OPTIMISATION OF TENNIS STRING PRODUCTION FROM BOVINE INTESTINE

A thesis presented in partial fulfilment of the requirements for the degree of Master in Technology at Massey University, Palmerston North, New Zealand

Kanda Komolwattanachai

1999

ERRATA

Page 1, para 3, line 22	much longer string [11.4 m (38 ft)]
Page 2, para 1, line 4	Pacific USA web page is in the Reference list, number 12.
Page 2, para 4, line 27	The relevance of hog intestine is on page 21, paragraphs 3 and 4. These reveal some studies on improving hog casing by enzyme and chemical treatments.
Page 22, para 3, line 12	0.5% Teric solution (see footnote on page 25)
Page 33, para 5, line 19-20	The shrinkage testing is in 2.2.11 on page 30-31. The official method defines the shrinkage temperature as that temperature at which the apparatus dial moves 1 scale division. A scale division corresponds approximately to 1° of angle rotation. No correlation between percent shrinkage and angular rotation of the scale was determined, as this was irrelevant.
Page 35, Table	The quantities total protein, ash and fat when summed give the composition of the thread sample on a dry matter basis. The summation of protein, ash and fat should be 100%. However, because of experimental error in each of the quantities, the sum could well exceed the theoretical 100%.
Page 40, whole page	The terms "strong" and "weak" were based on subjective observations on the handling qualities made by personnel at the Pacific Natural Gut Company who provided these test samples. The explanation is in the note on page 24.
Pages 45/56	From the results, there was not much change in shrinkage temperature between pH 7-11, as stated in paragraph 3 on page 45. At pH 12.4, more non-collagenous proteins were indeed extracted (about 2 times) than at pH 11.2, and the shrinkage temperatures of the treated samples did significantly drop. However, this does not imply a direct correlation between protein extracted and shrinkage temperature. A possible explanation for the shrinkage temperature drop is that the alkalinity of the sodium hydroxide bath disrupts internal hydrogen and salt bonds. On lowering the pH in subsequent process stages the original hydrogen and salt bonds of the starting material are not reformed.

ABSTRACT

The collagen and elastin content of the beef thread samples ranged from between 47-70% and 1.2-2.5% respectively. Amino acid analysis showed that the collagen present was probably collagen Type I while the non-collagenous proteins predominantly were globulins with a small amount of albumins. Putative "strong" and "weak" batches of threads could not be differentiated on the basis of collagen content or mechanical properties such as ductility, ultimate tensile strength or Young's modulus.

Treatment of "strong" or "weak" threads with three different processes, sodium carbonate, sodium carbonate/EDTA and sodium hydroxide, gave no significant differences in products

The sodium carbonate/EDTA process can remove 31.9% of non-collagenous proteins over the three stages of the process. The shrinkage temperature and ductility were lowered while the ultimate tensile strength, Young's modulus and diameter are increased by the processing.

Threads given three successive trypsin treatments had 47.4% (2% trypsin) and 36.2% (0.6% trypsin) of non-collagenous proteins had removed. Properties of the treated threads from this treatment gave similar trends to threads from the sodium carbonate/EDTA process except that enzyme treatment resulted smaller thread diameters. Moreover, when the treated threads from the second and third high concentration trypsin treatments were heated, they stretched rather than shrank. This phenomenon was unexpected and apparently has not been previously reported in the literature.

On subjecting threads which had had three successive trypsin treatments to the sodium carbonate/EDTA process, the stretch temperature phenomenon was abolished and the normal shrinkage temperature property of collagen was restored. However, the shrinkage temperature of the thread from the integrated trypsin –sodium carbonate/EDTA process was significantly lower than that from the sodium carbonate/EDTA process alone. This integrated process does not affect the tensile

strength properties, but the diameter of the treated threads using the higher trypsin rate is significantly smaller than the starting materials. However, threads from the integrated process using lower trypsin seemed to show a trend toward smaller diameters but this observation could not be shown to be statistically significant.

It is suggested that two trypsin treatments integrated into a sodium carbonate/EDTA process could be an optimum process provided the smaller diameter trend of wet thread can translate into smaller diameter in dry string.

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to my supervisor, Assoc. Prof. Robert Chong for his valuable guidance, patience, encouragement and continual assistance throughout all stages of this study. My deep gratitude is to Dr. Brian Wilkinson my co-supervisor, who kindly provided helpful guidance and has always been available to offer assistance when required. Without both of them, this research would not have been possible.

I am thankful to Mrs. Ann-Marie Jackson, Mr. Michael Sahayam, Mr. John Sykes and Mr. Steve Glasgow for their technical assistance during experimental work.

Thanks are also extended to the Pacific Natural Gut String Company for supplying beef thread samples and to Industrial Research Limited for giving the hydroxyproline determination procedure which has been used in this research.

My appreciation is to the New Zealand Government for granting the scholarship.

Lastly, special thanks go to my mother for her continual support and encouragement.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

"Natural" gut tennis strings are prepared from the small intestines of a number of animals, especially prime beef cattle. The tennis string industry has to compete with other potential industries for this valuable by-product, especially the sausage casings industry. The technology is also used to prepare a number of related products, which includes: strings for musical instruments, strings for sports rackets, and catgut for surgery. This thesis will concentrate on the optimisation of one part of the tennis string production process, namely the "wet" portion of the process. The aim is to optimise this "wet" stage of the process.

The original tennis string process was based on the utilisation of the innermost layer of sheep and lamb small intestine, called the submucosa as the raw ingredient for the process. In this layer, collagen fibres are laid randomly, which make for a very elastic membrane, but lacking in tensile strength. The texture of this tubular structure varies from smooth on one side of the circumference to rough on the mesenteric attachment side. In order to make consistent strings; the intestine is cut in half along its length in a process called "slitting". Top quality strings come from the smooth side, lower quality product from the rough, mesenteric side.

Wooden rackets, with their small surface area required a very much shorter tennis string [7.3 m (24 ft)] which could be made from the sub-mucosa of a sheep. Modern day rackets with their very much larger surface areas require very much longer strings which can only be obtained from the small intestines of cattle. Moreover, strings derived from sheep and lamb casings did not have the durability or tensile strength to cope with the power that can be generated in modern day rackets. Consequently, all modern tennis strings are produced from the small intestines of cattle, particularly prime beef cattle. Natural gut strings, produced by Pacific Natural Gut Strings Company in

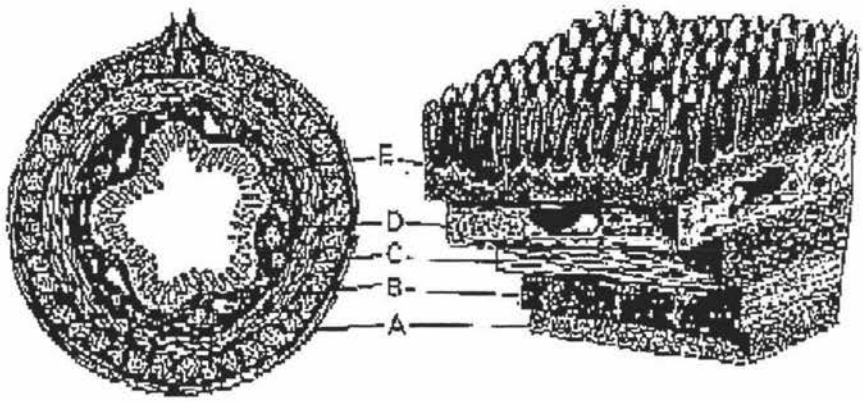
New Zealand, are made from the outermost layer, called the serosa, of bovine small intestines. The serosa is a sandwich of a highly oriented collagen layer and a very random one. The highly oriented layer contributes the tensile strength, and the random one elasticity (Pacific USA web page, 1999).

The process of tennis string manufacture is as follows: the intestine is first split by forcing the clean small intestine over a stuffing horn, which has two knives on each side of the horn to cut the intestine in half. The circular and longitudinal muscles together with the mucosa are then removed in a finishing machine to leave the connective tissue layers known as the serosa and sub-mucosa. The collagen layers then receive various chemical treatments (caustic soda, hydrogen peroxide and enzymes) to remove colouring material and as much of the non-collagenous material as possible. After the chemical treatments, the threads are spun using 1-10 threads, usually 6 smooth beef threads or 4 whole gut threads is normal, to give the rough tennis string, and then attached either end to a frame where the strings are further twisted to control the tension during drying. The humidity of the drying rooms is very carefully controlled to ensure that the strings dry at a precise rate and to prevent "case" hardening. Drying can take between 3-15 days. The dried strings are then passed through a circular grinding machine to produce smooth strings of a known and uniform diameter. Finally, in order to improve abrasion and water resistance, a special polyurethane coating is normally applied to the string (Pacific USA, web page, 1999). The strings are tested for tensile strength, uniformity of diameter and for stretching ability (Ockerman and Hansen, 1988).

The quality of the strings may be influenced by raw material e.g. breed, sex or age and by the variations in the process. This project will concentrate on elucidating variations in final tennis string quality due to the process.

1.2 Intestinal Tract Structure

The cattle and hog intestinal tracts, together with the structural pattern of its various divisions are very similar. Although varying in diameter and wall thickness, the entire intestinal tract has similar tissue architecture through out its length. It is composed of five distinct tissue layers (Price and Schweigert, 1971). As can be seen from Figure 1.1.



A. Serosa layer
B. Longitudinal muscle layer
C. Circular muscle layer
D. Submucosa layer
E. Mucosa layer

Figure 1.1 Diagrammatic cross-section of the intestines (Ockerman and Hansen, 1988)

1.2.1 Mucosa which surrounds the intestinal lumen and consists of

- *Mucosal membrane* composed entirely of epithelial cells.
- *Muscularis mucosae*, which are strands of smooth muscle cells, usually arranged longitudinally, and which are not continuously present throughout the entire tract.
- *Laminar propria* comprising a small amount of connective tissue cell and fibres closely associated with the smooth muscle cells.
- *Lymphatic tissues* present near the basal portion of the mucosa in the form of nodules.
- *Blood vessel and nerve plexuses*

1.2.2 Submucosa consists mainly of collagenous and elastic fibres. Blood vessels and adipose tissue are frequently embedded in it.

1.2.3 Circular muscle layer contains smooth muscle cells arranged with their longer axes around the digestive tube. These smooth muscle cells are spindle-shaped, uninucleate, comparatively short, and devoid of banding. They are supported by a reticulum of collagenous fibres, which make further connections with the collagenous fibres of the submucosa and those of the longitudinal muscles. In hog intestine this layer is noticeably thicker than the longitudinal layer. This is less noticeable in cattle intestine.

1.2.4 Longitudinal muscle layer consists of cells oriented at right angles to those of the circular layer and has a high content of collagenous fibres.

1.2.5 Serosa or external layer of the intestine is a thin layer composed of primarily of collagenous and elastic fibres and loose connective tissue cells. The serosa in hog intestine is thinner than in cattle, and is much less tightly bound to the adjacent layer.

Due to the serosa layer being used as the tennis strings production raw material, the chemical composition of this layer should be considered. The main composition of animal tissue is proteins, thus; clearly understanding the nature of proteins particular collagenous and elastic fibres is necessary.

1.3 Protein Chemistry

Proteins are bio-polymers that consist of linear chains of amino acids residues joined by peptide bonds. All organisms use the same 20 amino acids as building blocks for the assembly of protein molecules. Therefore these 20 amino acids are often cited as the common or standard amino acids. The polypeptide chain of a protein is not simply linear but folds into a biologically active shape. Depending upon their complexity, individual protein molecule may have up to four levels of structure (Moran *et al*, 1994).

1.3.1 Primary structure

This refers to the manner in which the amino acids residues are linked in sequence.

1.3.2 Secondary structure

This structure involves regularities in local conformations, maintained by hydrogen bonds formed between amide nitrogen and carbonyl oxygen of peptide bonds. The common secondary structures are the α helix and the β sheet.

1.3.3 Tertiary structure

Tertiary structure is the compacting of a polypeptide into one or more globular units, or domains. These structures are stabilised by interactions of side chains of non-neighbouring amino acid residues. The formation of tertiary structure brings distant portions of the primary structure close together. For instance, the active-site cleft of an enzyme may be composed of residues widely separated in the sequence.

1.3.4 Quaternary structure

It is the association of two or more polypeptide chains into a multisubunit protein. The chain may be the same or different.

On the basis of both their physical characteristics and functions, most proteins can be divided into two major classes, globular and fibrous (Moran *et al*, 1994).

- *Globular proteins*

  Most of these proteins are soluble in water and dilute salt solutions; compact, roughly spherical macromolecules whose polypeptide chains are tightly folded. Globular proteins characteristically have a hydrophobic interior and a hydrophilic surface. Globular proteins include enzymes, which are the biochemical catalyst of cells, and a large number of proteins that serve noncatalytic roles. They possess indentation or clefts, which specifically recognise and transiently bind other compounds. By selectively binding other molecules, these globular proteins serve as dynamic agents of biological action.

  The globular proteins can be further subdivided into albumins and globulins. The albumins are soluble in water, coagulated by heat, and usually deficient in glycine. They can be precipitated from solution by saturation with ammonium sulphate. Globulins are insoluble in pure water, but they are soluble in dilute neutral solutions of salts of alkalis and acids and are heat coagulable. They can be

precipitated from solution by half saturation with ammonium sulphate. They generally contain glycine.

- *Fibrous proteins*

They are water-insoluble, elongated molecules that are usually physically tough. They provide mechanical support to individual cells and to entire organisms. Typically, fibrous proteins are built upon a single, repetitive structure, assembled into cable or threads. Examples of fibrous proteins are α-keratin, the major component of hair and nails, and collagen, and the major protein component of tendons, skin, bones and teeth.

The serosa layer is thought to be composed of collagen and elastin. Two fibrous proteins provide the strength and flexibility of connective tissue (Price and Schweigert, 1971).

1.4 Collagen

Collagen is the major protein component of the connective tissue of animals and the most abundant vertebrate protein, constituting about 25-35% of the total protein in mammals. There are at least 15 different types of collagen proteins with remarkably diverse functions and forms. Collagen has a helical structure that differs from the common α helix. In bone, collagen and calcium-phosphate polymers are components of the hard substance. Collagen in tendons forms stiff, ropelike fibres of tremendous tensile strength. In skin, collagen takes the form of loosely woven fibres, permitting expanding expansion in all directions, and in blood vessels, collagen fibres are arranged in elastic networks.

1.4.1 Collagen structure

Native collagen is a molecule consisting of three chains having left-handed helices coiled around each other to form a right-handed supercoil. The collagen supercoil is stabilised by interchain hydrogen bonding and by the opposing twist of the helices and the supercoil. A typical collagen molecule is a rod 300 nm long and 1.5 nm in diameter. Within each collagen chain, the left-handed helix has three amino acid residues per turn and a pitch of 0.94 nm, giving a distance along the axis of 0.31 nm per residue. Thus, the collagen helix is not as highly coiled as an α helix. The left-handedness of the

collagen helix and much of the rigidity of collagen arises from steric constraints imposed by many proline residues. The ends of collagen molecules contain nonhelical sections, which appear to be necessary for proper alignment and cross-linking of the collagen molecules in the formation of fibrils.

Table 1.1 Amino aid composition of the α-chains of the main collagen types in meat connective tissues (Bailey and Light, 1989)

Amino acid	Type I		Type III	Type IV		Type V	
	α1 (I)	α2 (I)	α1 (III)	α1 (IV)	α2 (IV)	α1 (V)	α2 (V)
Hyp	102	87	127	146	127	107	108
Asp	43	46	43	48	52	55	51
Thr	17	19	13	18	28	27	22
Ser	39	36	39	34	26	34	26
Glu	74	69	72	77	62	90	99
Pro	139	123	109	79	69	92	119
Gly	352	350	354	318	313	318	320
Ala	121	113	97	31	49	59	46
Val	21	34	14	30	24	30	19
Cys	-	-	2	-	-	-	-
Met	7	5	8	15	12	10	7
Ile	7	15	13	34	42	18	20
Leu	21	33	22	54	59	37	40
Tyr	2	4	3	5	6	-	1
Phe	13	12	8	24	37	12	12
Hyl	5	9	5	61	42	25	36
His	2	11	6	6	8	10	7
Lys	31	23	30	5	6	18	19
Arg	51	53	47	20	45	57	48

Since the collagen helix has 3.0 residues per turn, every third residue of a given strand in a collagen triple helix makes close contact with the other two strands along the central axis of the triple helix. Since only the small side chains of glycine residues can fit at these positions, collagen helices typically contain glycine residues in every third position. There are no intrachain hydrogen bonds in the collagen helix. Characteristic of collagen is the repeating sequence –Gly-X-Y-, where X is often proline and Y is often 4-hydroxyproline (Hyp), a covalently modified derivative of proline. Together, proline and hydroxyproline account for about one-fourth of the residues in collagen molecules. For each –Gly-X-Y- triplet, one intermolecular hydrogen bond forms between the amide hydrogen atom of a glycine in one chain and the carbonyl oxygen atom of residue X in

an adjacent chain. The amino composition of the α-chains of the main collagen types in meat connective tissues is shown in Table 1.1

Both proline and hydroxyproline residues play a vital role in the structure of collagen. The limited conformational flexibility of these residues not only prevents the formation of α helices but also makes collagen helices and the collagen fibre itself somewhat rigid. Further stabilisation of the collagen triple helix seems to be provided by hydrogen bonds involving the hydroxyl group of hydroxyproline.

Covalent cross-links also contribute to the strength and rigidity of collagen fibres. The ε-$CH_2NH_3^{\oplus}$ groups of the side chains of some lysine residues are converted enzymatically to aldehyde groups (–CHO), producing allysine. Allysine residues and their hydroxy derivatives react with the side chains of lysine and hydroxylysine residues to form Schiff bases, complexes formed between carbonyl group and amines. These Schiff bases usually form between collagen molecules. Allysine residues also react with other allysine residues by aldol condensation to form cross-links, usually within collagen molecules. Both types of cross-links are converted to more stable bonds during the maturation of tissues, but the chemistry of these conversions is unknown. Reduction of the double bonds, which would make the cross-links more stable, does not occur (Moran *et al.*, 1994).

1.4.2 Collagen distribution

The localisation of the collagens within the vertebrate organism is the best information available about biological function.

The gross distribution of the collagens in the major connective tissue is show in Table 1.2. It is apparent that types I, II, and IV collagen comprise the majority of the collagen in selected tissues. There is some correlation between type and gross tissue properties. Thus the elements of the less extensible connective tissues, tendon and bone, are composed almost exclusively of type I collagen. In tissues located in regions most likely to encounter compressive forces such as the nucleus pulposus and hyaline cartilage structural elements comprised of type II collagen and proteoglycans predominate. Type IV collagen constitutes major units utilised in the structural scaffold of basement membranes.

Table 1.2 Macroscopic distribution of the collagens (Piez and Reddi, 1984)

Collagen	Tissue
Type I	Bone, cornea, dentin, fibrocartilage, tendon (>95%) Dermis, gingiva, heart valve (85%) Intestinal, large vessel, uterine wall (50-60%)
Type I-trimer	Dentin, dermis, tendon (<2-3%)
Type II	Notochord, nucleus pulposus, hyaline cartilage, vitreous (>95%)
Type III	Intestinal, large vessel, uterine wall (35-45%) Dermis, gingiva, heart valve (10-15%)
Type IV	Endothelial, epithelial basement membrane (>95%)
Type V	Cornea, placental membrane, large vessel wall (5%) Bone, gingiva, heart valve, hyaline cartilage (<5%)

In contrast, structural elements containing type III or V collagen are not predominant in any given tissue. However, type III collagen comprises a significant proportion of the collagen in the more distensible connective tissues such as dermis, heart valves and large blood vessel and intestinal uterine walls. Moreover, the level of type III present in these tissues increases with the degree of distensibility required in normal function with low level present in the compliant dermis and heart valves and much higher levels in the more distensible vessel and uterine walls. This apparent correlation implies that type III fibrils play a different role from type I, which present in the same tissue.

1.4.3 Collagen solubility

Collagen, when it is in the solid phase, usually cannot be dissolved in any solution without hydrolysis, however some of the collagen in certain tissues can be extracted in native form from the skin of young mammals.

Three solvents systems are commonly used for the extraction of native collagen molecules (Piez and Reddi, 1984).

- *Neutral salt-soluble collagen*

Very little or no collagen in mature muscle is soluble in neutral salt solution (1.0 *M* sodium chloride, 0.05 *M* Tris-hydrochloric acid pH 7.4). However, small quantities of non-cross-linked collagen can be extracted from homogenised foetal or new-born muscle samples.

- *Acid-soluble collagen*

Collagen cross-linked only by the acid-labile aldimine group can be solubilised by extraction with 0.5 *M* acetic acid. The best sources are placenta or foetal skin but small quantities can be extracted from the tendon and intramuscular connective tissue of foetuses or very young animals. The reason that only small amounts of collagen can be extracted from tendon and muscle is the prominence of the acid stable oxo-imine cross-link in these tissues. The major product from these sources is Type I collagen

- *Pepsin-soluble collagen*

Collagen can be extracted from most foetal, pre-mature and mature connective tissue by solubilising the source tissue, after preliminary washing with salt, acid, chaotropic or detergent solutions, with the enzyme pepsin. The action of this protease is to release whole soluble collagen molecules from the insoluble matrix (Bailey and Light, 1989).

1.4.4 Thermal properties of collagen

Collagen monomers can be denatured by heat, the rigid triple helical molecules denature over a narrow range of temperatures, the mid-point of which is referred to as the denaturation temperature, T_D. The long rod-like helical molecules produce a highly viscous solution possessing a high optical rotation, but at the denaturation temperature all structure is lost and there is a dramatic decrease in viscosity and optical rotation. There are two stages in denaturation; first is separation of the polypeptides and denaturation of their helical form as the second.

Generally the denaturation temperature is dependent on the genetic type of collagen and the species of origin and can range from 15°C for cod fish skin to 50°C for Ascaris cuticle.

In collagen fibres thermal denaturation occurs when the fibre structure collapses and consequently shrinks. The phenomenon of shrinkage provides a measure of the thermal transition and is determined as the temperature at which the fibre, if it is restrained, shrinks to one-quarter of its original length. This shrinkage temperature (T_s) is usually about 27°C higher than the T_D of the molecule due to the increased energy of crystallisation derived from the interaction between the close packed molecules in the fibre. The T_s of mammalian collagen is about 65°C compared to the 39°C of the molecule in solution. The variation of T_s with species naturally follows the hydroxyproline content of the molecule. The precise temperature of the denaturation varies with the environmental conditions at which it is determined.

The exogenous factors, which have been shown to affect Ts, include pH, heating rate and solute concentration amongst others. Other important factors are the endogenous components such as glycosaminoglycans and proteoglycans.

The properties of the denatured fibre depend on the nature of the polymerising cross-links. In young tissues, with a low content of the heat-stable mature collagen cross-links, the amount of collagen liberated as gelatin by heating is related directly to the proportion of heat-labile aldimine cross-links stabilising the collagen. If this proportion is high then above 70-75°C, at which temperature the aldimine bonds break, most of the collagen will dissolve and form gelatin. The collagen in muscles from older animals such as old ewes (mutton), culled cows and bulls contains a high proportion of the heat-stable mature cross-links. Consequently, these tissues are much less liable to dissolve following thermal denaturation and remain intact and insoluble even at high temperatures. In these cases the insoluble inextensible fibre is converted to an insoluble highly extensive 'rubber-like' material (Bailey and Light, 1989).

1.5 Elastin

Elastin is the major structural protein found in tissues whose functions require rapid extension and complete recovery upon relaxation. Whereas collagens and α-keratins

impart structural integrity and rigidity, elastin provides elasticity and flexibility to tissues such as those found in the lungs, skin and arteries.

1.5.1 Elastin structure

Elastin is a three-dimensional network of cross-linked molecules that lack a well-defined secondary structure. Elastin is extremely durable, and a single fibre can last the lifetime of an individual. The aorta (the large artery leading from the heart) contains roughly twice as much elastin as collagen, giving this vessel the elasticity required to undergo a billion or so stretch-relaxation cycles during a lifetime of heartbeats.

Elastin is a highly water-insoluble protein with a large proportion of hydrophobic amino acids, containing slightly less than 50% valine (V) + proline (P) + alanine (A), approximately 33% glycine (G), and smaller amounts of isoleucine (I), leucine (L), and phenylalnine (F). Elastin is usually purified using a variety of solvents that do not hydrolyse its peptide bonds but do dissolve and remove all other proteins and biomolecules. Elastin contains a number of cross-linked residues, including modified lysine residues like those found in collagen, and related cross-linked structures called desmosine and isodesmosine, which are derived from three allysine residues and one lysine residue. Normally, 15-17 such cross-links occur within the long (~800-residue) elastin chains. The cross-links of elastin are spread widely enough to allow extension but closely enough to give the fibres strength. Lysyl oxydase is a copper-containing enzyme that catalyses the oxidative deamination of lysine residues to allysine residues involved in covalent cross-linking.

Whilst the cross-linked areas of elastin have a rigid structure, the intervening sequences are responsible for the elastic properties of elastin. It has been suggested that the proline and glycine residues of the intervening sequences cause the polypeptide to turn and adopt a spiral conformation in the relaxed state. However, it is more likely that these hydrophobic regions between the cross-links have an amorphous or nonrepetitive structure (Moran *et al*, 1994).

1.5.2 Elastin isolation

Elastin is virtually insoluble unless the peptide bonds are cleaved. This has proved, in effect, a ready method of isolation in that elastic tissue can be extracted with 0.1 *N*

sodium hydroxide at 100°C or autoclaved in water, leaving in an insoluble residue, which is pure elastin. Although very little damage is incurred by the insoluble elastin through peptide bond cleavage with this method, milder techniques have to be employed when the detailed structure is being investigated. These methods have included extraction of other proteins with guanidine hydrochloride and β-mercaptoethanol, and, if necessary, cyanogen bromide and trypsin treatment. Elastin is resistant to all of these reagents and thus remains insoluble (Bailey and Light, 1989).

After outlining some general properties of collagen and elastin, the following section will deal with sample preparation and analytical methods of collagen and elastin determination.

1.6 Protein Hydrolysis

As hydroxyproline is a unique amino acid component of collagen, the amount of collagen can be determined from the hydroxyproline content in a sample. Since hydroxyproline is incorporated via peptide bonds, samples containing hydroxyproline must be hydrolysed to release the hydroxyproline for analysis.

Since the precision of quantitative amino acid analysis will depend upon the completion of protein hydrolysis, many protein hydrolysis methods have been evaluated. Lucas and Sotelo (1982) introduced the high-temperature, short-time hydrolysis method (HTST). This method required a much shorter hydrolysis time, and gave comparable amino acid composition when compared with the standard method. Their experiments used 6 *N* hydrochloric acid at 110°C for 24 h (the traditional method) and at 145°C for 4 h (the HTST method) for three groups of sample: pure proteins, protein concentrates and vegetable sample with a high carbohydrate content. In the protein concentrates group differences in amino acid composition was evident for only three amino acids; higher values by HTST method for valine; and lower values by the HTST method for aspartic acid and serine. Their results indicated that the HTST hydrolysis method was equivalent to the classic method and could be used for any type of proteinaceous material, especially foods.

Csapo and his co-workers (1986) studied the proteolysis of food and feed products. They determined the amino acid composition of maize, soybeans, milk powder and

meat meal using 6 *M* hydrochloric acid (105°C for 24 h), the 3 *M* p-toluenesulphonic acid and the 3 *M* mercaptoethanesulphonic acid methods of protein hydrolysis. They also studied the decomposition of the individual amino acids due to the effect of hydrolysis and the processing of the hydrolysate. It was found that hydrolysis with 6 *M* hydrochloric acid at 105°C for 24 h, followed by neutralisation with 4 *M* sodium hydroxide gave the optimum result for all amino acids with the exception of tryptophan. In acidic conditions tryptophan was almost entirely decomposed, while 20-25% of the cysteine was decomposed. For tryptophan determination, hydrolysis with sulphonic acid was the most suitable method.

In the two studies described above, satisfactory results were obtained by hydrochloric acid hydrolysis. There was another study which combined enzymatic digestion and acid hydrolysis to overcome the loss of tryptophan or decreased yields of serine and threonine problems (Church *et al.,* 1984). They used various kinds of immobilised proteases: pronase, proteinase K, carboxypeptidases A and B, aminopeptidase M, intestinal mucosa exopeptidases and prolidase, to study the total enzymic hydrolysis of proteins. Their results of combined immobilised enzymatic and acid hydrolysis, for assessment of protein quality, gave a more accurate chemical score than that afforded by acid hydrolysis alone. Amino acid analysis of enzymic hydrolysates of native protein substrates (β-lactoglobulin and insulin) yielded 92% of the theoretical values and 103% of the values observed for standard acid hydrolysates. Their results suggested that using a combination of immobilised proteases in concert with acid hydrolysis ensured total hydrolysis of protein substrates in a time period (18-24 h) comparable to conventional acid hydrolysis methods.

1.7 Hydroxyproline Determination

Measurement of hydroxyproline in various tissues, plasma and urine for the investigation of collagen is possible by colorimetric methods, high performance liquid chromatography, gas chromatography/mass spectrometry and enzymatic methods. In general most of the procedures are laborious and involve many time consuming steps. The colorimetric method was of interest due to its simplified procedure and availability of instruments.

Many hydroxyproline colorimetric determination methods have been developed. Most of the published methods for hydroxyproline determination involve the oxidation of the imino acid (proline and hydroxyproline) to pyrrole-2-carboxylic acid or pyrrole and then the formation of a chromophore with p-dimethylaminobenzaldehyde.

Neuman and Logan (1950 b) introduced a hydroxyproline determination method, which used hydrogen peroxide as an oxidising agent in basic solution and Ehrlich's reagent to form the chromophore while reading the absorbance at 540 nm. Unlike the conventional acid hydrolysis, this procedure hydrolysed the sample in 6 *N* hydrochloric acid using an autoclave at 222.4 Newton pressure for 3 h. and the hydrolysate was neutralised before testing for hydroxyproline. This method can be used for the estimation of collagen in mixtures of proteins including animal tissues. This method has been used by The Industrial Research Limited, New Zealand, but they reduced the final total volume from 10.0 ml in the original procedure to 1.0 ml instead. The reliability of this method when applied to determine hydroxyproline in hydrolysates of 40 to 100 μg of collagen gave ± 2% of reproducibility and accuracy, the optimum determination range is between 0-20 μg hydroxyproline.

The method of Neuman and Logan (1950 b) was fairly specific. Only two other amino acids, tyrosine and tryptophan, give colour with p-dimethylaminobenzaldehyde. The interference due to tyrosine is 2% and that of tryptophan is 1.3%. When small amounts of hydroxyproline are to be estimated in protein hydrolysates, there is a problem of removing this interference.

Prockop and Udenfriend (1960) developed a specific method for the analysis of hydroxyproline in tissue and urine. The imino acid is oxidised with chloramine-T and the mixture is extracted with toluene to remove the interfering substances. The product of oxidation is then transformed into a toluene-soluble pyrrole, extracted with toluene, and reacted with p-dimethylaminobenzaldehyde.

With respect to the interference problem, Serafini-Cessi and Cessi (1964) observed that the product of oxidation of hydroxyproline is easily distilled under suitable conditions. This property allows the separation of the chromogen from other non-volatile product like those occurring from tyrosine and tryptophan. Therefore, after the acid hydrolysate was oxidised, based on the Neuman and Logan (1950 b) method, the content was

distilled with Ehrlich's reagent. The colour developed immediately and was read at 550 nm. This method seems to be laborious because of the distillation step.

The method reported by Glick (1977) and Cunningham and Frederiksen (1982), is suitable for detecting very small amount of hydroxyproline (as low as 1 in 10,000), in samples containing a large amount of contamination. In addition, this method can also be applied to various kinds of connective tissues, cells in tissue culture and purified collagen. The samples were hydrolysed overnight at 118°C in 6 *N* hydrochloric acid. The hydrolysed samples then were evaporated at 65°C under vacuum. The evaporated samples were oxidised with chloramine-T at pH 8.7, and Ehrlich's reagent was added to form the chromogen, which was extracted with toluene and absorbance was read at 565 nm.

A method for the detection of collagen and collagen peptides in the presence of other proteins was described by Huszar *et al.* (1980). The procedure detected collagen during chromatography separation, the aliquots from column fraction were hydrolysed with 4 *N* sodium hydroxide in an autoclave at 120°C for 10 min, 1.4 *N* citric acid was added to bring pH to 6.0. The chromogen was developed as before, apart from Ehrlich's reagent being dissolved in n-propanol and perchloric acid mixture, and there was no toluene extraction stage. The absorbance of the chromogen was read at 550 nm. The advantages of this method are speed, sensitiveness and the colour yield of hydroxyproline is not affected by chromatography solvents. As all steps of the assay take place in a single test tube the method is suitable for batch processing of column fractions.

The recently established method by the International Standard (ISO 3496, 1994) should be used for meat and meat products containing less than 0.5% (m/m) hydroxyproline. In this method the homogenised sample is hydrolysed in 3 *M* sulphuric acid at 105°C for 16 h or overnight. The hydrolysate is then treated in the same manner as the alkaline hydrolysis method with no toluene extraction. Neutralisation of acid hydrolysate is not necessary in this method.

Reddy and Enwemeka (1996) introduced a simplified method for analysis of hydroxyproline in biological tissues. Their procedure is based on alkaline hydrolysis of the tissue homogenate and subsequent determination of the free hydroxyproline in hydrolysate. The range over which a linear relationship was observed between 0-20

μg/ml hydroxyproline. Although this procedure is similar to Huszar's method (1980), the total volume of sample was reduced to 1.0 ml including all reagent compared with the others, which have a total volume up to 15.0 ml. Moreover, the concentration of alkali used in hydrolysis was 2.0 *N* sodium hydroxide and the hydrolysis had taken place at 120°C in an autoclave for 20 min. This modified method omitted drying the sample before hydrolysis and treatment of the sample with citric acid for neutralisation after hydrolysis.

In comparison, the methods necessitating the use of chloramine-T as an oxidising agent seem to be more elaborate than the hydrogen peroxide method that gave acceptable reliability. Because the collagen content in bovine intestine serosa layer is quite high (~40%), the errors due to interference by tryptophan and tyrosine are small in comparison to the total concentration of hydroxyproline in the tissues. Consequently, the method, which was modified by Neuman and Logan (1950 b), has been used in this project.

1.8 Collagen and Elastin Content in Animal Tissues

Neuman and Logan (1950 a) reported the distribution of collagen and elastin content in several different tissues. In their report, the soluble protein was extracted by 20% urea solution, while collagen and elastin remained in solid matrix. The collagen was converted to the soluble form (gelatin) by hydrolysis with water in an autoclave at 110°C for 24 h and then extracted leaving elastin and other substances not dissolved by the process. The collagen content was then estimated from the hydroxyproline content of the acid hydrolysate extract. Elastin which contains 1.5 to 2.3% hydroxyproline, depending on the source, was estimated from the hydroxyproline content of the residue remaining after extraction of the collagen. Due to tyrosine interfering in the hydroxyproline determination, the results were corrected by the amount of tyrosine in the hydrolysates.

They used their modified method (Neuman and Logan, 1950 b) to determine the hydroxyproline content. They converted the hydroxyproline to collagen by using the factor 7.46, which assumes that there is 13.4% hydroxyproline in collagen.

To determine elastin, the remaining residues from the collagen determination were further autoclaved. Then the residues were acid hydrolysed and the hydroxyproline content determined. The factors, which they used to convert hydroxyproline to elastin content, were 66.7 for pig elastin, 52.3 for beef elastin and 43.4 for rat elastin.

The research results suggested that the collagen content of tissues ranged from 85% collagen for beef chordae to 0.22% for rat brain. The elastin content ranged 57% for pig aorta to 0.5% for rat kidney cortex.

Nguyen and Zarkadas (1989) compared the amino acid composition and connective tissue protein content in selected bovine skeletal muscles. They used the amounts of 5-hydroxylysine to indicate the content of collagen and collagen-like proteins and the connective tissue proteins from the 4-hydroxyproline content. In their experiment, they calculated the amount of collagen and elastin from 5-hydroxylysine and desmosine respectively in the unit of grams per kilogram of total protein by the equations as shown below

$$\text{Amount of collagen } (P_C) = \text{Amount of Lys (5-OH)} \times 63.3 \qquad (1.1)$$

$$\text{Amount of elastin } (P_E) = \text{Amount of Des} \times 62.4 \qquad (1.2)$$

Where; P_C is sum of collagen

P_E is sum of elastin

Lys (5-OH) is 5-hydroxylysine

Des is Desmosine

The results obtained from their calculation revealed that the total collagen content ranged from 1.9-3.6% in beef longissimus dorsi to 9.6% in cow sternomandiburalis muscle.

Zarkadas, *et al.* (1996) investigated collagen content in bovine and porcine cardiac muscle tissue. The procedure used was similar to that used for bovine skeleton muscle. They estimated the collagen content from the amount of 5-hydroxylysine found in the acid hydrolysis. The collagen content from their study was 3.9% in porcine and 4.8% in bovine hearts.

Amino acid, collagen and mineral composition of preruminant calves were studied by Williams (1978). The samples were from three groups of calves parts, (a) the head, hide,

feet and tail, (b) the internal organs (including kidneys, liver, lungs, hearts and empty alimentary tract) and blood and (c) the carcass, including bones. He analysed collagen content from the hydroxyproline content of the samples by the method of Goll, *et al.* (1963) using a correction factor of 7.25. The collagen composition of the three groups is in Table 1.3.

Table 1.3 Distribution of collagen N and total N in different components of the bodies of preruminant calves

Component	(a) Head, hide, feet and tail	(b) Internal organs and blood	(c) Carcass	(d) Whole body
Collagen N (g/100 g fresh wt.)	2.1 ± 0.03	0.2 ± 0.01	0.9 ± 0.05	1.0 ± 0.03
Total N (g/100 g fresh wt.)	3.7 ± 0.03	2.0 ± 0.05	3.0 ± 0.02	3.0 ± 0.02
Collagen N/Total N	0.57	0.10	0.30	0.33

Component (a) is particularly rich in collagen containing 43% of the total body collagen. Although the collagen content of bovine muscle, bone and various organs is known, this is the first direct estimate of the collagen content of the whole body of a calf. In fact, although the collagen content of mammals is often quoted as 30-35% of the total body protein, many factors affect the collagen content of tissue such as the size, age and nutritional status of the animal. These factors could account for the high collagen content of calf musculature.

According to Vognarova' and Böhm's (1968) study, increases in the amount of total connective tissue could be primarily attributed to an increase in the amounts of collagen, not to elastin, which tended to be relatively constant. In their study, the amount of collagen and elastin was estimated on the basis of the content of hydroxyproline, which is absent in other meat proteins.

Goll *et al.* (1963) investigated the relationship between age and bovine muscle composition. They studied the age-associated-changes in the chemical composition of bovine muscle. It was found that veal muscle had significantly lower Kjeldahl nitrogen and higher moisture contents than muscle from three older age groups. Muscle from veal and the oldest group (cows, 10 years) possessed less fat than muscle from two intermediate groups (steers, 1-2 years, and cows, 5 years). A modified procedure for determination of hydroxyproline (Goll *et al.*, 1963) failed to reveal any significant differences in the hydroxyproline content, and presumably the connective tissue content of muscle from the four groups. The tenderness of these muscles also decreased with age, which was revealed by the Warner-Bratzler shear-force values of cores.

1.9 Small Intestine Aspects

Since the small intestine was to be the source of the collagenous material for this study, some of its aspects deserve attention. However, information related directly to the serosa layer of bovine small intestine is rare. Sausage casing data has been used for comparative purposes because it is probably similar in composition to the sub-mucosal connective tissue layer.

There are many studies on the histology of the small intestine of human and mammalian animals. The ultrastructural distribution of fibronectin in the smooth muscle layer of mouse small intestine was studied by Kurisu *et al.* (1987). They found that fibronectin was present over the pericellular area extending from the cell membrane to the extracellular matrix beyond the basal lamina. Distribution of fibronectin over the pericellular area was heterogeneous; it was localised more abundantly in the narrow space between smooth muscle cells, the gap having a width of 60-80 nm where the two dense bands in adjacent cells matched each other. This localisation suggests that fibronectin contributes to cell adhesion. Fibronectin was also co-distributed with collagen fibres in the extracellular matrix. Within smooth muscle cells, this glycoprotein was observed on rough endoplasmic recticulum and secretory vesicle-like structures. These evidences suggested that smooth muscle cells synthesise fibronectin and secrete it as a component of the basal lamina and extracellular matrix.

Nakamura *et al.* (1991) studied the architecture of the collagen fibrillar network and the localisation of type I, III and IV collagen in the digestive organs of Holstein steer by

cell-maceration/scanning electron microscope (SEM) and by indirect immuno-fluorescence. Their results revealed that the SEM views of cross sections of the digestive duct showed the three-dimensional random network of collagen fibrillar bundles extends from the lamina propria to the tunica serosa including the muscularis mucosae, tunica submucosa and the tunica muscularis. In each digestive organ, the loose connective tissue collagen fibrillar bundles were fine but relative dense. The surface collagen fibrils of the lamina propria of the digestive duct presented a sheet-like structure and the geometrical figure resembled the specific morphologies of each part of the digestive duct cavity. In conclusion, the collagen in the digestive organs of the Holstein steer was quite varied concerning the three-dimensional fibrilogenesis and the localisation of its different types.

Apart from physiological studies, some research has been toward improving sausage casings. Hog small intestine and their utilisation as a sausage casing were studied by Kim *et al.* (1990). Several characteristics were investigated such as the length and diameter of intestine, chemical compositions and change of micro-organisms during preparation of casing. The results obtained were as follows; 1. The average length of hog small intestine was about 17.5 m and about 60% of samples belonged to the range from 16-19 m, 2. The average diameter of the hog small casing was about 3.16 mm and more than 70% of samples belonged to the range from 3.0-3.3 mm. 3. The contents of water, crude protein, crude fat and ash of casing were 85.1, 11.7, 1.6 and 0.5% respectively. 4. Collagen and salt concentrations of casing were 7.8 and 0.25% respectively. 5. In raw hog small intestine, total aerobic microbial counts were about 105.7/g and the flora was dominated by lactic acid bacteria.

Radhakrishnan and Ramamuthi (1987) attempted to improve the textural properties of hog sausage casings by treating them with chemical bleaching agents. The casings treated with 20% sodium carbonate solution for 24 h resulted in an improvement in the feel, appearance and strength of hog casings

Sakata *et al.* (1998) attempted to improve the tenderness of hog casings by treating them with a protease enzyme and/or organic acids. Each of the protease used in the study caused extensive degradation of the intrinsic collagen structure. Organic acid treatment did not increase the tenderness either. From all the proteases and organic acids tested, a combination of pepsin and lactic acid showed the desired tenderising effect under

specified treatment condition in respect of the physical and sensory characteristics of the sausage products.

1.10 The Process

Two wet process production processes are used by the Pacific Natural Gut String Company to produce tennis strings: a sodium hydroxide based process and an EDTA based process. The sodium hydroxide process is the original process and has been superseded recently by the EDTA process.

1.10.1 Sodium hydroxide process

In the sodium hydroxide process, the beef threads are given various treatments over a three-day time period. On the first day, the threads are pre-soaked in the 0.28% sodium hydroxide for 60 min at 25°C and then soaked in water for 30 min at 25°C, and finally transferred to an 0.5% Teric solution for an overnight soak. On the second day, the threads from the first day are put into an 0.075% sodium hydroxide solution for one hour, cold water for 20 min and the stripped into an 0.5% Teric solution at 25°C overnight. On the third day, the treated threads from the second day are then given a further series of treatments in the sequence outlined below:

1. 0.28% sodium hydroxide solution for 60 min,
2. water, for 20 min,
3. 0.3% hydrogen peroxide solution for 60 min,
4. 0.28% sodium hydroxide plus 0.3% hydrogen peroxide solution for 60 min,
5. 1% Teric solution overnight,
6. finally water for 60 min

As can be seen, the sodium hydroxide process is laborious and uses many chemicals in the entire process. The whole EDTA process can be accomplished in 24 hours overnight.

1.10.2 EDTA process

The beef threads are treated firstly in an of 0.25% sodium carbonate plus 0.2% ethylenediaminetetraacetic acid (EDTA) solution for 1 hour at 26-28°C, and then rinsed with water. In the third stage the threads are soaked in a mixture of 0.3% hydrogen peroxide, 0.25% sodium carbonate and 0.2% EDTA for 1 hour at 26-28°C, and rinsed again with water before going to the final stage. In the last step, the treated beef threads are soaked in an 0.45% Teric solution for 12-18 hours at 26-28°C, rinsed with water and then spun into threads to form tennis strings, usually 6-8 threads per string depending on thread diameter and desired tennis string diameter.

Both processes use alkali solutions to dissolve and hence remove proteins such as glycoproteins and mucopolysaccharides to leave only collagen fibres for tennis string production. It is hypothesised that this is one of the most important steps in the wet processing stage and as a consequence will form the focus of the current study.

From the information presented so far it would appear that the collagen content of a beef thread could be expected to be 7.8% on a wet weight basis or 52.3% on a dry weight basis if the data of Kim *et al.* (1990) for hog sausage casings is an indicator. However, their data is based on the collagen content of the sub-mucosa layer rather than the serosa layer. The texture improvement studies on hog sausage casings also should be relevant for this project, as it was found that treatment with a 20% sodium carbonate solution increased the strength of the casing (Radhakrishnan and Ramamuthi, 1987).

However, the study by Sakata *et al.* (1998) where they used a combination of pepsin and lactic acid to improve tenderness would suggest that there might be dangers in using such a treatment for tennis strings as it might reduce the tensile strength of the tennis strings. These studies suggest possible treatments which should be explored in the current study to see what effect they have on the physical and chemical properties of tennis strings.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Samples

All intestine samples were supplied by Pacific Natural Gut Strings Company, New Zealand. Five groups of samples were used in the experiment:

A "normal" batch of threads was received on 15/7/98

The first batch "weak" threads was received on 25/8/98

The second batch "weak" threads was received on 24/9/98

The first batch "strong" threads was received on 19/8/98

The second batch "strong" threads was received on 26/11/98

Note: "weak" and "strong" refer to the wet strength properties of the threads. An apparent anomaly is that weak wet strength turns into strong dry strength string while strong wet strength tends to translate into weak dry strength string.

2.1.2 Chemicals

2.1.2.1 0.1 *M* Hydrochloric acid

2.1.2.2 50% w/v Sodium hydroxide

2.1.2.3 4% w/v Boric acid

2.1.2.4 Kjeltabs s/3.5; source: Tecator (Höganäs, Sweden)

2.1.2.5 0.25% w/v Sodium carbonate

2.1.2.6 0.2% w/v Ethylenediaminetetraacetic acid di-sodium salt

2.1.2.7 0.28% w/v Sodium hydroxide

2.1.2.8 0.2% w/v Ethylenediaminetetraacetic acid tetra-sodium salt; prepared by dissolving ethylenediaminetetraacetic acid di-sodium salt (1.78 g) and 2.5% sodium hydroxide solution (15.3 ml) in distilled water (1 L).

2.1.2.9 0.25% w/v Sodium bicarbonate

2.1.2.10 0.45% v/v Teric LA 8[1]; gift of Pacific Natural Gut String Company

2.1.2.11 6 *M* Hydrochloric acid

2.1.2.12 0.01 *M* Copper sulphate

2.1.2.13 2.5 *M* Sodium hydroxide

2.1.2.14 6% w/v Hydrogen peroxide; 30% hydrogen peroxide (5 ml) was added to distilled water (20 ml); freshly prepared before each assay.

2.1.2.15 1.5 *M* Sulphuric acid

2.1.2.16 5% w/v p-Dimethylaminobenzaldehyde (DMAB); prepared by dissolving p-dimethylaminobenzaldehyde (1.25 g) in n-propanol (20 ml) and made up to a 25 ml volume with n-propanol; freshly prepare prior to each assay.

2.1.2.17 40% w/v or 10 *M* Sodium hydroxide

2.1.2.18 100 µg/ml Standard hydroxyproline

2.1.2.19 2.4 Anson Units/g Alcalase enzyme; source: Novo Nordisk (Bagsvaerd, Denmark)

2.1.2.20 690 U/g protein Trypsin enzyme

2.1.2.21 7450 U/g protein Pyrase enzyme; source: Chemcolour (NZ) Limited

---

[1] Teric LA 8 is a mixture of C_{12}-C_{15} ethoxylated ethers used as emulsifier, defoamer, wetting agent, dispersant, stabiliser, antistat, lubricant, detergent for household and industrial cleaners, textile, paper, leather, metal cleaning, coatings, ceramic and polishes.

2.1.2.22 1400 U/g protein Tanzyme enzyme; gift of NZ Leather and Shoe Research Association

2.1.2.23 0.1% w/v Sodium azide

2.1.2.24 0.1 *M* Sodium hydroxide

2.1.2.25 3:1 v/v Ethanol/ether

2.1.2.26 0.5 *M* Sodium hydroxide

2.1.2.27 1 *M* Phosphate buffer; prepared by dissolving potassium di-hydrogen phosphate (13.6 g) in distilled water (100 ml), labelled as solution A and by dissolving di-potassium hydrogen phosphate tri-hydrate (17.4 g) in distilled water (100 ml), labelled as solution B. Solution B was adjusted with solution A to pH 7.5 with a pH meter

2.1.2.28 0.1 *M* Buffered casein solution; a suspension of soluble casein (1 g) in distilled water (about 30 ml), mixed thoroughly with a magnetic stirrer until the casein was dissolved. Added 0.1 *M* Phosphate buffer (2.1.2.27) (10 ml) and adjusted pH to 7.5 with 1 *M* hydrochloric acid and made up to 100.0 ml volume.

2.1.2.29 0.05 *M* Hydrochloric acid

2.1.2.30 5 *mM* Tyrosine; prepared by dissolving tyrosine (45.3 mg) in 0.05 *M* hydrochloric acid (50 ml).

2.1.2.31 0.3 *M* Trichloroacetic acid; trichloroacetic acid (4.9 g) was dissolved in distilled water (100 ml).

2.1.2.32 *Folin-Ciocalteu's* phenol reagent; *Folin* solution (30 ml) was diluted with distilled water (60 ml).

Unless otherwise stated, all the chemicals used were analytical grade obtained from either BDH Chemicals (BDH Ltd., Poole, England) or Sigma Chemical Co. (St. Louis, MO, USA).

2.2 Experimental Procedures

2.2.1 Moisture determination

A sample of beef thread was cut into small pieces and heated in a moisture dish at 105°C until constant weight was obtained.

2.2.2 Fat content determination

An oven dried sample (1.5 g) of beef thread was weighed into a paper thimble and extracted with diethyl ether for 20 cycles by the Soxhlet method. Subsequently the extract was evaporated to dryness and then the weight of the residue determined.

2.2.3 Total protein determination (Kjeldahl method)

A sample (1g) of beef thread was digested with concentrated sulphuric acid (13 ml) plus Kjeltabs (2 tablets) by the Büchi digestion unit (Model 435) until the solution was clear and colourless (usually 1-3 h). The solution was allowed to cool, distilled water (30 ml) and 50% sodium hydroxide (60 ml) were added then distilled by the Büchi distillation unit (Model 323). The distillate was received in a 4% boric acid solution (30 ml) and titrated with 0.1 *M* hydrochloric acid by the Mettler titrator (Model DL 25). The protein content was determined using the following formula

$$\%\,\mathrm{Pr}\,otein = \frac{(V_2 - V_1) \times M \times 0.01401 \times 6.25 \times 100}{W}$$

Where V_2 = volume in mls of standard hydrochloric acid used

V_1 = volume in mls of standard hydrochloric acid used in the blank titration

M = the molarity of the acid

W = weight in g of the sample (or volume in ml)

2.2.4 Ash content

A dry sample (1 g) of beef thread was weighed into a porcelain crucible and placed in temperature controlled furnace preheated to 600°C and held at this temperature overnight. The crucible was transferred directly to desiccator, cooled and weighed.

2.2.5 Amino acid analysis

A dry sample (10 mg) of beef threads was hydrolysed with 6 *M* hydrochloric acid (500 µl) and phenol (0.5 µl) at 110°C for 24 h. The hydrolysate was vacuum dried then made up to volume (1.0 ml) with double distilled water. A sample (5 µl) was loaded onto the Pharmacia amino acid analyser (Model LKB 4151 Alpha Plus).

2.2.6 Sample hydrolysis

The sample was hydrolysed by two different methods, acid hydrolysis and enzyme plus acid hydrolysis.

2.2.6.1 Acid hydrolysis

- *Solid sample*

A dry sample (0.25 g) of beef threads was refluxed with 6 *M* hydrochloric acid (100 ml) at 108°C for 24 h. The hydrolysate was then neutralised with 10 *M* sodium hydroxide to pH 7.0 and made up to volume (200 ml).

- *Filtrate sample*

An aliquot (2.0 ml), from the enzyme hydrolysate, was pipetted into a Pyrex screwtop tube, concentrated hydrochloric acid (1.6 ml) was added and the mixture was autoclaved at 120°C for 3 h. The hydrolysate was then neutralised with 10 *N* sodium hydroxide to pH 7.0 and made up volume to 10.0 ml. Further appropriate dilution was required for hydroxyproline determination.

2.2.6.2 Combined hydrolysis

Distilled water (50 ml) was added to a chopped sample (20 g) of beef threads, boiled for 2 minutes, and then cooled immediately in an ice bath. The sample was adjusted to pH 8.5 with 0.1 *M* sodium hydroxide and Alcalase enzyme (0.6 ml) was added. The mixture was then placed in a 50°C water bath with a magnetic stirrer. The pH of the mixture was maintained at 8.5 with 0.1 *M* sodium hydroxide until no more sodium hydroxide was consumed. The enzyme hydrolysate was then neutralised to pH 7.0 with 0.1 *M* hydrochloric acid and made up to volume (200 ml).

The enzyme hydrolysate (3.0 ml) was then pipetted into a Pyrex screwtop tube, concentrated hydrochloric acid (2.4 ml) was added, the mixture was hydrolysed in an autoclave at 120°C for 3 h. After that, the acid hydrolysate was adjusted to pH 7.0 with 10 *M* sodium hydroxide, and made up to 50.0 ml volume. For hydroxyproline determination the hydrolysate was further diluted to give an appropriate dilution.

2.2.7 Collagen content [Industrial Research Limited method modified from Neuman and Logan (1950 b)]

A sample hydrolysate was diluted to contain hydroxyproline 20-30 μg/ml. Standard hydroxyproline solutions were prepared to give a hydroxyproline concentration in the range 10 to 40 μg/ml. Both standard and sample were treated sequentially as follows

A neutralised sample of hydrolysate (0.5ml) or standard hydroxyproline was added to a tube with 0.01 *M* copper sulphate, 2.5 *M* sodium hydroxide and 6% hydrogen peroxide (0.5 ml each). The mixture was incubated at 80°C for 5 min, cooled rapidly in ice water and then 1.5 *M* sulphuric acid (2.0 ml) was added together with 5% p-dimethylaminobenzaldehyde (1.0 ml). The mixture was incubated at 70°C for 15 min and cooled in ice water. Absorbance was read at 540 nm in the Philips spectrophotometer (Model PU 8625).

The hydroxyproline content was calculated from the standard curve.

Note: A new calibration curve was prepared for each series of analyses.

2.2.8 Elastin content (Bailey and Light, 1989)

A chopped sample (3 g) of beef threads was placed in a conical flask and 0.1 *M* sodium hydroxide (50 ml) was added. The sample was hydrolysed for 1 h at 100°C, then cooled, centrifuged and the supernatant was discarded. The residue was washed twice with 0.1 *M* sodium hydroxide (10 ml) then washed twice with distilled water. The residue was then dehydrated in 3:1 ethanol/ether, freeze dried overnight and oven dried to constant weight.

The elastin content was calculated from the amount of residue.

2.2.9 Beef threads treatment

The samples of beef threads were treated with different chemical solutions for 1 hour and overnight.

Two groups of sample were investigated:

"Normal" batch beef thread

Putative "weak" and "strong" batches beef threads

The following chemicals were used into the treatment.

- *Water*
- *0.25% Sodium carbonate + 0.2% ethylenediaminetetraacetic acid di-sodium salt*
- *0.28% Sodium hydroxide*
- *0.25% Sodium bicarbonate*
- *0.25% Sodium carbonate*
- *0.25% Sodium bicarbonate + 0.25% sodium carbonate (10:1)*

The filtrates were analysed for protein content by the Kjeldahl method and the treated samples were tested for diameter and shrinkage temperature. Also the mechanical properties were tested for tensile strength using the Instron.

2.2.10 Diameter

A 5 cm long sample was placed on a slide and the diameter was measured through an Olympus (Model BH-2) microscope, ×40 magnification, 10 measurements were taken for each thread.

2.2.11 Shrinkage temperature

A sample was cut into 70 mm length, attached to the hooks (J) of the temperature measurement apparatus (Official Methods of Analysis, SLP. 18, 1965) in 50 mm length

from the ends along the centreline (E). This apparatus was put into a beaker (A) of stirred distilled water at 50 ± 5°C. Heat was applied through the hotplate of the magnetic stirrer so that temperature rose as nearly as possible at 2°C per minute. The shrinkage temperature was recorded as being the temperature at which sample had shrunk by one scale division (F). A diagram of the device to measure the shrinkage temperature is shown in Figure 2.1.

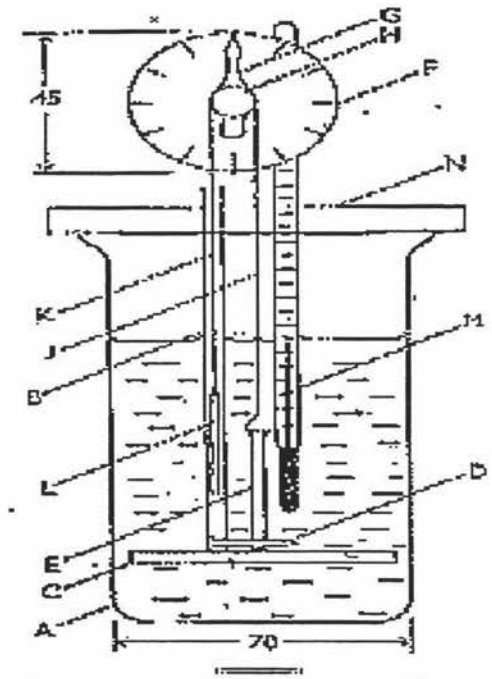


Figure 2.1 Diagram of shrinkage temperature apparatus (Official Methods of Analysis, 1965)

2.2.12 Tensile strength

A 20 cm long sample of beef thread was gripped between two sets of jaw of the Instron Universal Testing Machine (Model 4502, Instron Ltd., High Wycombe, UK) with 100 mm of grip distance. The sample was tested on a 1 KN load cell, cross head speed 100 mm/min and sample rate 10 pts/s. The sample was stretched until it was broken. The data were recorded on a computer and analysed.

2.2.13 Process treatment

The samples, i.e., nominally called "strong" and "weak" beef threads were subjected to conditions corresponding to the actual company process; three steps were applied as follow:

- First stage: beef threads (65 g) were soaked in a 0.25% sodium carbonate plus 0.2% ethylenediaminetetraacetic acid tetra-sodium salt solution (1 L) at 26-28°C for 1 hour. The threads were then rinsed with 26-28°C distilled water (1L) to remove the first soaking solution.
- Second stage: the beef threads from the first stage was then soaked in a second solution (1L) which was composed of 0.25% sodium carbonate, 0.2% ethylenediaminetetraacetic acid tetra-sodium salt and 0.3% hydrogen peroxide for 1 hour then rinsed with distilled water (1L).
- Third stage: the sample from stage 2 was soaked in 0.45% Teric solution (1L) at 26-28°C for 12-18 h, then rinsed with cold water.

After the third treatment, the filtrates from each of the stages were analysed to determine the extracted protein. The treated threads were tested to determine their chemical and physical properties.

2.2.14 Beef threads classification

The nominal "strong" and "weak" batches of beef threads were first tested for their tensile strength. The tested samples then were placed in a number of categories depending on their ductility results. They were classified into the following groups:

Group I – "strong" (first batch) beef threads with peak ductility of 20-25 mm

Group II – "strong" (first batch) beef threads with peak ductility of 30-35 mm

Group III – "strong" (second batch) beef threads with peak ductility of 20-25 mm

Group IV - "strong" (second batch) beef threads with peak ductility of 30-35 mm

Group V - "weak" (first batch) beef threads with peak ductility of 25-30 mm

Group VI – "weak" (first batch) beef threads with peak ductility of 40-45 mm

Group VII – "weak" (second batch) beef threads with peak ductility of 20-25 mm

Group VIII – "weak" (second batch) beef threads with peak ductility of 30-35 mm

Each group was then analysed for collagen and elastin content, and also the shrinkage temperatures were determined.

2.2.15 Trypsin treatment

A sample (25 g) of the second batch of "strong" beef threads was stirred with 0.1% sodium azide solution at room temperature for 0.5 h to prevent bacterial spoilage. After 0.5 h, the solution was decanted, distilled water (50 ml) was added and the pH adjusted to 8.0 with 0.1 *M* sodium hydroxide. Trypsin (0.5 g) was added and stirred, then the sample was incubated at 37°C (without stirring) for 16 h. Every 0.5 h, the sample pH was taken and 0.1 *M* sodium hydroxide was used to maintain the pH at 8.0. After 3 h, the sample was left to stand at 37°C overnight. A final amount of 0.1 *M* sodium hydroxide was added to bring up the pH to 8.0, and the total volume of sodium hydroxide noted.

The contents were then filtered through Miracloth, the filtrate was made up to volume and an aliquot was freeze-dried. The freeze-dried material was analysed for hydroxyproline content after hydrolysis with 6 *M* hydrochloric acid in the autoclave for 3 h.

In a larger scale experiment, a 150 g sample, was treated with trypsin and then put through the manufacturing process as described above.

After each stage, the treated beef threads were tested for tensile strength and shrinkage temperature. The treated beef threads were also freeze-dried and analysed for hydroxyproline content.

2.2.16 Enzyme treatments

Samples (25 g) of the second batch of "strong" beef threads were treated with different enzymes. The procedure was the same as the trypsin treatment apart from pH, which was set at 8.5 and the experiment was carried out at both 4°C and 37°C. The amount of enzyme varied according to enzyme activity; as shown below:

Trypsin	0.5 g;	690 U/g protein
Pyrase	50 µl;	7450 U/g protein
Tanzyme	0.25 ml;	1400 U/g protein

Again after enzyme treatment, the filtrate and treated beef threads were tested for the same parameters as described for the trypsin treated threads.

Note: U is catalytic activity unit; µmole/l

2.2.17 Enzyme activity

Trypsin (1%), pyrase (1:400) and tanzyme (1:250) solutions were prepared to give final solutions with equivalent enzyme activity. The procedure used to measure the enzyme activity of each enzyme preparation followed that described by Bergmeyer (1974) using casein substrate.

2.3 Data analysis

The mean and standard deviation values were determined for each parameter and the paired comparison t-test method at 95% confidence interval was used to establish whether there were significant differences between and within treatments.

The correlation between parameters of different batch samples were compared by using Excel programme to analyse the results.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Beef Thread Properties

3.1.1 Chemical composition

The chemical composition of a representative sample of a "normal" batch of beef threads is shown below; all results are on a dry-matter basis.

Moisture	76-86%
Total protein (Kjeldahl method)	98.3%
Ash	1.1%
Fat	1.8%
Collagen	57.6%
Elastin	1.8%

The moisture content of samples varied widely and depended on the sample itself and the way it had been prepared. As a result, the moisture content of each sample needed to be determined for each experiment.

Since the main component of beef threads was protein, an amino acid analysis was undertaken as the next step.

From the strength and flexibility of the beef thread sample, and from the literature, collagen was suspected as being the major protein in this tissue. Colorimetric determination of hydroxyproline content led to a calculated value, based on collagen Type I, of 57.6% for collagen. Thus, about half of the total proteins of beef threads would appear to be non-collagenous protein, which will be considered later. The amount of elastin (1.8%) seemed to be insignificant even though it is thought to contribute to the elasticity of tissues.

The amino acid composition of a "normal" batch of beef threads is shown in Table 3.1. It should be noted that neither 3-hydroxyproline nor 4-hydroxyproline was determined because of overlap with the aspartic acid peak, which was the first peak in the chromatogram (Appendix 7).

Table 3.1 Amino acid content in "normal" batch beef thread sample

Amino acid	Amino acid content (nmole/mg)	Amino acid content[2] (residues/ total AA residues)	Collagen Type I[3] (residues/total AA residues) $[\alpha 1\ (I)]_2\ \alpha 2\ (I)$	Collagen Type III[2] (residues/total AA residues) $\alpha 1\ (III)$
Hyp	n/d	(97.0)*	97.0	127.0
Asp	448.2	52.1	44.0	43.0
Thr	180.9	21.0	17.7	13.0
Ser	50.3	5.8	38.0	39.0
Glu	836.5	97.2	72.3	72.0
Pro	852.4	99.0	133.7	109.0
Gly	1276.0	148.3	351.3	354.0
Ala	766.6	89.0	118.3	97.0
Cys	0	0	0	2.0
Val	308.4	35.8	25.3	14.0
Met	138.3	16.0	6.3	8.0
Ile	255.2	29.7	9.7	13.0
Leu	449.3	52.2	25.0	22.0
Tyr	79.9	9.3	2.7	3.0
Phe	215.7	25.0	12.7	8.0
Hylys	47.3	5.5	6.3	5.0
His	116.3	13.5	5.0	6.0
Lys	362.2	42.0	28.3	30.0
Arg	479.5	55.6	51.7	47.0

The observed amino acid composition does not match the amino acid content of any of the known collagen types, which have been reported in the literature (refer to

[2] The number of amino acid residues/ total amino acid residues was calculated from the MW of one α chain collagen = 116160 (assumed that average MW of amino acids = 110 and one α chain contains 1056 amino acid residues).

[3] Collagen Type I and Type III refer to the main collagen types in meat connective tissues (Bailey and Light, 1989). A weighted average was used because there are two α1 (I) chains and one α2 (I) chain in the tropocollagen molecule. The hydroxylysine content is 6.3 residues per total amino acid residues.

n/d = not determined

* (97) is based on the literature, average of the values in two collagen α1 (I) chains and one collagen α2 (I) chain.

Table 3.1). The total amino acid content per α collagen chain (i.e. total amino acid residues in Table 3.1) was 797 residues compared to the expected figure of 1056 residues. Presumably this lower amino acid residue content could be traced to the loss of some amino acids during acid hydrolysis, and also to cumulative experimental error. Even allowing for the 97 hydroxyproline residues, the total residue figure per α-chain was short of the presumed 1056 residues per α-chain.

In several studies, it was reported that under acidic conditions tryptophan was almost entirely decomposed, while 20-50% of the cysteine was decomposed (Csapo *et al.*, 1986). Furthermore, the yields of serine and threonine were decreased and also cleavage of the amide glutamine and asparagine was reported (Church *et al.* 1984). Consequently, the amount of tryptophan, cysteine, serine and threonine obtained in this analysis was probably lower than the actual amounts in the "normal" beef threads supplied for testing.

As the beef threads contained 42% non-collagenous protein one would not expect the amino acid profile obtained in this study to exactly match one of the native collagen amino acid sequences reported in the literature. Clearly a separate amino acid analysis should be conducted on the purified collagen fraction and on the non-collagenous fraction – something that was not done for this study.

The amino acid composition of Type I and III collagen α-chains is shown in Table 3.2 as well as a predicted value for the beef threads assuming that 57.6% of the total protein was Type I collagen. As can be seen, the hydroxylysine content in the beef thread sample was 5.5 residues/total amino acid residues which is far greater than either Type I and Type III collagen. This unfortunately means that a definite decision as to whether the collagen in the beef thread sample was either Type I or Type III cannot be made. However, the closer correspondence to the 3.6 hydroxylysine/total amino acid residues per α–chain, suggests that Type I may be the predominant collagen in beef threads. As a result, subsequent collagen content results in this report have been based on the hydroxyproline content of bovine collagen Type I, 13.1% (Bailey and Light, 1989), and the factor used to convert hydroxyproline to collagen Type I was 7.63.

An examination of Table 3.2, shows that the aspartic acid, glutamic acid, methionine, histidine, lysine and arginine values, which are polar amino acids, are much higher

when compared with collagen Type I and III values. This would suggest that the non-collagenous proteins of the beef thread sample are highly polar, and that they may be either globulins and/or albumins. Globulins tend to be deficient in glycine.

Table 3.2 Comparison of amino acid composition in sample containing 57.6% collagen

Amino acid	Amino acid content (residues/total AA residues)	Collagen Type I[4] (residues/total AA residues)	Collagen Type III (residues/total AA residues)
Hyp	(97.0)*	55.9	73.2
Asp	52.1	25.3	24.8
Thr	21.0	10.2	7.5
Ser	5.8	21.9	22.5
Glu	97.2	41.6	41.5
Pro	99.0	77.0	62.8
Gly	148.3	202.3	203.9
Ala	89.0	68.1	55.9
Cys	0	0	1.2
Val	35.8	14.6	8.1
Met	16.0	3.6	4.6
Ile	29.7	5.6	7.5
Leu	52.2	14.4	12.7
Tyr	9.3	1.6	1.7
Phe	25.0	7.3	4.6
Hylys	5.5	3.6	2.9
His	13.5	2.9	3.4
Lys	42.0	16.3	17.3
Arg	55.6	29.8	27.1

Due to the complicated technique and cost of amino acid analyses, no further amino acid analyses were conducted on the beef thread samples. However, future studies should explore the amino acid content of the purified collagen and non-collagenous proteins to precisely define the type of collagen that forms the basis of the collagenous fraction and to also precisely define what type of protein(s) make-up the non-collagenous fraction.

[4] Collagen Type I is the average of amino acid composition of two α1 (I) chains and one α2 (I) chain

* (97) is based on the literature, average of the values in two chains collagen α1 (I) and one chain collagen α2 (I)

n/d = not determined

Besides chemical composition, the physical properties of intestine samples should be examined. It is possible that the final physical properties of tennis strings are dependent on both the amino acid profile of the strings, and proportion of collagenous and non-collagenous protein, and to their physical properties, such as tensile strength etc. The physical characteristics, which were investigated in this study, are shrinkage temperature and tensile strength.

3.1.2 Physical properties

Four different batches of sample were tested for their physical properties and the results are shown in Table 3.3.

Table 3.3 Comparison of the properties of the different batches beef thread

Parameter	1st strong batch beef thread	2nd strong batch beef thread	1st weak batch beef thread	2nd weak batch beef thread	N
Shrinkage temperature (°C)	72.7 ± 0.3[a]	71.4 ± 0.5	71.9 ± 0.4	71.1 ± 0.4	6
Ductility (mm)	25.8 ± 5.8	25.2 ± 5.1	31.6 ± 6.7	28.9 ± 4.2	10
Ultimate tensile strength (N)	11.4 ± 2.5	10.5 ± 3.6	14.6 ± 4.3	15.5 ± 4.4	10
Young's modulus (N/mm)	0.8 ± 0.1	0.8 ± 0.3	1.1 ± 0.3	1.2 ± 0.3	10
Collagen (%)	58.6 ± 3.7	55.1 ± 0.8	69.8 ± 1.5	47.2 ± 2.9	3

[a] Mean value ± standard deviation, N = number of determinations

Significant differences in shrinkage temperature were obtained with the first strong batch being significantly different ($P \leq 0.05$) from the other three batches. Moreover, there was a significant difference between the first weak and the second weak batch ($P \leq 0.05$), but no significant difference between the second strong batch and either the first or the second weak batches.

Considering the ductility of the four different batches, the results showed a significant difference between the second strong batch and the first weak batch ($P \leq 0.05$). There also seemed to be a significant difference between the first strong batch and the first

weak batch ($p \leq 0.054$). However, no significant difference was observed between the second weak batch and the other three batches. Therefore, one cannot say that there is a systematic difference in ductility between the "strong" and " weak" beef threads.

For the ultimate tensile strength data the so-called "weak" beef threads had a higher raw tensile strength than the "strong" batches. This ranking in tensile strength was reversed once the threads had been processed and this is where the "strong" and "weak" designations for the strings arose. A comparison between the "strong" and the "weak" group showed that the second "weak" batch was significantly stronger than the first and the second "strong" batches ($P \leq 0.05$), and the first "weak" batch was significantly stronger than the second "strong" batch ($P \leq 0.05$) and barely significant when compared with the first "strong" batch ($P \simeq 0.06$). It would appear therefore, that the "weak" batches had a higher unprocessed raw tensile strength than the "strong" beef threads.

The Young's modulus results revealed that there were no significant within batch differences between the two "strong" and the two "weak" batches of threads ($P \leq 0.05$). However, there were significant between group differences. The Young's modulus of the "weak" group was significantly higher than the "strong" group in both batches ($P \leq 0.05$).

Overall, the ductility, the ultimate tensile strength and the Young's modulus of the "weak" group tended to have significantly higher values for the respective tests than the "strong" group. However, more tests should be carried out to establish whether there is a more clear cut difference in tensile strength between the "weak" and "strong" groups.

In Table 3.4 a correlation matrix of the various physical measurements has been presented. As can be seen from this Table the collagen content was moderately correlated ($r = 0.52853$) with the shrinkage temperature. The ductility of the samples was not correlated with the per cent collagen content of the sample, ($r = 0.47182$), but significantly related to the ultimate tensile strength ($r = 0.85936$), and the Young's modulus ($r = 0.83277$). The ultimate tensile strength was highly correlated with Young's modulus ($r = 0.98742$) increasing in a linear fashion with increasing tensile strength. The Young's modulus was correlated with ductility ($r = 0.83277$) and highly correlated with ultimate tensile strength ($r = 0.98742$).

Table 3.4 Correlation matrix of various physical measurements on raw beef threads

	Shrinkage Temperature	Ductility	Ultimate tensile strength	Young's modulus	Collagen Content %
Shrinkage Temperature	1				
Ductility	-0.18360	1			
Ultimate tensile strength	-0.39921	0.85936	1		
Young' modulus	-0.53750	0.83277	0.98742	1	
Collagen Content (%)	0.52853	0.47182	-0.02682	-0.09519	1

3.1.3 Chemical treatment

To investigate what effect the various chemical treatments had on the physical properties of beef threads; one batch of beef threads was treated with different chemicals simulating the first stage of the process. The treatment times were overnight and one hour.

3.1.3.1 Overnight treatment

- *Normal batch beef thread*

There are historically two processes, the now discarded caustic process and the present EDTA process. This experiment simulated these two processes to investigate whether there was any significant differences in beef thread properties as a consequence of being processed by the two methods.

Table 3.5 Comparison of the effects of various overnight alkali treatments on a "normal" beef thread sample

Treatment	pH	Extracted Proteins % (dry starting material basis)	Shrinkage Temperature (°C)	Diameter (mm)
Control	7.0	n/d	70.9 ± 0.5[a]	1.4 ± 0.4
Distilled water	6.9	2.9 ± 0.1	71.4 ± 0.4	2.5 ± 0.4
0.28% NaOH	12.4	41.5 ± 0.3	65.1 ± 0.7	4.7 ± 0.8
0.25% Na_2CO_3 + 0.2% di-Na EDTA	10.0	19.4 ± 0.2	71.6 ± 0.2	5.4 ± 1.3
0.25% $NaHCO_3$	8.2	6.2 ± 0.0	71.0 ± 0.6	2.1 ± 0.3
0.25% $NaHCO_3$ + 0.25%Na_2CO_3 (10:1)	9.0	7.3 ± 0.2	71.7 ± 0.3	2.7 ± 0.2
0.25% Na_2CO_3	11.2	20.3 ± 0.0	71.6 ± 0.5	5.7 ± 0.5
N	1	2	6	6

[a] Mean value ± standard deviation, N = number of determinations, n/d = not determined

It can be seen from Table 3.5 that the amount of extracted protein on a dry matter basis increased with increasing pH of the treatment solution, illustrated in Figure 3.1. The pH and the amount of extracted protein were highly correlated (r = 0.94456) and a linear regression of the data showed that the relationship accounted for 0.892 of the variance in the data.

The amount of extracted proteins increased sharply from pH 11 to 12. As fibrous proteins are insoluble in water and salt solution, and as the fibrous structures at the macroscopic level are resistant to acid, alkalis and protease (Jakubke and Jeschkeit, 1977), the extracted proteins obtained from the experiment are most probably non-collagenous or non-fibrous proteins. This is supported by the amino acid profile of the protein illustrated earlier in section 3.1.1. The results suggest that the non-collagenous proteins were globulins which can be dissolved in salt solution and dilute alkali, and only a small proportion were possibly albumins.

Bowes *et al.* (1958) studied the collagen in calf skin; they extracted calf skin with 0.1 *M* sodium phosphate buffer, pH 9.0, which removed some 8-10% of the total protein of the tissue. This was considered to consist mainly of plasma proteins. The soluble collagenous protein fraction was then extracted with acetic acid and the residue was treated with dilute sodium hydroxide solution, pH 12.0, which dissolved 10-15% of the total protein. The composition of this alkaline-soluble protein was quite different from that of collagen, and it was considered possible that it was derived from the interfibrillary ground substance.

On the basis of Bowes *et al.*'s (1958) results, the proteins extracted by sodium hydrogen carbonate and the sodium hydrogen carbonate/sodium carbonate solutions may be viewed as plasma proteins while the more basic solutions extracted ground substance.

As to analytical work on collagen, most of the data was obtained from analysis of collagen which had been treated in alkali solutions for unhairing or which had been enzymatically treated in order to remove elastic fibres, accessory proteins of the globular type, and reticular tissue. The alkali treatment modified the collagen. It particularly affected the total nitrogen content, since deamidation occurs readily at the high degree of alkalinity corresponding to pH 12 to 13, which is usually employed in the liming process (Gustavson, 1956). This in turn significantly alters the isoelectric point of the collagen.

The result of lime, and of alkali generally, on collagen leads to purely chemical changes such as deamidation and the minor destruction of the arginine residue, with possible cleavage of some peptide bonds, yielding polypeptides to the solution. Lime also opens up the fibre structure, making it accessible and permeable to large molecules. This process may involve the breaking of cross-links in the form of chondroitin sulphate. Another important reaction is the activation of ionic groups by breaking up of the salt links through neutralisation of the charged amino groups by the hydroxy ions, which on prolonged liming (swelling) partly involves irreversible changes. Indirectly, interchain links of the hydrogen bond type, are disrupted by the swelling. The increase in the number of carboxylic groups resulting from the deamidation process allows greater ionic reactivity. The increase in collagen reactivity is supported further by the physical alteration in the hide caused by the liming, and also by chemical changes in the non-ionic protein groups, since the swelling of hide leads to the rupture of some of the co-

ordinate (hydrogen) bonds between adjacent protein chains (lowered cohesion of the hide). These hydrogen bonds are not completely reformed in the subsequent deliming process, which reduces the swelling by adding neutral salt such as sodium chloride. The peptide bond is therefore made more accessible to various agents (Gustavson, 1956).

The native collagen fibres are always associated with some amorphous ground-substance components comprised of non-collagenous proteins, sugars, mucopolysaccharides, polynucleotides and lipids, the actual amounts of each depending upon the particular tissue or organ involved. The major portion of each of these components can be removed by appropriate solvents and numerous extractions (Ramachandran, 1967).

Proteoglycans are a minor part of intramuscular connective tissue in terms of their proportionate dry weight in the tissue; there is a significant quantity present in cartilage. The proteoglycans possess a protein core to which glycosaminoglycans (GAG) chains are covalently bonded by specific carbohydrate sequences of three or four monosaccharides. The aggregation of the proteoglycans sterically hinders their movement within the collagen framework, at the same time conferring elasticity to the tissue.

The presence of large glycoproteins in the extracellular matrix has been known for a long time, but their contribution to the stabilisation of the various components is just beginning to be explained. In multicellular organisms glycoproteins appears to play a role in anchoring cells to basement membranes or the connective tissue and in controlling the highly organised movement of cells through the matrix.

There are two classes of glycoproteins, fibronectin and laminin. Fibronectin has a multi-domain structure and is present in a soluble form in plasma but also exist in an insoluble fibrous form in the extracellular matrix. Laminin is an important and major constituent of basement membranes (30% by weight). The protein has a high molecular weight (600 000) and binds specially to Type IV collagen and to heparan sulphate, one of the glycosaminoglycans. The glycoproteins, like the small proteoglycans, have been reported to affect the properties of collagen fibres. Modification of these properties could lead to a change in the shrinkage properties and ultimately of the denatured fibres of collagen after heating (Bailey and Light, 1989).

There is another fibrous protein present in connective tissues – elastin. Elastin is completely distinct from the collagen family. These fibres are distinctly elastic in behaviour, and occur where elasticity and high mechanical strength are required in combination. On the whole, elastins are less widely distributed in the organism than the collagens. The high stability of elastin under hydrothermal condition is mainly accounted for by its very low content of polar side chains (Ramachandran, 1967)

It can be assumed that the sodium hydroxide treatment, which was the most effective extraction, removed some non-collagenous proteins that were necessary for the strength of the beef threads. In addition, changing the ionic strength caused the swelling and disrupted the hydrogen bonds in collagen fibres. As a result, the shrinkage temperature of the beef thread samples treated with sodium hydroxide had a larger drop in comparison to other treatments as shown in Table 3.5 and Figure 3.2.

The shrinkage temperature was significantly increased ($P \leq 0.05$) when the pH of the treatment solution increased from 7.0 to 9.0 to 11.0 (in sodium hydrogen carbonate/sodium carbonate (pH 9.0), sodium carbonate/EDTA (pH 10.0) and sodium carbonate (pH 11.2) solutions) compared with the control beef thread. As less non-collagenous protein was extracted in this pH range as compared with higher pHs, the bonds strengths of the collagen as measured by shrinkage temperature was unaffected or slightly increased by the extracting solution became more basic. However, a solution pH of 12.4 resulted in a significant drop in shrinkage temperature.

The treatment of beef threads with sodium carbonate alone and with sodium carbonate/EDTA di-sodium salt gave very similar results in all properties. It could be assumed therefore that sodium carbonate is the dominant chemical responsible for changes in properties rather than the EDTA di-sodium salt.

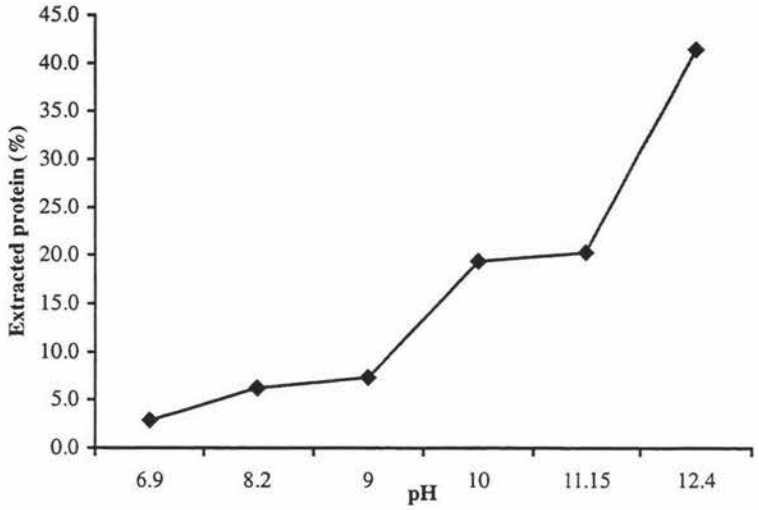


Figure 3.1 Relationship between pH and the amount of extracted proteins from the normal batch beef thread after overnight treatment

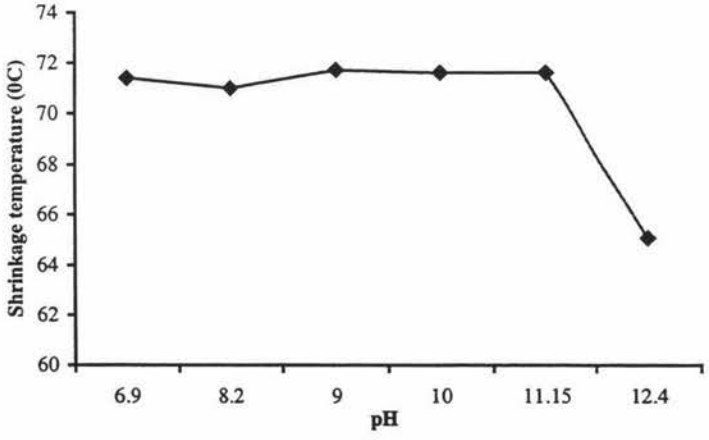


Figure 3.2 Relationship between pH and shrinkage temperature of the normal batch beef thread after overnight treatment

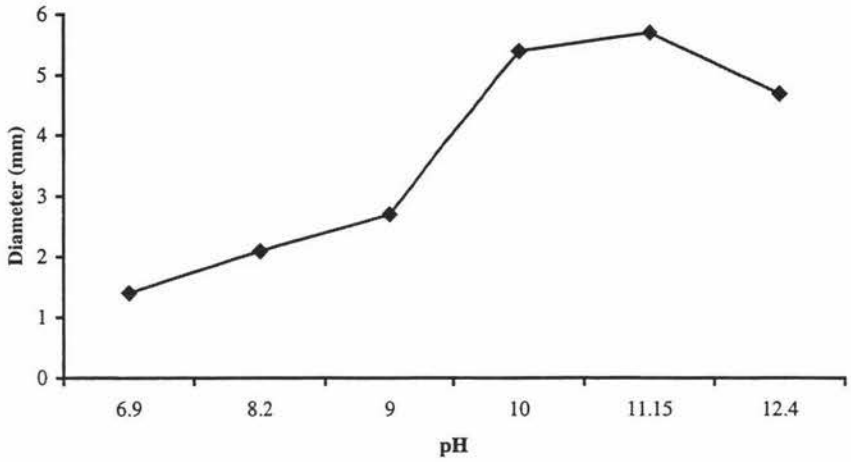


Figure 3.3 Relationship between pH and beef thread diameter of the normal batch beef thread after overnight treatment

The swelling of treated beef threads increased sharply from pH 9.0 to 10.0, this can be explained by the collagen properties. Normally, there are two types of swelling, osmotic and lyotropic. The main swelling is osmotic because the effect is associated with the concentration of ions within the fibril structure. The swelling takes place as a result of high concentrations of fixed non-diffusible ions within the fibril structure, this happens when the pH is moved away from the isoelectric point at low ionic strength (Ramachandran, 1967). At pH 2 and 12 the collagen is extensively swollen and becomes permanently damaged if left in these conditions for too long. The result of these varying osmotic forces as a function of pH can be expressed in the accompanying swelling curve, Figure 3.4

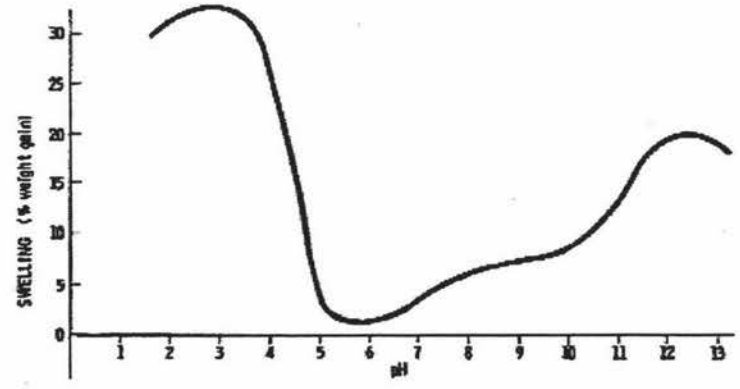


Figure 3.4 Relationship between pH and swelling of collagen (Thorstensen, 1969)

After soaking in the test solutions the diameter of beef threads increased with increasing pH as seen in Figure 3.3. The diameters of the samples treated with sodium carbonate plus EDTA di-sodium salt (pH 10.0) and sodium carbonate solution (pH 11.2) were not significantly different ($P > 0.05$). But the diameter of beef threads after treatment with sodium hydroxide (pH 12.4) was significantly smaller than those treated with sodium carbonate solution (pH 11.2). The result contrasted with the swelling curve in Figure 3.4, where swelling was shown to continue to increase with increasing pH. This can be explained by the large amount of non-collagenous protein removed (41.5%), so that the lesser mass countered the osmotic swelling.

In the current process, the beef threads are soaked successively in two different chemical solutions for only one hour. However, the final pHs obtained after one hour were identical to those that are obtained in the overnight experiment, so the pH effects are probably the same for the overnight and one hour processes.

- *Processing of "strong" beef threads*

The experimental conditions were the same as the normal batch beef thread and the results are shown in Table 3.6.

The results from the normal and the first strong batch of beef threads followed the same pattern, as can be seen from Figure 3.5, 3.7 and 3.9. The amount of extracted protein from the first strong batch was higher in the sodium hydrogen carbonate, sodium hydrogen carbonate/sodium carbonate and sodium carbonate treatments (refer to Table 3.5) and also in distilled water than was obtained from the "normal" threads: this probably occurred because of differences in protein composition between the "normal" and "strong" beef threads even though they were given the same treatments. The diameter of the first strong batch after treatment with sodium carbonate/EDTA tetra-sodium salt, sodium hydrogen carbonate and sodium hydrogen carbonate/sodium carbonate indicated more swelling than the normal batch beef thread. Even though the EDTA used in the latter experiment was different from the former, the final ionic strength of both solutions was not very different. The ionic strength of the sodium carbonate/EDTA di-sodium salt solution was 0.1192 molal for the "normal" beef threads, and 0.1234 molal for the "strong" beef threads. This difference should not affect the swelling of the samples.

Table 3.6 The effect of overnight treatments on the first strong batch beef thread

Treatment	pH	Extracted Proteins % (dry starting material basis)	Shrinkage Temperature (°C)	Diameter (mm)
Control	7.0	n/d	72.7 ± 0.3	1.5 ± 0.4
Distilled water	6.8	9.7 ± 0.4[a]	71.3 ± 0.4	2.1 ± 0.3
0.28% NaOH	12.5	42.1 ± 10.8	64.4 ± 0.5	4.3 ± 0.4
0.25% Na_2CO_3 + 0.2% tetra-Na EDTA	10.4	17.6 ± 3.4	71.5 ± 0.4	7.4 ± 1.0
0.25% $NaHCO_3$	8.2	16.4 ± 2.1	72.4 ± 0.4	3.1 ± 0.4
0.25% $NaHCO_3$ + 0.25% Na_2CO_3 (10:1)	8.7	17.0 ± 0.7	72.6 ± 0.1	3.3 ± 0.3
0.25% Na_2CO_3	10.7	24.6 ± 3.6	72.4 ± 0.1	5.7 ± 0.8
N	1	2	6	6

[a] Mean value ± standard deviation, N = number of determinations, n/d = not determined

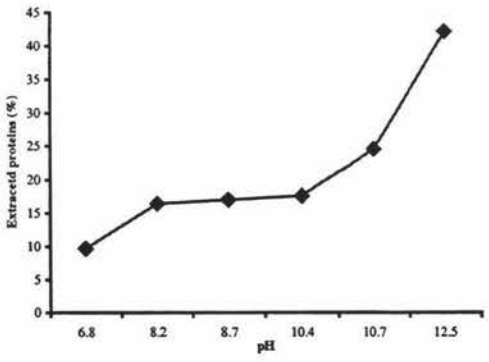


Figure 3.5 Relationship between pH and amount of extracted proteins from the first strong batch beef thread after overnight treatment

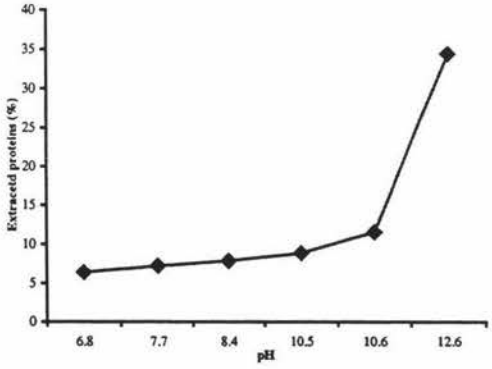


Figure 3.8 Relationship between pH and amount of extracted proteins from the first strong batch beef thread after one hour treatment

The shrinkage temperatures of the first "strong" batch were significantly higher after treatment with sodium hydrogen carbonate, sodium hydrogen carbonate/sodium carbonate and sodium carbonate treatments than the "normal" beef threads after the same treatments. This difference in shrinkage temperature between the two batches of

beef threads would not appear to be due to the collagenous fraction as both the "strong" and "normal" threads had very similar collagen compositions, i.e. 58.6% and 57.6% respectively. Its possible that the differences might be related to the non-collagenous fraction, i.e. differences in the proportion of albumin and globulins, but this is merely speculation since the tests for relevant proteins were not carried out. At this stage no definitive explanation for the differences in shrinkage temperature between the batches can be provided.

3.1.3.2 One hour treatments

- *First strong batch*

In this experiment, the first strong batch of beef threads were treated with the same chemicals as used in the previous section, but the soaking period was for 1 hour rather than overnight as occurred in the previous experiment. A one hour soak was selected to match the actual process conditions. The results have been summarised in Table 3.7.

The pattern for the one hour and overnight treatments was similar and these have been compared in Figures 3.5 - 3.10. The amount of extracted proteins from the overnight treatment was higher due to the longer soaking period.

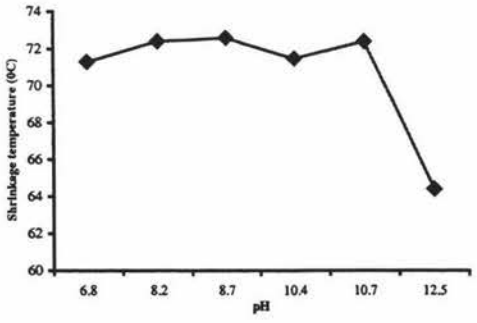


Figure 3.6 Relationship between pH and shrinkage temperature of the first strong batch beef thread after overnight treatment

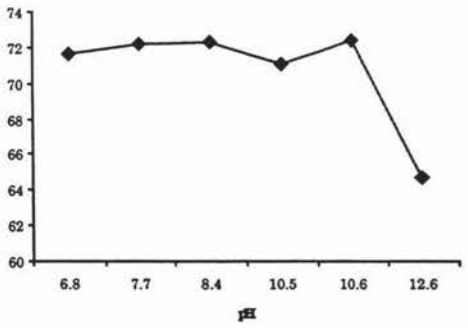


Figure 3.9 Relationship between pH and shrinkage temperature of the first strong batch beef thread after one hour treatment

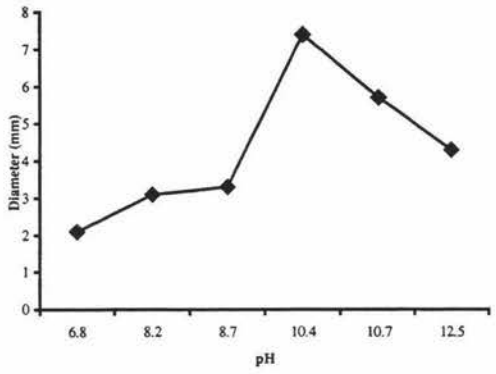


Figure 3.7 Relationship between pH and diameter of the first strong batch beef thread after overnight treatment

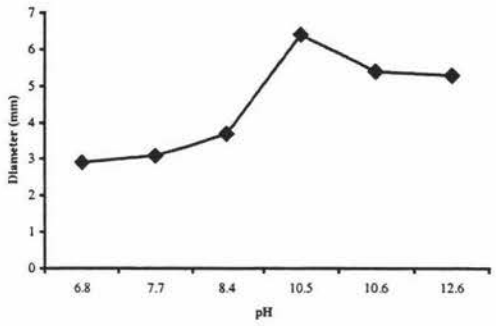


Figure 3.10 Relationship between pH and diameter of the first strong batch beef thread after one hour treatment

Table 3.7 The effect of one hour treatments on the first strong batch of beef thread

Treatment	pH	Extracted Proteins % (dry starting material basis)	Shrinkage Temperature(°C)	Diameter (mm)
Control	7.0	n/d	72.7 ± 0.3	1.5 ± 0.4
Distilled water	6.8	6.4 ± 0.7[a]	71.7 ± 0.4	2.9 ± 0.6
0.28% NaOH	12.6	34.4 ± 1.7	64.7 ± 1.4	5.3 ± 0.5
0.25% Na_2CO_3 + 0.2% tetra-Na EDTA	10.6	11.6 ± 0.4	71.1 ± 0.4	6.4 ± 0.3
0.25% $NaHCO_3$	7.7	7.2 ± 0.0	72.2 ± 0.4	3.1 ± 0.4
0.25% $NaHCO_3$ + 0.25% Na_2CO_3 (10:1)	8.4	7.9 ± 0.1	72.3 ± 0.3	3.7 ± 0.7
0.25% Na_2CO_3	10.5	8.9 ± 0.0	72.4 ± 0.5	5.4 ± 0.6
N	1	2	6	6

[a] Mean value ± standard deviation, N = number of determinations, n/d = not determined

Although the amount of extracted non-collagenous proteins from the overnight sodium hydroxide treatment was higher than the one hour treatment, the shrinkage temperatures from the two different soaking periods were not significantly different ($P > 0.05$).

Therefore, from this stage and the results from Table 3.3, it may be concluded that the amount of non-collagenous material that is extracted by these alkali treatments has a minimal effect on the shrinkage temperature. A regression of shrinkage temperature versus per cent non-collagenous protein extracted only accounted for 27.93% of the variance in the data ($R^2 = 0.2793$). It would also appear that any changes in hydrogen bonding, which plays a vital role in the stabilisation of proteins, had occurred within one hour of soaking as shown by the similarities of the shrinkage temperatures for beef threads given either a one hour soak or an overnight soak

The experiment also studied the chemical and physical properties of the "strong" and "weak" batch beef threads after treatment with different chemicals.

- *First strong batch and first weak batch*

In this experiment only three treatments were studied, distilled water, 0.28% sodium hydroxide and 0.25% sodium carbonate + 0.2% EDTA tetra-sodium salt. The procedure was the same as the previous experiment but applied to both the "strong" and "weak" batches of beef threads. The results are shown in Table 3.8.

From the results, the "weak" beef threads had a significantly lower shrinkage temperature than the "strong" beef threads for the control samples and the sodium hydroxide treated samples ($P \leq 0.05$). If the shrinkage temperature is related to the strength of the hydrogen bonds, which maintain the collagen structure, it can be assumed then that the "weak" beef threads might have fewer or weaker hydrogen bonds than the "strong" threads. Moreover, the amount of protein extracted from the "weak" threads by each solution was lower than from the "strong" batch. This is because the weak beef thread had less non-collagenous protein than the "strong" threads and/or a higher collagen. But whether this accounted for the difference in wet strength of the "weak " and "strong" threads is unknown.

After treatment with the sodium carbonate/EDTA solution, the diameter of the "strong" beef threads was significantly greater than that of the "weak" beef threads ($P \leq 0.05$), although in the raw, untreated beef threads the "weak" beef threads had a greater diameter. It showed that the "strong" beef thread underwent more swelling under the same conditions – this could be due to the fact that the "strong" threads had less non-

collagenous material extracted by each treatment than the "weak" threads. Again how this may relate to "strong" wet strength is unknown.

Table 3.8 Comparison of beef thread properties after one hour treatments

Treatment	Parameter	1st strong batch beef thread	1st weak batch beef thread	N
Control	pH	7.0	6.6	1
	Extracted proteins % (dry starting material basis)	n/d	n/d	n/d
	Shrinkage temperature (°C)	72.7 ± 0.3[a]	71.9 ± 0.4	6
	Diameter (mm)	1.5 ± 0.4	2.0 ± 0.5	6
Distilled water	pH	6.8	6.6	1
	Extracted proteins % (dry starting material basis)	6.4 ± 0.7	0.65 ± 0.01	2
	Shrinkage temperature (°C)	71.7 ± 0.4	71.6 ± 0.2	6
	Diameter (mm)	2.9 ± 0.6	2.5 ± 0.3	6
0.28% NaOH	pH	12.6	12.5	1
	Extracted proteins % (dry starting material basis)	34.4 ± 1.7	30.5 ± 0.6	2
	Shrinkage temperature (°C)	64.7 ± 1.4	61.8 ± 1.3	6
	Diameter (mm)	5.3 ± 0.5	5.9 ± 0.9	6
0.25% Na_2CO_3 + 0.2% tetra-Na EDTA	pH	10.6	10.6	1
	Extracted proteins % (dry starting material basis)	11.6 ± 0.4	8.0 ± 0.3	2
	Shrinkage temperature (°C)	71.1 ± 0.4	71.4 ± 0.8	6
	Diameter (mm)	6.4 ± 0.3	4.7 ± 0.4	6

[a] Mean value ± standard deviation, N = number of determinations, n/d = not determined

3.2 Treatment Process

3.2.1 Chemical process

The aim of this experiment was to compare the properties of the "strong" and "weak" beef threads when run through the whole wet process. Three different stages (including the Teric stage) were included in this portion of the experimental programme. The sodium carbonate plus EDTA, the sodium carbonate alone and the sodium hydroxide process were investigated. The results, shown in Table 3.9 were obtained after the samples had been run through the final Teric stage.

Table 3.9 Comparison of different processes on the "strong" and "weak" batch beef threads

Process	Parameter	1st strong batch	1st weak batch	N
Control	Shrinkage temperature (°C)	72.7 ± 0.3[a]	71.9 ± 0.4	6
	Ductility (mm)	25.8 ± 5.8	31.6 ± 6.7	10
	Ultimate tensile strength (N)	11.4 ± 2.5	14.6 ± 4.3	10
	Young's modulus (N/mm)	0.8 ± 0.1	1.1 ±0.3	10
	Diameter (mm)	1.5 ± 0.4	2.0 ± 0.5	6
	Collagen (% dry-basis)	58.6 ± 3.7	69.8 ± 1.5	3
	Elastin (% dry-basis)	0.64 ± 0.03	0.74 ± 0.01	3
0.25% Na_2CO_3 + 0.2% Na-tetra EDTA process	Shrinkage temperature (°C)	72.0 ± 0.8	71.8 ± 0.5	6
	Ductility (mm)	21.2 ± 2.4	22.6 ± 3.9	10
	Ultimate tensile strength (N)	13.8 ± 4.8	14.5 ± 3.5	10
	Young's modulus (N/mm)	1.0 ± 0.3	1.1 ± 0.3	10
	Diameter (mm)	7.1 ± 0.3	6.1 ± 0.9	6
NaOH process	Shrinkage temperature (°C)	65.0 ± 0.9	64.8 ± 0.8	6
	Ductility (mm)	46.8 ± 14.9	46.9 ± 5.1	10
	Ultimate tensile strength (N)	8.1 ± 2.6	10.3 ± 1.4	10
	Young's modulus (N/mm)	0.5 ± 0.1	0.6 ± 0.1	10
	Diameter (mm)	4.3 ± 0.5	5.4 ± 0.9	6
Na_2CO_3 process	Shrinkage temperature (°C)	72.9 ± 0.4	72.8 ± 0.4	6
	Ductility (mm)	20.6 ± 1.6	24.2 ± 4.0	10
	Ultimate tensile strength (N)	12.1 ± 3.0	11.3 ± 1.6	10
	Young's modulus (N/mm)	0.9 ± 0.2	0.9 ± 0.1	10
	Diameter (mm)	4.6 ± 0.5	5.5 ± 0.9	6

[a] Mean value ± standard deviation, N = number of determinations

Note: the tensile strength curves of the control and the treated beef threads are shown in Appendix 1-6

The sodium carbonate/EDTA and the sodium carbonate processes gave very similar results for most of the measured parameters except the diameter of the sample. After being treated with the sodium carbonate/EDTA process the diameter of the first "strong" threads was significantly greater ($P \leq 0.05$) than the thread diameter from any other alkali treatments. This could be explained by the ionic strength of the different solutions. The ionic strength of the sodium carbonate/EDTA solution was 0.12 molal while the ionic strength of the sodium carbonate solution alone was 0.07 molal. This might account for the difference in osmotic swelling that was recorded for the "strong" beef threads when compared with the "weak" beef threads after treatment with the above solutions. However, this change did not affect the other parameters, as there were no significant differences in shrinkage temperature, ductility, ultimate tensile strength and Young's modulus between these two alkali treatments.

The shrinkage temperatures, ultimate tensile strength and Young's modulus of the first "strong" batch of beef threads treated with sodium carbonate gave greater values than the sodium hydroxide process. In contrast, the ductility of the treated thread from the sodium hydroxide process was higher than the sodium carbonate process. The diameter of the threads soaked in sodium hydroxide and sodium carbonate was similar but significantly different from the sodium carbonate/EDTA treated threads. The results in this experiment were similar to the results in section 3.1.3.1.

For the first "weak" sample, there were significant differences of the shrinkage temperature and the ultimate tensile strength after being treated with the two sodium carbonate base processes ($P \leq 0.05$). The ductility, Young's modulus and diameter of the "weak" threads, were not significantly different between these two sodium carbonate base processes. The sodium carbonate process significantly increased the shrinkage temperature but lowered the ultimate tensile strength. The sodium hydroxide process gave the lower shrinkage temperature and Young's modulus but higher ductility when compared with the "weak" threads from the two sodium carbonate base processes, the results are similar to those reported in section 3.1.3.1.

When comparing the "strong" and the "weak" batches with regard to control sample, the shrinkage temperature of the starting samples were significantly different ($P \leq 0.05$) - the "strong" batch had a higher shrinkage temperature than the "weak" batch. However, after the three wet treatment processes, there were no significant differences between

shrinkage temperatures between the "strong" and "weak" samples. It is possible that the three wet stages of the process produced similar alterations to the structure of both the "weak" and "strong" threads, and as a consequence, there were no detectable differences in the shrinkage temperature of the resulting threads. The sodium carbonate/EDTA process resulted in the "strong" and "weak" threads having similar ductility, ultimate tensile strength and Young's modulus. The sodium carbonate/EDTA process might have affected the tensile strength properties, since the Young's modulus values of the starting samples and the processed threads were significantly different. The "weak" and "strong" threads were affected in a significantly different manner by the sodium hydroxide process as evidenced by the differences in the ultimate tensile strength, Young's modulus and diameter ($P \leq 0.05$).

Significant differences ($P \leq 0.05$) between threads treated by sodium hydroxide and the other two carbonate based processes were observed. The sodium hydroxide process decreased the shrinkage temperature, the ultimate tensile strength and the Young's modulus but increased the ductility of the beef threads. This tendency agreed well with the previous experiment, due to the large amount of non-collagenous proteins being extracted and the fact that a strong alkali can disrupt the cross-links of collagen molecules. The bond strength of the collagen molecules was lessened and this effect was translated into the macroscopic strength properties.

The diameters of the treated beef threads from sodium hydroxide process were smaller than the beef threads after treatment with the sodium carbonate/EDTA process but similar to the diameters of the threads from the sodium carbonate process. The observed differences in diameter between the three treatments could have arisen as a consequence of the pH of the soaking solutions and also to differences in ionic strength of the respective treatment solutions. The ionic strength of the sodium hydroxide and the sodium carbonate solution was equal, 0.07 molal, while the ionic strength of the sodium carbonate/EDTA solution was higher, 0.12 molal. Consequently, the ionic strength of the treatment solution could be involved in the swelling of the samples.

The shrinkage temperature of the "strong" threads from the sodium carbonate/EDTA process was significantly higher than the sample treated by the same solution for one hour ($P \leq 0.05$), but without the additional processing steps. It is difficult to ascribe any chemical reaction, which could occur during the subsequent pair of processing steps that

would account for the differences in shrinkage temperature. The total process extracted more non-collagenous protein than the one hour treatment, and this could explain some of the observed difference in the shrinkage temperature as it was shown earlier that shrinkage temperature and collagen content were moderately correlated (r = 0.529). Shrinkage temperature can be considered to be a measure of the (hydrogen) bonds' strength, which maintains the collagen structure. It is possible that the last stage (a soak in Teric solution overnight) might have caused some changes in the fibre cross-links and thus given rise to the higher shrinkage temperature. A more detailed study of collagen or intestine tissue chemical structure after being treated with Teric is required to confirm this possibility.

3.2.2 Extracted protein

The filtrates from the sodium carbonate/EDTA process were analysed for total protein by the Kjeldahl method. The filtrates included the soaking and rinse solutions. The experiment compared the first "strong" batch and the first "weak" batch.

Table 3.10 Amount of extracted proteins from EDTA process filtrates

Filtrate	1st strong batch (% total protein on dry starting material)	1st weak batch (% total protein on dry starting material)	N
Stage 1 Na_2CO_3/EDTA	15.8 ± 0.0[a]	8.6 ± 0.7	2
Stage 2 rinse water	10.7 ± 0.2	5.8 ± 0.1	2
Stage 3 Na_2CO_3/EDTA/H_2O_2	7.2 ± 0.2	1.0 ± 0.1	2
Stage 4 rinse water	4.4 ± 0.1	2.8 ± 0.2	2
Stage 5 Teric	6.9 ± 0.1	6.1 ± 0.1	2
Stage 6 rinse water	2.1 ± 1.1	2.6 ± 0.2	2
Accumulative proteins	47.0 ± 1.2	26.9 ± 0.8	

[a] Mean value ± standard deviation, N = number of determinations

The results in Table 3.10 show the amount of extracted proteins from the "strong" and "weak" samples after being run through the sodium carbonate/EDTA process. The total amount of protein extracted from the "strong" threads was consistently greater than that extracted from the "weak" threads for all stages of the process. Figure 3.11 illustrates the results.

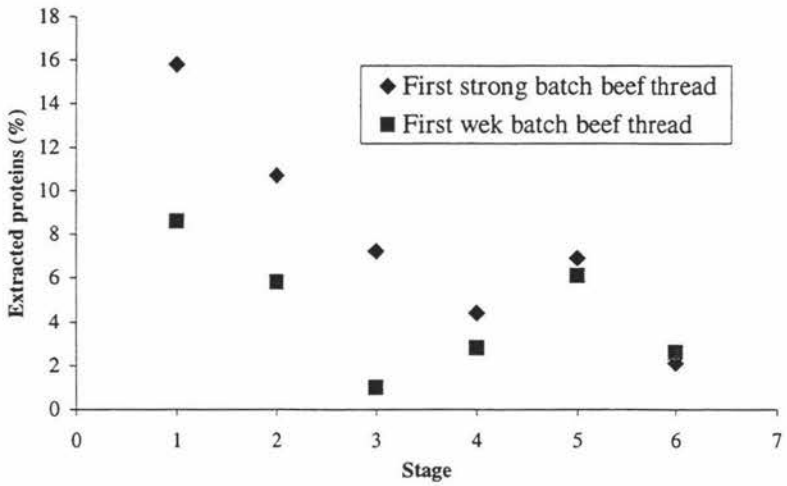


Figure 3.11 Comparison amount of extracted proteins from the strong and weak batch beef thread in the sodium carbonate/EDTA process

As can be seen from Table 3.10 the amount of extracted proteins was highest in the first stage. Around one third of the total protein was extracted in this stage. In the fifth stage, Teric soaking, the amounts of extracted protein from the both "strong" and "weak" beef threads gave similar amounts, unlike the results in the previous stages. It can be assumed that the two soakings in the sodium carbonate/EDTA solution removed almost all the non-collagenous proteins from both "strong" and "weak" beef threads.

Assuming that the rinse solutions contained protein from the previous sodium carbonate/EDTA stages, then the first sodium carbonate/EDTA stage actually removed 26.5% (15.8% + 10.7%) of the total protein, i.e. (26.5/47.0) × 100 = 56.4% of the protein actually extracted in the case of the "strong" threads. Similarly for the "weak" batch, 14.4% (8.6% + 5.8%), i.e. (14.4/26.9) × 100 = 53.5% of the total non-collagenous protein was removed. The first sodium carbonate/EDTA stage of the process extracted very similar proportions of soluble proteins from both the "weak" and "strong" threads.

In the second and the third stage treatments, the proportion of the extracted protein from the "strong" and the "weak" beef threads gave a different pattern from the first stage. The proportion of the protein removed from the "strong" and the "weak" beef threads were 24.7% and 14.1% of the total extracted protein respectively. In contrast, the proportion of extracted protein from the final stage of the "strong" and the "weak" beef thread were 19.1% and 46.1% of the total soluble protein respectively.

The pattern of non-collagenous protein extraction throughout the process was clearly different and depended on the nominal tensile strength of the raw, unprocessed threads, with the exception of the first sodium carbonate/EDTA stage where similar amounts of non-collagenous protein were extracted from the "weak" and "strong" threads. Clearly, more protein was extracted from the "strong" threads compared with the "weak" threads, and this difference occurred at the second sodium carbonate/EDTA soak and in the final Teric soak. A mass balance on the amount of collagen and extracted protein for each type of beef thread was determined. The sum of the collagen content and the extracted protein of the "strong" and the "weak" batches were 105.6% (58.6 + 47.0) and 96.7% (69.8 + 26.9) respectively.

Note that the factor used to convert hydroxyproline to collagen was 7.63, which is based on only bovine collagen Type I. As a result, the obtained collagen values were higher than 100%. In native tissues, Type III collagen is normally physically associated with Type I collagen and is prominent in tissues, which require a degree of flexibility (Bailey and Light, 1989). Thus, collagen Type III could be involved in the beef thread samples, but the proportion was unknown and it has not been studied in this project. If the factor is corrected for collagen Type III, it will give more accurate results (the factor will be lower than 7.63).

The filtrates from each stage were also analysed for hydroxyproline, which is an index of the collagen content in the filtrates. In all cases, the hydroxyproline content was below the detection limit (5 μg/ml) of the method, less than 0.04% on a dry starting material basis, or less than 0.1% on total collagen basis for both the "weak" and "strong" threads. This suggests that there was no or very little collagen in the extracted protein. Consequently, it can be concluded that the wet process causes little or no degradation of the collagen. Moreover, it can be assumed that the first batch of "weak"

beef threads contained less non-collagenous protein than the first batch of "strong" beef threads.

It would appear therefore that measurements of the physical properties and the chemical components of raw beef threads cannot be used to predict the physical and playing characteristics of tennis strings, because of the inherent biological variability of the raw materials.

3.2.3 Collagen and elastin distribution

The aim of this investigation was to establish whether there was a correlation between collagen content and the strength/elasticity of beef threads. The following classification method was used.

A batch identified as weak or strong by the company was divided into three subgroups on the basis of ductility. The subgroups of threads with the highest and lowest ductility were subjected to analysis for collagen and elastin content. The collagen and elastin content of the different subgroups are shown in Table 3.11.

Table 3.11 Collagen and elastin distribution

Batch	Group	Ductility (mm)	Collagen (% dry basis)	Elastin (% dry basis)
1st Strong	I	20-25	50.7 ± 8.4[a]	1.6 ± 0.5
	II	30-35	55.0 ± 2.4	1.4 ± 0.4
2nd Strong	III	20-25	58.2 ± 0.5	1.8 ± 0.5
	IV	40-45	51.1 ± 5.1	1.2 ± 0.8
1st Weak	V	25-30	64.9*	2.1 ± 1.3
	VI	40-45	67.2 ± 10.3	2.5 ± 0.9
2nd Weak	VII	20-25	51.9 ± 10.3	2.3 ± 0.6
	VIII	30-35	44.7 ± 0.9	1.8 ± 0.7
N		10	4	2

[a] Mean value ± standard deviation, N = number of determinations, * only one determination

There was only a significant difference in collagen content between two ductility subgroups in the second "strong" batch of beef threads ($P \leq 0.05$). For the other batches,

there were no significant differences in collagen between the ductility subgroups within the same batch. In addition, the average collagen content between the four batches did not significantly differ due to the large standard deviations of each batch. As a result, the collagen content of either within or between batches cannot be used to distinguish the characteristic of the strong and weak beef threads. It would appear from this experiment that the amount of collagen in beef threads ranges from 48 to 66%.

It is interesting to note that the trend of the collagen content of the first "strong" and the first "weak" batch significantly reflect the values for the undivided batches shown in Table 3.3 i.e. the first "weak" batch does indeed have a larger collagen content than the first "strong" batch. Moreover, the amount of collagen is reversed from the first batch to the second batch compared between the strong and the weak groups. There is higher collagen content in the second strong batch of beef thread but lower in the second weak batch of beef thread.

The results show a similar trend in the elastin content. There were no significant differences either within or between batches ($P > 0.05$). The elastin content ranged from 1.5 to 2.3%.

At this stage, it may be concluded that the amount of collagen and elastin varied widely from batch to batch and did not correlate with ductility.

3.2.4 Trypsin treatment

The second batch of "strong" beef threads was used in this section. In this study, the beef threads were digested with 2% trypsin (wet thread weight-basis) at 37°C overnight, then the filtrate and the treated beef threads were analysed for collagen. The results are shown in Table 3.12

3.2.4.1 Chemical properties

From the results below, trypsin can remove 22.4% of non-collagenous protein. No significant attack by the enzyme on collagen was observed in the extracted filtrate. The collagen content of the treated beef thread was found to be 86.7% collagen, which means there was still 13.3% (on dry treated beef thread basis) of non-collagenous proteins in the treated beef thread which was not removed by trypsin. Since the sodium

carbonate/EDTA process can remove about 47% protein (Table 3.10), a combined sodium carbonate/EDTA/trypsin procedure may be capable of extracting more non-collagenous proteins than the sodium carbonate/EDTA process and thus produce beef threads that were "richer" in collagen. The effect of removing more matrix protein on the beef threads' strength properties remains to be determined.

Table 3.12 Effect of trypsin on protein distribution of the second strong batch beef thread

Tested portion	Lyophilised residues (% dry starting material basis)	Protein content (% dry starting material basis)	Collagen content (% dry-basis)
Filtrate	23.6*	22.4 ± 0.4*	0.6 ± 0.1[a]
Treated beef thread	72.3	n/d	86.7 ± 6.1[b]
N	1	2	3

* Corrected for added alkali and trypsin, [a] Mean value ± standard deviation based on dry starting material, [b] Mean ± standard deviation based on dry treated beef thread basis, n/d = not determined, N = number of determinations

3.2.4.2 Physical properties

The treated beef thread from the trypsin treatment (3.2.4) was investigated for its physical properties, shrinkage temperature and tensile strength. The results are shown in Table 3.13.

Table 3.13 Effect of trypsin on the physical properties of the second strong batch beef thread

Parameter	Control	Treated sample	N
Shrinkage temperature (°C)	71.4 ± 0.5[a]	70.1 ± 0.4	6
Ductility (mm)	25.2 ± 5.1	23.4 ± 2.0	10
Ultimate tensile strength (N)	10.5 ± 3.6	13.6 ± 4.5	10
Young's modulus (N/mm)	0.8 ± 0.3	0.9 ± 0.4	10

[a] Mean value ± standard deviation, N = number of determinations

After trypsin treatment, only the shrinkage temperature significantly decreased ($P \leq 0.05$). No significant differences between treatments where observed for the other parameters.

In the sodium carbonate/EDTA treated beef threads, the shrinkage temperature was not significantly decreased but the ductility significantly decreased ($P \leq 0.05$). However, a more systematic study using the enzyme/sodium carbonate/EDTA process was subsequently carried out to determine whether enzymes could be used to improve the beef thread properties.

Proteolytic enzymes such as pronase, trypsin, chymotrypsin and pepsin in general do not disrupt the native collagen structure (Ramachandran, 1967). However, these enzymes are able to hydrolyse globular segments in the telopeptide region. The peptides released accounted for about two per cent of the amino acids of the intact collagen.

In general, the differing extent of digestibility of proteins by trypsin is a function of their molecular structure and organisation. Native collagen of mammals is not attacked by trypsin. On the other hand, collagen, which has been swelled by pre-treatment in a solution of acids, alkalis and lyotropic agents, or by thermal denaturation, is easily digested by trypsin (Gustavson, 1956). However, a pH of 8.5, the pH of the sodium carbonate/EDTA soak solution, should not significantly swell the collagen. (Refer to the collagen swelling curve in Figure 3.4.)

Trypsin is the most specific of the enzymes mentioned above. It catalyses the hydrolysis of peptide linkages which involve the carboxyl functions of lysine or arginine. The specificity of the enzyme is further increased if the amino group of lysine is blocked with a benzyloxycarbonyl or other amino protecting group, under these conditions only arginyl bonds are attacked. Cysteinyl peptide bonds can be rendered susceptible to trypsin degradation by reaction with ethylenamine, to give an S-β-aminoethyl derivative (Jakubke and Jeschkit, 1977) but these modification do not pertain to the experiment under discussion.

From the literature above, it is clear that the trypsin treatment probably led to the extraction of proteins from the non-helical ends of tropocollagen, but mainly from the glycoprotein in the ground substance of intestinal tissues.

3.2.5 Sequential trypsin treatments

The aim of this experiment was to assess whether intensive exposure to trypsin could digest more non-collagenous protein from samples of beef thread.

In this experiment the threads were given an initial trypsin treatment which was followed by a further two successive fresh trypsin solutions for 16 hours at 37°C. The filtrates and treated beef threads from each step were analysed as shown in Table 3.14.

Table 3.14 Effect of sequential trypsin treatments on the second strong batch beef thread

Parameter	Control	1st Trypsin treatment	2nd Trypsin treatment	3rd Trypsin treatment	N
Filtrate residues (% dry starting material)*	n/d	22.1	18.4	6.9	1
Total protein in filtrate (% dry starting material)	n/d	21.2 ± 0.3[a]	15.6 ± 0.1	4.5 ± 0.2	2
Dry treated beef thread (% dry starting material)	n/d	74.6	66.1	53.4	1
Shrinkage Temperature (°C)	71.4 ± 0.5	70.8 ± 0.4	67.8 ± 0.4[c]	65.3 ± 0.6[c]	6
Ductility (mm)	25.2 ± 5.1	22.7 ± 1.5	20.4 ± 1.7	20.7 ± 1.7	10
Ultimate tensile Strength (N)	10.5 ± 3.6	14.3 ± 5.0	10.0 ± 2.2	12.6 ± 3.0	10
Young's modulus (N/mm)	0.8 ± 0.3	0.9 ± 0.3	0.8 ± 0.2	0.8 ± 0.2	10
Collagen in filtrate (% dry starting material)	n/d	0.64 ± 0.04	0.74 ± 0.03	0.79 ± 0.04	3
Collagen in treated beef thread (% dry treated beef thread)	55.1 ± 0.8[b]	73.1 ± 0.6	104.2 ± 0.8	103.6 ± 1.4	3
Treated beef thread diameter (mm)	2.3 ± 0.5	2.1 ± 0.2	1.4 ± 0.3	1.2 ± 0.3	6

[a] Mean value ± standard deviation, [b] Mean value ± standard deviation based on dry treated beef thread, N = number of determinations, n/d = not determined, * Corrected for added alkali and trypsin, [c] Stretched temperature

The cumulative extracted protein from the three treatments was 41.3% (based on a dry starting material). This was close to the amount extracted by the sodium carbonate/EDTA process (47.0%). However, the diameter of the beef threads after the three trypsin treatments was apparently smaller than the threads from the sodium carbonate/EDTA process. As the trypsin treatment was carried out at pH 8.5 there was no marked pH-related swelling.

Since 0.6% to 0.8% of collagen (dry starting material basis) was degraded/extracted by each stage of the trypsin treatments, the shrinkage temperature of the threads decreased after the first treatment compared to the controls and the threads actually stretched on heating after the second and third trypsin treatments. It should be noted that the amount of collagen removed by each stage of the trypsin treatment was significantly higher compared with the sodium carbonate/EDTA process which can extract less than 0.04% on a dry starting material basis from each stage of treatment. This indicates major structural changes to the collagen fibrils, which cannot yet be explained. The observation that collagen fibrils actually stretch on heating has not been previously reported to the best of the author's knowledge. It suggests that a different process of denaturation was occurring in the threads given successive trypsin treatments, compared to the expected thermal shrinkage normally associated with collagen.

The ductility and the diameter of the beef threads after being treated with a second trypsin treatment were also significantly lower than the controls. There were no significant differences in most physical properties between beef threads after the second and the third trypsin treatments.

The fact that similar decreases in thread ductility were observed after treatment in sodium carbonate (see Table 3.9) and trypsin suggests that the non-collagenous proteins provide a matrix (for the collagen fibres) and that this matrix plays an important role in the ductile properties of the beef threads. The fact that the pH of the respective solutions were dissimilar, i.e. pH 8.5 (enzyme) and pH 10.5 (sodium carbonate), suggests that the decrease in observed ductility cannot be ascribed to a pH effect on the collagen fibres.

As can be seen, the collagen content of the beef threads after the second and third trypsin treatments were very similar and exceeded 100% collagen. Thus it may be assumed that all the non-collagenous proteins had been removed by the second

treatment. Collagen contents in excess of 100% can be ascribed to the fact that the conversion factor to convert hydroxyproline to collagen was based on the premise that the predominant collagen type in beef threads was bovine Type I collagen.

There are some similarities between the chemical reactions occurring in liming and bating of the pre-tanning stage of the leather making process and a combined enzyme/sodium carbonate/EDTA process, which was proposed for investigation. The main purpose of liming skins is to bring about alkali swelling and removal of non-collagenous proteins. The major chemical modification of collagen during liming is the hydrolysis of some of the amide groups attached to aspartic and glutamic acid residues:

$$-CONH_2 + HOH \rightarrow -COOH + NH_3 \cdot$$

As the carboxyl groups can ionise:

$$-COOH \leftrightharpoons COO^- + H^+$$

a greater potential for increasing the number of negatively charged centres exists, so that the isoelectric pH of collagen is invariably reduced by liming. A very small proportion of the arginine residues are also converted to ornithine and urea:

$$-CH_2CH_2CH_2NH-\underset{\displaystyle NH_2}{\overset{\displaystyle |}{C}}=NH \rightarrow -CH_2CH_2CH_2NH_2 + CO(NH_2)_2 \cdot$$

Apart from these reactions, lime liquors may lead to some modification of the covalent cross-links, especially of the ester-type, which are thought to join the chains together in the triple-chain unit, but the changes involved are not known with certainty. The total effect is that liming produces a pelt, which swells more at all pH values than native, unlimed skin. The forces of swelling lead to a general loosening of the fibre-network layer and to the splitting of the larger collagen fibres. In normal tannery liming, apart from the breakage of a few polypeptide main chains by hydrolysis (leading to the solubilisation of a small number of collagen fibrils), the fibrils themselves merely swell and do not show any marked changes in their general appearance. They are, however, much cleaner, as the interfibrillary material (mostly globular proteins and mucopolysaccharides) is largely removed by the lime liquor. Thus liming helps to prepare a clean system of fibrils ready for further operations leading to tannage. Removal of additional protein material, loosened by liming, is achieved by using enzymic digestion by proteases - the operation of bating (Reed, 1966).

Bating is a further step in the purification of the hide prior to tanning. The unwanted components consist of some of the protein degradation products, epidermis, hair, and the "scud" (surface dirt) on the surface of the skin and in the hair follicle and pores. Some of the chemically resistant, fibrous protein must also be removed in order to obtain proper grain (upper region of dermis) texture and leather softness. Bating refers to the action of enzymes on these components (Thorstensen, 1969).

The bating process is quite effective for the removal of fat from the skins through the adjustment of pH, temperatures and through a detergent action of the natured soaps. An additional effect claimed by some manufacturers is the action of the bating enzymes in the breakdown of the fat cells. By the time the bating has proceeded to the point desired, the differential swelling effects have been overcome and the skin can withstand mechanical action. It is desirable to wash out the chemicals and the degradation products after the bating application and to lower the temperature to stop further action by the enzymes (Thorstensen, 1969). The use of trypsin in the current experiment may cleave some of the ester interaction crosslinks mentioned previously. The prolonged contact time with trypsin, which has well documented esterase activity as well as protease activity, may cause more extensive ester hydrolysis than can be obtained under liming conditions. A "de-crosslinked" triple chain may well stretch rather than shrink upon thermal denaturation.

A decision was made to integrate trypsin digestion with the sodium carbonate/EDTA process as this potential process has strong resemblances to the conventional tanning process where liming is followed by bating. It was thought that such a process could result in increased protein extraction compared to the current company process where sodium carbonate/EDTA are used to extract the non-collagenous material from the threads. It was hoped that a combined enzyme/alkali process might overcome the anomalous stretching phenomenon observed when threads were treated with two or more trypsin treatments.

3.2.6 Combined sodium carbonate/EDTA and enzyme treatment

The trypsin treated beef threads from the previous experiment were given a subjected to a full EDTA process. The results of the measurements conducted on the threads given the trypsin treatment alone and the trypsin plus EDTA process are shown in Table 3.15 and Table 3.16.

Table 3.15 Effect of combined treatment on protein distribution of the second strong batch beef thread

Treatment	Extracted residues (% dry starting material)*	Dry treated beef thread (% dry starting material)	Collagen in treated beef thread (% dry treated beef thread)
Control	n/d	n/d	55.1 ± 0.8[b]
1st trypsin treatment	22.1	74.6	73.1 ± 0.6[a]
2nd trypsin treatment	40.5[c]	66.1	104 ± 1
3rd trypsin treatment	47.4[c]	53.4	103 ± 1
EDTA treatment	31.9	47.0	100 ± 2
1st trypsin + EDTA treatment	44.3[c]	59.6	101 ± 1
2nd trypsin + EDTA treatment	56.4[c]	47.6	111 ± 1
3rd trypsin + EDTA treatment	56.5[c]	33.2	108 ± 2
N	1	1	3

[a] Mean value ± standard deviation, [b] Dry starting material basis, [c] Accumulative extracted residues, n/d = not determined, N = number of determinations, * Corrected for added alkali and trypsin

As can be seen, the total amount of extracted residues, from the successive trypsin treatments was higher than the sodium carbonate/EDTA process. A 22% improvement in non-collagenous protein extraction was observed when the trypsin treated beef threads were given an additional sodium carbonate/EDTA treatment when compared

with just trypsin alone. There appeared to be little point in giving beef threads more than two trypsin treatments followed by a sodium carbonate/EDTA treatment as little extra protein was extracted. Due to the large number of stages in the integrated sodium carbonate/EDTA process, cumulative errors in the dry matter results, have led to results in excess of 100%. In the two cases where the total came to less than 90%, transfer losses would be a significant factor.

Table 3.16 Effect of combined treatment on the physical properties of the second strong batch beef thread

Treatment	Shrinkage temperature (°C)	Ductility (mm)	Ultimate tensile strength (N)	Young's modulus (N/mm)	Diameter (mm)
Control	71.4 ± 0.5[a]	25.2 ± 5.1	10.5 ± 3.6	0.8 ± 0.3	2.3 ± 0.5
1st trypsin treatment	70.8 ± 0.4	22.7 ± 1.5	14.3 ± 5.0	0.9 ± 0.3	2.1 ± 0.2
2nd trypsin treatment	67.8 ± 0.4[b]	20.4 ± 1.7	10.0 ± 2.2	0.8 ± 0.2	1.4 ± 0.3
3rd trypsin treatment	65.3 ± 0.6[b]	20.7 ±1.7	12.6 ± 3.0	0.8 ± 0.2	1.2 ± 0.3
EDTA treatment	70.1 ± 0.4	19.9 ± 1.4	13.5 ± 3.7	1.0 ± 0.2	2.6 ± 0.7
1st trypsin + EDTA treatment	68.6 ± 0.5	21.4 ± 2.5	13.5 ± 3.1	0.9 ± 0.2	1.4 ± 0.4
2nd trypsin + EDTA treatment	68.1 ± 0.7	20.2 ± 1.6	14.6 ± 3.6	1.0 ± 0.2	1.3 ± 0.4
3rd trypsin + EDTA treatment	68.1 ± 0.5	20.4 ± 1.8	13.6 ± 5.6	0.9 ± 0.3	1.0 ± 0.2
N	6	10	10	10	6

[a] Mean value ± standard deviation, N = number of determinations, [b] "Stretched" temperature

The shrinkage temperatures of trypsin treated beef threads after being put through the sodium carbonate/EDTA process were improved (see Table 3.16) when compared with the threads after just a trypsin treatment. It will be recalled that the threads actually stretched on heating rather than shrinking after two and three trypsin treatments (see section 3.2.5). Restoration of the shrinkage phenomenon upon exposure to sodium

carbonate/EDTA and a surfactant is also novel and shows that the stretch phenomenon is reversible. The reason for the reappearance of "normal" shrinkage after thermal denaturation is unknown and needs further study. However, the beef threads treated with the sodium carbonate/EDTA process alone gave higher shrinkage temperatures compared with the combined process.

If the threads were given just a trypsin treatment it was observed that a statistically significant ($P \leq 0.05$) drop in ductility was observed between the first trypsin treatment and subsequent treatments. The same trend was evident in the trypsin/EDTA treated threads, but in this case the differences were not statistically significant. No differences in ductility were observed between the second and third trypsin treatments for either thread treated by trypsin alone or by threads treated by the trypsin/EDTA process.

If an examination is made of the ultimate tensile strength results for threads treated with just trypsin alone it can be seen that threads from the first trypsin treatment were significantly tougher than threads given additional trypsin treatments. The threads given two trypsin treatments were the weakest threads, with the threads given three trypsin treatments having an intermediate value. There were no significant differences in the ultimate tensile strength when trypsin treated beef threads from three stages were integrated into the sodium carbonate/EDTA process.

There were no significant changes in the Young's modulus after being treated with either trypsin or combined process.

The main effects of adding an EDTA stage after the trypsin digestion stages appeared to be the prevention of the thermal elongation of the threads after more than one trypsin treatment and a decrease in thread diameter following each trypsin treatment. No significant differences in either tensile strength or ductility could be found between threads given just a trypsin treatment and threads given an additional EDTA stage after treatment with trypsin. The decrease in thread diameter after the additional EDTA stage can be attributed to the increased removal of non-collagenous protein. This property might translate into smaller diameter tennis strings, which in turn might lead to improved "playability" without any loss in tensile strength when compared with strings produced by the existing company process. High tensile small diameter strings should

mean that players can put more "work" on the tennis ball and thus enable them to gain a competitive advantage.

Young's modulus is normally determined by measuring the slope of a stress versus strain graph where strain is the force/unit area. For tennis strings one normally uses the cross-sectional area. However, in this study it was observed that when the beef threads were clamped in the jaws of the Instron and upon subsequent pulling the cross-sectional area of the threads was grossly distorted. Therefore since the diameter could not be used in the calculation, a constant cross section area was assumed.

$$E = \frac{F / A_0}{\Delta L / L_0}$$

Where E is Young's modulus, F is tensile force, A_0 is initial cross-section area, ΔL is change of gage length and L_0 is initial gage length.

3.2.7 Enzyme activity

The enzyme activities of three protease enzymes, trypsin, pyrase and tanzyme, were determined using soluble casein as substrate (Bergmeyer, 1974) at pH 7.5. The results were shown in Table 3.17.

Table 3.17 Enzyme activity

Enzyme	Activity (Units/ml)[a]	Total protein[b] (% w/v)	Specific activity (U/g protein)
Trypsin	151.5*	21.8**	695
Pyrase	274.3	3.7	7414
Tanzyme	49.4	3.5	1414

[a] 1 unit = 1 μmole tyrosine equivalent /min, [b] Determined by Kjeldahl method, * Units/g, ** % w/w

As can be seen, pyrase had the highest specific activity, 7414 U/ml protein, tanzyme and trypsin had less respectively.

Subsequent estimates of the protein content of the trypsin, as determined by the Kjeldahl method, gave an overestimate of the supplier's value of 15% since the product

contained 85% lactose. A recommended conversion factor of 6.25 for meat products was used to convert total nitrogen to protein. This may not be an appropriate factor for enzyme samples. Moreover, possible non-protein content of the enzymes was not considered when estimating the protein content of the enzymes. According to Dintzis *et al.* (1988) and Lebet *et al.* (1994), in both of these studies the authors recommended a careful choice of the conversion factor used without neglecting the non-protein nitrogen fraction. However, because of time considerations, all the protein calculations in this study were based on 6.25 as the conversion factor for the Kjeldahl nitrogen value.

3.2.8 Enzymes digestion

Although trypsin gave satisfactory results in removing non-collagenous proteins, two cheaper alternate commercial grade enzymes were investigated as substitutes in the integrated process. Pyrase and tanzyme have been used in leather manufacture in the pre-tanning part of the process. Pyrase is a bacterial enzyme(s) while tanzyme is a mixture of enzymes prepared from bovine pancreas. Trypsin was a powder consisting of a mixture of lactose with trypsin from beef pancreas.

Table 3.18 Effect of different enzyme on the second strong batch beef thread

Enzyme	Enzyme charge	Enzyme units (Units/ml)	Enzyme units/g wet beef thread[a]	Extracted residues (% dry starting material)
Control	0	0	0	2.2
Trypsin	2% w/w	151.5*	3.0	37.3
Pyrase	0.02% v/w	274.3	0.05	32.7
Tanzyme	0.2% v/w	49.4	0.10	21.8

[a] 100% float = volume of liquid equal to wet assay weight, * Units/g

Note that the digestion was carried out at pH 8.5 while the enzyme activity determinations were carried out at pH 7.5. To study the activity of pyrase and tanzyme, the experiment was carried out under the same condition as trypsin treatment.

As shown in Table 3.18 the amount of extracted residues by trypsin was highest, 37.3% due to its higher enzyme ratio. Moreover, if a comparison is made of the amount of non-collagenous protein that was extracted in this trial (37.3%) compared with the results of

the previous trypsin trials (22.1%), it can be seen that the amount of non-collagenous protein was 15% higher in this trial. This difference in protein extraction arose because a float of 100% was used in this trial, whilst a float of 200% was used in previous trials, i.e. the trypsin concentration was higher in this latter trial than previous trials.

The concentration of pyrase and tanzyme used in this experiment was twice the optimum concentration recommended for bating. Moreover, the beef threads treated overnight at 37°C in the tanzyme and pyrase spoiled as evidenced by the foul odours emanating from the respective flasks.

To avoid bacterial spoilage, an experiment at 4°C was undertaken, with the concentration of pyrase increased to 0.2% and tanzyme to 1%. The amount of 0.1 *M* sodium hydroxide consumed at 4°C was only 30% of that consumed when the reactions proceeded at 37°C. Whilst bacterial spoilage could be avoided by carrying out the digestion at 4°C, such a temperature was clearly impractical from a protein digestion perspective. However, because pyrase costs less than trypsin, future work to optimise reaction conditions could be warranted.

An additional experiment aimed at reducing the amount of trypsin used in the digestion stage was carried out. A trypsin concentration of 0.6% (wet wt.) and a 100% float, which is equivalent to 0.9 U/g wet beef thread, was used in the experiment.

After three successive trypsin treatments, the treated beef threads from each stage were subjected to the sodium carbonate/EDTA process. The results are shown in Table 3.19.

If an examination of the amount of protein extracted by the various treatments is made it can be seen that 46.8% of the protein was extracted after just the first trypsin/sodium carbonate/EDTA stage. More protein could be extracted by increasing the number of trypsin treatments followed by a sodium carbonate/EDTA treatment. However, little extra protein was extracted after the second trypsin stage (60.1%) compared with three trypsin treatments (63.1%).

The shrinkage temperature, unlike the previous experiment (3.2.6), showed no significant change from that of the control sample probably because of the lower trypsin concentration. Also all the integrated treatments gave similar results. The interesting treatment is the two trypsin treatments plus sodium carbonate/EDTA process, because it

gave the results closest to the control sample, even though it removed a large amount of non-collagenous proteins compared with the sodium carbonate/EDTA process (60.7% vs 31.9%).

Table 3.19 Effect of reduced trypsin charge in combined treatment on the second strong batch beef thread

Treatment	Extracted residues* (% dry starting material)	Shrinkage temperature (°C)	Ductility (mm)	Ultimate tensile strength (N)	Young's modulus (N/mm)	Diameter (mm)
Control	n/d	71.4 ± 0.5[a,b]	25.2 ± 5.1	10.5 ± 3.6	0.8 ± 0.3	2.3 ± 0.5
1st trypsin treatment	4.9	72.0 ± 0.0	19.3 ± 2.2	13.6 ± 2.6	1.0 ± 0.2	2.8 ± 0.5
2nd trypsin treatment	24.3[c]	71.5 ± 0.6	19.2 ± 1.6	14.1 ± 3.9	1.1 ± 0.3	2.2 ± 0.4
3rd trypsin treatment	36.2[c]	71.0 ± 0.0	19.2 ± 1.9	13.0 ± 3.2	1.0 ± 0.2	1.4 ± 0.2
EDTA treatment	31.9	70.1± 0.4	19.9 ± 1.4	13.5 ± 3.7	1.0 ± 0.2	2.6 ± 0.7
1st trypsin + EDTA treatment	46.7[c]	70.2 ± 0.3	21.1 ± 2.1	11.2 ± 2.0	0.8 ± 0.1	1.6 ± 0.2
2nd trypsin + EDTA treatment	60.7[c]	71.0 ± 0.0	19.8 ± 1.5	15.2 ± 4.9	1.1 ± 0.4	2.1 ± 0.4
3rd trypsin + EDTA treatment	63.1[c]	71.5 ± 0.0	19.9 ± 1.8	11.8 ± 1.9	0.9 ± 0.2	1.9 ± 0.2
N	1	3	10	10	10	6

[a] Mean value ± standard deviation, [b] Number of determination = 6, , [c] Accumulative extracted residues, n/d = not determined, N = number of determinations, * Corrected for added alkali and trypsin

A comparison of the results from Table 3.16 with those in Table 3.19 to determine the effect of altering the trypsin ratio showed that there were some significant differences. The shrinkage temperature of beef threads treated with trypsin plus the sodium carbonate /EDTA process in the former experiment was significantly lower in all stages ($P \leq 0.05$). But there were no significant differences in the tensile strength between both experiments. Due to the large amount of non-collagenous protein removed from the former experiment, the beef thread diameter of the combined treatments was significantly smaller than the latter experiment.

This suggests that an integrated enzyme plus sodium carbonate/EDTA process may be an improvement over the existing process used by the company since a smaller diameter wet product is obtained. Whether this smaller wet diameter translates into smaller dry diameter will be crucial to the adoption of the "new" integrated process that has been developed during the course of this experimental work.

Another process variation, which more closely resembles the established pre-tanning process, could be investigated. The alternative process would require two sodium carbonate/EDTA stages followed by an enzyme stage and then a final Teric stage. This suggested process may give rise to slightly different properties in the final threads when compared with threads from either the existing process or the three trypsin stages followed by the sodium carbonate/EDTA stage used in the latter part of this study.

CHAPTER 4

CONCLUSIONS

The beef thread samples provided by The Pacific Natural Gut Strings Company, have a wide variation in chemical and physical properties due to their sources. Therefore, to improve the quality of this product, the properties of raw materials and also the production process needs to be studied.

4.1 Beef Threads Properties

The main composition of the beef thread is protein - 98% on a dry weight basis. Roughly half of these proteins is collagen, which ranged from 47 to 70%. Elastin, which is a minor component, ranged between 1.2-2.5%.

An amino acid analysis, using calculation by differences, showed that the non-collagenous proteins of beef thread contain high proportions of the polar amino acids. From this and subsequent processing results it could be deduced that these non-collagenous proteins are predominantly globulins (soluble in salt solution) with a small amount of albumins (water soluble).

Significantly higher amounts of non-collagenous protein was extracted from the first "strong" batch of threads compared with the first "weak" batch after treatment with sodium carbonate/EDTA. This result related to the amount of collagen content of each batch.

The physical properties of starting samples varied widely from batch to batch and within a batch. The shrinkage temperatures of the samples were moderately correlated to the amount of collagen but cannot be used to distinguish groups of beef thread samples.

The results from the tensile strength measurements revealed that there was no significant difference between "strong" and "weak" groups. But there were tendencies toward higher ductility, ultimate tensile strength and Young's modulus in the "weak"

group compared with the "strong" group. However, these results cannot be used to predict whether all putative beef threads will give the same pattern as these tested batches.

4.2 Treatment Process

No differences in "strong" or "weak" beef thread physical properties, with the exception of tensile strength and thread diameter, were evident after treatment in sodium carbonate/EDTA, sodium carbonate or sodium hydroxide solutions.

Since the sodium hydroxide process gave the lower strength and more swelling of the treated threads compared with the sodium carbonate/EDTA process it was decided that the sodium carbonate/EDTA process would become the basis for further trials aimed at improving the wet strength of beef threads. Trypsin was selected in this study because it has been used in the bating process in leather manufacture, for the purpose of removing protein degradation products; epidermis, hair and dirt on a skin.

The sodium carbonate/EDTA process can remove 31.9% of non-collagenous proteins, half of this amount was removed in the first stage of this treatment. This process lowered the shrinkage temperature and the ductility of the thread sample. In contrast, the sodium carbonate/EDTA process increased the ultimate tensile strength, Young's modulus and also the diameter of the treated threads.

Three successive trypsin treatments removed more non-collagenous protein than the sodium carbonate/EDTA process. Some 47.7% non-collagenous protein was removed when 2% (w/w) of trypsin was used or 36.2% when 0.6% (w/w) of trypsin was used. The 2% (w/w) trypsin treatments produced threads that had lower shrinkage temperatures, and in the case of threads soaked in two or more trypsin solutions the threads actually stretched. The diameter of the trypsin treated samples was apparently decreased in the third trypsin treatment step. However, the trypsin treatments produced threads with similar tensile properties, lower ductility, higher tensile strengths and Young's modulus than threads treated in the sodium carbonate/EDTA process.

The study showed that threads produced in a process with at least two trypsin treatments followed by a sodium carbonate/EDTA process had improved shrinkage temperature, and similar ductility, ultimate tensile strength and Young's modulus when compared

with threads treated by trypsin alone. Furthermore, the combined enzyme/sodium carbonate/EDTA process removed more non-collagenous protein resulting in strings with a greater collagen content. Moreover, the diameter of these threads was significantly smaller. If this advantage could be carried through to the final tennis strings then the strings could well have improved "playability".

It is suggested that the company proceeds with experiments using two trypsin treatments plus a sodium carbonate/EDTA to see whether the advantages evident for wet strings will translate into improvements in the final tennis strings. The recommendation is made on the basis that it extracts more non-collagenous proteins, has less effect on the shrinkage temperature, improves the tensile strength and decreases the diameter of the treated beef threads when compared with the company's existing process.

4.3 Enzyme Activity

Alternative protease enzymes, pyrase and tanzyme, were studied to see whether they could be used to replace trypsin in the non-collagenous protein removal step.

Pyrase gave the highest enzyme activity, 7414 U/g protein; tanzyme and trypsin gave 1414 and 695 U/g protein respectively. Pyrase and tanzyme concentration in the experiment was twice the recommended optimum for use in tanning. Pyrase and tanzyme removed less protein from the beef threads than trypsin. In addition, when the reaction was carried out at 37°C, the reaction mixture with tanzyme and pyrase were bacterially spoiled. Consequently, these two enzymes are not recommended as replacements for trypsin.

In conclusion, it is suggested that an integrated trypsin plus sodium carbonate/EDTA process should be investigated as it may be an improvement over the existing company production process. The recommendation is based on the following advantages compared with strings produced by the current process: the diameter of the treated beef thread is smaller and more non-collagenous proteins are extracted while the other properties of the threads are similar to the product from the current company sodium carbonate/EDTA process. Whether this smaller wet diameter translates into dry diameter will be crucial. It needs more study in this integrated process to confirm the results. Moreover, the treated beef thread from the integrated process should be carried on to a finished product to study the dry product properties.

REFERENCES

1. Bailey, A. J. and Light, N. D. (1989). *Connective Tissue in Meat and Meat Products*. London; New York: Elsevier Applied Science.

2. Bergmeyer, H. U. (1974). *Methods of Enzymatic Analysis*. 3rd ed., New York: Verlag Chemie; Academic Press, 270-276.

3. Bowes, J. H., Elliott, R. G. and Moss, J. A. (1958). *Recent Advances in Gelatin and Glue Research*. London: Pergamon Press.

4. Church, F. C., Swaisgood, H. E. and Catignani, G. L. (1984). Compositional Analysis of Proteins Following Hydrolysis by Immobilized Proteases. *Journal of Applied Biochemistry* **6** (4): 205-11.

5. Csapo, J., Toth-Posfai, I. and Csapo-Kiss, Z. (1986). Optimization of Hydrolysis at Determination of Amino Acid Content in Food and Feed Products. *Acta Alimentaria* **15** (1): 3-22.

6. Cunningham, L. W. and Frederiksen, D. W. (1982). *Methods in Enzymology*. New York: Academic Press.

7. Dintzis, F. R., Cavins, J. F., Graf, E. and Stahly, T. (1988). Nitrogen-to-Protein Conversion Factors in Animal Feed and Fecal Samples. *Journal of Animal Science* **66** (1): 5-11.

8. Glick, D. (1977). *Methods of Biochemical Analysis*. New York: John Wiley & Sons.

9. Goll, D. E., Bray, R. W. and Hoekstra, W. G. (1963). Age-Associated Changes in Muscle Composition. The Isolation and Properties of a Collagenous Residue from Bovine Muscle. *Journal of Food Science* **28**: 503-9.

10. Gustavson, K. H. (1956). *The Chemistry and Reactivity of Collagen.* New York: Academic Press.

11. Hodge, A. J., Highberger, J. H., Deffuer, G. G. J. and Schmitt, F. O. (1960). *Proceedings of the National Academy of Sciences of the United States of America* **46**: 197.

12. http://www.sover.net/~schwacke/pacific1999.h. (1999). "Pacific the Power of Your Game" Web page.

13. Huszar, G., Maiocco, J. and Naftolin, F. (1980). Monitoring of Collagen and Collagen Fragments in Chromatography of Protein Mixtures. *Analytical Biochemistry* **105** (2): 424-29.

14. Industrial Research Limited, New Zealand. Collagen Estimation by Hydroxyproline Measurement (personal communication).

15. International Standard. (1994). Meat and Meat Products: Determination of Hydroxyproline. Switzerland: International Organization for Standardization.

16. Jakubke, H. D. and Jeschkeit, H. (1977). *Amino Acids, Peptides and Proteins: an Introduction.* London: Macmillan.

17. Kim, H R., Lee, K T. and Kataoka, K. (1990). Studies on the Preparation and Utilization of Hog Small Intestine Casing. Quality Characteristics as a Raw Material for Sausage Casing. *Korean Journal of Animal Sciences.* **32** (6): 334-38.

18. Kurisu, K., Ohsaki, Y., Nagata, K., Kukita, T., Yoshikawa, H. and Inai, T. (1987). Immunoelectron Microscopic Localization of Fibronectin in the Smooth Muscle Layer of Mouse Small Intestine. *Journal of Histochemistry & Cytochemistry.* **35** (4): 411-18.

19. Lebet, V., Schneider, H., Arrigoni, E. and Amado, R. (1994). A Critical Appreciation of the Protein Determination by Kjeldahl's Method Based on the Amino Acid Analysis. *Mitteilungen Aus Dem Gebiete Der Lebensmitteluntersuchung Und Hygiene* **85** (1): 46-58.

20. Lucas, B. and Sotelo, A. (1982). Amino Acid Determination in Pure Proteins, Foods and Feeds Using 2 Different Acid Hydrolysis Methods. *Analytical Biochemistry* **123** (2): 349-56.

21. Moran, L. A. and Rawn, J. D. (1994). *Biochemistry*. 2nd ed. Upper Saddle River, N.J.: Prentice-Hall.

22. Nakamura, F., Kitamura, K., Okamura, K. and Kondo, K. (1991). A Histological Study of the Collagen in Bovine By-Products Particularly in Digestive Organ. *Memoirs of the Faculty of Agriculture Hokkaido University* **17** (4): 451-64.

23. Neuman, R. E. and Logan, M. A. (1950 a). The Determination of Collagen and Elastin in Tissues. *Journal of Biological Chemistry* **186**: 549-56.

24. Neuman, R. E. and Logan, M. A. (1950 b). The Determination of Hydroxyproline. *Journal of Biological Chemistry* **184**: 299-306.

25. Nguyen, Q. and Zarkadas, C. G. (1989). Comparison of the Amino Acid Composition and Connective Tissue Protein Contents of Selected Bovine Skeletal Muscles. *Journal of Agricultural and Food Chemistry* **37** (5): 1279-86.

26. Ockerman, H. W. and Hansen, C. L. (1988). *Animal By-Product Processing*. Chichester (England): VCH.

27. Piez, K. A. and Reddi, A. H. (1984). *Extracellular Matrix Biochemistry*. New York: Elsevier.

28. Price, J. F. and Schweigert, B. S. (1971). *The Science of Meat and Meat Products.* 2nd ed. San Francisco: W. H. Freeman.

29. Prockop, D. J. and Udenfriend, S. (1960). A Specific Method for the Analysis of Hydroxyproline in Tissues and Urine. *Analytical Biochemistry* **1**: 228-39.

30. Radhakrishnan, K. T. and Ramamurthi, R. (1987). Studies on the Quality of Hog Casings Treated with Chemicals. *Cheiron* **16** (3): 123-24.

31. Ramachandran, G. N. (1967). *Treatise on Collagen.* London: Academic Press.

32. Reddy, G. K. and Enwemeka, C. S. (1996). A Simplified Method for the Analysis of Hydroxyproline in Biological Tissues. *Clinical Biochemistry* **29** (3): 225-29.

33. Reed, R. (1966). *Science for Students of Leather Technology.* Oxford: Pergamon Press.

34. Sakata, R., Segawa, S., Morita, H. and Nagata, Y. (1998). Tenderization of Hog Casings-Application of Organic Acids. *Fleischwirtschaft* **78** (6): 703-4.

35. Serafini-Cessi, F. and Cessi, C. (1964). An Improved Method for Determination of Hydroxyproline in Protein Hydrolyzates. *Analytical Biochemistry* **8**: 527-28.

36. Society of Leather Trades' Chemists. (1965). *Official Methods of Analysis.* 4th rev. ed. Redbourn, England: Society of Leather Trades' Chemists.

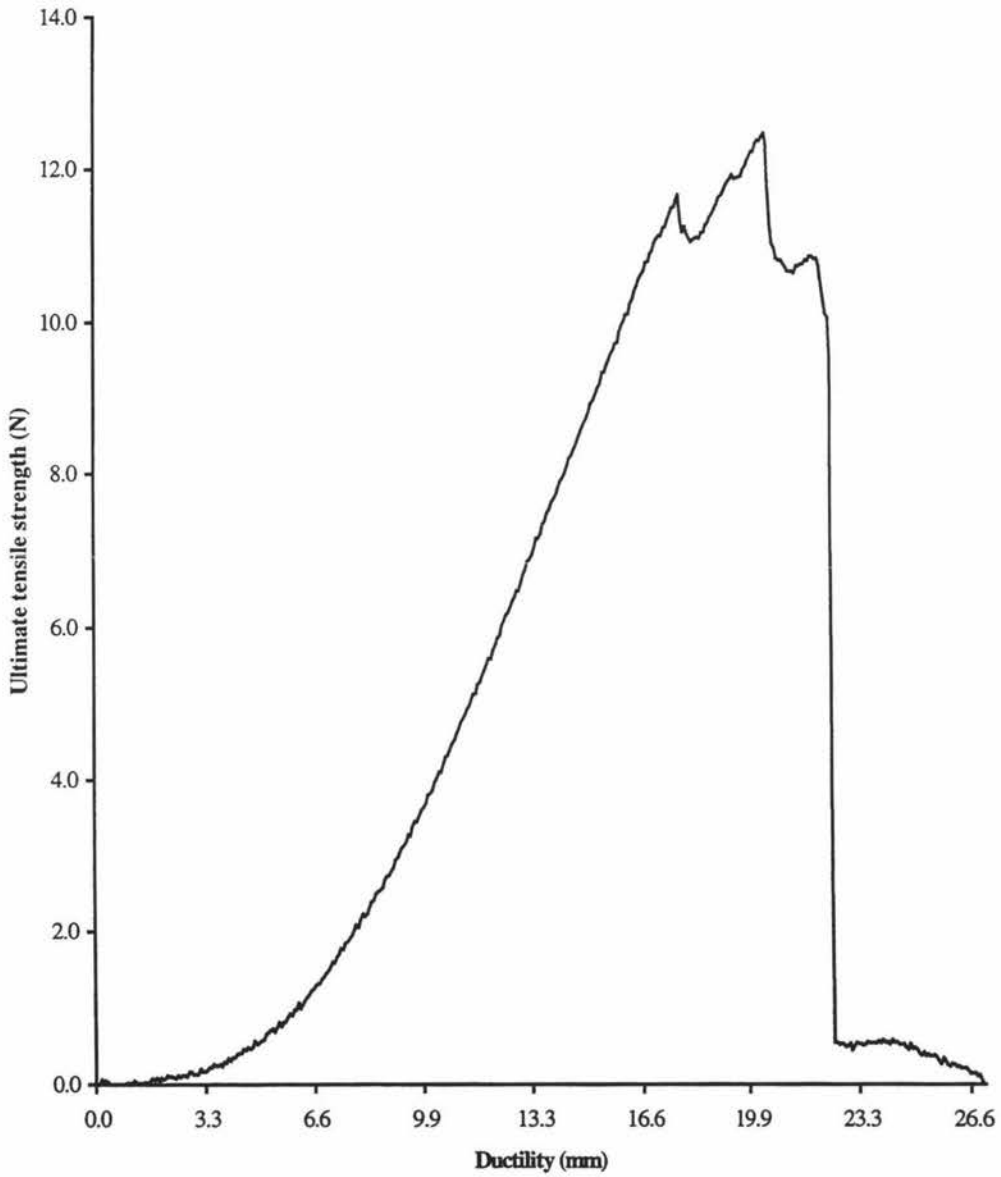
37. Thorstensen, T. C. (1969). *Practical Leather Technology.* New York: Van Nostrand Reinhold Co.

38. Vognarova, I., Dvorak, Z. and Bohm, R. (1968). Collagen and Elastin in Different Cuts of Veal and Beef. *Journal of Food Science* **33**: 339-43.

39. Williams, A. P. (1978). The Amino Acid, Collagen and Mineral Composition of Preruminant Calves. *Journal of Agricultural Science* **90**: 617-24.

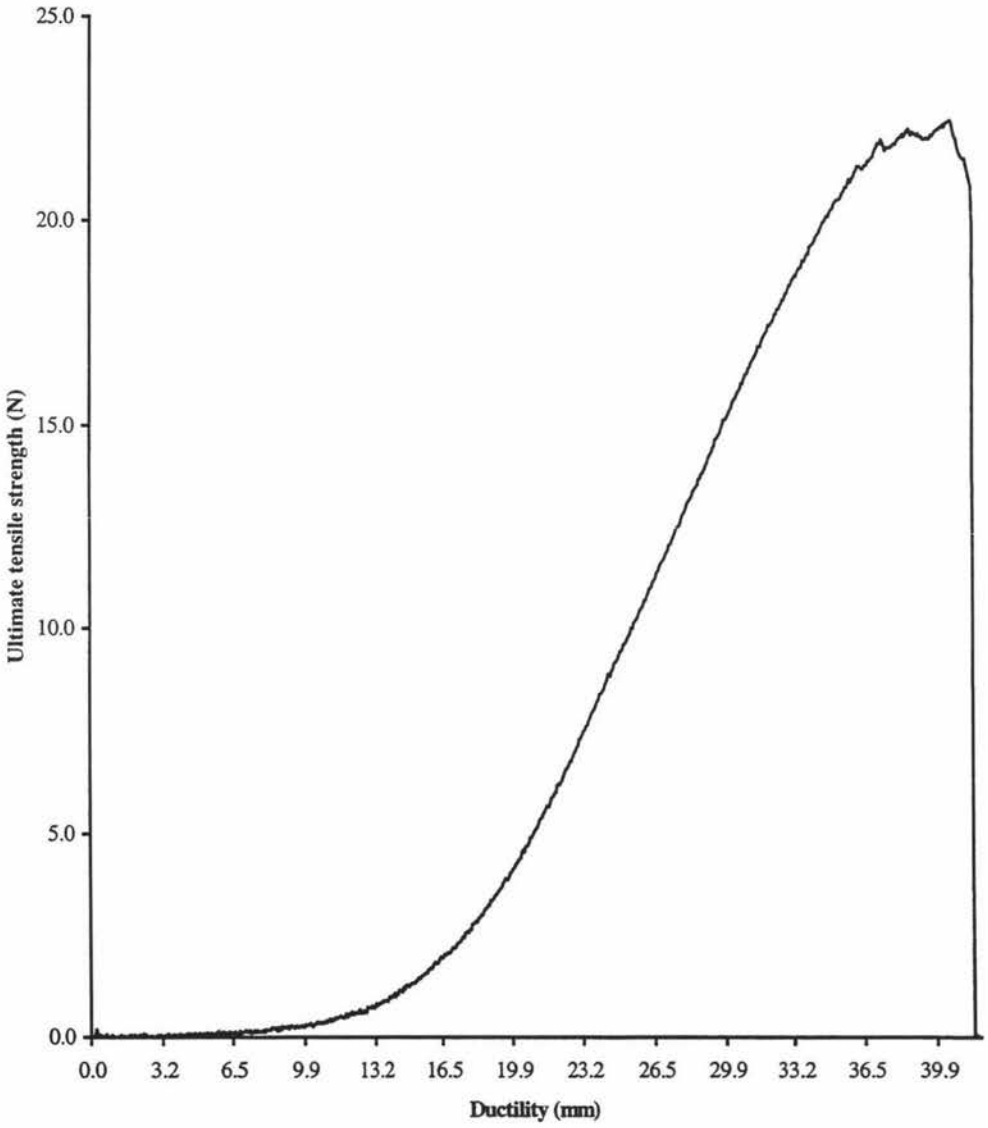
40. Zarcadas, C. G., Nguyen ,Q. and Khalili, A. D. (1996). Amino Acid Composition and N-Methylhistidine Contents of Bovine and Porcine Cardiac Muscle Tissues. *Journal of Agricultural and Food Chemistry* **44** (3): 661-71.

APPENDIX 1



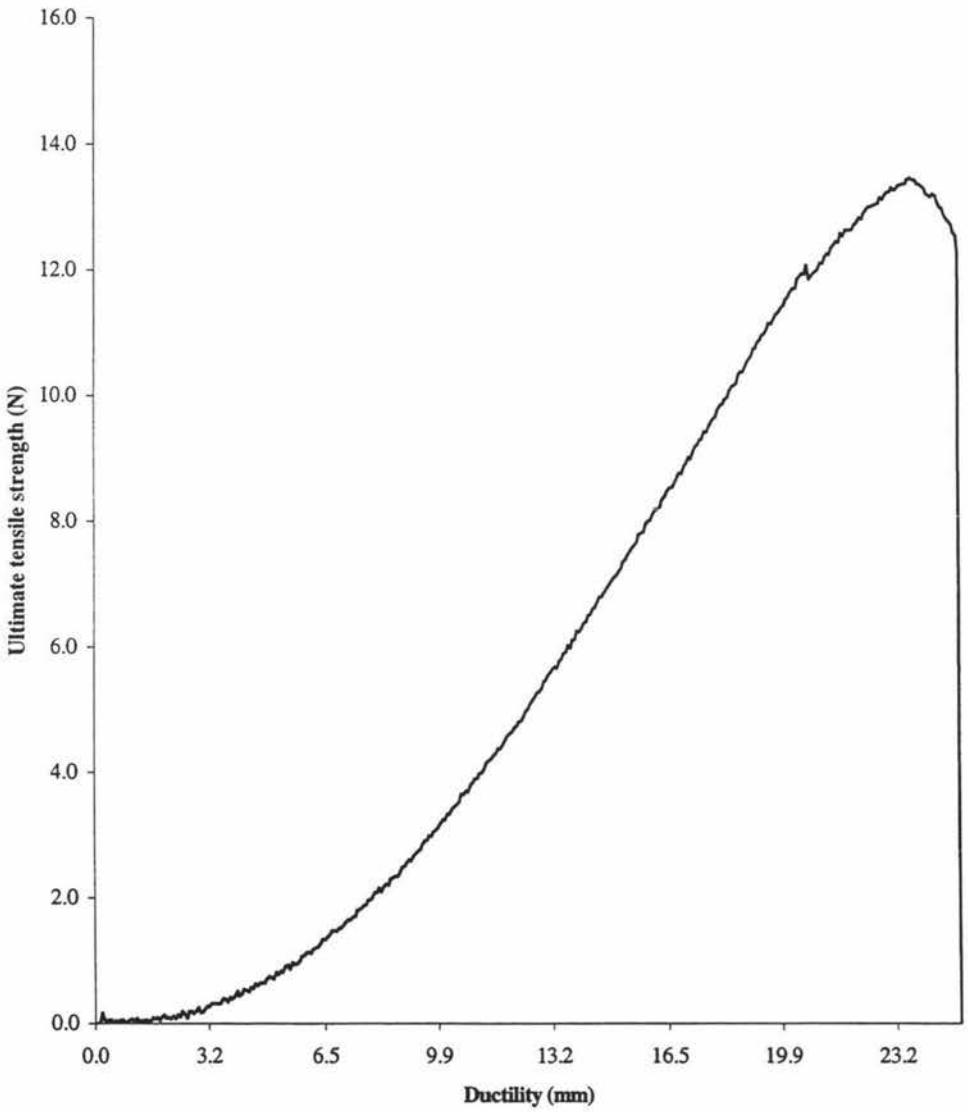
Ultimate tensile strength-ductility curve of the first strong batch beef thread

APPENDIX 2



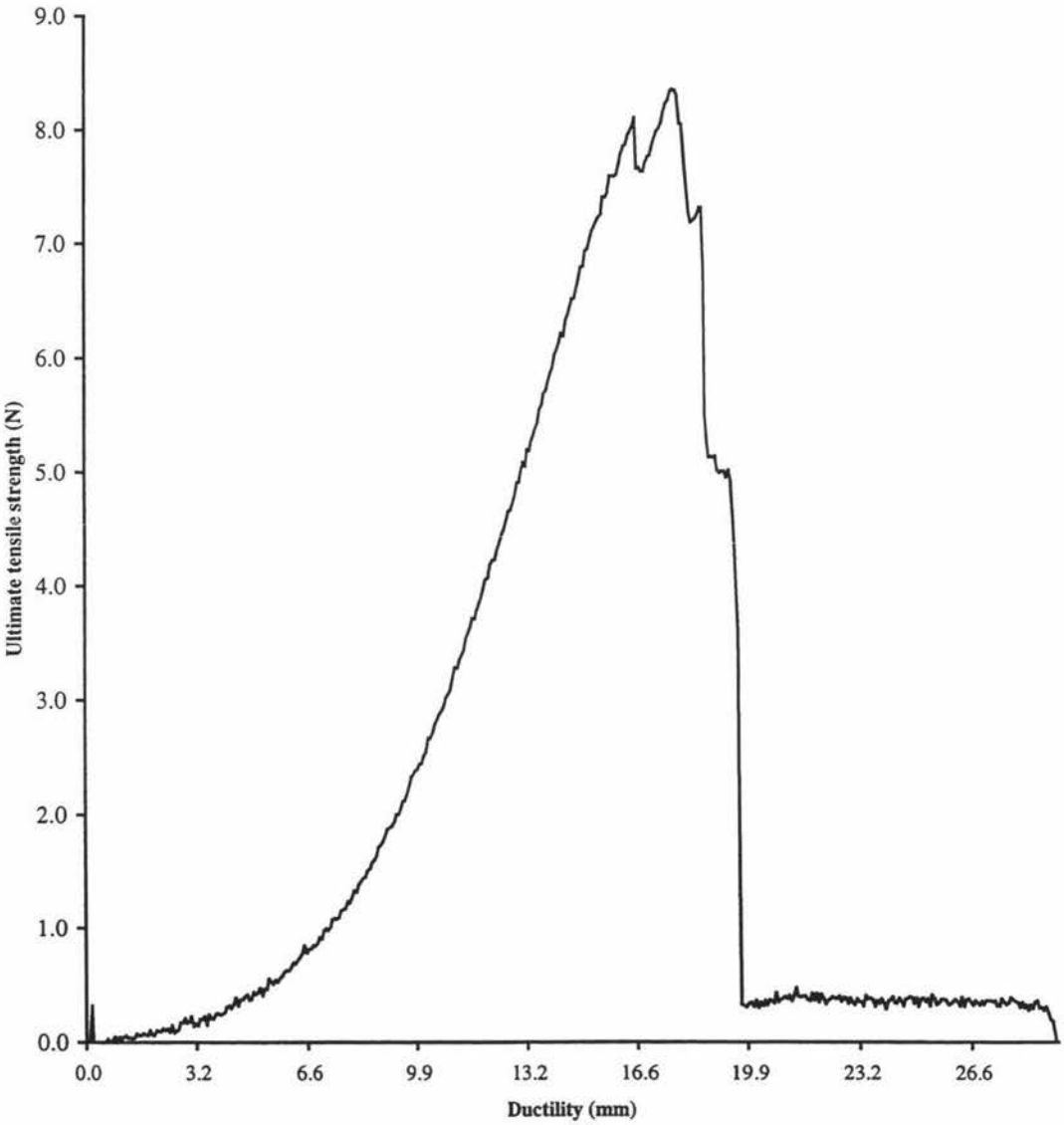
Ultimate tensile strength-ductility curve of the first weak batch beef thread

APPENDIX 3



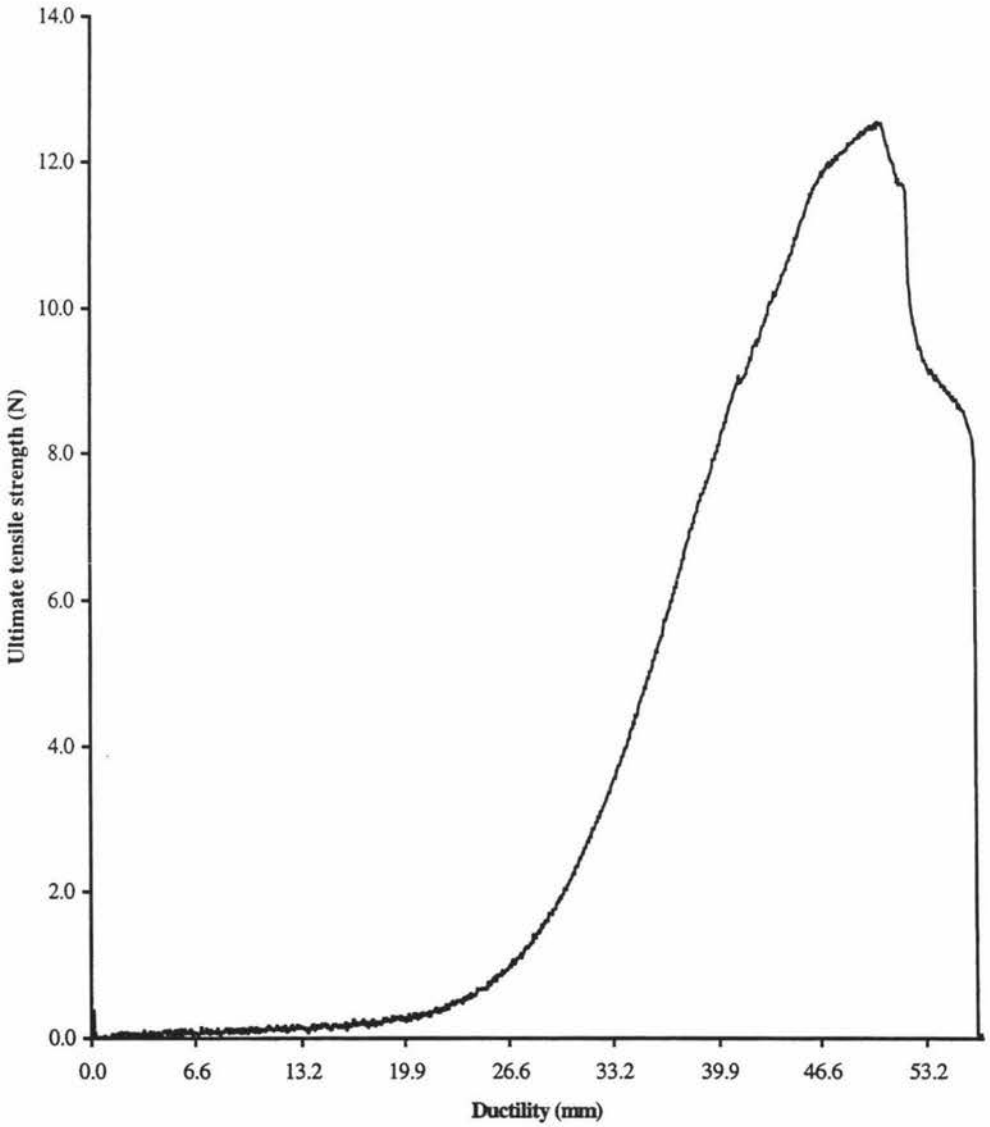
Ultimate tensile strength-ductility curve of the first strong batch beef thread after treated with sodium carbonate/EDTA process

APPENDIX 4



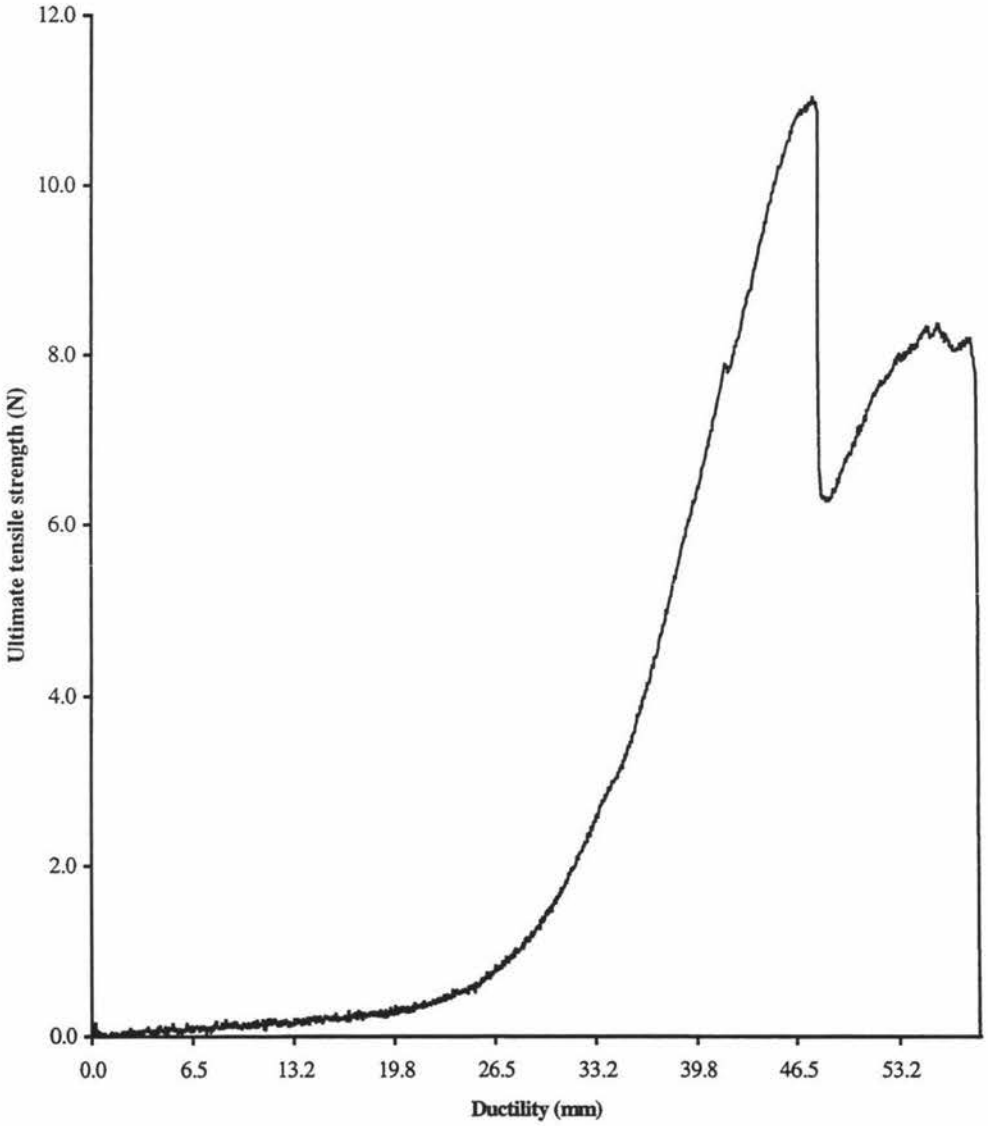
Ultimate tensile strength-ductility curve of the first weak batch beef thread after treated with sodium carbonate/EDTA process

APPENDIX 5



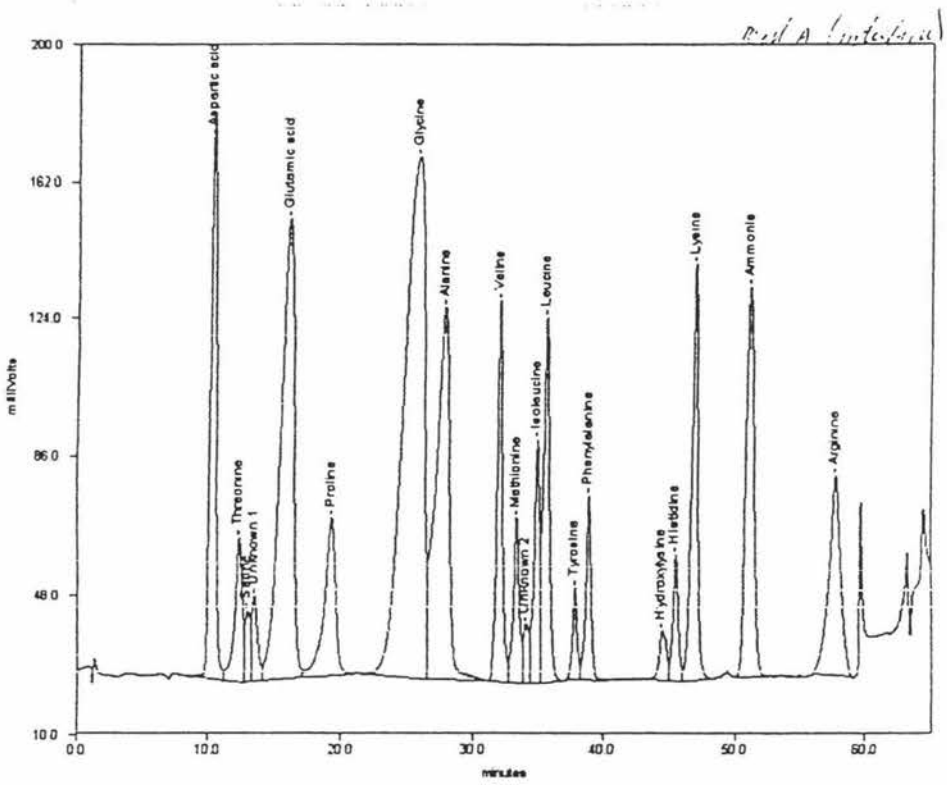
Ultimate tensile strength-ductility curve of the first strong batch beef thread after treated with sodium hydroxide process

APPENDIX 6



Ultimate tensile strength-ductility curve of the first weak batch beef thread after treated with sodium hydroxide process

APPENDIX 7



Amino acid chromatogram of "normal" batch casing