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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

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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.

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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

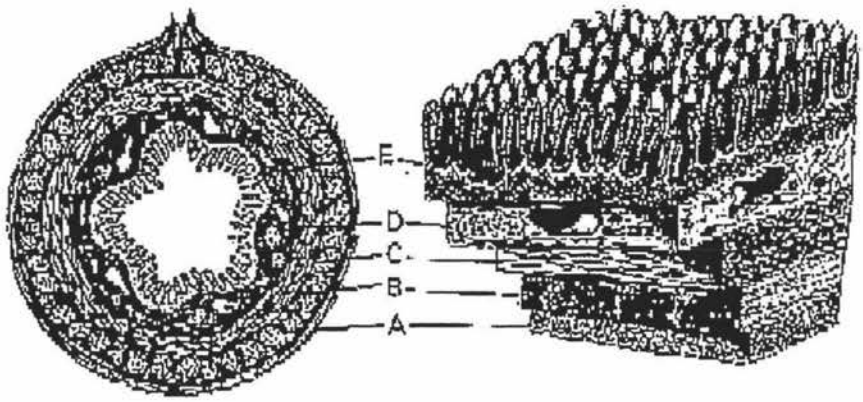
New Zealand, are made from the outermost layer, called the serosa, of bovine small intestines. The serosa is a sandwich of a highly oriented collagen layer and a very random one. The highly oriented layer contributes the tensile strength, and the random one elasticity (Pacific USA web page, 1999).

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.