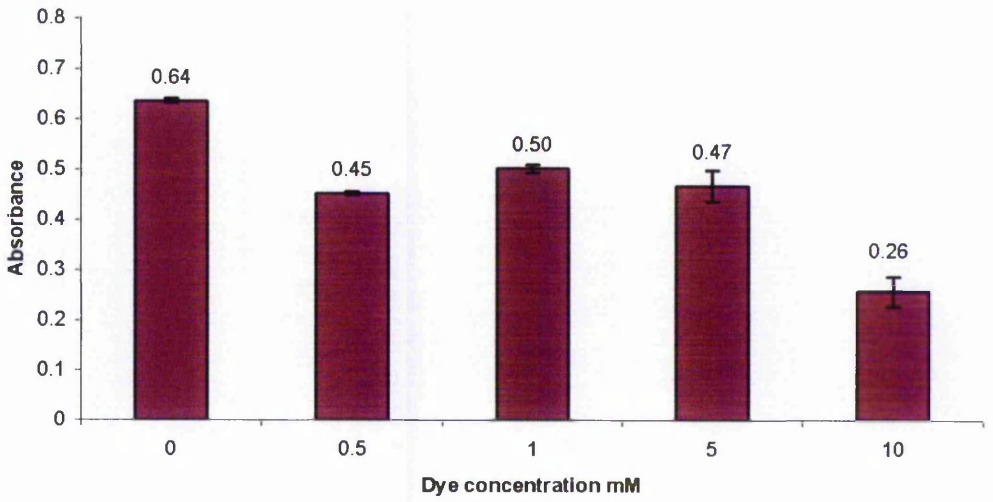


Figure 37. Dye effect on fluorescein cadaverine incorporation by mTG into N,N'-dimethylcasein.

The x-axis represents the concentrations of dye assayed. The y-axis absorbance measured at 450 nm. Higher absorbance values mean higher level of incorporation. The results are the average of three replicates of the same experiment. The error bars show the standard error.

4.3 Conclusion

The method adopted to incorporate the five carbon aliphatic chain into the dye was successful. However, the yield of the reaction was low due to the formation of the N-acylurea. The alternatives found in the literature to prevent this side reaction would not be suitable in the current case. It would be very unlikely that the phenolic group of the azo dye could displace the t-BOC 6-aminohexanoic acid intermediate suggested by Takeda *et al.* (1983) due to its relative poor nucleophilicity. Since there are no suitable alternatives to improve the current method, future work should rely on the chemistry suggested by Lewis *et al.* (1991). This method converts the monochlorotriazine ring of the dye to the aminoalkylaminotriazine form by means of an aromatic nucleophilic substitution with an alkyl diamine. The wrong geometry of the five aliphatic carbon chain appeared to be the reason why the modified dye was not a mTG substrate. This might be resolved by incorporating the amine moiety in a dye position that is more accessible to the active site. If this does not overcome the problem, incorporation of amines with longer chains should be investigated.

Chapter 5. Microbial transglutaminase mediated incorporation of keratin hydrolysate and its effect on dyeing

5.1 Introduction

The aim of the work was to investigate the effect of mTG mediated incorporation of keratin hydrolysate on the dyeing properties of the leather. Since there is evidence that other mechanisms, alongside ionic interaction, are involved in dye fixation (Muralidharan *et al.*, 1990; Britten, 1994), the use of other compounds to improve dyeing performance, beside cationic fixatives has to be considered. Amongst these compounds collagen hydrolysate and keratin hydrolysate have shown their ability to improve dyeing performance in textiles such as wool (Winkler, 1999; Cortez, 2004). Furthermore, keratin hydrolysate is a waste product in the leather industry which highly contributes of the total COD generated in leather processing, and this is an issue that needs to be adressed (Landmann, 1990). Therefore, it appears plausible to investigate the potential of using mTG mediated incorporation of keratin hydrolysate in the retanning operations and its consequent effect on the dyeing properties of the leather. Initially work has been undertaken with a commercially available keratin hydrolysate to facilitate standarisation and control of experiments.

Since crust leather, which has been chrome tanned, fatliquored and air dried, is the substrate to be dyed, preliminary work was carried out to determine whether crust leather was suitable for both the efficient uptake of mTG and also crosslinking. In the light of these results, further work was undertaken to assess the potential of mTG mediated incorporation of keratin hydrolysate into crust leather. Having confirmed that mTG was able to include keratin hydrolysate within the leather matrix, the experiments were focussed on investigating the effect of mTG mediated inclusion of keratin hydrolysate into crust leather on dyeing performance.

5.2 Microbial transglutaminase uptake by crust leather and potential for crosslinking

Experiments were undertaken to determine whether crust leather, either the dry or wet state would be the preferred material to apply mTG treatments, as differences between the dry or wet state had been observed with bated hide (freeze drying bated significantly increased the enzyme uptake as discussed in Chapter 3). Whilst crust leather is commonly traded and stored in the dried state and easy to deal with because it is soft and pliable, the application of mTG at this stage would overcome the inconvenience of using freeze dried bated hide. Freeze drying is not a commercially viable process and is difficult to deal while dried bated hide is hard and not sufficiently pliable. As shown in Figure 38, better penetration was observed in the dried substrate. However, as shown in Figure 39, when an identical experiment was performed using crust leather that had been prepared without the fatliquoring process, better uptake into the fibril bundles was observed (Figures 39 A and 39 B). Whilst these results strongly suggest that the use of a substrate without fatliquor should be more amenable, the option of crust leather with fatliquor was continued because again this was a substrate much easier to deal with due to better softness, pliability and better wetback ability. Crust leather without fatliquor is very susceptible to undergo over drying which results in a substrate very hard, not pliable and poor wetback ability properties.

With respect to glutamine availability (a measure of potential crosslinking), data provided by Nottingham Trent University showed that mTG incorporated 1.7 $nmolmg^{-1}$ of [^{14}C] putrescine into crust leather, compared with 4.7 $nmolmg^{-1}$ into bated hide.

5.3 Microbial transglutaminase mediated incorporation of keratin hydrolysate into crust leather

Investigations were undertaken to determine the feasibility of keratin hydrolysate incorporation into crust leather by means of mTG. These examined the penetration of keratin hydrolysate alone or in the presence of mTG through crust leather. The penetration of keratin hydrolysate through crust leather was assessed using biotinylated keratin hydrolysate. As shown in Figure 40 A, biotinylated keratin hydrolysate was

found to penetrate well through crust leather, incorporation of mTG in the process (Figure 40 B) improved the uptake of keratin hydrolysate. This work was carried out only by Nottingham Trent University.

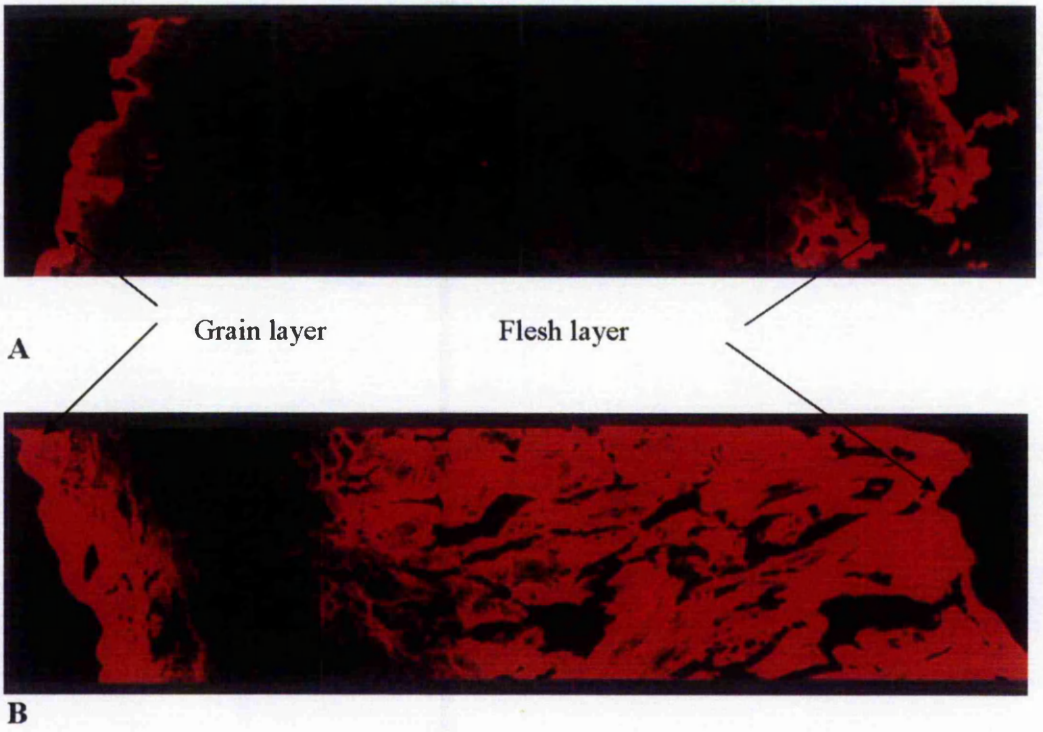
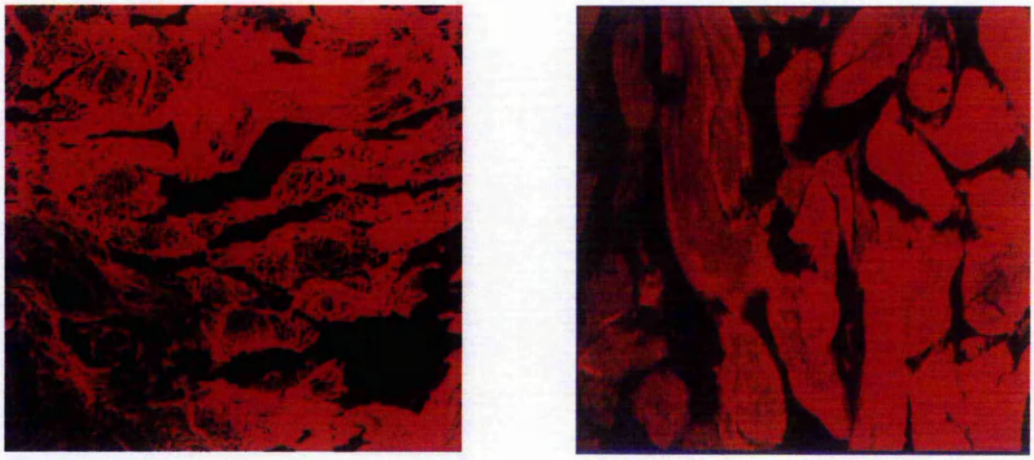


Figure 38. Uptake of biotinylated mTG into bovine crust leather. A is incorporation after soaking into wet crust leather B shows incorporation after soaking into dried crust leather. The experiments were undertaken as described in Chapter 2, section 2.10 and 2.14. The experiments were carried out by Nottingham Trent University



C D

Figure 39. Uptake of biotinylated mTG into dry bovine leather. C fatliquored crust leather. D not fatliquored crust leather. The experiments were undertaken as described in Chapter 2, section 2.10 and 2.14. The experiments were carried out by Nottingham Trent University.

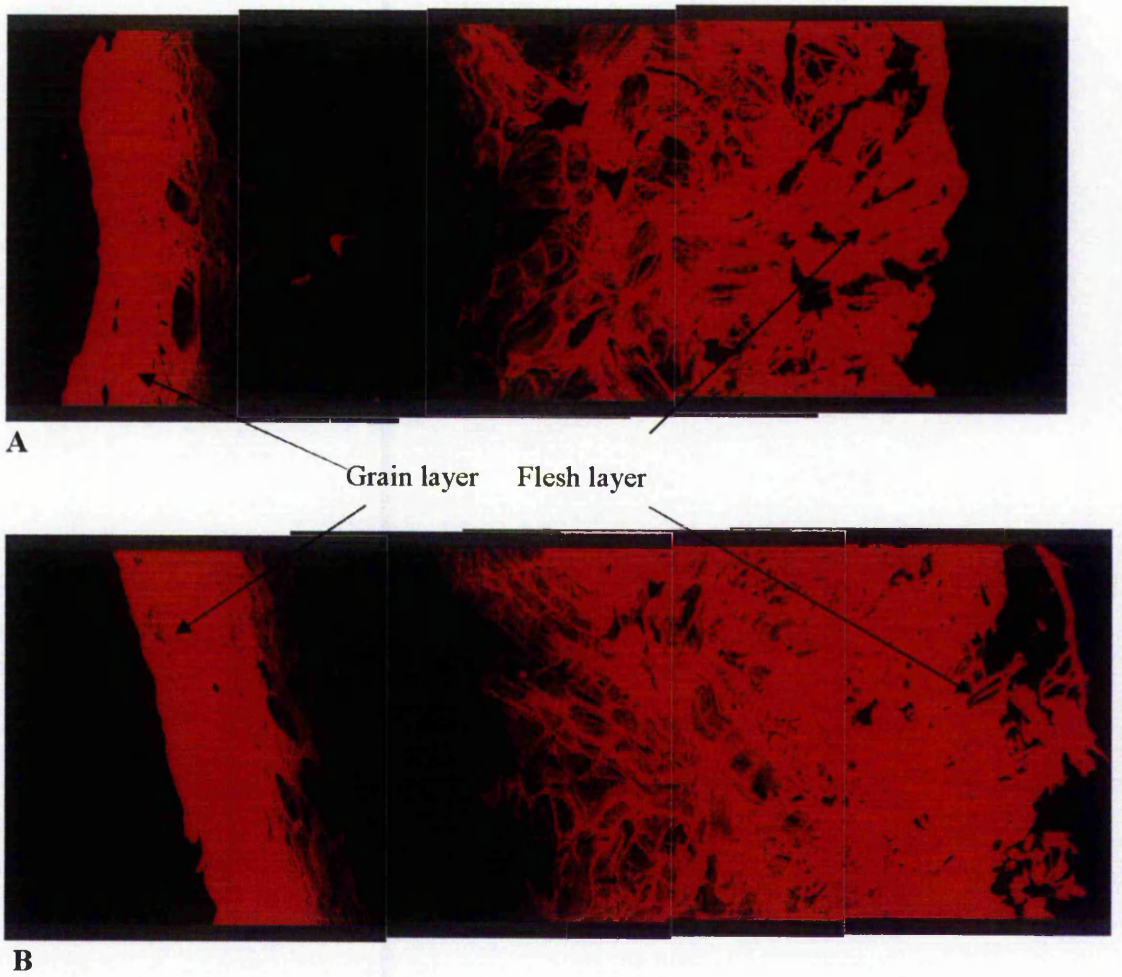


Figure 40. Uptake of biotinylated keratin hydrolysate into crust leather. A biotinylated keratin hydrolysate. B biotinhylated keratin hydrolysate + 100 μgml^{-1} mTG. The experiments were carried out as described in Chapter 2, section 2.15. The experiments were carried out by Nottingham Trent University.

5.4 Initial investigations of mTG mediated incorporation of keratin hydrolysate into leather and its effect on dyeing

Freeze dried bated leather samples of approximately 1/4 of A4 size were treated in a range of enzyme reaction solutions, containing one or more of mTG, keratin hydrolysate or putrescine at 4 °C until complete wet back was achieved. After wetting back, the treatment was continued for a further 2 hours at 37 °C to allow for enzyme incorporation of the keratin hydrolysate and/or putrescine into the leather matrix. The samples were then separately processed and then dyed with an acid dye. The lightness of the leathers was measured as an indicator of the strength of colour and the samples were then assayed for dye release in an artificial perspiration solution. By fastness of colour of leather is meant its resistance to the action of an artificial perspiration solution which is supposed to simulate the action of human perspiration. The data are shown in Figures 41 and 42.

With the exception of putrescine treatment, all the treatments increased the depth of shade of dyeing (measured as reduced lightness of shade) on the grain surface of the bated leather, compared to the control (Figure 41). A similar pattern was observed on the flesh surface of the samples although the greatest increase in the depth of shade was achieved after treatment with mTG alone (Figure 42). Analysis of variance (ANOVA) of the dye released by perspiration solution showed a significant difference ($p<0.005$) between the treatment groups with a trend for increasing dye release with increasing degree of treatment when compared to the control. The overall effect (grain +flesh) is shown in Figure 43, which indicates that mTG + keratin hydrolysate significantly increased the depth of shade.

Further work was undertaken with crust leather samples of approximately 1/4 of A4 size and the previously described solutions. The lightness of the leathers was measured as an indicator of the strength of colour and assayed for dye release in an artificial perspiration solution. The data are shown in Figures 44 and 45. Treatment with putrescine, putrescine + keratin hydrolysate, mTG + keratin hydrolysate, or mTG + keratin hydrolysate + putrescine increased the depth of shade on the grain surface compared to the control, with the greatest increase measured in the sample treated with

mTG + keratin hydrolysate (Figure 44). Similarly, treatment with putrescine + keratin hydrolysate, mTG, mTG + keratin hydrolysate, or mTG + keratin hydrolysate + putrescine increased the depth of shade on the flesh surface of the samples (Figure 45). As for the grain surface, the greatest increase in depth of shade was measured after treatment with mTG + keratin hydrolysate. Analysis of the dye release by perspiration solution showed a significant difference between the treatment groups. However, in contrast to the bated samples, treatment with mTG + keratin hydrolysate was found to significantly decrease the dye release in the perspiration solution. The data showed that pre-treatment of crust leather with mTG and keratin hydrolysate could increase the depth of colour of dyeing with an acid dye and also improved dye fastness when exposed to an artificial perspiration solution.

Analysis of the data displayed in Figures 45 and 49 provides evidence that there is no correlation between an increase in depth of shade and higher dye uptake, as the dye remaining in solution after the process was completed did not vary with the different treatments. These results suggest the varying treatments increased the reactivity of the outer layers of the leather, which led to a build up of dye in those layers with the consequent increase in depth of shade. As shown in Figures 43 and 47, it is also important to notice that addition of putrescine to mTG + keratin hydrolysate gave rise to a reduction in depth of shade. Since putrescine is a good substrate for mTG, this decrease would suggest that the presence of putrescine is inhibiting the incorporation of keratin hydrolysate. This is true in both cases, when carrying out the biopolymer with the bated leather and crust leather. Consequently, the data indicate the keratin incorporation is a mTG mediated event. Whilst treatment with keratin hydrolysate and mTG brought about the higher increase in depth of shade, this was reduced when carrying out the treatment with keratin hydrolysate alone which would again suggest that the effect observed was a mTG mediated event (see Figures 43 and 47). This is true for both treatments with bated hide and crust leather. In the light of these results, and assuming that the enhancement of the depth of shade was brought about by mTG in combination with keratin hydrolysate, further investigations were directed towards increasing the effect of increasing keratin offers. As shown in Figure 49 10 % (w-keratin hydrolysate/dry-w of hide) appeared to be the optimum offer.

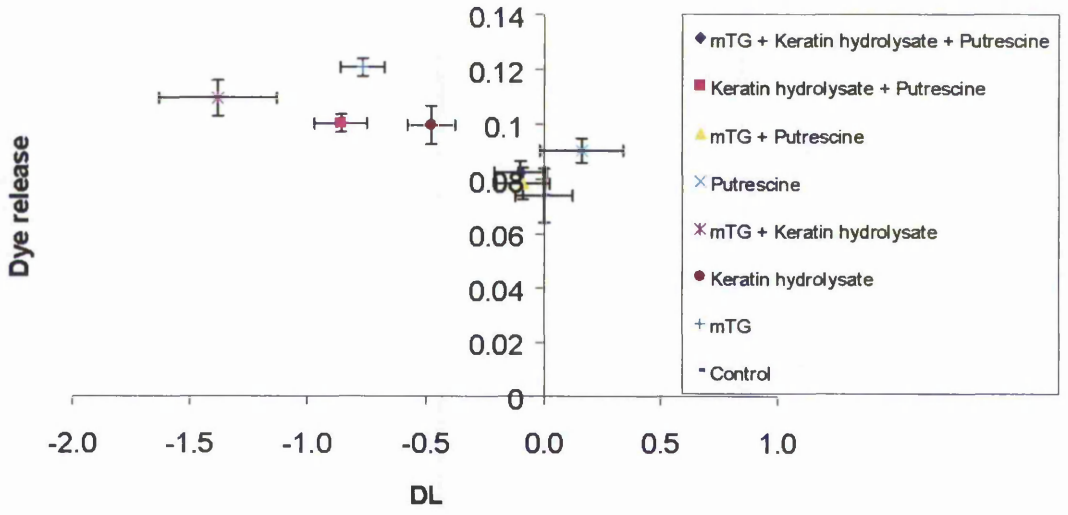


Figure 41. Dye release from freeze-dried bated leather samples treated with mTG and/or biopolymer (putrescine and/or keratin) by perspiration solution treatment vs differences in lightness of the leather grain surface (n=4). The x axis shows the strength of colour (grain side) expressed as the difference in lightness of the leather due to the varying treatments and the untreated control (the value of the untreated control is zero). Lower values are indicative of deeper shades. The y axis shows the dye release in solution due to perspiration solution treatments by uv-vis Spectrophotometer at 574 nm. Higher values are indicative of poorer fastness to perspiration solutions. The results are the average of 4 replicates of the experiments. The error bars show the Standard Error. The dyeing protocol was carried out as described in Chapter 2, Section 2.16, Table IX. The colour and fastness measurements were carried out as described in Chapter 2, Section 2.17 and Section 2.18 respectively.

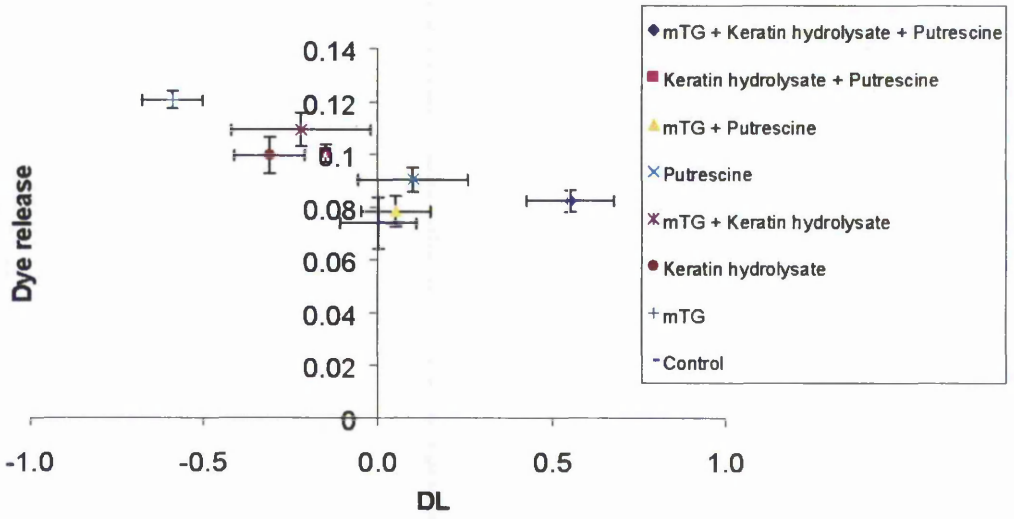


Figure 42. Dye release from freeze-dried bated leather samples treated with mTG and/or biopolymer (putrescine and/or keratin) by perspiration solution treatment vs differences in lightness of the leather flesh surface (n=4). The x axis shows the strength of colour expressed (flesh side) as the difference in lightness of the leather due to the varying treatments and the untreated control (the value of the untreated control is zero). Lower values are indicative of deeper shades. The y axis shows the dye release in solution due to perspiration solution treatments by uv-vis Spectrophotometer at 574 nm. Higher values are indicative of poorer fastness to perspiration solutions. The results are the average of 4 replicates of the experiments. The error bars show the Standard Error. The dyeing protocol was carried out as described in Chapter 2, Section 2.16, Table IX. The colour and fastness measurements were carried out as described in Chapter 2, Section 2.17 and Section 2.18 respectively.

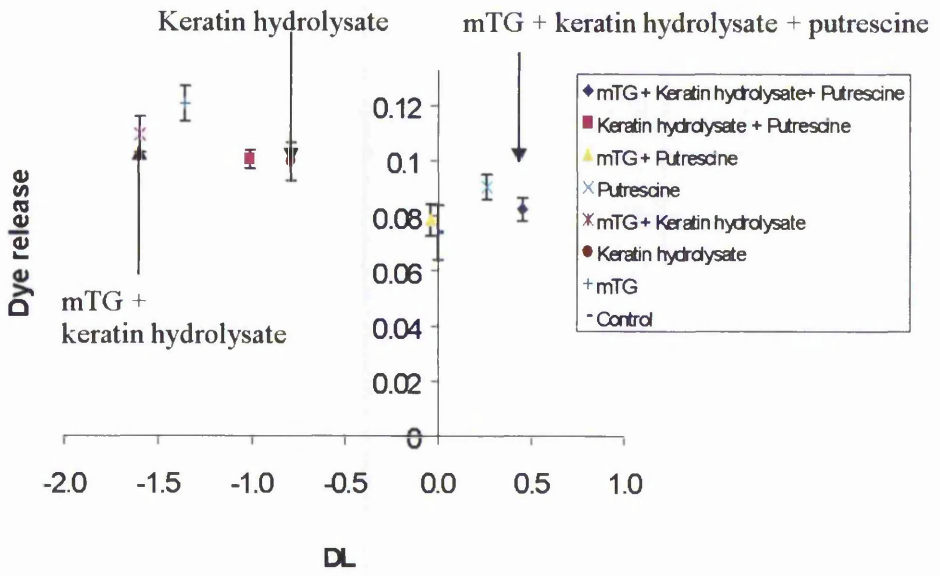


Figure 43. Dye release from freeze-dried bated leather samples treated with mTG and/or biopolymer (putrescine and/or keratin hydrolysate) by perspiration solution treatment vs differences in lightness of the leather grain + flesh surface (n=4). The x axis shows the strength of colour expressed as the difference in lightness of the leather (grain + flesh side) due to the varying treatments and the untreated control (the value of the untreated control is zero). Lower values are indicative of deeper shades. The y axis shows the dye release in solution due to perspiration solution treatments by uv-vis Spectrophotometer at 574 nm. Higher values are indicative of poorer fastness to perspiration solutions. The results are the average of 4 replicates of the experiments. The error bars show the Standard Error. The dyeing protocol was carried out as described in Chapter 2, Section 2.16, Table IX The colour and fastness measurements were carried out as described in Chapter 2, Section 2.17 and Section 2.18 respectively.

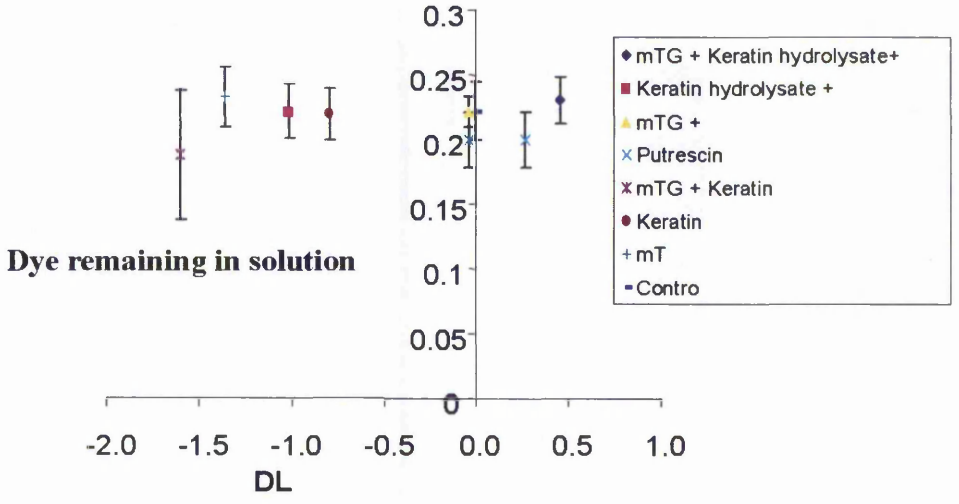


Figure 44. Dye remaining in solution at the end of the dyeing process, bated leather samples treated with mTG and/or biopolymer (putrescine and/or keratin hydrolysate) vs differences in lightness of the leather grain+ flesh surface (n=4). The x axis shows the strength of colour expressed as the difference in lightness of the leather (grain + flesh side) due to the varying treatments and the untreated control (the value of the untreated control is zero). Lower values are indicative of deeper shades. The y axis shows the dye remaining in solution at the end of the dyeing measured by uv-vis Spectrophotometer at 574 nm. Lower values are indicative of higher dye-uptake. The dyeing protocol was carried out as described in Chapter 2, Section 2.16, Table IX.

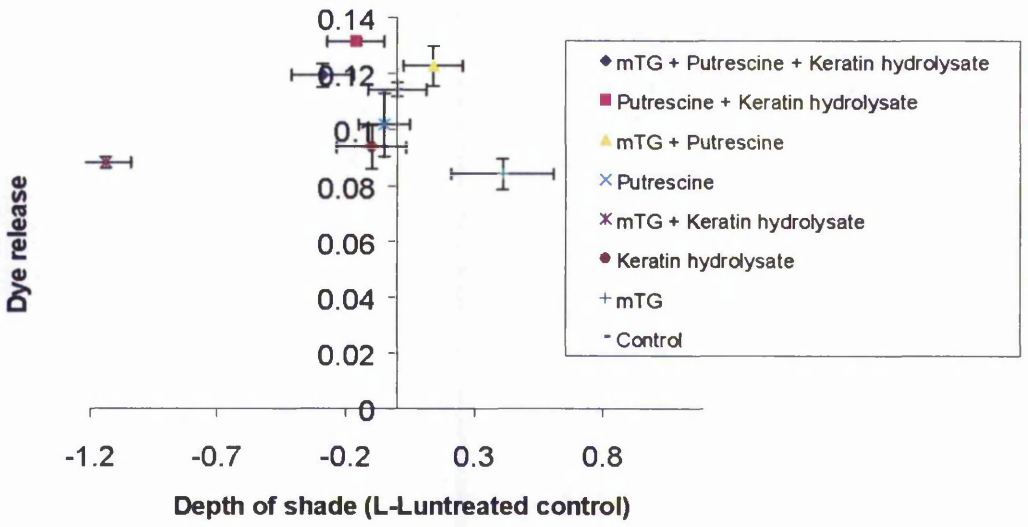


Figure 45. Dye release from crust leather samples treated with mTG and/or biopolymer (putrescine and/or keratin) by perspiration solution treatment vs differences in lightness of the leather grain surface (n=4). The x axis shows the strength of colour (grain side) expressed as the difference in lightness of the leather due to the varying treatments and the untreated control (the value of the untreated control is zero). Lower values are indicative of deeper shades. The y axis shows the dye release in solution due to perspiration solution treatments by uv-vis Spectrophotometer at 574 nm. Higher values are indicative of poorer fastness to perspiration solutions. The results are the average of 4 replicates of the experiments. The error bars show the Standard Error. The dyeing protocol was carried out as described in Chapter 2, Section 2.16, Table IX. The colour and fastness measurements were carried out as described in Chapter 2, Section 2.17 and Section 2.18 respectively.

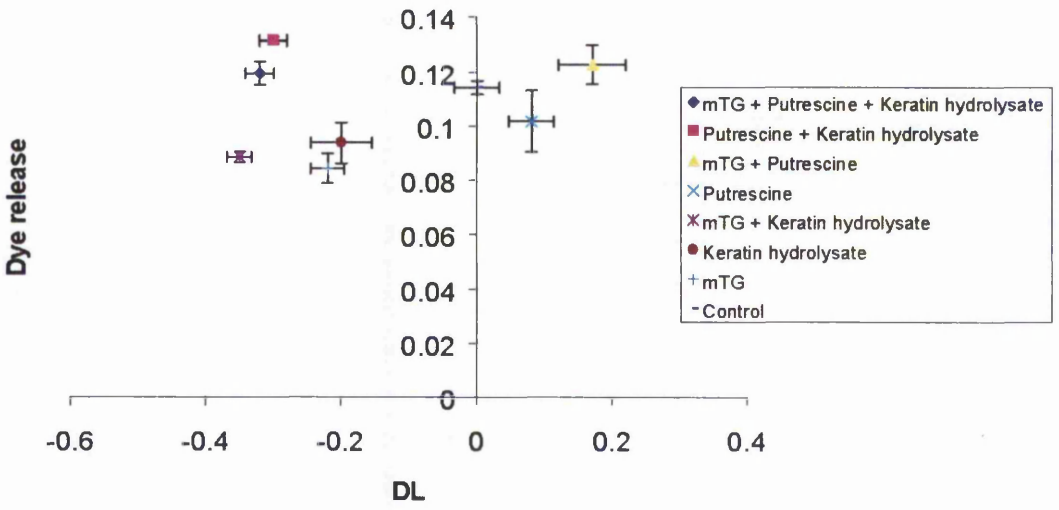


Figure 46. Dye release from crust leather samples treated with mTG and/or biopolymer (putrescine and/or keratin) by perspiration solution treatment vs differences in lightness of the leather flesh surface (n=4). The x axis shows the strength of colour (flesh side) expressed as the difference in lightness of the leather due to the varying treatments and the untreated control (the value of the untreated control is zero). Lower values are indicative of deeper shades. The y axis shows the dye release in solution due to perspiration solution treatments by uv-vis Spectrophotometer at 574 nm. Higher values are indicative of poorer fastness to perspiration solutions. The results are the average of 4 replicates of the experiments. The error bars show the Standard Error. The dyeing protocol was carried out as described in Chapter 2, Section 2.16, Table IX. The colour and fastness measurements were carried out as described in Chapter 2, Section 2.17 and Section 2.18 respectively.

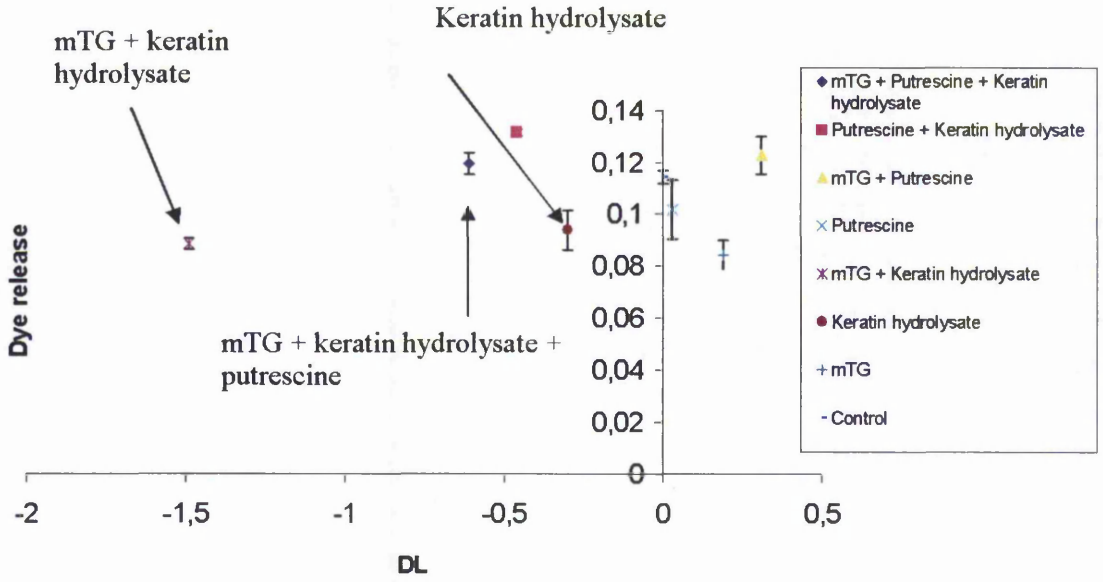


Figure 47. Dye release from crust leather samples treated with mTG and/or biopolymer (putrescine and/or keratin) by perspiration solution treatment versus differences in lightness of the leather grain + flesh surface (n=4).

The x axis shows the strength of colour (grain + flesh side) expressed as the difference in lightness of the leather due to the varying treatments and the untreated control (the value of the untreated control is zero). Lower values are indicative of deeper shades. The y axis shows the dye release in solution due to perspiration solution treatments measured by uv-vis Spectrophotometer at 574 nm. Higher values are indicative of poorer fastness to perspiration solutions. The dyeing protocol was carried out as described in Chapter 2, Section 2.16, Table IX. The colour and fastness measurements were carried out as described in Chapter 2, Section 2.17 and Section 2.18 respectively.

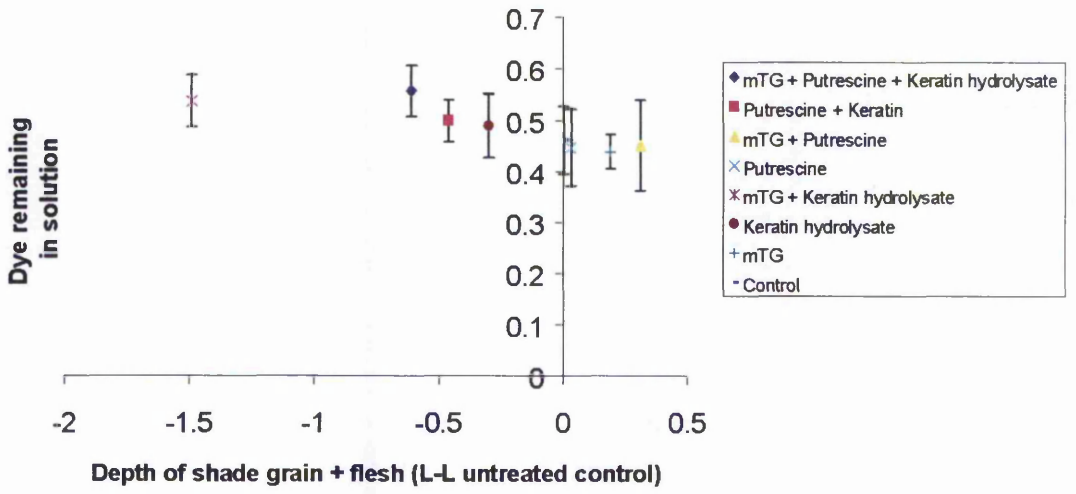


Figure 48. Dye remaining in solution at the end of the dyeing process crust leather samples treated with mTG and/or biopolymer (putrescine and/or keratin hydrolysate) vs differences in lightness of the leather grain+ flesh surface (n=4). The x axis shows the strength of colour expressed as the difference in lightness of the leather (grain + flesh side) due to the varying treatments and the untreated control (the value of the untreated control is zero). Lower values are indicative of deeper shades. The y axis shows the dye remaining in solution at the end of the dyeing measured by uv-vis Spectrophotometer at 574 nm. Lower values are indicative of higher dye-uptake. The dyeing protocol was carried out as described in Chapter 2, Section 2.18, Table IX.

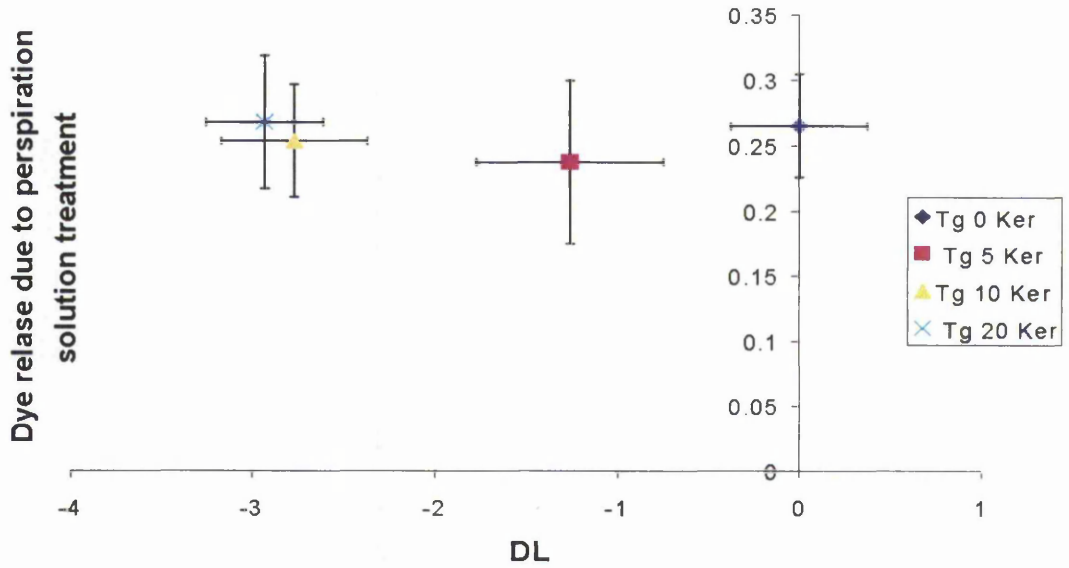


Figure 49. Effect of the keratin offer on the increase in depth of shade of leather and fastness to perspiration solution n=4 The x axis shows the strength of colour (grain + flesh side) expressed as the difference in lightness of the leather due to the varying treatments and the untreated control (the value of the untreated control is zero). Lower values are indicative of deeper shades. The y axis shows the dye release in solution due to perspiration solution treatments measured by uv-vis Spectrophotometer at 574 nm. Higher values are indicative of poorer fastness to perspiration solutions. The results are the average of 4 replicates of the experiments. The results showed that an increase from 10 to 20 % keratin offer, did not result in a significant increased in depth of shade. The dyeing protocol was carried out as described in Chapter 2, Section 2.16, Table IX. The colour and fastness measurements were carried out as described in Chapter 2, Section 2.17 and Section 2.18 respectively.

5.5 Microbial transglutaminase mediated incorporation of keratin hydrolysate in crust leather and its effect on dyeing process at a larger scale

In the light of the results observed in the initial investigations further work was undertaken on a larger scale (A4 size pieces) with only crust leather. Crust leather was the preferred substrate instead of freeze dried bated hide, as better results were observed in terms of fastness to perspiration solution and the problems associated with freeze drying on a larger scale. In order to design a process that was operational to current tannery conditions, the step of soaking the crust leather with the enzyme and keratin hydrolysate solutions overnight at 4 °C was omitted, and soaking of dry crust leather with the treatment solutions was only carried out for 2 hours at 37 °C. Subsequent treatments were carried out in the same way. The experiments looked at the effect of mTG mediated incorporation of keratin hydrolysate on the depth of shade, dye fastness to perspiration solutions and dye fastness to washing solutions. By fastness of the colour of the leather to washing is meant its resistance to washing under domestic conditions. In order to validate the effects observed in the preliminary work and ensure that it was not just an artefact introduced by the variability of the dyeing process, the number of dyeing processes was increased up to 12 for each treatment with the subsequent analysis of fastness to perspiration and washing. The results are shown in Figure 50, Figure 51 and Figure 52. As shown in Figure 50, treatment with mTG and keratin hydrolysate resulted in a significant increase in depth of shade on the grain side of the leathers. However, the increase observed was reduced one and half fold in respect with the results from preliminary investigations. (-0.8 DL units in Figure 50 units compared with –1.5 DL units in Figure 45). Also observed was an improvement in the fastness to washing solution, as a deeper shade did not result in a significantly higher fade of the shade due to treatment with wash fastness solutions. Unlike the previous experiments, mTG mediated incorporation of keratin hydrolysate did not significantly increase the depth of shade in the flesh side as shown in Figure 51. (-0.04 DL units in Figure 51compared with -0.4 DL units in Figure 46). As shown in Figure 52, mTG mediated incorporation of keratin hydrolysate improved the fastness to perspiration solution, as a deeper shade did not give rise to a significant increase ($p>0.005$) in dye released following to perspiration solution treatment.

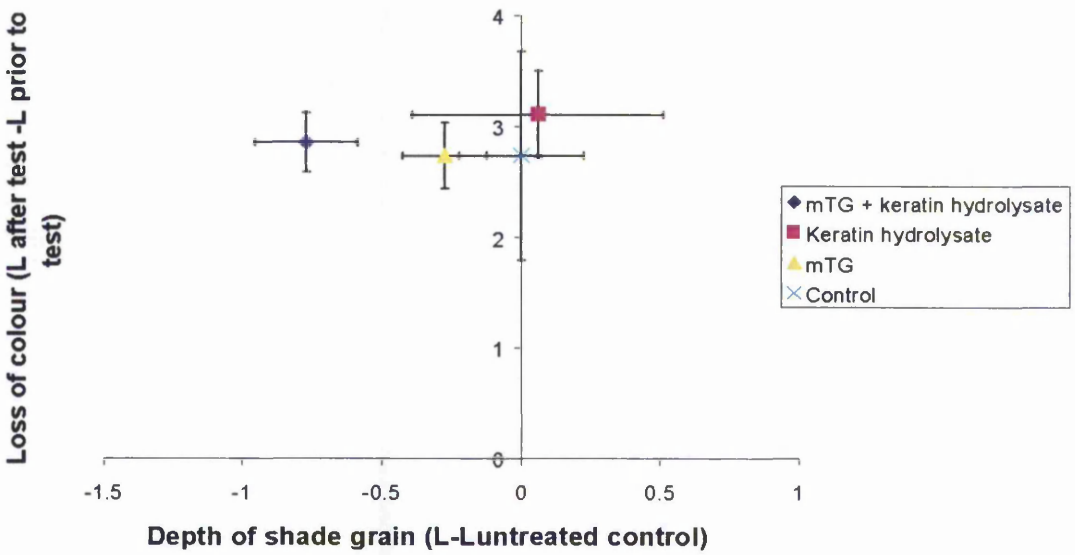


Figure 50. Loss of colour due to wash fastness treatment solution vs differences in lightness of the leather grain side n=12

The x axis shows the strength of colour (grain side) expressed as the difference in lightness of the leather due to the varying treatments and the untreated control (the value of the untreated control is zero). Lower values are indicative of deeper shade. The y axis shows the loss of colour due to the washing solution treatment (L after the test minus L before the test). Higher values indicate poorer resistance to washing fastness solutions. The results are the average of the 12 replicates of the same experiment. The error bars show the Standard Error. The dyeing protocol was carried out as described in Chapter 2, Section 2.16, Table IX. . The colour and fastness measurements were carried out as described in Chapter 2, in section 2.17 and 2.19 respectively.

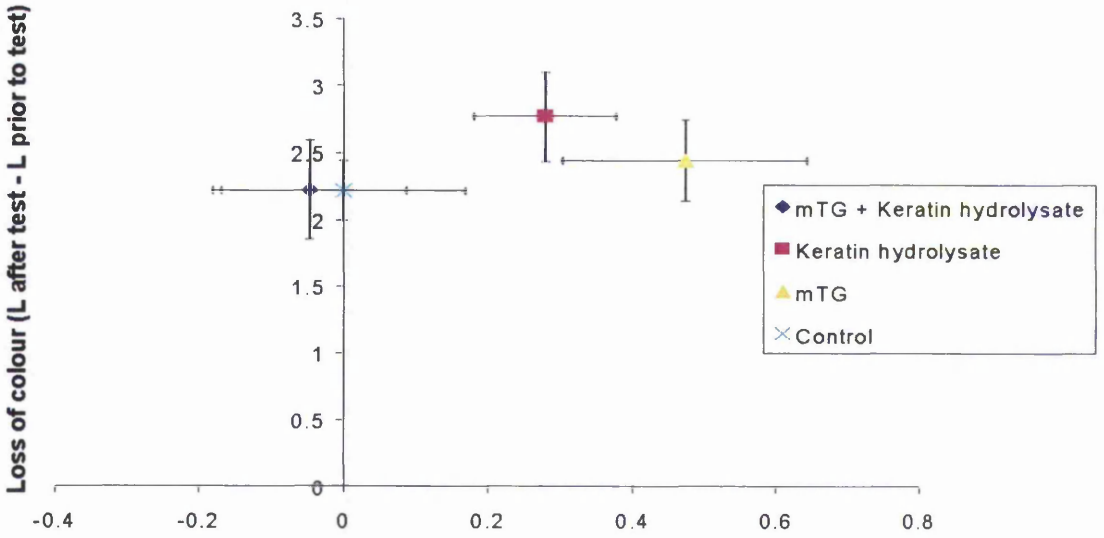


Figure 51. Loss of colour due to wash fastness treatment solution vs differences in lightness of the leather flesh side n=12

The x axis shows the strength of colour (flesh side) expressed as the difference in lightness of the leather due to the varying treatments and the untreated control (the value of the untreated control is zero). Lower values are indicative of deeper shade. The y axis shows the loss of colour due to the washing solution treatment (L after the test minus L before the test). Higher values indicate poorer resistance to washing fastness solutions. The results are the average of the 12 replicates of the same experiment. The error bars show the Standard Error. The dyeing protocol was carried out as described in Chapter 2, Section 2.16, Table IX. The colour and fastness measurements were carried out as described in Chapter 2, in section 2.17 and 2.19 respectively.

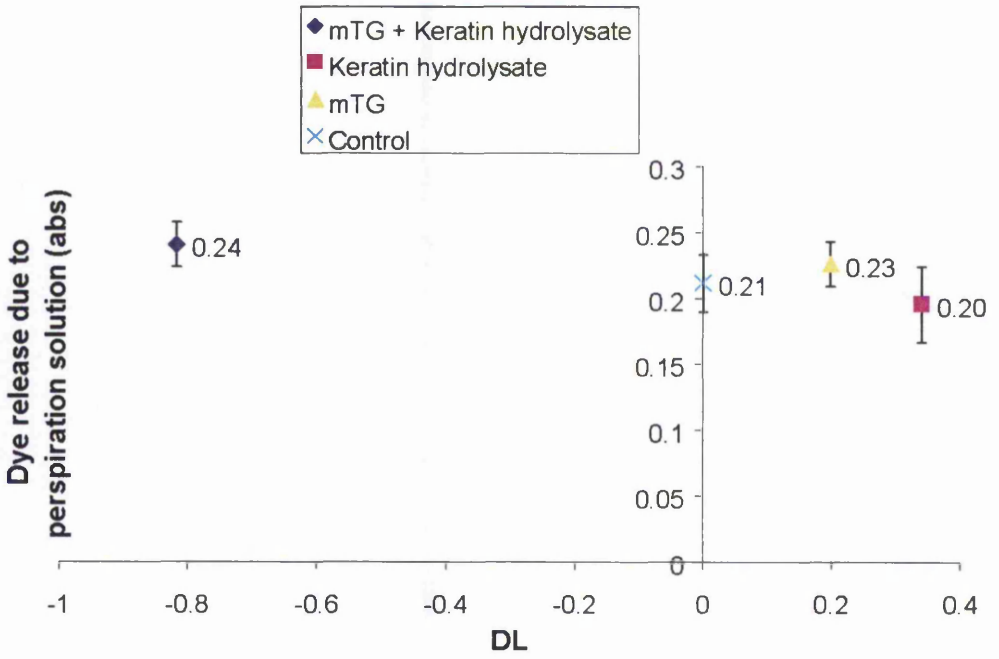


Figure 52. Dye release from crust leather samples treated with mTG and biopolymer by perspiration solution treatment vs differences in lightness of the leather grain + flesh surface (n=12). The x axis shows the strength of colour (grain + flesh side) expressed as the difference in lightness of the leather due to the varying treatments and the untreated control (the value of the untreated control is zero). Lower values are indicative of deeper shades. The y axis shows the dye release in solution due to perspiration solution treatments measured by uv-vis Spectrophotometer at 574 nm. Higher values are indicative of poorer fastness to perspiration solutions. The results are the average of 12 replicates of the experiments. The dyeing protocol was carried out as described in Chapter 2, Section 2.16, Table IX. The colour and fastness measurements were carried out as described in Chapter 2, Section 2.17 and Section 2.18 respectively.

5.6 Microbial transglutaminase mediated incorporation of keratin hydrolysate in wool on sheepskins and its effect on dyeing.

Further work investigated the ability of mTG to incorporate keratin hydrolysate into wool on crust slink skins on which the wool is still attached and its effect on dyeing and on the wool properties. Since this was the substrate produced by one of the partner tanneries in the consortium, initial investigations were carried out on a small scale leather production. Microbial transglutaminase mediated incorporation of keratin hydrolysate was undertaken as in the experiments described in the previous section. The effect mTG mediated incorporation of keratin hydrolysate on the depth of shade, the resistance to wash fastness solutions, the resistance to perspiration fastness solutions and the wool resistance to abrasion were investigated. The latter variable was considered since work carried out by (Cortez *et al.*, (2002); Cortez *et al.*, (2004)) has provided evidence that mTG + keratin hydrolysate treatment of wool improves its physical properties. As shown in Figures 53 and 54, mTG mediated incorporation of keratin hydrolysate into "wool on" slink crust skins significantly ($p<0.01$) enhanced the depth to about 1.75 DL units. However, neither the fastness to washing nor the fastness to perspiration was improved. A significant increase in the loss of dye due to treatments with wash fastness solutions and also higher dye release by means of perspiration solutions can be seen in Figure 53 and Figure 54. Analysis of the data displayed in Figure 55 provides evidence that the treatment resulted in a significant enhancement of the wool's resistance to abrasion ($p<0.01$). The results indicate that the effect provided by mTG is further increased when adding keratin hydrolysate.

In the light of these results, the consortium agreed to carry out further work on full scale. The results of these trials (see Figure 56 and Figure 57) correlated to a certain extent with what had previously been found at a laboratory scale, as the treatment with mTG and keratin resulted in leathers with a significantly deeper ($p<0.01$) shade (1 DL units against the untreated control). However, the fastness to perspiration also appeared to be improved since a significant reduction in dye release due to treatment with artificial perspiration solution (from approximately 0.2 to 0.15). As in the previous

trials an increase of dye loss due to wash fastness was associated with the enhancement in depth of shade, see Figure 58, which allows one to conclude that wash fastness was not improved. Figure 58 shows that mTG mediated incorporation of keratin in crust leather significantly increased ($p<0.005$) the resistance of wool to abrasion (the percentage of wool lost was lowered from 1.06% in the untreated control to 0.64 % in the mTG/keratin sample).

With regards to the results reported in this chapter it can be concluded that mTG mediated incorporation of keratin hydrolysate significantly increases the depth of shade of the grain side of bovine crust leather. Some inconsistencies have been observed when evaluating the data from the flesh side and it appears unlikely that the same treatment enhances the depth of shade in the flesh side, and if an increase is occurring this is very little. The results provide good evidence that the mTG mediated incorporation of keratin hydrolysate improves both the fastness to washing and perspiration solutions.

The increase in depth of shade is also observed for both small scale tannery production and full scale with wool on slink crust leather. However, a reduction in the enhancement of depth of shade was measured during the scale up (from –1.8 DL to –1 DL units). Unlike bovine crust leather, the results indicate that deeper shades are not associated with better fastness. This is true for both fastness to wash and perspiration solutions. Preliminary investigations on the effect of mTG mediated incorporation of keratin hydrolysate suggest that higher wool resistance to abrasion can also be achieved.

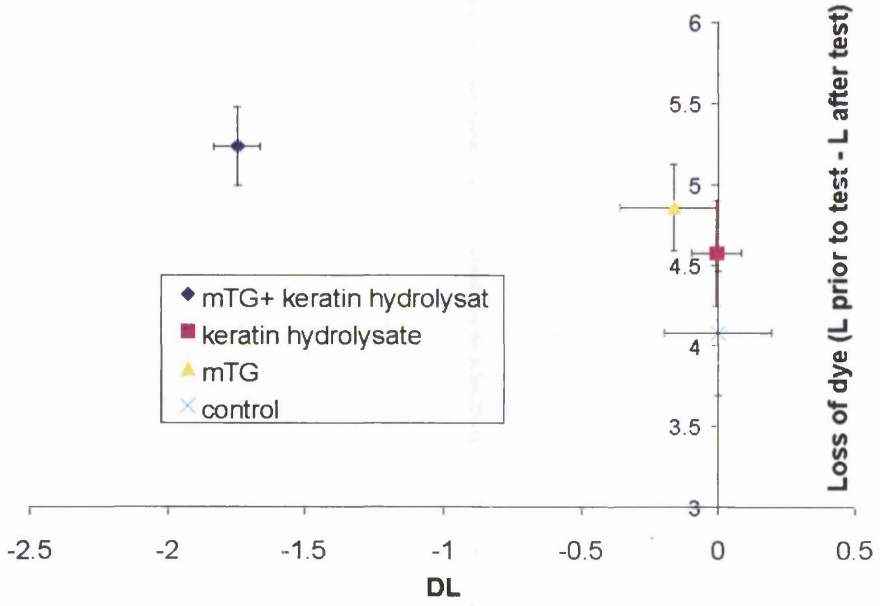


Figure 53. Loss of colour due to wash fastness treatment solution vs differences in lightness of the leather (n=12 wool on slink skin crust leather)

The x axis shows the strength of colour (flesh side) expressed as the difference in lightness of the leather due to the varying treatments and the untreated control (the value of the untreated control is zero). Lower values are indicative of deeper shade. The y axis shows the loss of colour due to the washing solution treatment (L after the test minus L before the test). Higher values indicate poorer resistance to washing fastness solutions. The results are the average of the 12 replicates of the same experiment. The error bars show the Standard Error. The dyeing protocol was carried out as described in Chapter 2, Section 2.16, Table X. The colour and fastness measurements were carried out as described in Chapter 2, in section 2.17 and 2.19 respectively.

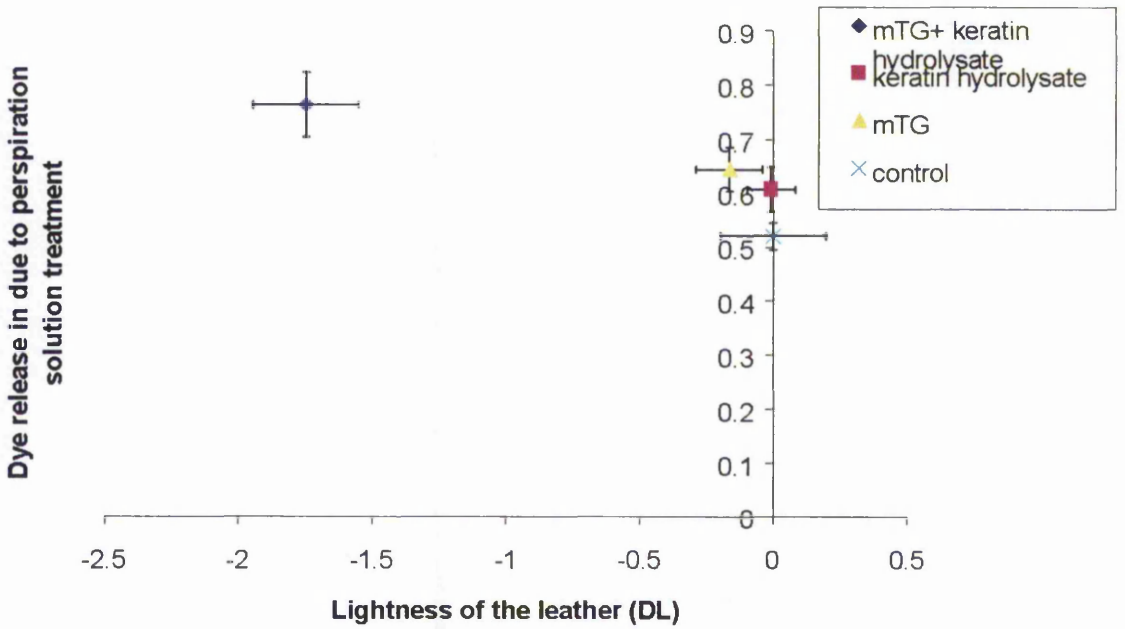


Figure 54. Dye release from crust leather samples treated with mTG and biopolymer by perspiration solution treatment vs differences lightness of the leather(n=12 wool on slink skin crust leather)

The x axis shows the strength of colour (flesh side) expressed as the difference in lightness of the leather due to the varying treatments and the untreated control (the value of the untreated control is zero). Lower values are indicative of deeper shades. The y axis shows the dye release in solution due to perspiration solution treatments measured by uv-vis Spectrophotometer at 415 nm. Higher values are indicative of poorer fastness to perspiration solutions. The results are the average of 12 replicates of the experiments. The dyeing protocol was carried out as described in Chapter 2, Section 2.16, Table X. The colour and fastness measurements were carried out as described in Chapter 2, Section 2.17 and Section 2.19 respectively.

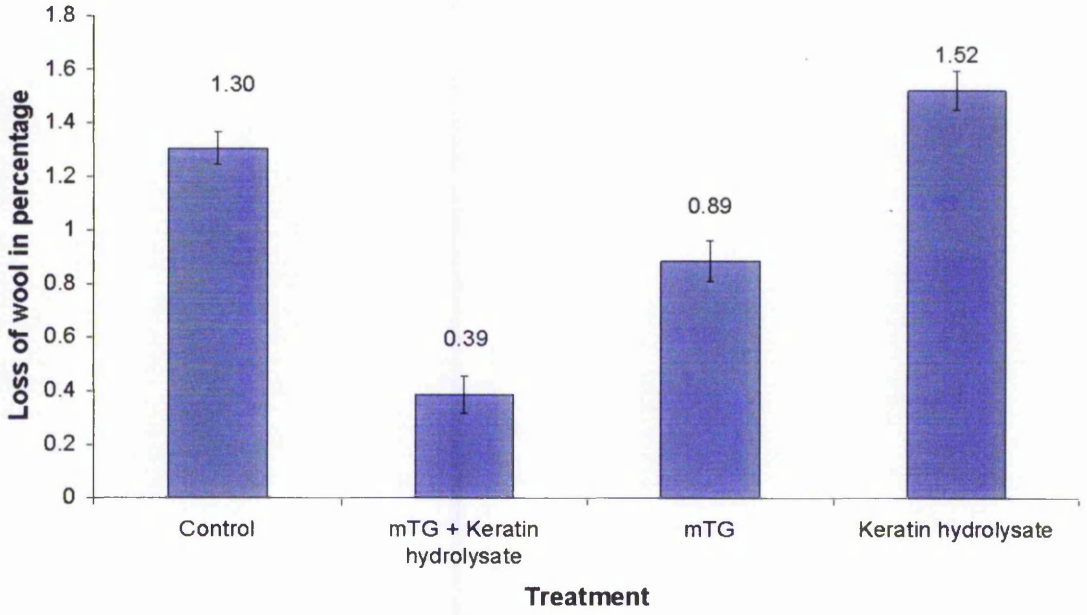


Figure 55. Wool resistance to abrasion after 1000 rubbing cycles (n=12). A higher percentage of wool loss is indicative of less resistance to abrasion. The results are the average of the replicates of 12 experiments. The y-error bars show the standard error.

A circular test specimen was taken from the sheep leather sample, weighed and placed in the specimen holder with the wool facing downwards. The test conditions were 12 K Pa and 1000 cycles. Once the test was completed, the weight of the samples was measured again. The resistance of wool to abrasion was expressed as the relative lost in weight. The wool resistance to abrasion was carried out as described in Chapter 2, section 2.20.

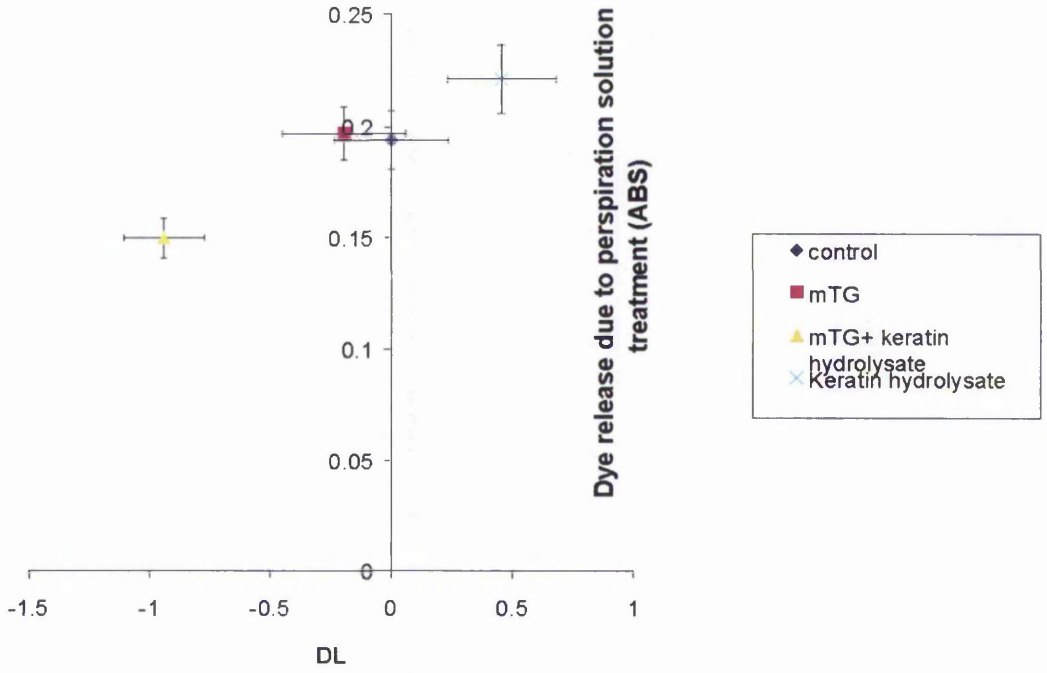


Figure 56. Dye release from crust leather samples treated with TG and biopolymer by perspiration solution treatment vs differences in lightness of the leather(n=12 wool on slink skin crust leather. Results from the industrial trials.

The x axis shows the strength of colour (flesh side) expressed as the difference in lightness of the leather due to the varying treatments and the untreated control (the value of the untreated control is zero). Lower values are indicative of deeper shades. The y axis shows the dye release in solution due to perspiration solution treatments measured by uv-vis Spectrophotometer at 415 nm. Higher values are indicative of poorer fastness to perspiration solutions. The results are the average of 12 leathers from one load full scale dyeing process carried out in one of partner's tanneries. The dyeing protocol was carried out as described in Chapter 2, Section 2.16, Table X. The colour and fastness measurements were carried out as described in Chapter 2, Section 2.17 and Section 2.18 respectively.

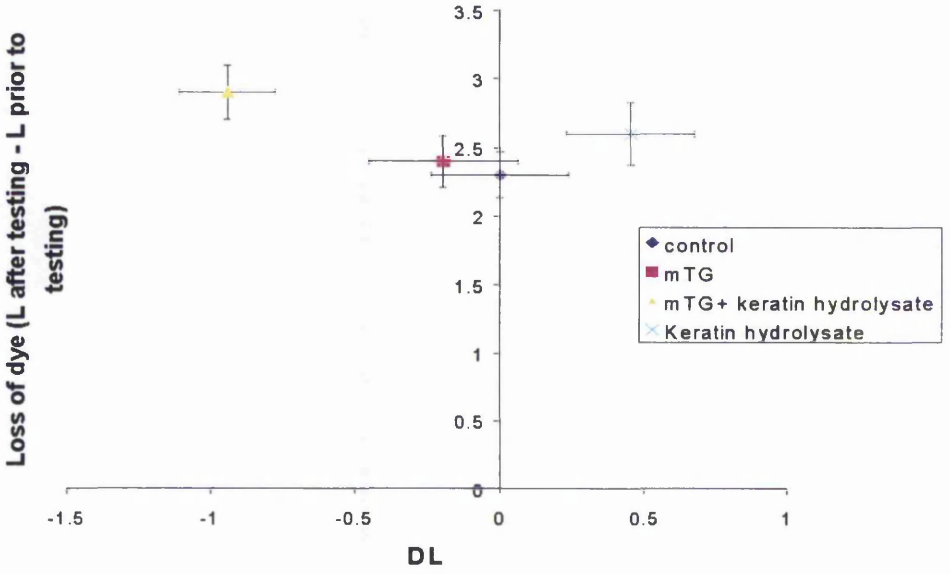


Figure 57. Loss of colour due to wash fastness treatment solution vs differences lightness of the leather (n=12 wool on slink skin crust leather). Results from the industrial trials.

The x axis shows the strength of colour (flesh side) expressed as the difference in lightness of the leather due to the varying treatments and the untreated control (the value of the untreated control is zero). The y axis shows the loss of colour due to the washing solution treatment (L after the test minus L before the test). Higher values indicate poorer resistance to washing fastness solutions. The results are the average of 12 leathers from one load full scale dyeing process carried out in one of partner's tanneries. The dyeing protocol was carried out as described in Chapter 2, Section 2.16, Table X. The colour and fastness measurements were carried out as described in Chapter 2, Section 2.17 and Section 2.19 respectively.

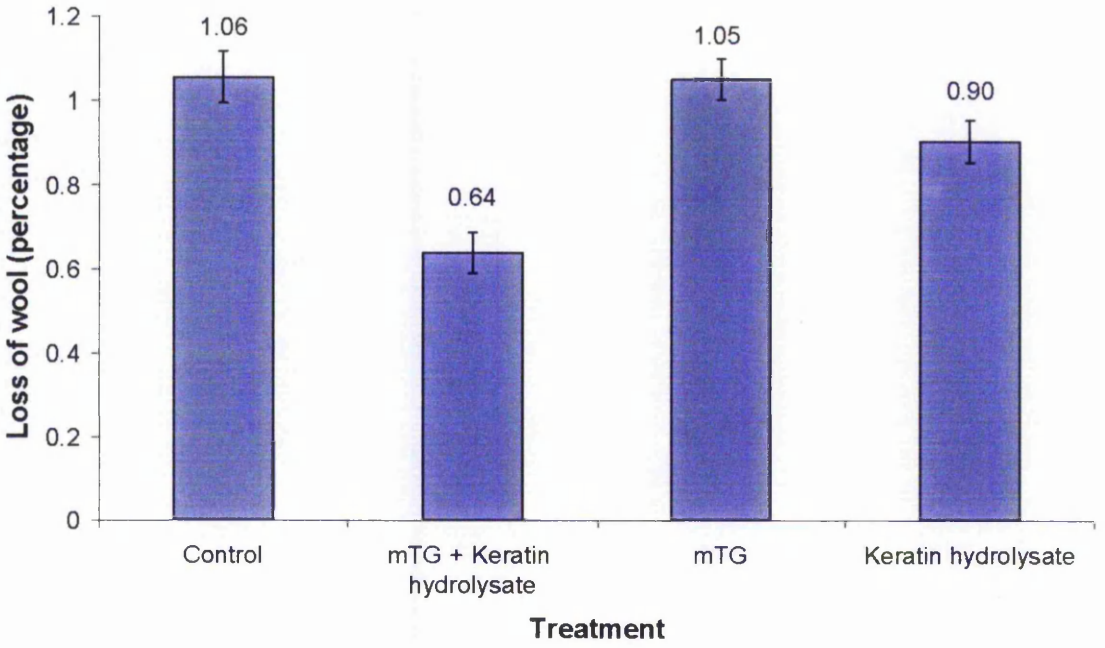


Figure 58. Wool resistance to abrasion after 1000 rubbing cycles (n=12). A higher percentage of wool loss is indicative of less resistance to abrasion. Results from the industrial trials.

A circular test specimen was taken from the sheep leather sample, weighed and placed in the specimen holder with the wool facing downwards. The test conditions were 12 K Pa and 1000 cycles. Once the test was completed, the weight of the samples was measured again. The resistance of wool to abrasion was expressed as the relative lost in weight. The results are the average of 12 leathers. The wool resistance to abrasion was carried out as described in Chapter 2, section 2.20.

5.7 Discussion

The results reported in this chapter suggest that there is a huge scope to apply biopolymers, in particular keratin hydrolysate, during the post tanning operations in a view to improving the dyeing performance of the leather. They match to a certain extent with previous findings that investigated the modification of physical and aesthetic properties of the leather by means of protein hydrolysates (Cabeza, 1999; Cantera, 2000; Ma S., 1999; Ma J., 2004).

It must be acknowledged that the concept of mTG mediated incorporation of keratin hydrolysate in the post-tanning operations, has been applied more empirically than scientifically, and there are plenty of questions that have yet to be answered. Furthermore, very little has been done in this area and it is difficult to find papers in the literature to compare the outcome of this work with the findings arising from similar research. The intention of this discussion is to hypothesise about the reasons why mTG mediated incorporation of keratin hydrolysate results in a leather with deeper shade and how this might be further proven to be correct.

The first factor that should be considered is the chemical composition of keratin hydrolysate and the consequent implications on the modification of the reactivity of leather towards dyestuffs by incorporation of such substrate. Table XVI shows the theoretical amino acids incorporated using the keratin hydrolysate and their characteristics in terms of polarity and capacity to interact with anionic dyes.

Since the literature has provided evidence that the dye fixation mechanism with proteins is extensively more complex than just a simple ionic interaction between the cationic protonated lysine and the sulphonate residue in the dye molecule (Haurowitz, 1963), it is very likely that as shown in Table XV, that incorporation of keratin hydrolysate within the leather matrix potentially increases the sites for the fixation of dyes whatever the type of mechanism. If the effect observed is due to crosslinking of keratin through the collagen lysines via formation of ε(γ-glutamyl) lysine cross-link

between proteins with the consequent reduction of free lysines and hence the potential reduction for dye affinity, the literature has also provided evidence that although binding of the dye to the protein is initiated by the long range electrostatic forces (e.g. ionic bonding), the short-range Van der Waals' forces are more efficient after binding has been established (Muralidharan, 1990)

Table XV. Amino acid composition of wool keratin (g amino acid in 100 g of proteins)

Amino acid	Composition*	Chemical characteristics**
Glycine	5.2	-
Alanine	3.4	Apolar-hydrophobic interactions
Valine	5	Apolar-hydrophobic interactions
Leucine	7.6	Apolar-hydrophobic interactions
Isoleucine	3.1	Apolar-hydrophobic interactions
Proline	5.3	Apolar-hydrophobic interactions
Phenylalanine	3.4	Apolar-hydrophobic interactions
Tyrosine	4	Polar-hydrogen bonding capacity
Tryptophan	1.8	Apolar-hydrophobic interactions
Serine	7.2	Polar-hydrogen bonding capacity
Threonine	6.6	Polar-hydrogen bonding capacity
Cysteine	22.4	Polar-hydrogen bonding capacity
Methionine	0.5	Apolar-hydrophobic interactions
Arginine	9.2	Basic-cationic
Histidine	2.8	Basic-cationic
Lysine	0.7	Basic-cationic
Aspartic acid	6.4	Acidic-anionic-hydrogen bonding capacity
Glutamic acid	13.1	Acidic-anionic-hydrogen bonding capacity
Hydroxyproline	0	Polar
Hydroxylysine	0.2	Polar/basic-cationic

* (Haurowitz, 1963)

**(Lehninger, 1975)

Unlike cationic fixatives, where the increase in depth of shade is accompanied with a higher dye uptake, it is worth noting that this was not observed in the current study. This would further suggest that the effect observed is due to other types of interaction, particularly of the hydrophobic type, since the literature also reports dye build up in the outer layers of the leather with increasing hydrophobicity of the dyes (Britten, 2004). This might explain why mTG mediated incorporation of keratin hydrolysate increases the depth of shade of crust leather. To confirm this further work should be undertaken with dyes with a more hydrophobic character, if the hypothesis is correct application of dyes with increasing hydrophobicity should result in leathers with deeper shades. This thesis does not provide sufficient data to further discuss why mTG and keratin hydrolysate treatment of crust leather results in leather with deeper shades.

The second big question that this thesis does not answer is if crosslinking between the collagen and the keratin hydrolysate is taking place. The apparent answer to this question would be given by quantification of ε(γ-glutamyl) lysine cross-link of crust leather. That was the intention of this thesis, however this was not possible because complete digestion for crosslinks quantification is impeded by the high stability provided by chrome tanning. Therefore alternative protocols should be developed for crosslink quantification. Despite the fact that there is not direct evidence of mTG mediated crosslinking of keratin hydrolysate into the leather matrix, some of the data discussed in this study suggest that at least the effect is a mTG mediated event. Firstly because the qualitative analysis of the uptake of biotinylated keratin hydrolysate indicated a significant increase in the presence of mTG (as shown in Figure 40). Secondly, the reduction observed in the presence of putrescine, supports the hypothesis that mTG is involved to some extent to incorporate the protein hydrolysate into the leather matrix (as shown in Figures 43 and 47). The last argument is that the effect is a combination between mTG and keratin hydrolysate, and this was not observed when applying either mTG or keratin hydrolysate alone (as shown in Figures 43, 47, 50, 51, 52, 53, 54, 56 and 57). Evaluation of the effect of mTG mediated incorporation of dimethylcasein and/or N-N' dimethylcasein in which the free glutamines have been blocked by mTG mediated incorporation of putrescine into crust leather on its dyeing properties might one way of answering this.

Despite the fact that further research is necessary to understand the chemistry and biochemistry behind the improvement of the dyeing properties of the leather by means of mTG and keratin hydrolysate, the outcome of this work has provided evidence that there is a huge scope to explore novel applications based on the use of biopolymers in the leather industry with a view to optimising the post tanning operations. Further work is currently underway, this is investigating the potential of other biopolymers and also exploring the possibility of recovering keratin from the liming process and its consequent application during the post tanning operations.

Chapter 6. General discussion and future work

The initial objective of this thesis was to investigate the potential for using the protein cross-linking enzyme mTG to increase the shrinkage temperature of bated hide to produce a tanning effect, in a similar manner to the chemical crosslinking agents currently in use as tanning agents.

Initial work was done to optimise the process conditions with a view to maximising the amount of mTG mediated cross-linking. Since the literature has reported hydrolysis of the collagen amides (asparagine and glutamine) to their acid form (aspartic and glutamic acid) due to the basic conditions encountered during liming (Highberger *et al.*, 1941; Warner, 1942; Bowes, 1949; Menderes *et al.*, 1999) the extent of amide hydrolysis occurring under artificial and real liming conditions was quantitated. The results indicated that after incubating bovine tendon Type I collagen for 16 hours in the presence of Ca^{2+} at pH 12.5, only 10.6% of the amides were hydrolysed (as shown in Table XI) . Surprisingly hydrolysis was not observed with the same pH and ionic strength conditions in the presence of Na^{+} instead of Ca^{2+}. The degree of deamidation of mechanically dehaired raw hide in real liming conditions was determined to be 27% of the total collagen amides (as shown in Table XI). The effect of Ca^{2+} on the mechanism of deamidation was investigated using pure glutamine as a model substrate, to remove the possibility of other collagen or hide components being involved. Although hydrolysis was observed in the presence of either Na^{+} or Ca^{2+}, a higher degree of hydrolysis was observed with Ca^{2+}, which would further suggest that Ca^{2+} is directly involved in catalysing deamidation of glutamine (the results are shown in Figure 10). The outcome of this work correlates to a certain extent with what is found in the literature, since 27% amide hydrolysis is a figure within the range that has been previously reported (Higberger *et al.*, 1941; Warner, 1942; Bowes, 1949; Menderes *et al.*, 1999). The possibility that Ca^{2+} catalyses the deamidation reaction has been suggested by some authors. Buckingham (1977) and Houghton (1979) reported metal catalysed hydrolysis of parathion, methyl parathion and alidcarb. Warner *et al.* (1947) investigated the formation of ammonia from egg albumin, β-lactoglobulin and edestin

at varying temperatures in alkaline solution. They showed that considerable ammonia may also originate from other amino acids such as arginine. The possibility exists, therefore, that the ammonia given off during liming is due partly or wholly to amino acids other than glutamine or asparagine. A review paper published by Bowes (1949) states that only 19.6 % of amides are lost. Previous work carried out by Highberger *et al.* (1941) state that 25 % of the amides are hydrolysed during liming conditions, although unlike the data provided in this thesis, the effect was not favoured by Ca^{2+}. Menderes *et al.* (1999) have recently investigated amide hydrolysis by measuring the ammonia released in solution during liming. They have reported 42.5% hydrolysis of the total amides, a figure much higher than what has been found in this project.

Although the data suggested that 73% of the collagen glutamines remained intact after liming, the possibility of designing an unhairing process at neutral conditions as an alternative to conventional liming was investigated. Dispase has been used to separate the epidermis from the dermis in biomedical applications (Stenn *et al,* 1989; Paul *et al.*, 2001) and it is believed that the specific activity of the enzyme on collagen type IV underpins the mechanism to remove the epidermis leaving the epithelial cells intact (Paul *et al.*, 2001) . However, it has also been reported that using proteases with low collagenase and elastase activity does not necessarily result in leather without grain damage (Germann 1997). The literature also provides evidence of processes based on the combination of enzymes and lyotropic reagents (Frendrup, 2000). Urea has also been applied in chemical unhairing systems, to partially substitute the changes introduced in collagen by conventional liming (Thorstensen, 1964). Furthermore, Gustavson (1960) investigated the ability of 8 M urea solutions to dissolve hair. In light of the above, unhairing at neutral conditions was performed with Dispase, a proteolytic enzyme from *Bacillus Polymixa* (Takekawa *et al.*, 1991) and urea to bring about the lyotropic effect. The swelling effect that occurs during liming was introduced by carrying out a salt free pickling process. The data demonstrated that complete unhairing was achieved (as shown in Figure 12), and that it was likely to be effected by a combination of the enzyme, the urea and the acid swelling (as shown in Table XII). However, the degree of grain damage was such that the process was not suitable for full

grain leather production (data presented in Figure 12). Furthermore, mTG mediated incorporation of [^{14}C] putrescine indicated that the substrate was not suitable for the enzyme, as little amine incorporation was observed (data shown in Figure 15). Paul *et al.* (2001) have also used Dispase for unhairing purposes and showed that incubation of bovine hide with Dispase resulted in separation of the epidermis at the point of the epidermal/dermal junction with consequent hair loosening. Examination of the finished leathers by Scanning Electronic Microscopy did not show grain damage. However, unlike the results reported in this thesis, complete hair removal was not observed. It is difficult to make a direct comparison between their work and the work reported in this study, since the experimental conditions were changed by the introduction of a lyotropic reagent and acid swelling. However, the data presented in Chapter 3 suggested that the grain damage was associated with the urea (micrographs in Figure 12). Unlike the work carried out by Paul *et al.* (2001) results showed that complete hair removal might be achieved. Although again, this appeared to be caused by the combination of Dispase, urea and acid swelling (as shown in Table XII).

Collighan *et al.* (2004) investigated the possible inhibitory effects of the chemicals applied in the early stages of the process prior to mTG treatment. $CaCl_2$ and $(NH_4)_2SO_4$ particularly inhibited the enzyme activity whilst Na_2S inhibited mTG activity to a much lesser extent. The protease preparation used for bating did not affect mTG activity. A preliminary literature survey has not found other authors that report the inhibitory effects of $CaCl_2$ and $(NH_4)_2SO_4$. In light of this, $Ca(OH)_2$ was substituted by NaOH, and $(NH_4)_2SO_4$ by H_2SO_4 in the experimental beamhouse process. Comparison of the level of mTG mediated-[^{14}C] putrescine incorporation into powdered bated hides processed with either $Ca(OH)_2$ liming and $(NH_4)_2SO_4$ deliming, NaOH liming and H_2SO_4 deliming, or Dispase unhairing (without urea or acid swelling) showed that a much greater level of amine incorporation could be achieved with the NaOH limed and H_2SO_4 delimed hide powder (data presented in Figure 15). As a pure conjecture, higher [^{14}C] putrescine incorporation might have been observed because the inhibitory effect of residual Ca^{2+} and $(NH_4)_2SO_4$, and/or hydrolysis of glutamine occurred to a higher extent with traditional liming. The first hypothesis would be explained by the results

from the inhibitory experiments carried out by Collighan *et al.* (2004) and would match with the inhibitory effect of ammonia on transglutaminases activity reported by Folk *et al.* (1966).The second would match with the higher degree of hydrolysis observed with $Ca(OH)_2$ liming solutions with respect to NaOH solutions.

With the leather processing conditions optimised to ensure maximum amount of cross-linking, the results showed that the enzyme could incorporate 4 nmol of crosslink.mg^{-1} protein when used alone. In conjunction with a cross-linking facilitator, such as the glutamine-rich casein, this can be further increased up to 9 nmol-crosslink.mg^{-1} (data presented in Figure 20). However, incorporation of this amount of cross-linking did not bring about any changes in the shrinkage temperature of bated hide (as shown in Table XIII and Figures 19 and 21). However, mTG-mediated cross-linking of bated hide modified some of the physical properties of bated hide, such as an increase in the resistance towards microbial collagenase (Figure 16), an increase towards acid hydrolysis (Figure 17) and a reduction of the tensile strength of chrome tanned (Figure 23) and glutaraldehyde tanned leather (Figure 24). However, the increase in resistance towards acid hydrolysis and microbial collagenase observed was lower than that of glutaraldehyde crosslinked hide powder.

Since glutaraldehyde covalently cross-links collagen via the ε-amino group of lysines to produce the tanning effect (Nicholls *et al.*,1971), the degree of crosslinking provided by glutaraldehyde was estimated by measuring the free lysines after acid hydrolysis. Although the stability to 6M HCl hydrolysis is rather complex (Cater, 1963), the results were the best possible approximation since they estimated the lowest degree of glutaraldehyde mediated crosslinking. As shown in Table XIII, the data indicated that the amount of cross-linking incorporated by glutaraldehyde would be approximately 5 fold (compared with the best case scenario achieved by means of mTG mediated cross-linking). As already discussed in Chapter 3, it is probably too simplistic to consider the tanning effect of glutaraldehyde by the formation of a covalent bond between two lysines. Nicholls (1971) reported the formation of a α,β-unsaturated polymeric aldehyde via an aldol type polymerisation of the glutaraldehyde monomer. He stated

that both monomer glutaraldehyde and the polymer have crosslinking ability (Nicholls ii, 1971). Work carried out by Hardy *et al.* (1969) advocated that glutaraldehyde in solution existed as an equilibrium of three forms of hydrate. Milch (1964) suggested that glutaraldehyde would only have cross-linking ability when total hydration occurred. Heidemman (1993) stated that the reaction between protein amines and the monomer glutaraldehyde gives rise to the formation of cyclic compounds. The above discussion suggests that, apart from the higher degree of cross-linking involved in glutaraldehyde tanning, the type of cross-linking is more complex than that provided by mTG. This may explain why mTG-mediated cross-linking does not provide a dose-dependent increase in the denaturation temperature of bated hide in the same way that glutaraldehyde does.

Work carried out by Balian *et al.* (1971) reported that the denaturation temperature of bovine mature collagen (bull collagen) was only a few degrees higher than that of immature bovine collagen (calf collagen) but the isometric tension generated by the denaturation transition was much higher, which was indicative of formation of intermolecular cross-links rather than intramolecular cross-links. Although mTG-mediated crosslinking of bovine bated hide did not impart any change in the denaturation temperature of bovine bated hide, the results reported in this thesis showed that mTG-mediated cross-linking of bated hide increased the isometric tension generated during the shrinking transition at a certain temperature (as shown in Figure 22). This may suggest that the mTG can only incorporate intermolecular cross-links. This thesis has provided evidence of the ability of mTG to cross-link bated hide and the consequent modification of some of the physical properties of bated hide or the subsequent tanned leather. However, despite its ability to cross-link bated hide, mTG has failed as a tanning agent.

In light of these results, further work investigated the ability of mTG to improve the dyeing properties of leather. The work was undertaken following two different routes. The first route made use of the ability of mTG to incorporate primary amines into glutamine residues of a protein (Collighan *et al.*, 2002; Cortez *et al.*, 2002; Cortez *et al.*

2004). Thus, a primary amine with a five aliphatic carbon chain was linked to an acid dye via esterification of the hydroxyl group of the dye and the carboxylic acid of 6-aminohexanoic acid. Although dye modification was proved by NMR, mTG-mediated incorporation of the dye into N-N'dimethyl casein as a model system failed, suggesting that the modified dye was not a mTG substrate. The cosmetics industry (Richardson *et al.*, 1996) has made use of the same approach to impart colour into skin, hair or nails. Cortez *et al.*(2002) followed the same route to dye wool. However, besides the information provided by these patents, further details about the chemistries of the modified dyes have not been found in the literature. Consequently, a comparison between the Chapter 4 results and the work carried out in cosmetics and textiles appears to be rather difficult.

Although crust leather is a chemically modified protein substrate, the literature has shown the ability of mTG to incorporate casein into tanned leather (Rasmussen *et al.*, 1996). Consequently, the same concept could be applied to incorporate other proteins, e.g. keratin hydrolysate. Furthermore, mTG mediated incorporation of keratin hydrolysate protein has been shown to improve the performance of wool (Winkler *et al.*, 1999 ; Cortez *et al.*. 2002; Cortez et al., 2004). Some of the methods to improve the dyeing properties of leather rely upon application of a polymer during leather retanning which increases the sites for dye fixation, particularly cationic sites for anionic dyes (Schaffer *et al.*, 1986; GB 2 184739, 1987; Burkinshaw *et al.*, 1993; Hudson, 2000). In this way, the ionic bonding between the dye molecule and the leather matrix is increased, improving the depth of shade and the dye fastness. Although the literature provides extensive evidence about the ionic interaction between anionic dyes and the positively charged groups in the leather matrix (Heidemann, 1993 (b)), it is also acknowledged that dye and leather interaction is rather more complex (Knight, 1980; Muralidharan *et al.*, 1990; Britten, 1994) and other type of interactions such as hydrophobic, ionic bonding and dipolar interactions have also to be considered. Beside that, the literature also reports that application of other biopolymers, e.g. collagen (Cantera, 2000) or grafted biopolymers (Ma S., 1999; Ma J. *et al.*, 2004) may improve the dyeing performance of leather. Consequently, the second route investigated the

ability of mTG to incorporate keratin hydrolysate into crust leather (bovine and wool on slink) and its effect on dyeing. The results have shown that mTG-mediated incorporation of keratin hydrolysate increases the depth of shade as expressed as lightness of the leather (Figure 43, Figure 48, Figure 52, Figure 53 and Figure 56). This is true for mTG-mediated incorporation of keratin hydrolysate in both substrates. The treatment in bovine crust leather also improved its fastness to washing and perspiration solutions (Figure 51 and Figure 52). Unlike the bovine substrate, that was not observed for wool-on slink crust leather (Figure 56 and Figure 57).

Although the results do not provide direct evidence that the observed effect is due to mTG-mediated crosslinking of the keratin hydrolysate into the leather matrix, they suggest that it is a mTG-mediated event. Firstly, because confocal microscopy suggested that the leather uptake of keratin hydrolysate was increased by mTG (Figure 40). Secondly, the effect was only observed when the keratin hydrolysate treatment was done in conjunction with mTG (Figure 43, Figure 48, Figure 52, Figure 53 and Figure 56), the same effect was not seen with keratin hydrolysate alone. And lastly, because the effect was reduced in the presence of the competitive crosslinking inhibitor, putrescine (Figure 43 and Figure 47), indicating that the keratin hydrolysate was being incorporated into the leather matrix via a mTG mechanism.

The results do not provide data to elucidate the mechanism that takes place between the modified leather substrate and the dye with the consequent increase in depth of shade. However, since the increase in the depth of shade was not accompanied with higher dye uptake (Figure 44 and Figure 48), it is likely that mTG mediated incorporation of keratin hydrolysate modifies the physical and chemical environment of the leather matrix increasing its dye affinity in the surface (Britten, 1994). This is only an hypothesis and needs to be proven. To achieve this end, a comprehensive study of the effect of mTG-mediated incorporation of keratin hydrolysate into crust leather on its reactivity towards dyestuffs needs to be done. The dyestuffs would be selected on the basis of their polarity and hydrophobicity. Beside this study, the physical and chemical changes (e.g. hydrophobicity and polarity) introduced in the leather by mTG mediated

incorporation need also to be investigated. In this way, it should be possible to correlate the depth of shade and the chemical nature of the dyes. The results discussed in this thesis suggest that is very likely that the dyeing performance of leather can be improved by means of mTG in conjunction with a biopolymer that is a suitable substrate for the enzyme.

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