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Changes in Bovine Intramuscular Collagen during Extended Post-mortem Aging of Beef

Heather Lee Bruce

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosopy

Department of Agricultural, Food and Nutritional Science

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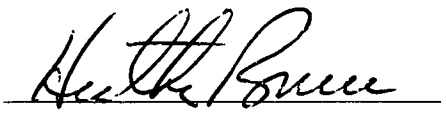

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ABSTRACT

Controlled atmosphere packaging (CAP) allows storage of fresh beef in CO_2 atmospheres at -1.5°C for up to 21 weeks. Beef stored in CO_2-CAP develops pores along the perimysium upon cooking. Packaging beef in 0.25 M $NaHCO_3$ duplicated pore formation. Dialysis with distilled water removed dissolved CO_2 from beef stored in CO_2 or sodium bicarbonate solution, because pores were absent and gas production during cooking was reduced. This supports the hypothesis that heating releases dissolved CO_2 in the meat, which gathers and expands at the weakest point, the perimysium, producing pores.

CAP was used to preserve beef in order to investigate collagen structural changes during extended aging. Perimysium and endomysium were isolated from normal and high 24 h post-mortem pH roasts after CAP storage for 0, 4, 8, 12 and 15 weeks. Collagen and protein contents of perimysium and endomysium were estimated from hydroxyproline and nitrogen analysis, respectively. Collagen structural changes were assessed using heat solubility measurements, differential scanning calorimetry and SDS-PAGE. Perimysium collagen content increased significantly during storage (55% to 74% collagen), while endomysium collagen content did not change. Perimysium protein content increased from 14% to 17% during storage, while endomysium protein content remained unchanged. Existence of non-collagen protein and non-protein contamination suggested the presence of glycoproteins. Perimysium non-protein material decreased during storage, possibly due to post-mortem degradation. Perimysium heat solubility increased slightly during storage. Differential scanning calorimetry showed that perimysium T_m values decreased constantly through storage, while endomysium T_m values decreased only from [illegible] weeks of

storage. Perimysium mean denaturation enthalpies remained constant during aging; however, endomysium mean denaturation enthalpies varied widely. Results suggested that covalent bonds were degraded in both collagens, with more degradation in the perimysium than the endomysium. SDS-PAGE profiles revealed that both collagens contained Type I and III. Type III of the perimysium appeared preferentially degraded. The results of these studies show that bovine collagen was unaffected by high concentrations of CO_2 and that perimysium heat lability increased during storage, probably by damage to the telopeptides where covalent bonds are located, possibly contributing to beef tenderization during storage.

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1. INTRODUCTION

Toughness is one of the primary characteristics influencing consumer satisfaction with beef (Bailey 1972). The toughness of beef is partly determined by the effects of ante-mortem and post-mortem factors on the two major structural components of muscle: the myofibrillar ultrastructure and the extracellular collagen network. The changes in the myofibrillar component that affect the toughness of beef are well established (Pearson and Young 1989); however, the changes in collagen which may occur during carcass conditioning are not fully understood. This chapter presents a review of the structure and function of the various collagens and their association with muscle, in order to explore the relationship between collagen structure and beef toughness.

1.1. COLLAGEN STRUCTURE

Collagen, the major component of connective tissue, is usually perceived as "gristle" in cooked beef, and normally composes approximately 2% of total muscle protein (Bailey and Light 1989). In muscle, collagen is found in three structures: the endomysium, perimysium and epimysium. The endomysium, which encompasses each muscle fibre, is contiguous with the perimysium, which encircles bundles of muscle fibres. The perimysium continues into the epimysium, a thick sheath of connective tissue which encompasses whole muscles. At muscle insertions and attachments, the epimysium and perimysium taper into tendons, anchoring muscle to bone.

To date, approximately fourteen genetically distinct collagens have been identified, and were summarized by van der Rest and Garrone (1991) as fibrillar collagens (Types I, II,

III, V and XI) and non-fibrillar collagens (Types IV, VI, VII, VII, IX, X, XII, XIII and XIV). The family of collagens is characterized by each molecule having at least one triple helical domain. The molecular triple helix is formed from three α-chains that contain a repetitive glycine-X-Y sequence, in which X and Y can be any amino acid except tryptophan, which is not found in collagen. Usually, the amino acids located at the X and Y are proline and hydroxyproline, respectively. The regularity of glycine, which occurs every third amino acid, places it in the middle of the triple helix. Its small size allows the helix to be shallow and tightly coiled. The pyrrole ring of the adjacent proline stabilizes the helix by preventing rotation about the N-C bond which reduces the entropy of unfolding and precludes the helix folding into a globular shape. The hydroxyproline preceding the glycine imparts thermal stability to the collagen molecule, possibly through water bridges involving its hydroxyl group (Piez 1984). Studies of the thermal stability of synthetic collagens showed that collagens with proline in the X position and hydroxyproline in the Y position were the most heat-stable (as reviewed by Bailey and Light 1989; Kuhn 1987). Increasing the concentration of hydroxyproline in the helix confers thermal stability on the collagen molecule. Collagen from fish found in very cold waters has less hydroxyproline residues per 1000 amino acid residues than that from fish of warm waters (data reviewed by Bailey and Light 1989). Collagen contains a higher proportion of hydroxyproline than any other protein, with hydroxyproline present in collagen from the same species in a constant proportion; consequently, hydroxyproline is used as an index of collagen concentration. Hydroxyproline is formed intracellularly through a post-translational modification of proline, with the hydroxylation by prolyl-4-

hydroxylase occuring at the fourth carbon (Pearson and Young 1989; Kuhn 1987).

The triple helical collagen molecules associate with each other to form either fibrils, in the case of fibrillar collagen types, or other aggregates if non-fibrillar. Fibrillar or non-fibrillar aggregations appear to occur non-enymatically, and may be guided by the hydrophobicity and charges of the amino acids along each collagen molecule (Piez 1984). Fibrillar collagen molecules align into bundles referred to as fibrils. Two structural models have been proposed specifying the arrangement of collagen molecules within a fibril. Smith (1968) proposed a five-stranded microfibril model in which five collagen molecules were staggered by 67 nm) and rotated by one-fifth of a circle to form a rope-like, helical structure. The second model is a refinement of the first by Trus and Piez (1980) and Piez and Trus (1981) in order to incorporate the three-dimensional crystal model proposed by Hulmes and Miller (1979) and the micofibrillar packing model postulated by Miller and Tocchette (1981). Piez and Trus (1981) hypothesized that the five collagen molecules associated into microfibrils in a flattened ellipse rather than a circle.

Fibrils form fibres through hydrophobic interactions on their surfaces. Collagen fibrils of tendon can be more than several hundred nanometers in diameter (Parry et al. 1978) and are organized in bundles which are visible in the light microscope as fibres. These fibres exhibit a characteristic planar crimp which can be straightened with extension of the collagen structure in order to improve its shock absorbing properties (Gathercole and Keller 1991). Collagen fibre size varies with the strength of fibre required, with long, thick fibres being found in tendon (Bailey and Light 1989). Collagen fibre size required

also determines the type of fibrillar collagen found in a physiological region (Bailey and Light 1989).

All collagen type molecules are synthesized intracellularly as individual procollagen molecules that, once the propeptides are removed, may become part of the organized collagen network. Intermolecular covalent bonds stabilize the molecular assembly (Kuhn 1987). Of all the types that exist, only Types I, III, IV, V and VI are found in muscle. Consequently, only these types will be discussed in detail according to their molecular organization.

1.1.1. Fibrillar Collagens

The fibrillar collagens found in muscle (Types I, III and V) have molecular weights of approximately 360 kilodaltons and are 300 nm long with a diameter of about 1.4 nm Kuhn 1987). Type I collagen was the first isolated and characterized; hence, its nomenclature. It is found in major connective tissues such as the skin, tendon, bone, dentine, intra-organ and muscle (Kuhn 1987). It is comprised of two α-1(I) chains and one α-2(I) chain, each of which is approximately 1050 amino acid residues long (Bailey and Light 1989). Of the total amino acid residues in each chain, 1014 are found in the triple helix (Kuhn 1987). The remaining residues are found at either end of the molecule, with 16 at the N-terminus and 25 at the C-terminus of the α1(I) chain (Kuhn 1987) and 9 at the N-terminus and 6 at the C-terminus in the α2(I) chain (Glanville and Kuhn 1979). The non-triple-helical telopeptides of this heteropolymer contain no cysteine (Bailey and Light 1989). Type I forms the thickest fibrils of the collagen family (Kuhn 1987).

Type III collagen is found throughout the body except in mineralized bone and dentin,

particularly in the vascular system, embryonic tissue, scar tissue, organs and muscle perimysium (Bailey and Light 1989). Type III monomers consist of three α-1(III) chains (Kuhn 1987), and form very fine fibrils which were once referred to as "reticulin" (Pearson and Young 1989). These fibres are often physically associated with Type I fibrils (Bailey and Light 1989). Unlike Type I, Type III collagen molecules contain cysteine at the C-termini which may form a disulphide bonds with another Type III molecule (Bailey and Light 1989).

Bailey and Light (1989), in their review, noted that Type V is poorly characterized but appears to be fibrillar. It is associated with muscle basement membranes and has high concentrations of hydroxyproline and hydroxylysine. It is a heteropolymer with two α-1(V) chains and one α-2(V) chain. An α-3(V) chain has been identified, so this collagen may form heterotrimeric or heterodimeric molecules (van der Rest and Garrone 1991).

1.1.2. Non-fibrillar Collagens

Type IV collagen is a non-fibrillar collagen that forms sheets and is found in basement membranes surrounding muscles and nerves (Pearson and Young 1989). The Type IV collagen molecule is a heterotrimer consisting of 2 α-1(IV) and one α-2(IV) and is approximately 420 nm long (Pearson and Young 1989). Type IV assembles into tetrameric aggregates by anti-parallel overlapping of the amino-terminal domains (Timpl et al. 1981), a region which has been termed the 7S domain (Figure 1.1). The tetramers are thought to assemble in pairs through the carboxy-terminal globular region to form a "chicken-wire" network that allows great extensibility (Figure 1.1). The molecule is quite flexible because its triple helix is discontinuous. These internal, non-triple-helical regions,

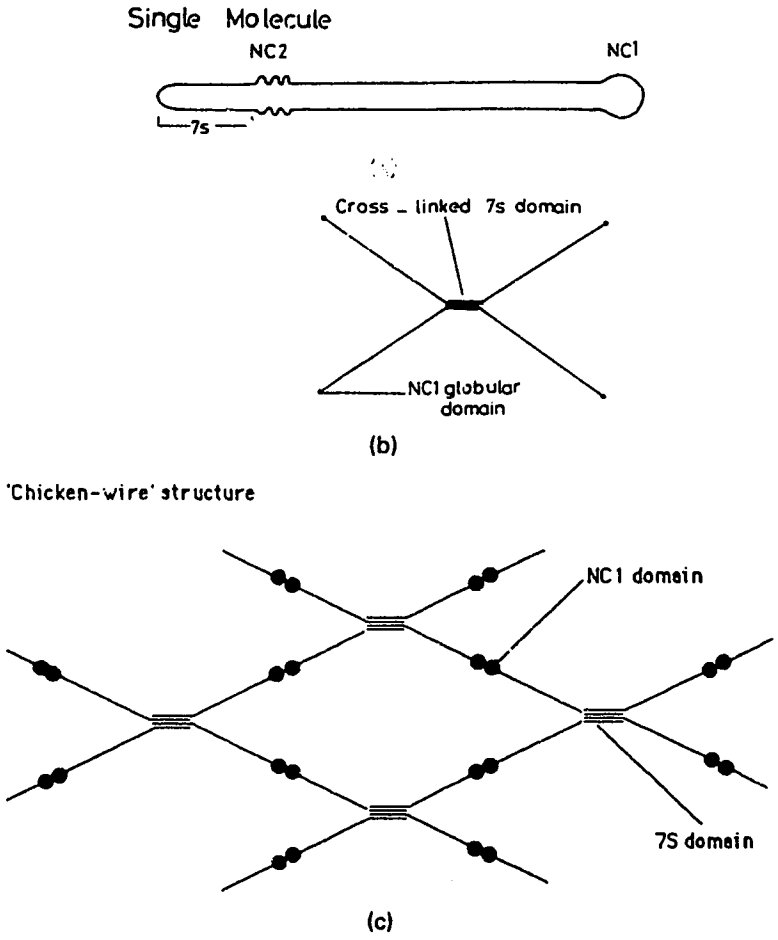


Figure 1.1. Diagram of (a) the Type IV collagen molecule, (b) the Type IV collagen tetramer and (c) the hypothetical "chicken wire" network. From Bailey and Light (1989) as described by Timpl et al. (1981).

however, may predispose the molecule to proteolysis (Timpl et al. 1981).

Type VI, although classified as non-fibrillar, is actually a filamentous collagen that has been found in the perimysium and in many other tissues along with Type I collagen (Bailey and Light 1989). It consists of three polypeptide chains: α-1(VI), α-2(VI) and α-3(VI)(Pearson and Young 1989). It is approximately 10 to 20 nm in diameter (Pearson and Young 1989) and appears to connect collagen fibres to elastin (von der Mark et al. 1984). Type VI collagen forms beaded filaments of molecular dimers, associated anti-parallel to each other, that aggregate laterally at the globular or "bead" region to form tetramers (van der Rest and Garrone 1991). This lateral association is stabilized by disulphide bonds and filaments are formed from serial alignment of the tetramers. The filaments then aggregate laterally into bundles. Very little is known of the contribution of this collagen to meat toughness.

1.1.3. Basement Membrane Components

Collagen types III, IV and V are associated with the basement membrane, which is an amorphous layer covering the sarcolemma of each muscle cell. As well as collagen, the basement membrane contains non-collagenous glycoproteins and proteoglycans.

Glycoproteins of the basement membrane are proteins that have oligosaccharides with a mannose core N-glycosidically linked to an asparagine. These disaccharides modify the physical properties of protein to which they attach by altering susceptibility to proteolysis and increasing resistance to denaturation (Hakomori et al. 1984). The two most common glycoproteins found in the basement membrane of muscle are fibronectin and laminin. Fibronectin binds to collagen and mediates cell to collagen adhesion (Hakomori et al.

1984). This 220 kd glycoprotein preferentially binds to Type III collagen (Engvall and Ruoslahti 1977; Jilck and Hormann 1979), and is capable of binding to heparan sulfate (Jilek and Hormann 1979). It can covalently crosslink to collagen in a reaction catalyzed by transglutaminase (Mosher 1980). Laminin, with a molecular weight of more than 600 kd, is located in the lamina rara and may be involved in the attachment of epithelial cells to Type IV collagen (Terranova et al. 1980). It is a major constituent of the basement membrane, and binds to heparan sulphate and dermatan sulfate but not hyaluronic acid, which are glycosaminoglycan found in muscle (Hakomori et al. 1984). Glycoproteins constitute a large portion of the basement membrane and could increase meat toughness by increasing collagen resistance to proteolysis (Etherington 1977).

Proteoglycans consist of glycosaminoglycan (GAG) chains covalently attached to a protein core (Heinegard and Paulsson 1984) and may modulate collagen fibrillogenesis (Vogel et al. 1984). GAGs are linear polymers of repeated disaccharides, with an average number of repeats being fifty (Heinegard and Paulsson 1984). Hyaluronic acid, dermatan sulphate and heparan sulphate are the GAGs found in muscle basement membrane. The constituent monosaccharides are the basis of differentiating the various GAGs.

Hyaluronic acid is the largest GAG, having 50 to 1000 repeating disaccharide units (Pearson and Young 1989). Its disaccharide unit consists of the monosaccharides β-D-glucuronic acid (β-D-GlcUA) and β-N-acetylglucosamine (β-D-GlcNAc) bonded with a β-linkage.

Dermatan sulphate is the name given to GAGs that contain α-L-iduronic acid (β-L-IdUA) β-linked to N-acetylgalactosamine (β-D-GalNAc). Dermatan sulphate can contain

β-D-GlcUA linked to β-D-GalNAc as well and the two types of disaccharides often appear in the same polymer, the polymer averaging 50 disaccharides (Pearson and Young 1989). Dermatan sulfate can bind to Type IV collagen and has approximately one sulfate in each disaccharide (Laurie et al. 1986).

Heparan sulphate is the most sulphated GAG, with approximately two sulphates in each disaccharide. Heparan sulphate consists of two types of disaccharides: β-D-GlcUA with α-D-GlcNAc and β-L-IdUA with α-D-GlcNAc; thus it is very similar to heparin. Heparin, paradoxically, is more sulphated and has a higher concentration of β-L-IdUA than heparan sulphate (Pearson and Young 1989).

The GAGs are anchored to core proteins in proteoglycans such as aggrecan, biglycan and decorin. Decorin, which in muscle has a small protein core with a single dermatan sulphate chain, is associated with almost all connective tissues (Bianco et al. 1990). Vogel et al. (1984) and Vogel and Trotter (1987) showed that decorin modified collagen fibrillogenesis by decreasing fibril size. Decorin could, in this way, decrease meat toughness (Light et al. 1985; McCormick 1994).

1.2. COLLAGEN CROSSLINKING

With its ubiquitous presence in muscle, collagen has been attributed to providing the "background" toughness of beef (Bailey 1972), implying that its contribution to toughness occurs in concert with that of the myofibrillar proteins. Collagen is the major determinant of toughness in beef from carcasses compared across a wide range of maturity levels (Goll et al. 1964a,b,c; Hill 1966). The toughness of veal is paradoxical in that veal contains

more collagen per gram muscle than beef from a 2 year old steer, yet it is far more tender than beef. The difference lies in the extent to which the collagen has crosslinked, because crosslinking determines its mechanical and thermal strength.

Collagen molecules can be crosslinked intra- and inter-molecularly. Intramolecular covalent crosslinks involving cysteine and lysine lend only thermal resistance to the collagen fibril and not tensile strength. Intramolecular disulphide bonds are found in Type III and IV collagens (Bailey and Light 1989). In Type III collagen, the cysteines are located in the junction between the C-terminal telopeptide and the triple helix. Consequently, two cysteines may react to form a disulphide bridge, leaving the third to interact intermolecularly (Chung and Miller 1974). The formation of the disulphide bridge is catalyzed by disulphide isomerase during triple helix formation in the endoplasmic reticulum. In Type IV collagen, disulphide bonds are formed extensively in the 7S domain within and between molecules (Furthmayr et al. 1983).

Intramolecular covalent bonding involving lysine occurs in all the collagen types. These crosslinks arises from the lysines or hydroxylysines found in the telopeptides and triple helical regions of collagen molecules. Hydroxylysine is a post-translational modification of lysine in either the Y position of the triplet repeat or in the non-triple helical telopeptides of α-chains by lysyl hydroxylase (Kuhn 1987). Lysyl hydroxylase is located on the inner side of the rough endoplasmic reticulum membrane and requires Fe^{2+}, 2-oxoglutarate, O_2 and ascorbate to catalyze the hydroxylation. It acts only on the α-chains and cannot modify lysine once the triple helix has formed (Kuhn 1987).

In order to participate in intra- and intermolecular covalent crosslinking, lysine and

hydroxylysine must be converted to the aldehydes allysine and hydroxylallysine. Allysine and hydroxyallysine are produced when lysyl oxidase binds to the newly-forming collagen fibrils and oxidatively deaminates the ϵ-amino group of the lysine and hydroxylysine residues in the N- and C-terminal telopeptides (Siegel and Fu 1976). These reactions require copper, molecular oxygen and pyridoxal phosphate, and involve lysines and hydroxylysines in triple helical α-chains (Ricard-Blum and Ville 1988)(Figure 1.2). An intramolecular aldol crosslink can form between two allysine residues, an allysine and a hydroxyallysine residue or two hydroxyallysine residues in the non-triple-helical telopeptides of two α-chains of the same molecule (Ricard-Blum and Ville 1988).

Lysine residues are found in both N- and C-telopeptides of the α-chains, except the α-2(I) chains, which do not have a lysine residue in the C-telopeptide; therefore, five lysine aldehyde (allysine) or hydroxylysine aldehyde (hydroxyallysine) residues may exist in each collagen molecule (Bailey and Light 1989). Eyre et al. (1984b), in their review, noted that allysine is the ninth residue in the N-telopeptide and the sixteenth in the C-telopeptide in the α-1(I) chain. Of the allysines available in the telopeptides of the collagen molecule, two may react intramolecularly, leaving a third for an intermolecular link. The majority of the aldol linkages appear between α-1 and α-2 chains, although homodimers (β1,1) bonds are possible (Bailey and Light 1989). This aldol condensation appears to occur only between aldehydes in the N-terminal telopeptides. Steric hindrance may account for their absence between C-terminal telopeptides (Bailey and Light 1989).

Intermolecular covalent crosslinks involving cysteine and lysine contribute to the tensile strength, proteolytic resistance and thermal stability of fibrillar collagen (Bailey and

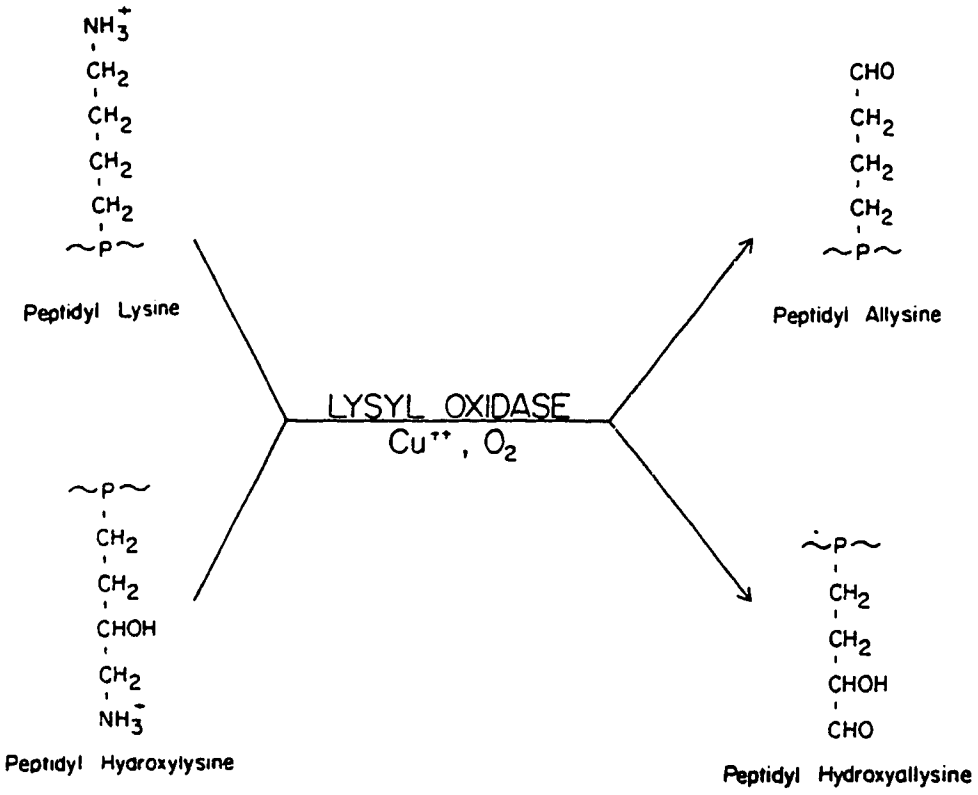


Figure 1.2. Reaction of lysyl oxidase with peptidyl lysine and peptidyl hydroxylysine to form peptidyl allysine and peptidyl hydroxyallysine, respectively, in the non-helical ends of the α-chains. From Pearson and Young (1989).

Light 1989). Intermolecular covalent crosslinks involving cysteine are found in aggregates of Type IV or Type III collagen fibres (Bailey et al. 1984) Disulphide bridging can occur between two Type III collagen molecules and extensive disulphide bridging occurs between the N-telopeptides of four Type IV collagen monomers to form the 7S domain of a tetramer (Chung and Miller 1974; Furthmayr et al. 1983)(Figure 1.2). Further disulphide bridging occurs at the globular C-terminal domains in a hexamer formation which allows Type IV collagen to achieve its "chicken wire" network (Furthmayr et al. 1983).

Allysine and hydroxyallysine are the residues involved in two different intermolecular covalent crosslink pathways (Eyre et al. 1984b). Eyre et al. (1984b) noted that the allysine or aldimine pathway predominated in the skin, whereas the hydroxyallysine or keto-amine pathway operated mostly in other tissues. Bailey and Light (1989), in their review, noted that, because of the difference in the hydroxylation of the components of the two pathways, collagen that displays low hydroxylation in the telopeptides has primarily aldimine crosslinking. Conversely, collagen that has many hydroxylated allysines will contain more keto-amine crosslinks. Bailey and Light (1989) also noted that the extent of hydroxylation of collagen in certain tissues may change with with maturity. They surmised that this would reflect a change in the hydroxylation of the allysines in the telopeptide domains only. Bailey and Light (1989) concluded that such differentiation would be possible if another hydroxylase catalysed the hydroxylation of the telopeptide lysines.

Intermolecular covalent crosslinks involving allysine and hydroxyallysine appear to involve two sites in the triple helix. Eyre et al. (1984b) noted in their review that

crosslinks may exist between the N-telopeptide allysine and the hydroxylysine at residue 930 in the triple helical region of the α-1(I) or α-1(III) chains of an adjacent molecule. As well, a crosslink may form between the C-telopeptide allysine or hydroxyallysine at residue 87. With this mode of intermolecular bonding, collagen molecules are staggered, overlapping by 67 nm.

In the aldimine covalent crosslink pathway, allysine from one collagen molecule can combine with lysine, hydroxylysine or histidine to produce an acid-soluble, and hence immature, intermolecular crosslink (Miller 1984). Dehydro-lysino-norleucine, the product of the condensation of an allysine from one collagen molecule with the lysine residue of another, and is a minor crosslink in collagen because most crosslinking involves hydroxylysine (Miller 1984). The most common aldimine crosslink then is dehydro-hydroxy-lysino-norleucine (dehydro-HLNL), which is the condensation product of allysine and hydroxylysine and is readily broken by acid (Miller 1984). These crosslinks are all reducible in sodium borohydride, which confers stability to the crosslinks and allows them to be quantitated. Reducibility in sodium borohydride indicates crosslink instability and, thus, crosslink immaturity.

Allysine is capable of combining with another allysine to form allysine aldol, but this crosslink occurs primarily intramolecularly (Miller 1984). The intramolecular allysine aldol can combine with a hydroxylysine from another collagen molecule to form dehydro-hydroxy-merodesmosine, an immature crosslink. Also, the allysine aldol may combine with a histidine from another collagen molecule to produce dehydro-aldol-histidine, another immature crosslink. Histidine residues eligible to participate in the formation of

these crosslinks are located at residues 89 and 932 in the triplet repeating portion of the α1(I) chains (Bernstein and Mechanic 1980; Miller 1984).

Tanzer et al. (1973) postulated that allysine may combine with a histidine to form aldolhistidine, which links with hydroxylysine to form the reducible crosslink dehydro-histidino-hydroxy-merodesmosine (dehydro-HHMD). Robins and Bailey (1973) concluded from their work that dehydro-HHMD was an artifact of sodium borohydride reduction. These researchers supposed this based upon their inability to isolate the crosslink after reduction of completely redissolved collagen fibres, which yielded only reduced aldolhistidine. Bernstein and Mechanic (1980) claimed, however, that histidino-hydroxy-merodesmosine (HHMD) was from a real, reducible crosslink in the allysine pathway. These researchers, using the methods of Robins and Bailey (1973), found HHMD, which they proposed was the reduction product of the in vivo crosslink dehydro-HHMD. Bernstein and Mechanic (1980) found that His-89 on the α-1 chain was the most probable histidine involved in the crosslink. Eyre et al. (1984b), in their review, cited HHMD as one of the two borohydride-reducible, immature crosslinks of the allysine pathway.

Mature intermolecular crosslinks that are not reduced by sodium borohydride are thought to develop from the reducible crosslinks because the concentration of reducible crosslinks decreases with age as that of mature crosslinks increases (Ricard-Blum and Ville 1988). Robins (1983) suggested that intermicrofibrillar crosslinking by further reaction of reducible crosslinks would form non-reducible, polyfunctional crosslinks. Bailey et al (1977) proposed that a mature crosslink of the allysine pathway was α-aminoadipic acid, which arose from oxidation of the aldimine form of dehydro-HLNL. Bailey et al. (1977)

isolated significant amounts of α-aminoadipic acid from acid hydrolysates of mature bovine dermal collagen. Eyre et al. (1984b) noted that no crosslink peptides have been isolated that contain this compound, and that it is unlikely that this is a bond found in vivo.

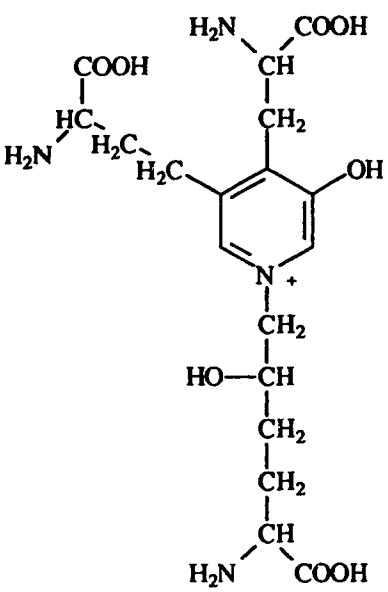
Yamauchi et al. (1987) identified a mature, non-reducible, trifunctional crosslink of the allysine pathway, histidino-hydroxy-lysino-norleucine (HHLNL), from human and bovine skin. These researchers believed it to be a mature crosslink because its concentrations in skin increased throughout maturation. These workers found that the crosslink is derived from the sidechain of histidine (His-92, α-2 chain), hydroxylysine (Hyl-16, α-1(I)) and lysine (Lys-16, α-1(I)) residues. Yamauchi et al. (1987) postulated from in vitro incubation studies that the bifunctional, reducible crosslink HLNL was its precursor. The allysine pathway, however, is found predominantly in skin, and probably does not affect meat toughness.

The hydroxyallysine pathway is the major pathway for collagen crosslinking in mammalian connective tissues. It appears in all connective tissues except the skin and cornea; consequently, its mature crosslinks may affect meat tenderness (Eyre et al. 1984a). Again, the mature crosslinks are thought to form from the immature keto-amine crosslinks. One immature crosslink is a product of the Schiff's base reaction between hydroxyallysine and hydroxylysine to form dehydro-dihydroxy-lysino-norleucine (dehydro-DHLNL), which spontaneously undergoes Amadori rearrangement to a keto-amine, hydroxy-lysino-5-keto-norleucine (Bailey and Light 1989). As well, hydroxyallysine may condense with a lysine residue to form dehydro-hydroxy-lysino-norleucine (dehydro-HLNL). The resulting structure undergoes an Amadori rearrangement to produce the stable keto-amine

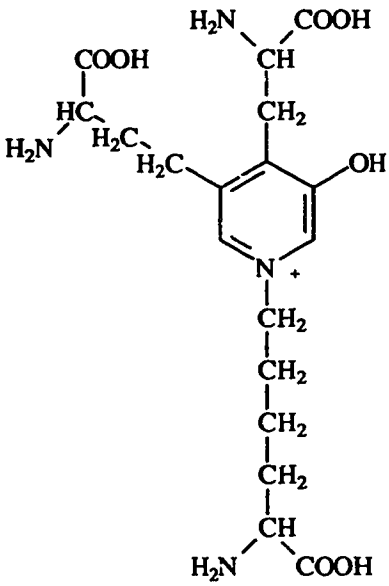
crosslink lysine-5-oxonorleucine (Ricard-Blum and Ville 1988).

Fujimoto et al. (1977) presented evidence that the mature crosslinks of the hydroxyallysine pathway are trifunctional 3-hydroxypyridinium residues. The 3-hydroxypyridinium crosslink is naturally fluorescent, which allows identification and quantitation according to its excitation at 295 nm and fluorescence at 395 nm (Fujimoto 1980). It is also easily destroyed by ultra-violet radiation; hence, its absence from skin and cornea (Eyre et al. 1984b). Fujimoto (1980) confirmed the in vivo presence of 3-hydroxypyridinium crosslinks by isolating them in peptides obtained in collagen digested with pronase.

Eyre et al. (1984b) reviewed the characteristics of the two forms of the 3-hydroxypyridinium crosslink identified in the literature. The most abundant is hydroxylysyl pyridinoline, which is believed to be derived from three hydroxylysines, and the second is lysyl pyridinoline, which is derived from two hydroxylysines and one lysine (Figure 1.3). Fujimoto and Moriguchi (1978) identified the residues involved to be located in the amino acid sequences of 99 to 106 and 1036 to 1049 in the α-1(I) chain and hypothesized that the reducible crosslink dehydro-dihydroxy-lysino-norleucine (dehydro-DHLNL) was the precursor of the pyridinoline crosslink because it is located in a similar location along the α-1(I) chain This hypothesis has been supported by other researchers (Siegel et al. 1982; Eyre et al. 1984b). From the literature, Eyre et al. (1984b) surmised that the residues involved from the α-2(I) chain appeared to be Hyl-87 and possibly Hyl-933. Light and Bailey (1985) used cyanogen bromide (CNBr or CB) digestion to determine that pyridinoline bound α-1CB5 to α-2CB3,5, not α-1CB6, as previously



HP



LP

Figure 1.3. Structural formulas of hydroxy-lysyl-pyridinoline (HP) and lysyl-pyridinoline (LP)(as described by Eyre et al. 1984a).

hypothesized by Fujimoto (1980) and Fujimoto and Moriguchi (1978). Light and Bailey (1985) suggested that pyridinoline was formed from the condensation of an N-terminal telopeptide hydroxylysine aldehyde with an keto-amine crosslink between the N-terminal non-helical telopeptide and the α-2(I)CB3,5 peptide from another molecule. Light and Bailey (1985) based this conclusion on their analysis of the CNBr peptides derived from Type I collagen, of which α-2(I)CB3,5 was the only peptide that contained pyridinoline. These researchers also concluded that the Type I collagen appeared to be stabilized primarily by poly-α-1CB6 crosslinks rather than pyridinoline, indicating that there was more than one mature crosslink.

Scott et al. (1981) isolated a three-chained peptide from collagen-rich tissues that reacted with Ehrlich's reagent at room temperature. This Ehrlich's chromogen (EC) was not present in neutral salt extracts, suggesting that it was associated with mature, crosslinked collagen. As well, their research provided evidence that the chromogen was a trifunctional crosslink, possibly a pyrrole, containing nitrogen. Further research using fluorescence suggested that pyridinoline also was present in the peptide (Scott et al. 1983). Scott et al. (1983) found that the EC was present in the 7S region of Type IV collagen. As well, Kemp and Scott (1988) found that the EC was also present in elastin, but could be differentiated from the EC derived from collagen because the collagen EC peptides contained glucose and galactose, whereas EC peptides from elastin did not.

Henkel and Glanville (1982) published evidence of intermolecular bonding between Type I and III fibrils. Using the bovine aorta and human leiomyoma, the workers isolated three peptides, two of which were trivalent crosslinks of N-terminal α-1(I) and α-1(III) to

C-terminal helical sites on α-1(I) and α-1(III), respectively. This data indicated that the bond between the N-terminals was between non-helical regions of Type I and III molecules that were in register. The data substantiate the hypothesis that heterogenous crosslinks occur between substructures in the collagen fibril, which are arranged in lateral register to each other. The existence of these heteropolymer crosslinks in muscle has not been verified, but they may occur in order to maintain the contiguity between the perimysium and endomysium (Henkel and Glanville 1982).

In the non-fibrillar collagen Type IV, the N- and C- terminals are linked by intermolecular covalent bonds (Bailey et al. 1984). In Type IV collagen isolated from young rat lens capsule, the main crosslink appears to be dihydroxy-lysino-norleucine (DHLN). Another crosslink, hydroxy-lysino-norleucine (HLNL), has also been identified in adult bovine lens capsule (Eyre et al. 1984b). These crosslinks are derived from allysine residues in the 7S and NC1 regions of the molecules (Eyre et al. 1984b). The sequence Hyl-Gly-His-Arg has been conserved in order to allow reaction with the hydroxylysine at each end of the 7S region (Bailey et al. 1984). Pyridinoline does not exist in Type IV collagen (Bailey et al. 1984), but a mature crosslink quite similar in function, the Ehrlich's chromogen, has been identified in the 7S region (Scott et al. 1983). The mature crosslinks of Type IV have yet to be identified in the globular regions (Eyre et al. 1984b).

1.3. ASSESSMENT OF COLLAGEN INTEGRITY

The collagen that ultimately influences the toughness of beef has usually been exposed

to heat during cooking. Consequently, early studies explored the thermal lability of collagen not as an indicator of collagen structure but as a measure of toughness. The solubility of collagen during cooking was related to tenderness by Goll et al. (1964a) and Hill (1966) who found that collagen solubilities decreased as shear force values of meat increased.

The method of Hill (1966) involved a very crude extraction of lyophilized, powdered meat with one-quarter strength Ringer's solution. Many researchers have used this method, possibly because of the simplicity of preparation, to assess collagen solubility differences between cattle of various ages and have confirmed the results of Hill (1966) which indicated that collagen solubility decreased significantly with age (Cross et al. 1973; Herring et al. 1967). The method of Goll et al. (1964a) was more rigorous, requiring the isolation of collagen from the myofibrillar proteins with 1.1 M KI in a neutral 0.1 M potassium phosphate buffer. This approach allowed for crude quantitation of the amount of collagen present in muscle samples (Goll et al. 1963), as well as direct perturbation of the collagen without myofibrillar interference (Goll et al. 1964b).

More intensive characterization of the thermal denaturation of collagen has been accomplished by using differential scanning calorimetry (McClain and Wiley 1972). DSC is a more precise tool for estimating the heat lability of collagen than quantitating the release of hydroxyproline into a neutral solution during cooking because it estimates the temperature at which it unfolds. An endothermic peak is produced that indicates the amount of heat required to disrupt the intramolecular hydrogen bonds. Myers (1990) explained that the thermal denaturation temperature was determined by entropic and

enthalpic forces. As the temperature of the protein increases from 15°C, entropic interactions such as hydrophobic bonds strengthen to a maximum between 60 and 80°C, then weaken as the temperature approaches 100°C. As well, enthalpic interactions such as electrostatic, van der Waals and hydrogen bonds weaken as the temperature increases. The endothermic effects of the enthalpic interactions are countered by the exothermic hydrophobic effects. The exothermic opposition is greatest between 60 and 80°C, and this lowers the enthalpy. As a result of the strong hydrophobic effects and the weak electrostatic, hydrogen and van der Waals forces, most muscle proteins unfold between 60 and 80°C (Myers 1990).

Myers (1990) reviewed the three thermal transitions found in meat analyses. The transition with a maximum between 54 and 58°C is the denaturation of myosin and its subunits and some sarcoplasmic proteins. The second transition maximum occurs between 65 and 67°C and is the denaturation of sarcoplasmic proteins, collagen, and some myosin subunits. The third transition maximum occurs between 80 and 85°C and is the denaturation of actomyosin and F- and G-actin.

Much of the DSC data collected has focused on purified collagen (Judge et al. 1981; Bernal and Stanley 1986: Kopp et al. 1989). Findlay and Barbut (1990) caution that thermodynamic data of isolated proteins are different from those found in situ because of the absence of protein-protein interactions. Consequently, thermodynamic data on the interaction of muscle proteins during the cooking of meat cannot be determined through examining the behaviour of isolated proteins in model systems. Nevertheless, DSC is very useful for characterizing thermal changes in proteins that would be obscured in the

complete meat system (Findlay and Barbut 1990).

The extent to which collagen is crosslinked has been related to its thermal stability (Horgan et al. 1990). The crosslinking density of collagen can be assessed by digesting collagen with CNBr and separating the resulting peptides using SDS-PAGE. A decrease in peptide size has been interpreted to indicate collagen degradation (Stanton and Light 1988; 1990a). As well, the efficacy of limited digestion of collagen with pepsin may indicate collagen resistance to proteolysis. Collagen that is less resistant to proteolysis of the telopeptides will exhibit increased concentrations of α-chains on SDS-PAGE, whereas mature collagen displays more β (two crosslinked α-chains) and γ (three crosslinked α-chains) components (Wu et al. 1982). Also, the electrophoretic profiles of the α, β and γ components are characteristic of the collagen types present (Glanville and Kuhn 1979); therefore, collagen types as well as the extent of crosslinking can be identified.

Quantitation of each component on SDS-PAGE gels is traditionally accomplished using a densitometer to estimate band density. The recent development of capillary electrophoresis (CE) has provided researchers with the technology to quantify the collagen components and to detect extremely low concentrations of polypeptides (Deyl et al. 1989).

Collagen exhibits behaviour anomalous to other proteins during SDS-PAGE. The apparent molecular weights following SDS-PAGE are 40% greater than those determined by other methods (Freytag et al. 1979; Butkowski et al. 1982). Electrophoretic mobility of globular and collagenous proteins is more indicative of the number of residues in the polypeptide rather than the molecular weight (Freytag et al. 1979). This large margin of error does not preclude the use of SDS-PAGE to identify collagen types and to separate

its components, but other methods such as equilibrium sedimentation should be used to estimate molecular weight (Freytag et al. 1979).

1.4. COLLAGEN AND MEAT TENDERNESS

Goll et al. (1964a) found that collagen from veal calves released hydroxyproline into solution sooner and at a lower temperature when heated than collagen from steers and aged cows, although total collagen isolated from each age group did not differ. This lead to the hypothesis that collagen crosslinking strengthened during growth, decreasing its susceptibility to heat. Eyre and Oguchi (1980) later supported this hypothesis when they found that concentrations of 3-hydroxypyridinium crosslinks in bovine articular collagen increased with age. As well, they noted that pyridinoline crosslinks were the major crosslink residue detected in collagen from mature skeletal tissues and cartilage. Nakano et al. (1985) found that pyridinoline concentration increased with age in the epimysium of skeletal muscle. These workers postulated that the concomitant increase in body weight and contractile force with age demanded more mechanical strength from existing collagen crosslinks and in response, stable, trifunctional crosslinks developed. Palokangas et al. (1992) also deduced that increased pyridinoline concentrations in skeletal muscle collagen were related more to functional load than muscle fibre composition. Their research, involving various muscle types, showed that pyridinoline concentrations were highest in slow-twitch postural muscles such as the soleus and longissimus dorsi, which are muscles that are constantly loaded. Fast-twitch "mixed" dorsiflexors differed in pyridinoline concentration with functional load, as dorsiflexors exposed to increased muscle stretch

exhibited more pyridinoline crosslinks than those dorsiflexors exposed to less stretch. Horgan et al. (1990) verified changes in crosslink density with load function when their data indicated variation in collagen shrinkage temperature along the length of calf tendons. The tendon from the biceps femoris showed an increase in thermal stability from bone attachment to muscle. Horgan et al. (1990) found strong linear relationships between pyridinoline concentration and both the thermal transition temperature (T_m) (r=.97) and thermal isometric tension (r=0.96). As well, these workers found that Ehrlich's chromagen (EC) was similarly related to T_m (r=.90) and isometric tension (r=.89). From the data, these researchers concluded that EC behaves identically to pyridinoline with respect to thermal stabilization, and that EC and pyridinoline were directly related to thermal response patterns of tendon. Smith and Judge (1991) also found that thermal stability of bovine intramuscular collagen directly paralleled pyridinoline concentration; however, the correlation was not strong (r = .34).

Although thermal lability of collagen has been found to be significantly different between maturity groups and indicative of toughness changes, it does not appear to be indicative of the toughness of beef carcasses within maturity classes. Herring et al. (1967) and Seideman et al. (1987) found that the solubilities of bovine collagen as measured by the method of Hill (1966) were not related to meat toughness within a maturity class. These results suggest that either collagen contributes very little to variations in toughness within a maturity class (Herring et al. 1967; Seideman et al. 1987) or the method of Hill (1966) is not sufficiently precise to detect less pronounced alterations in collagen solubility.

Inevitably, research interest focused on collagen changes during post-mortem aging. Stanley and Brown (1973) found that salt extractability of intramuscular collagen increased and its acid solubility decreased with aging. As well, the amount of guanidine-extracted collagen increased with days post-mortem, indicating that intramuscular collagen was being degraded during aging of the beef. Wu et al. (1982) showed that more α and β components of collagen were released after 24 h of aging as compared to after 12 h of aging, particularly if the beef was aged at 37°C. These researchers, however, did not find a difference in the thermal solubility of collagen from beef aged 12 and 24 h using the method of Hill (1966).

Few researchers have documented an increase in collagen thermal solubility during aging. Herring et al. (1967), using the method of Hill (1966), found that collagen solubility did not increase significantly after 5 days of aging the beef, but that it did increase significantly after 10 days. Mills et al. (1989a) quite definitively demonstrated that bovine collagen solubility increased significantly from 0 to 6 h post-mortem, whereupon the authors interpreted a halt to collagen degradation. The graph of the mean collagen solubilities for each sampling time post-mortem shows an asymptotic curve that indicates steadily increasing collagen solubility to 24 h and that the 6 h mean collagen solubility value is a variant from the curve. The mean collagen solubility values rise from 20% of the total collagen solubilizing at 4 h to 26% at 6 h only to fall to 22% at 8 h. Clearly, the variability of their data precludes their interpretation. A second experiment by Mills et al. (1989b), again measuring collagen solubility at various intervals post-mortem, showed that collagen solubilities increased until 8 h post-mortem, where they

remained at approximately 38% soluble collagen of total collagen. Neither of the experiments of Mills et al. (1989a and b) investigated the heat solubility of collagen past 24 h post-mortem. From the research reviewed, there appears to be no definitive pattern of collagen degradation post-mortem. Clearly, more research exploring the thermal stability of collagen during the early and late post-mortem aging is required.

Research has focused on the effect of collagen type on meat quality. Burson and Hunt (1986) concluded from their work with thermal solubilization of isolated collagen that Type I collagen was more soluble than Type III. They postulated that the intramolecular disulfide bonds of Type III collagen may have increased its thermal stability. Conversely, Stanton and Light (1990a) found that Type III collagen decreased significantly in the endomysium (22%) with aging, suggesting that it may have been more susceptible to postmortem degradation than Type I. Light et al. (1985) found that the proportion of collagen type was not related to meat tenderness.

Solubility research has typically involved the total muscle collagen isolated using non-selective procedures; thus individual contributions of the perimysium and endomysium to toughness are unclear. Light and Champion (1984) developed a method to separate and isolate the perimysium and endomysium from a muscle homogenate. Their method, however, involved using sodium dodecyl sulphate (SDS) to isolate the perimysial and endomysial collagens. Although a very clean collagen preparation resulted, the collagen was no longer in its native state and this limited the subsequent characterization analyses and eliminated the accurate assessment of collagen yields by weight. Stanton and Light (1987) addressed this problem by comparing the efficacy of various solutions at yielding

clean, native collagen. They selected 6 M urea buffered with 0.05 M Tris-Cl, pH 7.4, as the most effective. Unfortunately, the researchers were unable to obtain a clean endomysial preparation without the use of SDS, which prevented them from investigating the endomysium further.

Stanton and Light (1987) successfully isolated the perimysium and found that the perimysium isolated from aged beef yielded more urea-soluble collagen than perimysia derived from unconditioned beef. Evidence for degradation of endomysium during aging was presented by Stanton and Light (1990a). The authors noted that the solubility of the endomysium in 1% SDS increased with aging. Also, after aging, the endomysium not solubilized by urea showed a reduction in high molecular weight material after CNBr digestion, which the authors proposed was indicative of postmortem proteolysis. These results indicated that the perimysium and endomysium are susceptible to endogenous enzyme degradation during aging.

In an earlier study, Stanton and Light (1988) found that perimysium treated with crude spleen extract, deemed by the authors to be primarily catheptic, was extensively degraded as evidenced by a reduction in CNBr peptide size observed using SDS-PAGE. The changes in the electrophoretic profile of the treated perimysium were also shown to be similar to those effected by carcass aging. The authors concluded from their data that the perimysium was susceptible to catheptic action. As well, the authors found that cathepsins acted on the non-triple helical terminals of collagen because the electrophoretic profiles of cathepsin- and pepsin-digested perimysia were very similar. The data of Maciewicz and Etherington (1985) supported this hypothesis by indicating that cathepsins B and L attacked

the telopeptide region of the collagen molecule. Burleigh et al. (1974) showed that cathepsin B acted on the triple helix as well. Kopp and Valin (1980-81) found that collagen incubated in a lysosomal slurry exhibited increased hydrothermal solubility and decreased thermal denaturation temperatures. All of these studies demonstrated that the cathepsins were capable of acting on collagen.

The work of other researchers indicated that, although capable of degrading collagen, cathepsins may not be effective postmortem proteases in slaughter weight animals. Beltran et al. (1992) showed that pure cathepsin B caused the formation of a new denaturation peak in the thermograms of calf collagen at low temperatures, but had no effect on the enthalpy of steer collagen, although it decreased the peak denaturation temperature significantly (3°C). Beltran et al. (1992) also demonstrated that cathepsin L decreased the initial denaturation temperature of calf collagen, but had no effect on steer collagen. Etherington (1984) showed that pure cathepsin B had no effect on collagen tension and speculated that the cathepsins may require the symbotic action of β-glucuronidase to remove the proteoglycans from the collagen surface. Interestingly, Beltran et al. (1992) found that the effect of cathepsin L was enhanced by the addition of 20 mM $CaCl_2$, which prompted a 50% decrease in the denaturation enthalpy of calf collagen. The authors hypothesized that the $CaCl_2$ stripped the proteoglycans from the collagen molecules, exposing the collagen to the cathepsin.

Stanton and Light (1990b) showed that the rate of collagen solubilization was significantly increased by infusing the muscle with lactic acid which lowered the pH early post-mortem. This increased solubilization of collagen following the infusion of an acid

was also shown in work by Etherington (1977), who found that collagen degradation was rapid during the first 5 h following preparation with 0.5 M acetic acid. Etherington (1977) attributed this to the acetic acid swelling the collagen fibres which increased the availability of the telopeptide linkages to the cathepsins and broke acid-labile bonds. Asghar and Yeates (1978) noted in their review that lactic acid could swell collagen fibres, but whether it disrupts acid-labile bonds as well is unknown. Lactic acid in low pH carcasses, such as those that have been electrically stimulated, may increase the thermal lability of collagen (Judge et al. 1981).

Recent research suggests that molecular alterations to collagen occur early in aging (Mills et al. 1989b). These changes coincide with increased tenderness within the first 6 h post-mortem in conventionally-aged beef, a period noted for its collagen degradation by other researchers (Wu et al. 1982). Although cathepsins can clip collagen telopeptides, the early post-mortem muscle conditions would favour calcium-dependent protease activity (calpains). Kang et al. (1981) showed that crude enzyme preparations of calcium-dependent proteases can degrade native collagen. The involvement of calpains in collagen crosslink degradation seems unlikely, however, because collagen is extracellular and calpains are localized intracellularly near the sarcolemma, cytoplasm and Z-disks (Dayton and Schollmeyer 1981). Also, calpains are too large to permeate the muscle cell membrane (Dayton et al. 1976). Prohibitively, the muscle membranes remain intact for most of the early post-mortem period (Greaser 1986), well after the pH has declined into the optimum region for cathepsin activation.

Researchers in the medical field have characterized collagenases that are bound to the

extracellular matrix. Gross and Lapiere (1962) initially isolated a collagenase active at neutral pH from tadpole tail tissue which later researchers isolated from collagen matrices that were actively remodelling (Montfort and Perez-Tamayo 1975; Pardo et al. 1980; Weeks et al. 1976). From this early research, the study of matrix metalloproteinases (MMPs) has burgeoned. Eleven MMPs have been isolated, and seven are well-characterized (Woessner 1991).

Woessner (1991) and Emonard and Grimaud (1990), in their reviews, outlined the common characteristics of the MMP family. The most striking feature of an MMP is the zinc atom in the active site. The MMPs cleave one or more components of the extracellular matrix, and they require extrinsic Ca^{2+} for full activity. They are secreted from connective tissue cells such as fibroblasts as a zymogen, which is activated by other proteinases. In vitro activation is effected by trypsin or plasmin, by organomercurials, HOCl, SDS and chaotropic agents. These agents bind to the enzyme and change its structure, exposing the active site zinc by disrupting the contact between it and cysteine. Full activity of the enzyme is only achieved after the cysteine and zinc link is broken and the enzyme is cleaved by stromelysin (MMP-3) to expose an NH_2-terminal phenylalanine. Activation of the enzyme produces a molecular weight loss of 10 kd.

The interstitial collagenase first discovered by Gross and Lapiere (1962) is now denoted as MMP-1. MMP-1 is a true collagenase, a collagenase being an enzyme that clips the collagen molecule triple helix into two readily denatured parts, TC_A and TC_B. MMP-1 cleaves all α-chains of Types I, II, and III at residues 772-773 (Gly-Ile or Leu), which is three-quarters along the sequence (Emonard and Grimaud 1990). MMP-1 has

been isolated from ligament and tendon, although it is most abundant in organs and involuting uteri (Emonard and Grimaud 1990). MMP-1, because it appears ubiquitous, is most likely responsible for remodelling intramuscular collagen during growth and healing.

Type IV collagen is cleaved by MMP-2, which is also capable of cleaving Types V, VII, and X, as well as fibronectin and elastin (Woessner 1991). It is able to attack denatured Types I, II and III as well (Emonard and Grimaud 1990). This "gelatinase" has not been isolated from muscle tendons or ligaments (Emonard and Grimaud 1990).

MMP-3 degrades proteoglycans, basement membranes, Type IV collagen, laminin and fibronectin (Woesnner 1991). It is also a procollagen peptidase for Types I, II and III and has some activity against elastin (Emonard and Grimaud 1990). It has been found in ligament and tendon and appears to work in concert with MMP-1 as an activator of the MMP-1 zymogen (Emonard and Grimaud 1990).

MMP-4 cleaves the α_1-chain of Type 1 collagen in the C-terminal telopeptide between the triple helix and Lys-17 which is involved in intermolecular crosslinking (Nakano and Scott 1987). Nakano and Scott (1987) found it also digested fibronectin, facilitating its attack on the collagen molecule.

MMP-5 works in concert with MMP-1 by degrading the three-quarter collagen fragments resulting from MMP-1 cleavages of Types I, II and III (Emonard and Grimaud 1990). MMP-5 has been found in the gingiva and muscle ligaments (Emonard and Grimaud 1990) so it may exist intramuscularly as well.

The remaining MMPs are not well-characterized, nor do they appear to be important

in the intramuscular extracellular matrix.

MMPs are not specific in their activity, and Woesnner (1991) noted that almost all MMPs cleave the octapeptide sequence GPQGIAGQ. Their lack of specificity makes MMPs very dangerous if uncontrolled; consequently, all MMPs are inhibited by Tissue Inhibitor of Metalloproteinases (TIMP), a 28,500 d, glycosylated protein with 2 distinct domains (Woessner 1991). MMPs are inhibited by α_2-macroglobulin as well, which irreversibly binds to the collagenases to prevent them from cleaving collagen (Woessner 1991). Their lack of substrate specificity may be why the MMPs are not stored in most cells, except neutrophils and macrophages, and are not synthesized until induced.

The MMPs have been almost completely ignored by meat researchers. Brady et al. (1991) found that bovine placenta collagenase was very active at the pH and temperature f post-mortem muscle and the authors suggested that it could be used for meat tenderization. Bernal and Stanley (1986) administered a bacterial collagenase to bovine tendon collagen and found that thermal denaturation temperature decreased by 18°C. Suzuki et al. (1985) found evidence of a collagen-bound collagenase in rabbit skeletal muscle; however, no literature was found showing isolation of collagenase from bovine intramuscular tissue. The application of MMP to the degradation of collagen in beef during aging may warrant investigation.

1.5. EXTENDED STORAGE OF BEEF

Recently, extended storage of fresh beef has been made possible using Controlled Atmosphere Packaging (CAP). With CAP, fresh beef can be stored in carbon dioxide at

-1.5 ± 0.5°C for longer than 15 weeks (Gill 1990). The carbon dioxide atmosphere, maintained in foil laminate bags, retards the growth of gram-negative bacteria such as enterobacteria and lactobacilli populations, thus delaying spoilage (Gill and Penney 1988). Gill and Penney (1988) found that 2 L of CO_2 per kg of beef provided the longest viable storage at 1°C. At this temperature, gross spoilage of normal pH beef occurred after 24 weeks of storage, and high pH beef exhibited off-flavours after 7 weeks storage and gross spoilage by 15 weeks. Gill and Penney (1990) defined the optimal storage temperature to be -1.5°C for CAP beef.

Preliminary observations of CAP beef showed that the meat developed fissures and caverns along and between bundles of muscle fibres upon cooking (Gill and Penney 1990). This porous appearance was apparent only when the meat was cooked and only if it was stored for 24 h or more at an equilibrium partial pressure of carbon dioxide of approximately 40% or greater (Gill and Penney 1990). The porosity was most obvious in meat exposed to moderate cooking temperatures, with the pores occurring between the muscle fibre bundles along the perimysia (Gill and Penney 1990). Gill and Penney (1990) observed "membranes" torn in the caverns, with some pockets containing fluid. Gill and Penney (1990) suggested that the pores were due to an ionic effect of the dissolved CO_2 that solubilized the muscle proteins. This seems unlikely because myofibrillar damage would be more homogenous. Clearly, this issue needs to be resolved prior to marketing CAP beef in order to facilitate consumer education.

Controlled atmosphere packaging may be of great benefit to the beef industry. This technology will permit transport of fresh beef globally by sea rather than by air, thus

reducing expenses and eliminating discrimination faced by frozen beef exports. Also, CAP will facilitate a constant supply of fresh beef to the market, improving inventory and price controls. Despite its many possible advantages, the effect of CAP on the development of beef tenderness is not well-characterized. Understanding this process is crucial because the increased exposure of muscle proteins to proteolytic enzymes with extended aging may affect beef quality and subsequent consumer acceptance.

Controlled atmosphere packaging provides a scientific model from which to glean information regarding the aging of beef collagen. Few researchers have documented post-mortem changes in collagen solubility using extended aging. Sharp (1963) stored rabbit and bovine muscle aseptically at 37°C for 6 months, with no changes in collagen solubility in 0.1 M KCl solution. Sharp (1963), however, did not investigate the thermal lability of the collagen. Clearly, CAP technology presents an opportunity to investigate the structural changes in collagen in the late post-mortem period.

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2. POROSITY IN COOKED BEEF FROM CONTROLLED ATMOSPHERE PACKAGING IS CAUSED BY RAPID CO_2 GAS EVOLUTION

2.1. INTRODUCTION

Extended storage of fresh beef is possible through controlled atmosphere packaging (CAP)(Gill 1990). CAP, using carbon dioxide (1000 to 2000 mL · kg^{-1} meat), reduces the growth of spoilage bacteria by depriving aerobic bacteria of oxygen (Gill 1990; Gill and Penney 1988). Beef packaged in CAP can be stored unspoiled for up to 15 weeks for high pH beef (greater than pH 6.0) and 21 weeks for normal pH beef (pH 5.5 to 5.7)(Gill and Penney 1988).

Beef cooked after packaging in carbon dioxide develops pores between muscle fibres bundles along the perimysia (Gill and Penney 1990). This porous appearance is apparent only after the meat is cooked and only if it is stored for one day or more at an equilibrium partial pressure of carbon dioxide of approximately 40% or greater. Gill and Penney (1990) observed "membranes" torn in the caverns, with some pockets containing fluid.

Gaseous carbon dioxide dissolves readily in water, the carbon dioxide combining with water to produce carbonic acid (H_2CO_3) and/or bicarbonate (HCO_3^-)(Asada 1982). The pK_a of this reaction is 6.35 (Asada 1982). Bicarbonate then dissociates to carbonate (CO_3^{2-}), with a pK_a of 10.32 (Asada 1982). In water equilibrated with air, at standard temperature and pressure, at a pH of 5.5, the primary species of carbon dioxide is aqueous carbon dioxide (11.2 uM), with 1.58 uM of bicarbonate and 0.029 uM of carbonic acid (Asada 1982). At pH 6, the concentration of bicarbonate rises to 5.01 uM and at pH 6.5, it exceeds the concentration of aqueous carbon dioxide as it rises to 15.8 uM (Asada

1982). Hence, in post-rigor muscle with a pH below 6.3 stored in CAP, the primary species should be aqueous carbon dioxide, although the concentrations of each species should be substantially greater than those found in a system equilibrated with air because of the high equilibrium partial pressure of a pure CO_2 atmosphere.

Rapid evolution of carbon dioxide during cooking could cause pore formation, although Gill and Penney (1990) thought this unlikely. These authors predicted that bicarbonate would be the predominant phase of carbon dioxide present, and that it would dissolve muscle proteins to produce the pores. Collagen is a protein very resistant to dissolution in weak acid and salt solutions unless it is immature; consequently, bicarbonate solubilizing collagen seems unlikely. A more obvious hypothesis is that the porous appearance of cooked, CAP beef results from the rapid evolution of carbon dioxide gas due to the heat of cooking. This paper presents a series of experiments that were designed to investigate the effect of bicarbonate on beef in order to determine how storage in carbon dioxide contributes to pore formation during cooking.

2.2. MATERIALS AND METHODS

All samples were purchased from a local supermarket. The pH of bovine longissimus muscle (striploin) was measured at four points along its length with a glass electrode (Fisher Accumet Model 320, Fisher electrode 13-620-271, Fisher Scientific, Edmonton, Alberta). The muscle was cubed into twelve portions of relatively equal size (96.67 $\pm$ 10.08 g S.D.), and packaged in vacuum (control), N_2 (inert gas, laboratory grade)(Canox, Edmonton, AB)(approximately 10 mL $N_2 \cdot g^{-1}$ muscle), or carbon dioxide (laboratory

grade)(Canox, Edmonton, AB)(approximately 10 mL $CO_2 \cdot g^{-1}$ muscle), with four portions per treatment. Samples were packaged using a Multivac (Sepp Haggenmuller KG, West Germany) and stored for 70 h at 3 to 4 °C. Packages were then opened and pH of the meat samples measured. Samples were baked at 175°C to an internal temperature of 80°C, allowed to cool to a comfortable handling temperature, then sliced transversely to expose the internal structure and photographed.

In a second experiment, intact sections of bovine semitendinosus (eye of round) and psoas major (tenderloin) were sliced into nine portions (semitendinosus, 106.8 ± 13.5 g S.D.; psoas, 91.6 ± 20.1 g S.D.). Three portions of each muscle were vacuum-packaged (control), while six portions were packaged in carbon dioxide (approximately 10 mL $CO_2 \cdot g^{-1}$ muscle). Three of the samples packaged in carbon dioxide were unpackaged after 48 h, vacuum-packaged and stored for a further three days. All samples were then unpackaged, baked at 175°C to an internal temperature of 80°C, cooled, sliced transversely and photographed.

In a third experiment, a bovine semitendinosus (eye of round) was cut into nine samples (36.74 ± 12.0 g S.D.). Two of the samples were bagged in carbon dioxide gas (approximately 27 mL $CO_2 \cdot g^{-1}$ muscle) and one was vacuum-packaged to serve as the control. These samples were stored for three days at 3 to 4°C. Of the remaining six samples, two were placed in 0.01 M Na_2HPO_4, pH 5.6, in a 500 mL brown, screw-top bottle, while four were placed in identical bottles with 0.25 M $NaHCO_3$ (sodium bicarbonate), into which HCl was quickly added just before the bottle was capped to reduce the pH to approximately 5.6. This procedure produced a pressurized environment

under which the carbon dioxide would equilibrate. After three days of storage at 3 to 4°C, one buffered control and two sodium bicarbonate solution bottles were opened and the meat pieces baked at 175°C to an internal temperature of approximately 77°C to assess the porosity. The remaining bottled and bagged samples were immersed in distilled water and returned to refrigerated storage. The pH of all solutions emptied from the bottles during dialysis changes were recorded. The samples were dialyzed for 48 h with three changes of distilled water in order to remove the dissolved carbon dioxide. Following dialysis, the six samples were baked at 175°C to an internal temperature of approximately 77°C, cooled, sliced transversely and evaluated for pore formation.

In a fourth experiment, two bovine semitendinosus muscles (eye of round) were cut into thirty-six samples (37.9 ± 8.8 g S.D.). Each sample was randomly assigned to one of nine treatments: 1) immersion in 0.01 M phosphate buffer solution (PBS), pH 5.6, for 72 h; 2) immersion in 0.01 M PBS, pH 5.6, for 72 h and then dialysis against distilled water; 3) immersion in 0.25 M $NaHCO_3$, pH 5.6, for 72 h; 4) immersion in 0.25 M $NaHCO_3$, pH 5.6, for 72 h and then dialysis against distilled water; 5) storage in CO_2 (approximately 27 mL $CO_2 \cdot g^{-1}$ muscle) for 72 h; 6) storage in CO_2 (approximately 27 mL $CO_2 \cdot g^{-1}$ muscle) for 72 h and then dialysis against distilled water; 7) vacuum-packaged storage for 72 h; 8) vacuum-packaged storage for 72 h and dialysis against distilled water; or 9) storage in N_2 (approximately 27 mL $N_2 \cdot g^{-1}$ muscle) for 72 h. Samples soaked in sodium bicarbonate were stored in 500 mL, screw-cap bottles. Phosphate buffer samples were stored in covered beakers. Samples stored in CO_2, vacuum or N_2 were packaged on the Multivac.

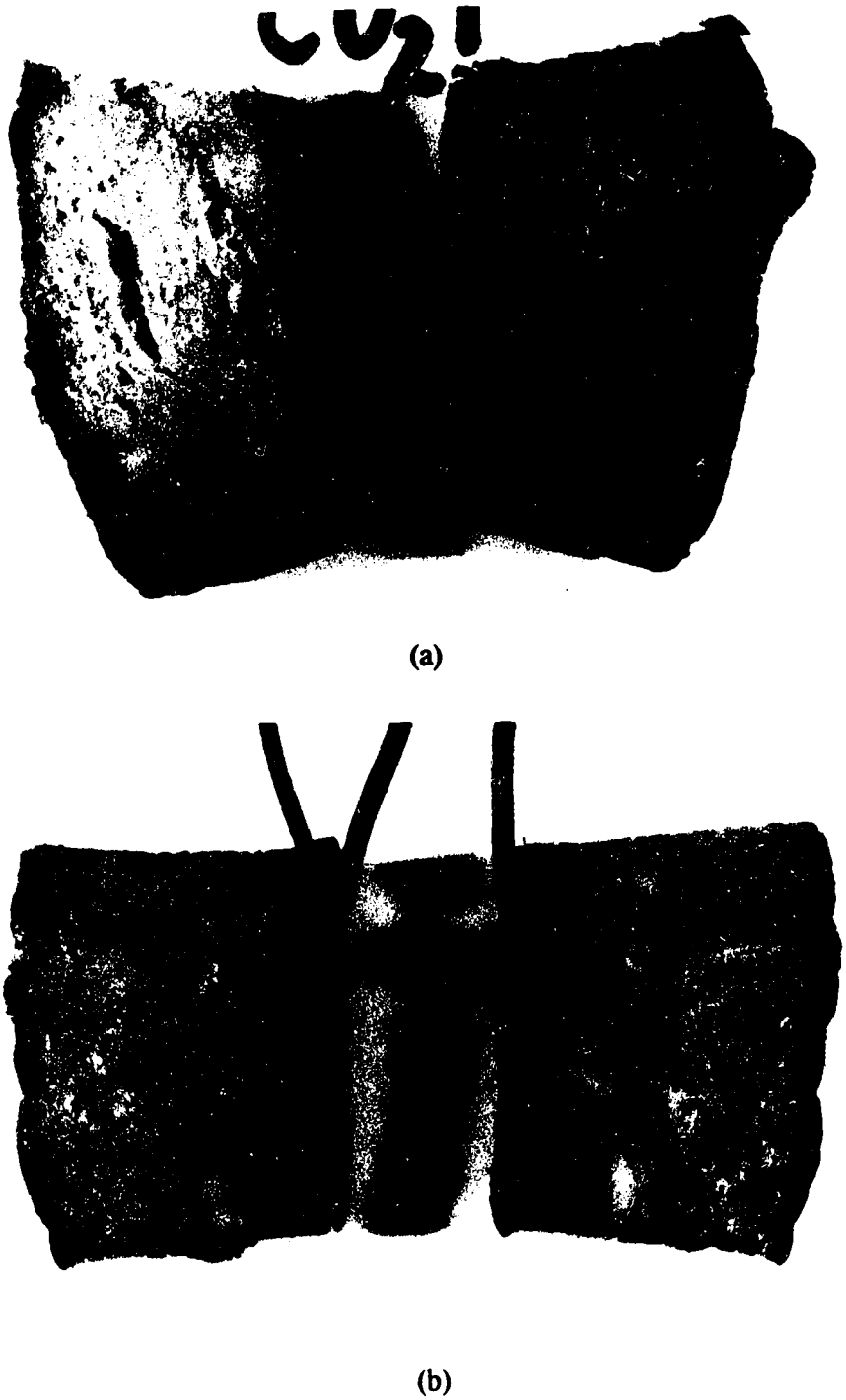
Following treatment, samples were vacuum-packaged and the volume measured by displacement of deionized water. The samples were cooked for 45 min in boiling water while in the bag. The displacement was measured following cooking, and the volume difference determined. Meat samples were then removed from the package, sliced transversely to observe pore distribution and photographed.

Volume difference data were expressed in $mL \cdot g^{-1}$ of uncooked sample and analyzed using the Statistical Analysis System (SAS Institute 1989). The data were found to be distributed normally (W=0.43, ranked data). Analysis of variance was performed on the data and differences between means of treatments that showed significant effects were assessed using Student-Newman-Keuls multiple range test. One-way regression was performed to investigate the relationship between sample fresh weights and volumes of gas generated during cooking in the sodium bicarbonate and CO_2-packaged samples.

2.3. RESULTS AND DISCUSSION

Beef packaged in carbon dioxide clearly exhibited pores once cooked (Plate 1a). Vacuum-packaged beef showed no evidence of pore development (Plate 1b). Cracking exhibited by meat samples appeared to be due to drying of the perimysium through overcooking. Beef sealed in nitrogen appeared similar to beef packaged with conventional vacuum. There was no significant effect of packaging atmosphere on pH ($P > 0.05$)(data not shown).

In the second experiment, meat samples were vacuum-packaged following 72 h of exposure to CO_2 to investigate whether vacuum storage would allow the CO_2 to withdraw



(a)

(b)

Plate 1. Cooked appearance of beef exposed to various storage treatments. Beef was stored in (a) CO_2; (b) vacuum-packaging.

from the meat. Vacuum-packaging the muscle following packaging in CO_2 did not reduce the pore formation of the samples. This indicated that CO_2 was dissolved in the aqueous fraction of the muscle or bound to muscle proteins (Mitsuda et al. 1975), as it would have left the meat under vacuum if it were present as a gas. The semitendinosus, which is primarily white fibres, showed pore formation more clearly than the psoas major, which is predominantly red. The psoas major developed cracks and fissures along the perimysia in control and treated samples; however, the cracks in the control samples were not as extensive as in the treated samples. None of the treatments affected the pH of the raw meat ($P > 0.05$)(data not shown).

In the third experiment, immersion of beef in 0.25 M $NaHCO_3$ in an enclosed container produced pores in the cooked sample (Plate 2a). Meat soaked in sodium bicarbonate or packaged in CO_2 and then dialyzed for 72 h did not show any pore formation (Plate 2b). The pH of the extracting or dialyzing solution decreased with each dialysis change even in controls (Fig. 2.1) as would be expected because of the release of H^+ ions from carbonic acid dissociation. These results indicated that the production of pores within the cooked meat was not due to an ionic solubilization of muscle proteins as suggested by Gill and Penney (1990), but rather an evolution of gaseous carbon dioxide during cooking, which gathered at the perimysial seams where fat may have been also. Carbon dioxide becomes more soluble in fat than aqueous solution as temperature is increased (Gill 1988). Also, the pores may be located at the perimysia because it is the weakest structure in cooked meat (Purslow 1985).

To disprove that the porous appearance may have been due to CO_2 gas evolution during

(a)

(b)

Plate 2. Cooked appearance of beef exposed to (a) 0.25 M $NaHCO_3$, pH 5.6, no dialysis; (b) 0.25 M $NaHCO_3$, pH 5.6, then dialyzed with distilled water.

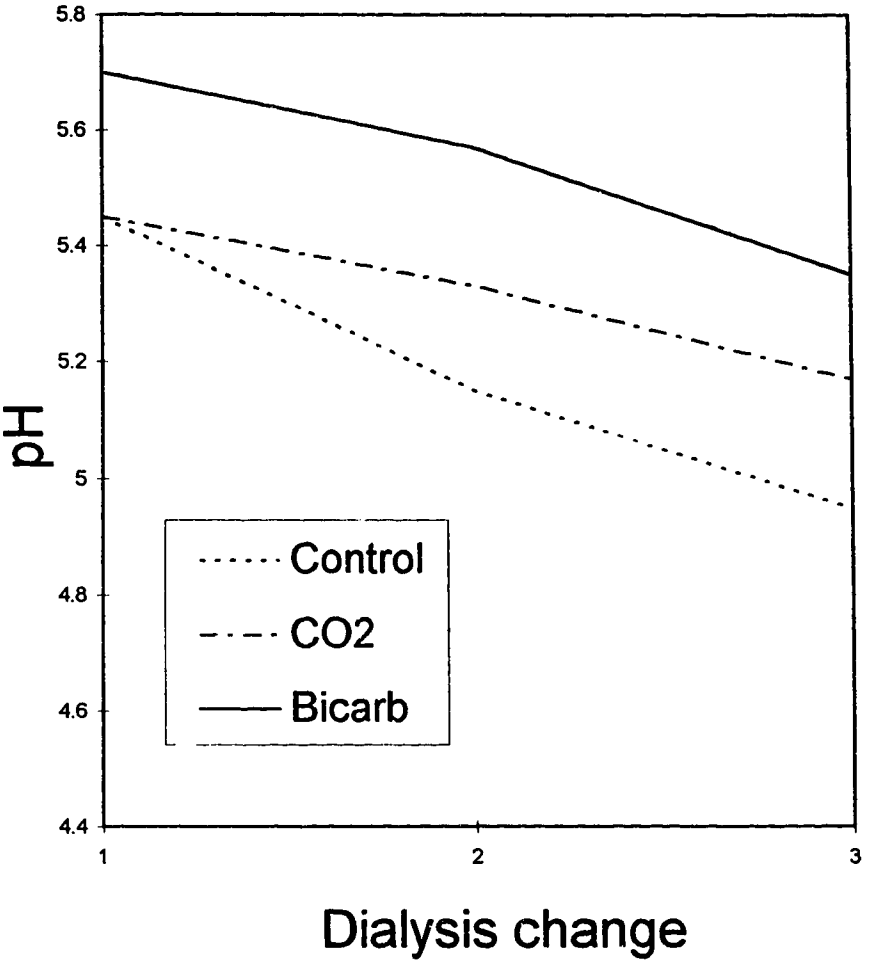


Figure 2.1. Changes in dialysis solution pH of samples from various treatments during dialysis. Treatments were: ----- control (vacuum-packaged); -·-·- CO_2 packaged samples; —— 0.25 M $NaHCO_3$, pH 5.6.

cooking, Gill and Penney (1990) allowed CO_2 to dissipate from meat previously packaged in 100% CO_2. By day four of dissipation, the meat was not releasing carbon dioxide in quantities different from that of fresh, unpackaged meat, suggesting that CO_2 levels in the meat samples were comparable. Upon cooking, however, this meat still produced a porous cooked product. Gill and Penney (1990) concluded that the pores were not a result of rapid gas expulsion during cooking but were from the denaturation of proteins holding adjacent perimysial sheaths together. The formation of bicarbonate was thought by them to denature the proteins, increasing the exudate found in the fluid pockets. Then, lateral shrinkage of the connective tissue proteins at 60°C (cooking temperature) caused pores to form along the perimysium. At higher temperatures, longitudinal shrinkage would occur, which would reduce pore size and make porosity less pronounced in the well-cooked periphery of meat and not visible in the centres that remained raw. If, however, the bicarbonate solubilized the muscle proteins, then the effect would have persisted following removal of the bicarbonate. This hypothesis was tested in our third experiment and found to be null, as indicated by the absence of pores following dialysis. Gill and Penney (1990) tested a similar hypothesis by allowing the CO_2 to escape as a gas to equilibrate with the atmosphere while in 12% perchloric acid. This method may not have depressed the pH to a level sufficient to drive all the aqueous CO_2 to the gas phase or the acid simply may not have permeated the meat completely. The depth to which the acid vapors penetrated the meat should have been visible in a cross-section of the muscle sections because the acid would have denatured the proteins to that depth, but Gill and Penney (1990) did not consider this. Our approach removed the CO_2 not as a gas but as aqueous CO_2 and

bicarbonate, the states in which it would be expected to be in a solution at pH 5.5 (Asada 1982).

Finally, in the fourth experiment, the gas evolved from the meat when cooked was estimated. As expected, the samples packaged in CO_2 or soaked in sodium bicarbonate produced significantly larger amounts of gas $\cdot g^{-1}$ sample than those of the other treatments ($P < 0.0001$)(Fig. 2.2). Dialysis of the beef samples packaged in CO_2 produced a result identical to dialysis of the sodium bicarbonate-treated samples in that it reduced gas evolution to values comparable to phosphate-soaked and vacuum-packaged samples. The absence of gas production and pore formation in the samples that had been exposed to CO_2 or sodium bicarbonate and subsequently dialyzed further supported the experimental hypothesis. There did not appear to be a significant relationship between gas evolved and sample weight, possibly because of differences in fibre type proportions throughout the samples.

Interestingly, meat packaged in CO_2 often had a frothy, bubbly exterior during cooking, which was not noted by Gill and Penney (1990), but was regularly associated with pore formation in our experiments. Conclusive evidence of the presence or absence of CO_2 in all treatments is not claimed in this paper or any other because of the difficulty of preparing an enclosed apparatus for total gas entrapment and quantitation. Nevertheless, these data present evidence that strongly suggests that pore formation in beef packaged in CO_2 is not a by-product of bicarbonate solubilizing structural proteins but that of rapid evolution of CO_2 gas during cooking.

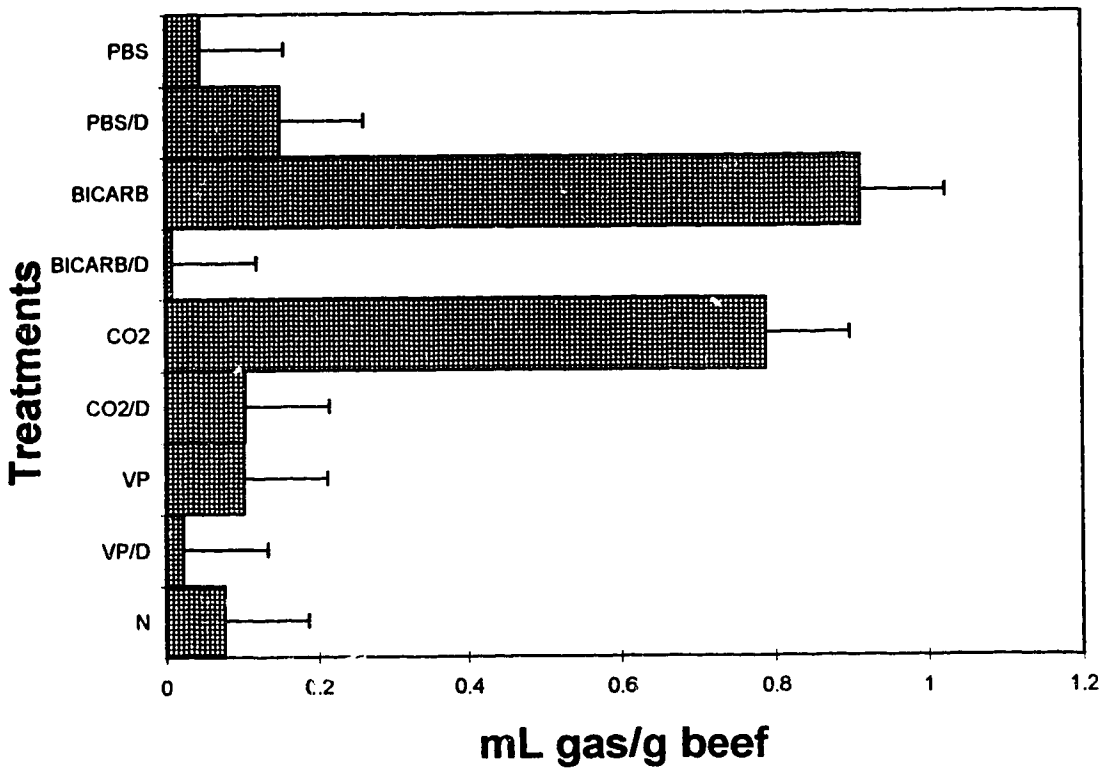


Figure 2.2. Gas evolved (mL · g^{-1} sample) by meat during cooking after various treatments. Treatments are: PBS, phosphate buffered solution, pH 5.6; PBS/D, phosphate buffered solution, pH 5.6, then dialyzed with dH_2O; BICARB, 0.25 M $NaHCO_3$, pH 5.6; BICARB/D, 0.25 M $NaHCO_3$, pH 5.6, then dialyzed with dH_2O; CO2, CO_2 gas atmosphere; CO2/D, CO_2 gas atmosphere then dialyzed in dH_2O; VP, vacuum-packaged; VP/D, vacuum-packaged then dialyzed in dH_2O; N, nitrogen gas atmosphere.

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3. MODIFICATIONS TO BOVINE INTRAMUSCULAR COLLAGEN DURING EXTENDED STORAGE OF BEEF IN CONTROLLED ATMOSPHERE PACKAGING

3.1. INTRODUCTION

Extended storage of fresh beef has been accomplished by depressing oxygen levels to less than 200 ppm with controlled atmosphere packaging (Gill 1990). With this technology, beef can be stored in a carbon dioxide atmosphere at -1.5 $\pm$ 0.5°C for up to 24 weeks with no bacterial spoilage (Gill 1990). This preservation system has not yet been widely accepted in the industry, possibly because of anticipated consumer dissatisfaction with the discoloration that beef exhibits following storage in CO_2 (Silliker et al. 1977). Nevertheless, because controlled atmosphere packaging reduces bacterial spoilage and the low storage temperature slows aging, this preservation system provides a model for the investigation of the effects of endogenous proteolytic enzymes on muscle proteins during aging.

One of the proteins that may be affected by proteolytic enzymes during aging of beef is collagen. Collagen, the major component of connective tissue, usually accounts for approximately 2% of muscle protein (Bailey and Light 1989). As collagen contributes the majority of background toughness of beef (Bailey 1972), its degradation during the aging of beef may decrease beef toughness. Collagen from aged beef has been found to have increased chemical and thermal solubility (Stanley and Brown 1973; Herring et al. 1967; Mills et al. 1989a,b; Stanton and Light 1987) and increased thermal lability (Judge and Aberle 1982), characteristics which have been interpreted as evidence of proteolytic

degradation. In contrast, other researchers have reported no change in the salt extractability (Chizzolini et al. 1977), crosslinking (Wu et al. 1982) or heat solubility (Jeremiah and Martin 1981) of beef collagen following aging. The attempt to clarify the effect of carcass aging on bovine collagen has led several researchers to investigate the various types of collagen that exist in beef muscle. Recently, they have begun to characterize the perimysium and endomysium from beef muscle (Light and Champion 1984; Stanton and Light 1987; 1988; 1990). The perimysium, which is the connective tissue surrounding the muscle fibre bundles, has been shown to contain Types I and III collagens, while the endomysium, which is the connective tissue around each muscle fibre, has been shown to contain primarily collagen Types I, III and IV (Duance et al. 1977). Stanton and Light (1987) showed that the solubility of the perimysium in urea increased with aging ($P = 0.1$). Due to the difficulty of obtaining clean endomysial preparations, Stanton and Light (1990) did not draw strong conclusions concerning the changes in the endomysium during aging, although they noted that the changes appeared to be similar to those found in the perimysium. The results of Stanton and Light (1990) indicated that Type III collagen was preferentially degraded relative to Type I. Clearly, the effect of aging on the perimysium and endomysium of beef requires further characterization in order to understand the contribution of collagen degradation to beef toughness.

The objective of this experiment was to investigate changes in solubility, thermal lability and structure of the perimysial and endomysial collagens during the aging process using CAP technology to extend aging for periods of up to 4 months.

3.2. MATERIALS AND METHODS

3.2.1. Storage

Eight Grade A beef steer carcasses were selected from Gainers Inc., Edmonton. Four of the carcasses had 24 h post-mortem pH values of 5.6 and were designated normal pH carcasses. The remaining four carcasses had pH values between 5.8 and 6.0, and were designated high pH carcasses. Approximately 48 h post-mortem, the sixteen loins (2.92 kg $\pm$ 0.09 kg S.E.) were separated from the rib sections and cut into 48 roasts (0.973 kg $\pm$ 0.017 kg S.E.). Approximately 72 h post-mortem, the roasts were placed in aluminum foil laminate bags that were filled and evacuated twice with 100% CO_2 gas by the Captron III (RMF, Grandview, MO) at Lacombe Research Station (Lacombe, Alberta) before filling the bags to a final concentration of 3 L $CO_2 \cdot kg^{-1}$ muscle. Five roasts from each pH designation were randomly assigned to a storage period of 4, 8, 12 or 15 weeks, and eight roasts were designated week 0 controls. Oxygen content of the packaging atmosphere was monitored by extracting bag atmosphere into a syringe plunged through a glue septum and analyzing it with a Mocon oxygen headspace analyzer (Modem Controls, Inc., Minneapolis, MN).

3.2.2. Isolation of Collagen

Roasts were removed from the aluminium foil laminate bags at 4°C after the allotted time of storage. Each roast was halved longitudinally and one half was trimmed free of epimysial tissue, sliced into 2 mm square cubes, vacuum-packaged and frozen at -30°C. Samples were thawed at 4°C for isolation of collagen and total wet weight of each chopped muscle sample was recorded.

Perimysium and endomysium were isolated using a modification of the method of Light and Champion (1984), with the endomysium separated from the myofibrillar proteins using the method of Helander (1957). Muscle pieces were combined with 150 mL of Hasselbach-Schneider (HS) solution (0.6 M KCl, 0.01 M $Na_4P_2O_7$, 0.001 $MgCl_2$, in 0.1 M KH_2PO_4, pH 7.4) in 50 mg lots and blended in three 3 second bursts. The blended homogenate was then filtered through a 1 mm square sieve, and the filtrate retained. The residue collected on the filter was combined with 150 mL of HS solution and blended again. The residue that remained on the sieve was designated perimysium and was rinsed in distilled water before drying on Whatman Filter Paper No. 4 (Fisher Scientific, Edmonton, Alberta). The endomysial collagen, still in the myofibrillar debris of the combined filtrates, was centrifuged at 2460 x g and the supernatant strained through cheesecloth before discarding. The precipitate was suspended in approximately 150 mL of 1.1 M KI, 0.1 M KH_2PO_4, pH 7.4, overnight with constant stirring during the extraction at 4°C. The extract was centrifuged at 3840 x g for 20 min and the supernatant strained through three layers of cheesecloth to trap floating collagen before being discarded. The trapped collagen was returned to the precipitate. The precipitate was extracted twice more for 3 h and 2 h, respectively. The resulting pellet was rinsed with 0.025 M NaCl, 0.002 M L-histidine, 0.01 M EDTA, pH 7.4, then immersed in distilled water before drying on Whatman Filter Paper No. 4 (Fisher Scientific, Edmonton). Following drying, perimysium and endomysium samples were stored at -30°C until further analysis.

3.2.3. Hydrolysis Time Determination

Two samples were taken from both the perimysium and endomysium. One sample was from a roast stored for 0 weeks and the other was from a roast stored for 12 weeks. All collagen samples were from the same animal, with the week 0 samples originating from one roast and the week 12 samples from another. Approximately 50 mg of lyophilized perimysium and endomysium were hydrolyzed in duplicate for 9, 12 and 24 h in 6 M HCl at 110°C in a test tube fitted with a teflon-lined cap. Test tubes were flushed with N_2 prior to capping and hydrolysis. Acid was removed from the samples by rotary evaporation (Buchi Rotovapor-R, Brinkeman Instruments, Rexdale, Ontario) at 45°C. The samples were reconstituted in 10 mL distilled water, neutralized with 1 N NaOH and analyzed for hydroxyproline concentration using the spectrophotometric method of Bergman and Loxley (1963) as described in Section 3.2.8. Results were expressed as percentage collagen found in the hydrolyzates by multiplying the percentage of hydroxyproline · g^{-1} dry collagen by 7.14 (Bailey and Light 1989).

3.2.4. Total Hydroxyproline Content

Perimysial and endomysial collagen samples were analyzed for total hydroxyproline content to assess their purity and to allow expression of heat solubility on the basis of g heat-soluble hydroxyproline/g hydroxyproline. Approximately 10 mg of lyophilized perimysium and endomysium were hydrolyzed in duplicate for 9 h in 6 N HCl at 110°C. Acid was removed by rotary evaporation and each sample was reconstituted to 5 mL with distilled water and neutralized with 6 N NaOH. The hydroxyproline concentration of each sample was estimated using the spectrophotometric method of Bergman and Loxley (1963)

as described in Section 3.2.8. Results were expressed as g soluble hydroxyproline · g^{-1} dry residue.

3.2.5. Nitrogen Content

Nitrogen content of perimysium and endomysium samples from roasts stored for 0 and 15 weeks was estimated using the Leco Nitrogen Analyzer (Model FP-428, Leco Corporation, St. Joesph, MI). Briefly, the sample is rapidly burned at 850°C in pure oxygen. Nitrogenous products (NO_x) were converted to N_2 through contact with hot copper and then separated from water and CO_2. The nitrogen was then measured by a thermal conductivity cell and expressed as percent nitrogen.

3.2.6. Heat Solubility Analysis

Heat solubilities of perimysial and endomysial collagens were estimated using the method of Goll et al. (1964). Approximately 300 mg of perimysial collagen (wet weight) and 50 mg of lyophilized endomysium were placed in 50 mL capped test tubes, two replicates for each roast. Forty mL of 0.1 M sodium phosphate, pH 7.0, were added to the perimysium and endomysium and the tubes were allowed to equilibrate at room temperature for 1 h. Three mL were taken from each test tube after equilibration before the test tubes were placed in a boiling water bath (96.5°C). Three mL fractions were removed from the test tubes after 15, 30, 60, 120 and 240 min in the water bath. The 3 mL fractions were placed in test tubes that were pre-cooled in crushed ice and stored at -30°C following completion of each sampling period until analyzed for hydroxyproline concentration as described in Section 3.2.8.

3.2.7. Hydrolysis of Soluble Collagen

One mL of heat-soluble collagen solution was combined with 1 mL of 12 N HCl and 3 mL of 6 N HCl in a test tube fitted with a teflon-lined cap. The test tubes were flushed with nitrogen prior to capping and were hydrolyzed for 9 h at 110°C. A preliminary experiment deemed 9 h as the hydrolysis time which allowed for maximum release of hydroxyproline from soluble collagen. Following hydrolysis, the tubes were cooled with cold water to room temperature. Hydrolysates were stored at -30°C prior to drying on a rotary evaporator at 45 °C, after which they were reconstituted to 5 mL with distilled water. The reconstituted sample was then neutralized with 6 N NaOH and frozen at -30°C until analyzed for hydroxyproline concentration as described in Section 3.2.8.

3.2.8. Hydroxyproline Analysis

Hydroxyproline concentrations of hydrolysates were estimated using the spectrophotometric method of Bergman and Loxley (1963). One mL of each neutral hydrolysate was used in the assay, in which the hydroxyl group of hydroxyproline is oxidized by chloramine-T and then allowed to react with p-dimethyl-amino-benzaldehyde to form a coloured product. The absorbance of each sample was read within 4 h at 558 nm on a Hewlett-Packard 8452A Diode Array Spectrophotometer (Hewlett-Packard, Toronto, Ontario). Hydroxyproline concentrations of samples were determined by comparing sample absorbancies to those of dilutions from a trans-4-hydroxy-L-proline standard (Sigma Chemical Co., St. Louis, Missouri).

3.2.9. Differential Scanning Calorimetry

The amount of water required for adequate hydration of collagen during thermal

analysis was determined using perimysium from roasts from the same animal stored for 0 and 15 weeks. The denaturation of perimysium was characterized between 35 and 110 °C at three moisture ratios: 1 mg collagen to 2.5 uL distilled water; 1 mg collagen to 5 uL distilled water; and 1 mg collagen to 7.5 uL distilled water. For each measurement, the dry collagen was weighed into an aluminum pan prior to the addition of water. Samples were sealed to prevent moisture loss and analyzed on a Du Pont 910 Differential Scanning Calorimeter (Du Pont, Wilmington, DE). Peak denaturation temperature (T_m) and enthalpy (ΔH) were recorded for three replicates of each treatment (Du Pont 990 Thermal Analyzer, Du Pont, Wilmington, DE). Enthalpy was calculated using the equation $\Delta H = (A/m)(60BE\Delta qs)$, where A is the peak area in cm^2, m is the sample mass in mg, B is the time base setting in min/cm, E is the cell calibration coefficient, Δqs is the Y-axis range setting in mV/cm.

Hydration time for collagen was assessed using a week 0 perimysial sample from a normal pH roast. The perimysium was soaked in distilled water and the pH adjusted to 7.4 before it was lyophilized and stored at -30 °C. Three replicates, each of approximately 1.5 mg of lyophilized perimysium sealed in aluminum pans with 11.5 uL of distilled water, were allowed to hydrate for 0, 30, 60, 120, 240 and 480 minutes at room temperature. Following hydration, the thermal denaturation curve of each sample was estimated between 35 and 100 °C using the Du Pont 910 DSC and recorded on the Du Pont 990 DTA.

Each endomysium sample was immersed in a covered beaker filled with distilled water and surrounded by crushed ice for 4 h at 4 °C. The pH of each sample was adjusted to pH

7.4 using NaOH and HCl, and the samples were allowed to equilibrate for 2 h at 4°C. The endomysium was then lyophilized and ground to small fragments with a glass rod. Approximately 1 mg of lyophilized endomysium was placed in an aluminum calorimeter pan (Westech Industrial Ltd., Calgary, Alberta) and 7.5 uL of distilled water added. Each sample was sealed with a hand press and allowed to hydrate for 1 to 2 h. The thermal denaturation curve of each sample was obtained using the Du Pont 910 DSC and was recorded between 35 and 110°C on the Du Pont 990 DTA. Three analyses were performed on perimysium and endomysium from each roast.

Collagen standards of Types I (acid-insoluble), III and IV (both acid-soluble)(Sigma Chemical Co., St. Louis, Missouri) were analyzed using the Du Pont 910 DSC, and the thermogram between 35 and 100°C recorded on the Du Pont 990 DTA. Approximately 1 mg of sample was combined with 10 to 15 uL of 0.025 M NaCl, 0.0025 M L-histidine buffer, pH 7.4, and hermetically sealed prior to thermal analysis. Three analyses of each standard were recorded.

3.2.10. Preparation of Collagen Types

Collagen types were separated by modifying the methods outlined by Miller and Rhodes (1982). Approximately 50 mg of lyophilized perimysium or endomysium were mixed with 10 ml of 0.5 M acetic acid containing 10 mg of pepsin. Pepsin was allowed to digest the non-helical portions of the collagens for 24 h at 4°C with constant stirring. The samples were then centrifuged (43,700 x g) and the precipitate discarded. The soluble collagen was precipitated overnight from the 0.5 M acetic acid solution by adding crystalline NaCl to a concentration of 7% (w/v). The precipitate was collected by

centrifugation (43,700 x g) and the supernatant discarded. The precipitate was resuspended in 20 mL of 1.0 M NaCl, 0.05 M Tris, 0.001 M NaN_3, pH 7.4, and the pH adjusted to 7.0 using 1 M NaOH after overnight solubilization. Collagen that was not solubilized was collected by centrifugation (43,700 x g) and discarded. Type III collagen was precipitated from the supernatant by adding 2 mL of 5 M NaCl to each tube to attain a concentration of 1.2 M NaCl. After allowing the Type III collagen to precipitate overnight, it was collected by centrifugation at 43,700 x g. The Type III collagen was dialysed (Spectro/por Molecular Porous Membrane standard dialysis tubing, 12000-14000 molecular weight cut-off, Fisher Scientific, Pittsburgh, PA) overnight against distilled water, lyophilized and stored until analyzed electrophoretically for α-chain profile. To the remaining supernatant, 1 23 g of crystalline NaCl were added to adjust the NaCl concentration to 2.5 M in order to precipitate Type I collagen. Type I collagen was collected using centrifugation (43,700 x g) and dialysed against water overnight, then lyophilized and stored for electrophoresis.

3.2.11. SDS-Polyacrylamide-Gel Electrophoresis

Lyophilized Type I and III collagen samples were solubilized in 6 M urea (2 mg collagen · mL^{-1} 6 M urea) in preparation for application to the Mighty Small Slab Gel SDS-PAGE gel electrophoresis apparatus (Hoefer Scientific Instruments, San Francisco, CA) and mixed with a tracking dye to a concentration of 1 mg collagen · mL^{-1} tracking dye. The tracking dye consisted of 9% (w/v) SDS, 0.01% (w/v) Bromophenol Blue (from stock solution with 1% w/v in distilled water), 150 mM Tris-HCl, pH 6.8, in distilled water. The samples mixed with the tracking dye were denatured for 10 min at 90°C and

frozen until analyzed.

Collagen constituents were separated using SDS-polyacrylamide gel electrophoresis as outlined in the method of Laemmli (1970). A discontinuous gel system was used, with a 4% acrylamide upper gel and a 5% (w/v) acrylamide lower gel, and a 0.3% (w/v) TRIS (base), 1.44% glycine, 0.1% SDS running buffer, pH 8.6. Twenty-five uL of each sample were applied to each well. A pepsin-digested Type I collagen standard (Sigma Chemical Co., St. Louis, Missouri) and a high molecular weight standard (Sigma Chemical Co., St. Louis, Missouri) were included in each gel profile. Slab gels were subjected to 75 volts for 15 min, then the unit was disconnected and 5 uL of reducing solution (30% (v/v) β-mercaptoethanol, 20% (v/v) glycerol, 10% (v/v) tracking dye and 40% (v/v) distilled water) were added to the sample lanes to reduce any disulphide bonds which existed. This was done to separate the Type I and III α-chains which otherwise appear in the same position if the sample is reduced prior to application of the voltage (Sykes and Bailey 1971). The apparatus was then reconnected, and allowed to continue at 75 volts for 10 min or until the samples had progressed out of the upper gel. The voltage was then increased to 150 volts for the remainder of the electrophoresis. Gels were stained overnight in an aqueous solution containing 50% (v/v) methanol, 10% (v/v) acetic acid and 0.25% (w/v) Coomassie Brilliant Blue R250. Gels were destained in an aqueous solution of 50% methanol and 10% acetic acid for 1.5 h and stored in an aqueous solution of 18.75% acetic acid and 12.5% methanol.

3.2.12. Proteoglycan Concentration Estimation

Proteoglycan concentration of the perimysial collagen was estimated by the method of

Lammi and Tammi (1988). Approximately 125 mg of lyophilized perimysium or endomysium were extracted overnight at 4°C in 5 mL of 4 M guanidine-hydrochloride with 2% (w/v) Triton X-100 in 0.1 M sodium acetate, pH 5.8. The supernatant was collected by centrifugation (43,700 x g) and stored in ice. A sheet of pre-cut nitrocellulose membrane (0.45 um pore size)(Pierce, Rockford, Illinois) was used to cover the 96 wells of a Pierce Easy-Titer ELIFA System (Pierce, Rockford, Illinois). The membrane was wetted with distilled water prior to placement in the ELIFA apparatus. The outlet valve to the vacuum was closed and 50 uL of standards and samples were pipetted into the wells. Following this, 400 uL of 0.02% Safranin-O in 50 mM sodium acetate buffer, pH 4.75, which had been filtered through a 0.2 um Nalgene cellulose acetate membrane syringe filter (Fisher Scientific, Edmonton, Alberta), were added to the wells containing standards and samples. The valve to the vacuum was opened and the contents of the wells were rapidly filtered. The precipitates remaining on the nitrocellulose filter were washed twice with 250 uL of distilled water, with the vacuum opened after each washing. The nitrocellulose membrane was allowed to dry in the air and the dots were scanned using a Bio-Rad Imaging Densitometer with Molecular Analyst™/PC Imaging Analysis Software (Model GS-670, Bio-Rad, Mississauga, Ontario) between 400 and 500 nm. Sample reflectance values were measured against various dilutions of heparin and chondroitin sulfate A standards (Sigma Chemical Co., St. Louis, Missouri) to estimate sample glycosaminoglycan concentration.

3.3. STATISTICAL ANALYSES

3.3.1. Hydrolysis Time Determination

The data from the hydrolysis time test were analyzed statistically using the analysis of variance from the General Linear Models procedure of the Statistical Analysis System (SAS 1989) program (SAS Institute Inc., Cary, NY). The data were tested for the effects of collagen source (endomysium and perimysium), storage time (0 and 12 weeks), time of hydrolysis (9, 12 and 24 h), and the various two and three-way interactions.

3.3.2. Total Hydroxyproline Content

Differences in total hydroxyproline content data of the perimysium and endomysium between the storage periods were assessed as above. The model partitioned hydroxyproline concentration according to muscle pH (normal and high), time of storage (0, 4, 8, 12 and 15 weeks) and the interaction. A Student-Newman-Keuls multiple range test determined mean differences between the storage periods.

3.3.3. Nitrogen Content

Differences in nitrogen content of the perimysium and endomysium from weeks 0 and 15 were tested with a two-way analysis of variance using the General Linear Models procedure (SAS 1989). Nitrogen content was expressed as collagen content and tested for the effect of collagen source (endomysium and perimysium) and time of storage (0 and 15 weeks).

3.3.4. Heat Solubility Analysis

Repeated measures analysis in the General Linear Models procedure of SAS (1989) was used to identify the effects of roast pH (normal and high) and storage time (0, 4, 8,

12 and 15 weeks for the perimysium and 0 and 15 weeks for the endomysium) on the heat solubility of the perimysium and endomysium. The model included the interaction of roast pH and storage time as well. Significance of the sources of variation were assessed using the Greenhouse-Geisser probabilities, which are adjusted for correlations between repeated measurements. Least square means were used to identify significant differences between the means.

3.3.5. Differential Scanning Calorimetry

The Analysis of Variance procedure (SAS 1989) was used to separate the effects of time of storage and ratio of collagen to water on the peak denaturation temperature of the perimysium as measured using differential scanning calorimetry. Least square means were used to identify significant differences between the means.

The temperature at which perimysium denatured as well as the enthalpy were compared across the hydration times using the Analysis of Variance procedure (SAS 1989). Significant differences between the means of the hydration times were tested using the Student-Newman-Keuls multiple range test (SAS 1989).

The effects of muscle pH and storage time on peak denaturation temperature and denaturation enthalpy of the perimysium and endomysium were tested as a 2 x 2 x 5 factorial using the General Linear Models procedure (SAS 1989) to generate an Analysis of Variance. The model tested the effects of collagen fraction (endomysium and perimysium), muscle pH (high vs normal), storage time (0, 4, 8, 12, and 15 weeks) and their interactions on the peak denaturation temperature and the enthalpy of denaturation. Each hypothesis used collagen fraction x pH x storage time as the error term.

3.4. RESULTS

3.4.1. Storage

Oxygen levels in the atmospheres of the foil-laminate bags remained below 200 ppm for the duration of the experiment. After 8 w :ks of storage, the average oxygen concentration of the atmosphere in three randomly selected bags was 88.5 ppm. The mean oxygen concentrations of randomly selected bags were 56 ppm and 84 ppm after 12 and 15 weeks of CAP storage, respectively (n=4). These data show that the aluminum foil laminate bags performed to specifications and that bag integrity was preserved for the duration of the experiment.

3.4.2. Hydrolysis Time

The concentration of hydroxyproline in the endomysial and perimysial collagen samples was unaffected by time of hydrolysis; therefore, nine hours of hydrolysis were deemed adequate for maximal hydroxyproline recovery from the collagen (Table 3.1).

3.4.3. Total Hydroxyproline Content

A Student-Newman-Keuls multiple range test showed that the g hydroxyproline $\cdot g^{-1}$ dry perimysium increased significantly with storage time (Fig. 3.1). Hydroxyproline concentration of the perimysial residue increased during the first 8 weeks of storage and then reached a plateau. These data indicated that the collagen content of the perimysium increased with CAP storage time. Analysis of variance showed that storage time had no significant effect on the g hydroxyproline $\cdot g^{-1}$ dry endomysium (Fig. 3.1).

3.4.4. Nitrogen Content

Results of the nitrogen content analysis of randomly selected perimysial and

Table 3.1. Collagen released (% collagen · g^{-1} dry collagen) from perimysium and endomysium from roasts stored for 0 and 12 weeks following hydrolysis for 9, 12 or 24 hours[1].

	Storage Time (weeks)					
	0			12		
	9[a]	12	24	9	12	24
Endomysium	36.1[b]	39.9	39.6	26.9	27.5	26.0
Perimysium	60.5	61.8	59.8	71.9	69.4	69.2

[1] n = 2
[a] Hours hydrolyzed.
[b]Standard Error of Means = 2.4%.

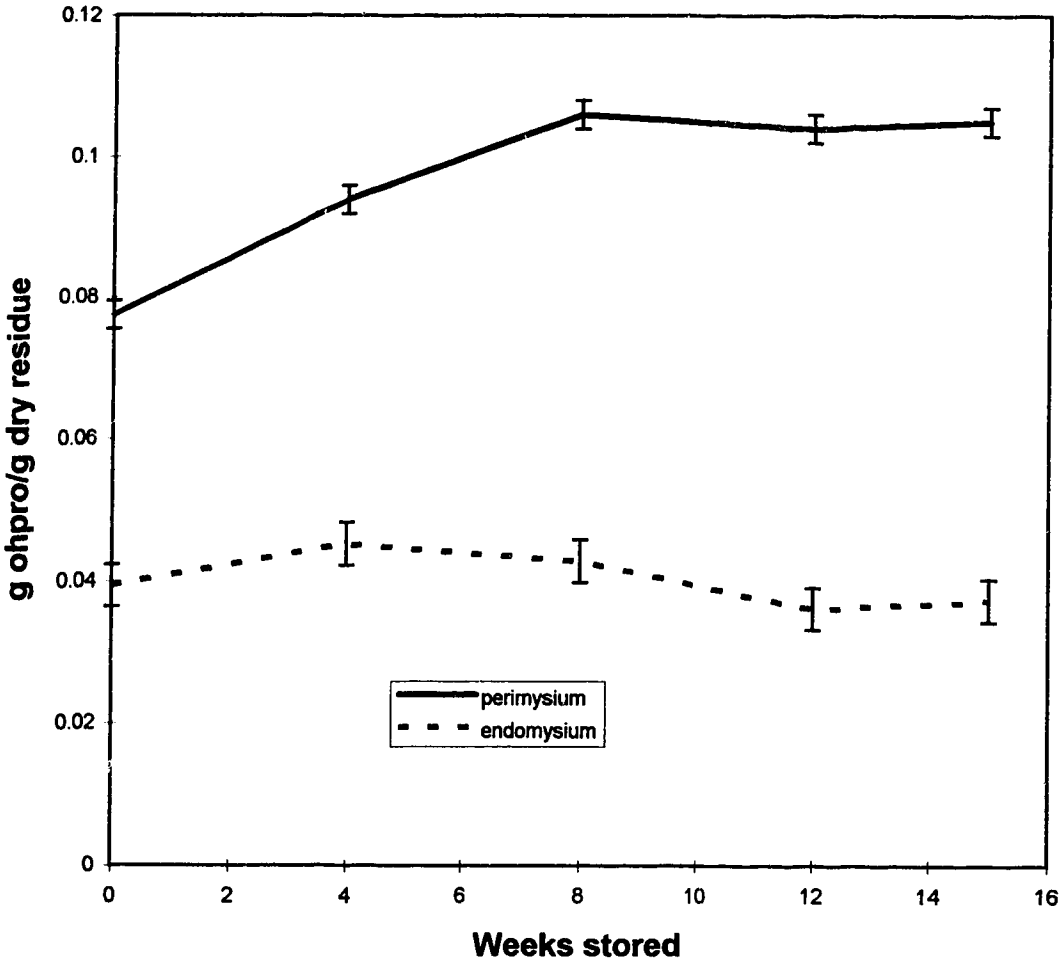

Figure 3.1. Hydroxyproline concentrations of lyophilized endomysium (--------) and perimysium (——)(g hydroxyproline · g^{-1} dry sample) isolated from beef roasts stored 0, 4, 8, 12 and 15 weeks in CAP at -1.5 ± 0.5°C.

endomysial collagen samples from high and low pH roasts stored for 0 or 15 weeks are shown in Table 3.2. Analysis of variance of the nitrogen values showed a significant interaction between collagen source and time of storage ($P = 0.0077$). The nitrogen content of the endomysium ($\%N_2$) did not change significantly with CAP storage. The nitrogen content of the perimysium, however, increased significantly, indicating that the concentration of its non-protein substances decreased with CAP storage.

3.4.5. Heat Solubility

Repeated measures analysis of the values of hydroxyproline released into solution from the perimysium during cooking showed that there was a significant interaction between weeks of storage and 24 h postmortem pH of roast ($P = 0.0001$). The means of the hydroxyproline solubilities at the various cooking times are presented in Table 3.3. Significant differences in pH were found at 0 weeks of storage, where the perimysia from the high pH roasts released more hydroxyproline into solution after 120 and 240 min of cooking than that from normal pH roasts. Another significant difference was found after 12 weeks of storage, with perimysia from normal pH roasts having a higher mean hydroxyproline solubility than perimysia from high pH roasts after 30 min of cooking, although this advantage disappeared after 60 min of cooking. The data indicated that the perimysia from high pH roasts contained more soluble collagen than the perimysia from normal pH roast at 0 weeks storage, but this difference was not sustained after 4 weeks of storage.

Comparison of the mean hydroxyproline solubilities at the various cooking times over the weeks of storage revealed few significant differences between the storage times for

Table 3.2. Mean nitrogen content (% nitrogen of lyophilized residue) of perimysium and endomysium from roasts stored for 0 and 15 weeks in CAP as estimated by nitrogen (N_2) analysis.

	Weeks Stored	
Collagen	0	15
Endomysium	10.8[a] (4, 0.6)[1]	8.5[a] (2, 0.9)
Perimysium	13.7[b] (3, 0.7)	16.7[c] (3, 0.7)

[a,b,c] Means with different superscripts are significantly different ($P < 0.05$).
[1] Values in parentheses are the n and standard error of the mean, respectively.

Table 3.3. Hydroxyproline release into 0.1 M sodium phosphate (pH 7.0) during cooking of perimysium isolated from roasts of normal and high 24 h pH after storage for various times in controlled atmosphere packaging (% of total hydroxyproline).

			Cooking Time (minutes)					
Storage Time	pH	n	0	15	30	60	120	240
0 weeks	Normal	4	0^{a}	13.1^{a}	26.2^{ab}	53.3^{abc}	74.4^{bc}	77.3^{c}
	High	4	0^{a}	14.3^{ab}	24.2^{a}	59.4^{a}	85.3^{a}	88.3^{a}
4 weeks	Normal	5	0.5^{b}	17.1^{bc}	28.8^{abc}	53.5^{abc}	74.1^{bc}	70.3^{de}
	High	5	0^{a}	15.1^{ab}	29.9^{bc}	56.8^{ab}	76.5^{bc}	79.2^{bc}
8 weeks	Normal	5	0.1^{ab}	16.7^{abc}	29.7^{bc}	48.2^{c}	73.8^{c}	81.2^{bc}
	High	5	0^{a}	18.2^{bc}	29.6^{bc}	51.1^{bc}	77.7^{bc}	76.2^{cd}
12 weeks	Normal	5	0.1^{ab}	17.1^{abc}	32.7^{c}	55.3^{ab}	79.1^{bc}	84.8^{ab}
	High	5	0^{a}	15.4^{ab}	25.6^{ab}	50.5^{bc}	79.4^{b}	83.1^{ab}
15 weeks	Normal	5	0^{a}	19.8^{c}	29.7^{bc}	53.5^{abc}	75.0^{bc}	75.6^{cd}
	High	5	0.3^{ab}	19.3^{c}	28.9^{abc}	53.3^{abc}	73.6^{c}	65.6^{e}
S.E.M.		4	0.2	1.5	1.9	2.6	2.2	2.4
		5	0.1	1.3	1.7	2.3	2.0	2.1

^{a,b,c,d,e} Means with different superscripts in columns are significantly different ($P < 0.05$).

hydroxyproline release during cooking (Table 3.3). The week 15 perimysia released significantly more hydroxyproline into solution after 15 min of cooking than the perimysia from week 0. This suggests that the perimysia from the stored roasts were slightly more soluble than perimysia from the unstored roasts.

Repeated measures analysis indicated that neither storage nor muscle pH exerted significant effects on the heat lability of the endomysium (Table 3.4).

3.4.6. Differential Scanning Calorimetry

Analysis of variance of the maximum thermal transition temperature (T_m) and denaturation enthalpy values measured during the assessment of the effect of water content on thermal behaviour showed a significant interaction between time of storage and concentration of water to sample ($P = 0.02$)(Table 3.5). The structural characteristics of collagen depend upon the water content of the protein (Pineri et al. 1978); therefore, adequate water must be present to obtain a true measurement of thermal characteristics (Luescher et al. 1974). The collagen from roasts stored for 0 weeks showed a significant increase in mean T_m as water was added to the dry collagen, whereas the collagen from the roasts stored for 15 weeks did not. There were no significant differences in the mean enthalpies of the collagen due to time of storage or water concentration (Table 3.6). The highest ratio of water to dry collagen (7.5 uL dH_2O to 1 mg collagen) was chosen to ensure results were representative of native collagen.

Analysis of variance showed a significant effect of hydration time on the T_m of collagen ($P = 0.006$)(Figure 3.2). T_m values increased with hydration time at room temperature; however, the change in T_m was insignificant after 1 h; therefore, hydration

Table 3.4. Hydroxyproline release into 0.1 M sodium phosphate (pH 7.0) during cooking of endomysium isolated from roasts of normal and high pH after storage for various times (min) in controlled atmosphere packaging (% of total hydroxyproline).

Weeks Stored	n	0 min cooked	15 min	30 min	60 min	120 min	240 min
0	8	0	7.4 (0.9)[a]	11.2 (1.0)	30.1 (2.6)	57.0 (4.2)	71.4 (4.6)
15	10	0	9.7 (0.8)	19.5 (0.9)	38.6 (2.3)	62.7 (3.8)	76.1 (4.1)

[a] Standard error of the mean.

Table 3.5. The effect of hydration on the thermal transition temperature (°C) of collagen (perimysium) isolated from roasts stored 0 and 15 weeks in CAP[1].

Ratio of collagen to dH_2O	0 weeks	15 weeks
1 mg : 2 ul	69.7[a]	68.5[d]
1 mg : 5 u[1]	70.7[b]	68.3[d]
1 mg : 7.5 ul	71.7[c]	68.3[d]

[1] Standard error of means = 0.33°C, n = 3.
[a,b,c,d] Values with different superscripts are significantly different ($P < 0.05$).

Table 3.6. The effect of hydration on the denaturation enthalpy (kj · kg^{-1}) of collagen (perimysium) isolated from roasts stored 0 and 15 weeks in CAP[1].

Ratio of collagen to dH_2O	0 weeks	15 weeks
1 mg : 2 ul	16.8	15.3
1 mg : 5 ul	17.2	18.8
1 mg : 7.5 ul	18.3	16.9

[1] Standard error of mean = 1.1 kj · kg^{-1}, n = 3.

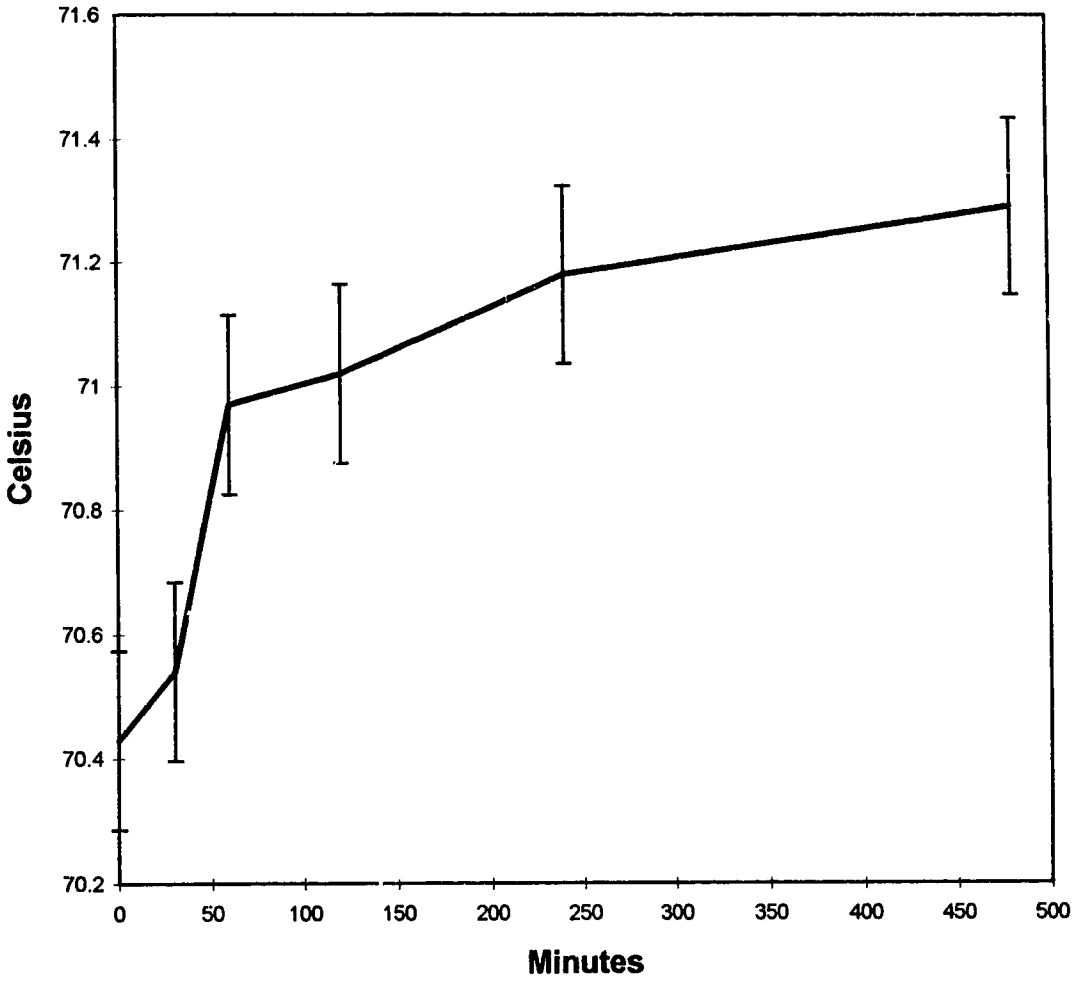

Figure 3.2. Means of maximum thermal denaturation temperatures (°C) of perimysium allowed to hydrate for various times at room temperature.

for one to two hours prior to analysis was deemed adequate. There was no significant effect of hydration time on collagen enthalpy values (Figure 3.3).

Comparison of the T_m values of the endomysium and perimysium revealed a significant interaction between collagen source and time of storage ($P = 0.0001$)(Figure 3.4). The mean T_m values of both collagen sources decreased with time of storage, but that those of the perimysium declined more rapidly than those of the endomysium ($P = 0.0001$)(Figure 3.4).

There was a significant interaction between collagen source and time of storage ($P = 0.0001$) for the mean denaturation enthalpies of the endomysium and perimysium (Figure 3.5). There was no significant change in the mean denaturation enthalpies of the perimysium with storage. The mean denaturation enthalpies of the endomysium, however, increased significantly from 0 to 4 weeks of storage, plateaued, then returned to a value similar to week 0 (Figure 3.5).

As well, the perimysium had a significantly greater mean denaturation enthalpy than the endomysium at each storage time (Figure 3.5).

The analysis of variance also showed a significant interaction for T_m between 24 h postmortem roast pH and time of storage ($P = 0.0115$)(Figure 3.6). Figure 3.6 illustrates the relationship, where the collagen from high pH roasts had significantly higher mean T_m values at 0 and 4 weeks of storage than collagen from normal pH roasts. This significant interaction indicated that the T_m values of the collagen from the high pH roasts were declining slightly faster than those of the collagen from normal roasts. The mean T_m values and denaturation enthalpies of the collagen standards showed that acid-

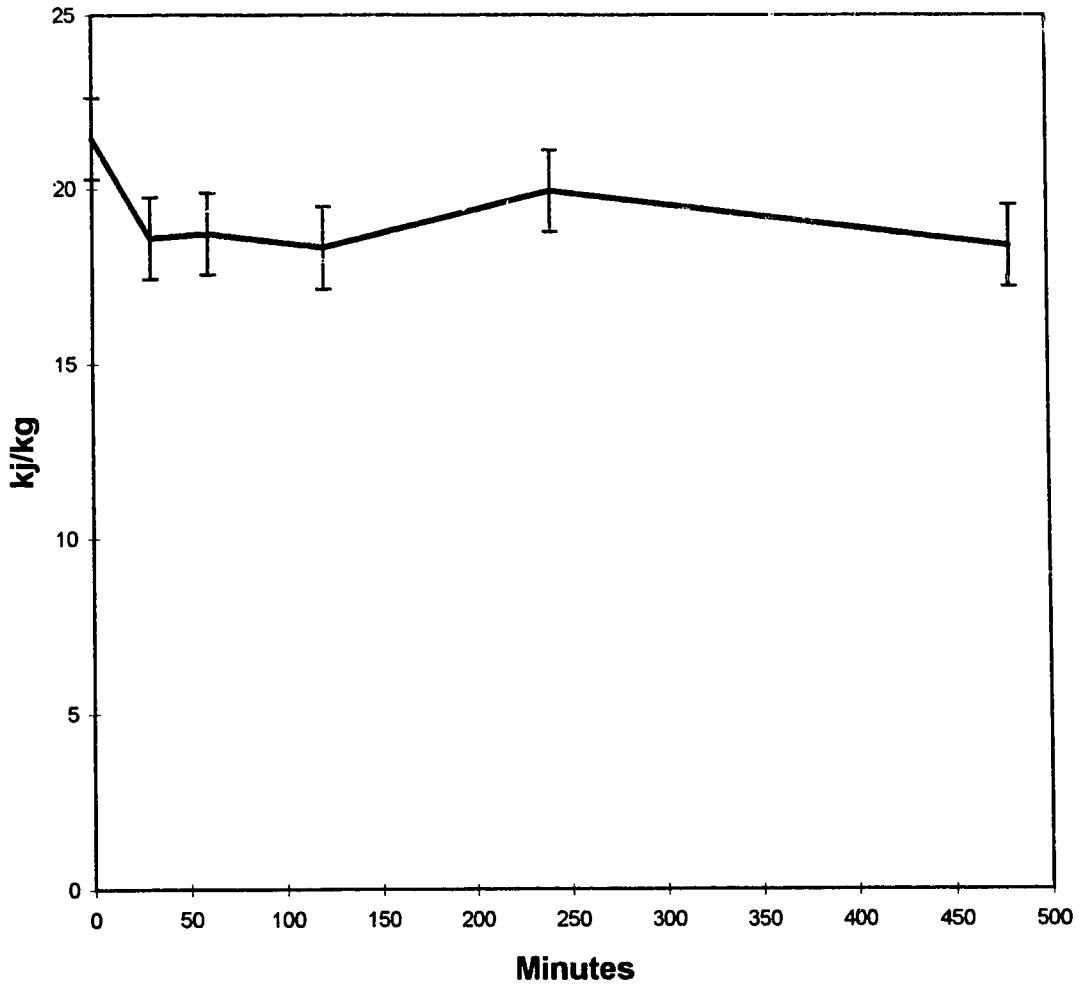

Figure 3.3. Mean denaturation enthalpies (kj · kg^{-1}) of perimysium allowed to hydrate for various times at room temperature.

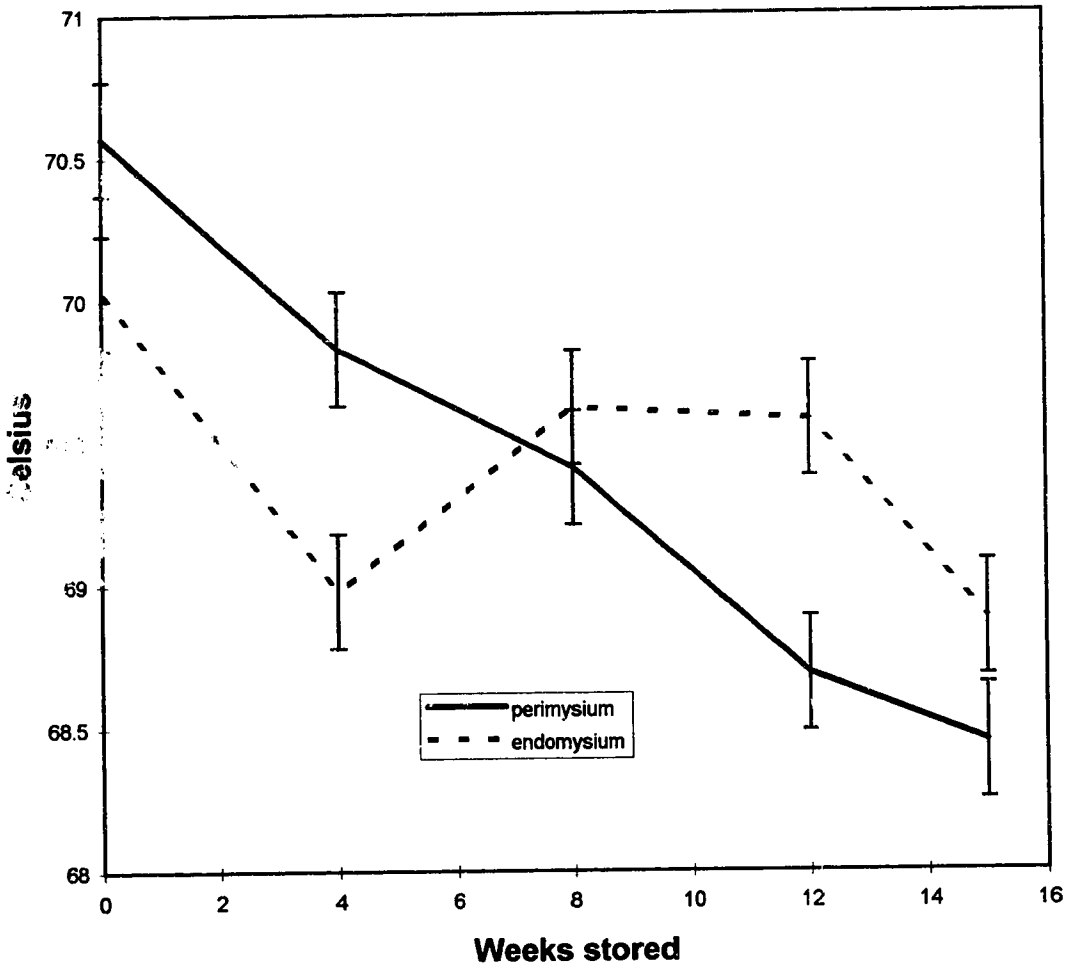

Figure 3.4. Means of thermal denaturation temperatures (°C) of perimysium and endomysium from roasts stored in CAP at -1.5 ± 0.5°C for 0, 4, 8, 12 and 15 weeks.

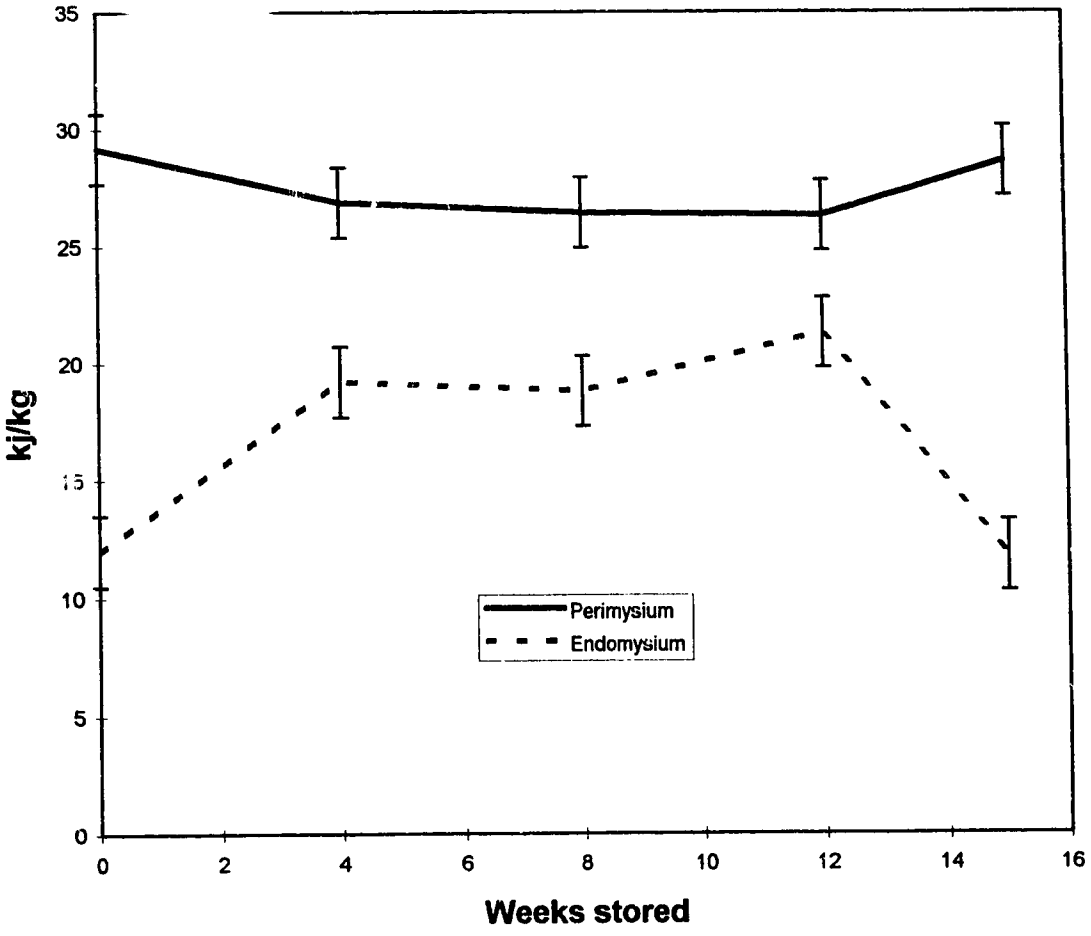

Figure 3.5. Means of thermal denaturation enthalpies (kj · kg^{-1}) of perimysium and endomysium from roasts stored in CAP at -1.5 ± 0.5°C for 0, 4, 8, 12 and 15 weeks.

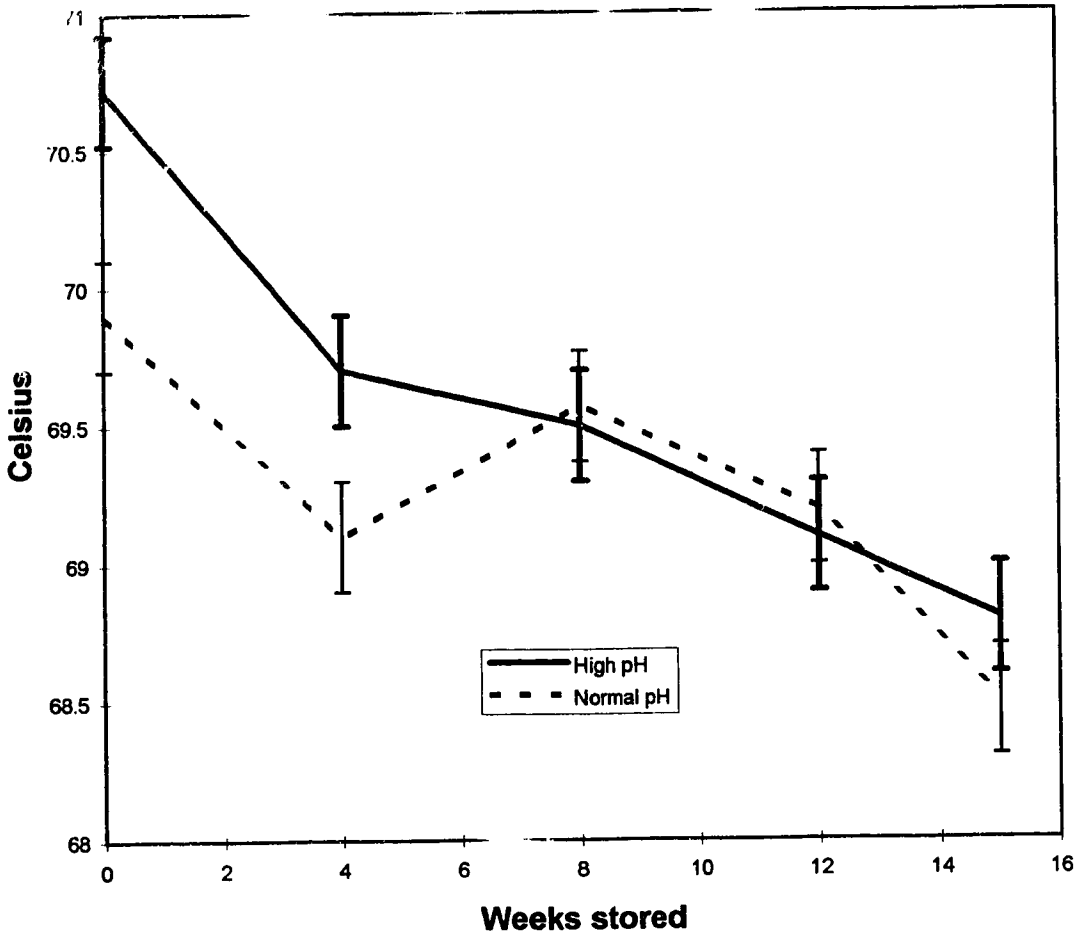

Figure 3.6. Means of collagen T_m values (°C) illustrating the interaction between roast pH and time of storage (weeks).

insoluble Type I had significantly higher mean T_m values than the acid-soluble Type III and IV collagen standards (Table 3.7). The mean denaturation enthalpies of the collagen standards were not significantly different.

3.4.7. SDS-Polyacrylamide-Gel Electrophoresis

The addition of β-mercaptoethanol was delayed 15 min during SDS-PAGE to separate α1(I) chains and disulphide crosslinked α1(III) chains (Plate 3, lane 2 (c) shows the α1(I)- and α2(I)-chain bands (large and small open arrows respectively) of perimysium from the 1.2 M NaCl fraction. Addition of β-mercaptoethanol to this fraction produced an α1(III)-chain band slightly above the α1(I)-chain band (Plate 3 (d), small closed arrow). Plate 3 (e) and (f) show no disulphide-linked α1(III) chains separated in the 2.5 M NaCl fraction of the perimysium.

The Type I collagen standard (Plate 3 (a)) exhibited two light bands (no arrows) below the α1(I)- and α2(I)-chain bands (large and small open arrows, respectively). These bands probably resulted from excessive pepsin digestion. Also, the α1(I)- and α2(I)-chain bands of the perimysium samples (Plate 3, large and small open arrows) do not exhibit the characteristic two to one ratio of density, indicating that the α1(I)-chain band contains α1(III)-chains separated by the pepsin digestion of the telopeptide in which their intramolecular disulphide bonds were located.

Pepsin-digested endomysium precipitated with 1.2 M NaCl consisted of Type III collagen (small closed arrow) with some Type I collagen (open arrows)(Plate 4 (c) and (d)). The α2(I)-chain band is barely detectable, indicating that most of the α1(I)-band is α1(III)-chains. In this fraction, there appeared to be a decrease in the quantity of

Table 3.7. Mean T_m (°C) and denaturation enthalpies (kj · kg dry collagen) of collagen standards[1].

Collagen Type	T_m	Denaturation Enthalpy
I	65.5[a]	16.5[a]
III	45.7[b]	16.3[a]
IV	45.1[b]	14.2[a]
Standard Error of Means	0.33	2.97

[1] n = 3.
[a,b] Means with different superscripts in columns are significantly different ($P \leq 0.05$).

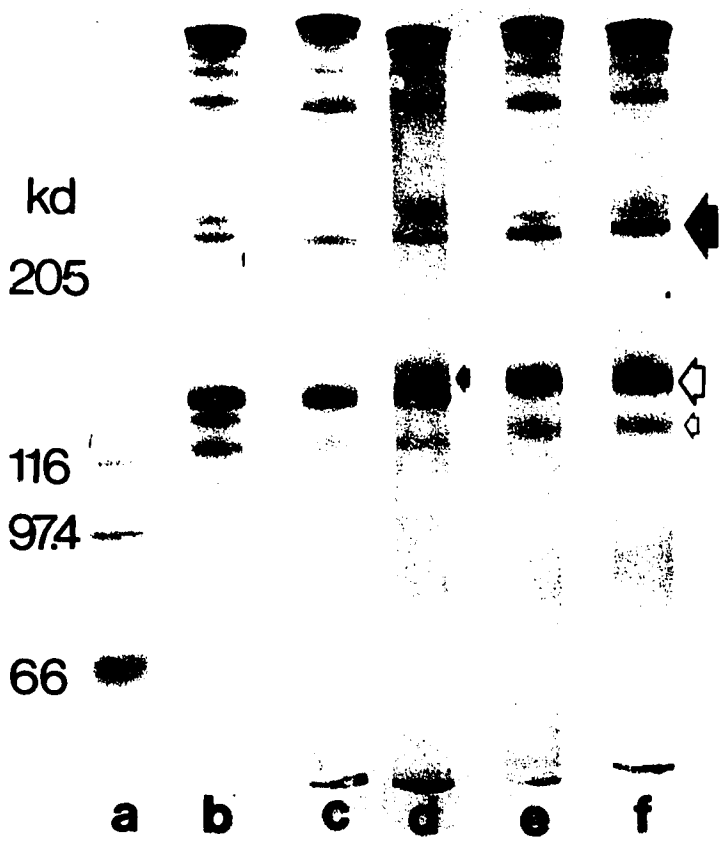

Plate 3. Various fractions of perimysium digested with pepsin loaded with or without β-mercaptoethanol (β-ME) during polyacrylamide gel electrophoresis: (a) high molecular weight standard, myosin heavy chain (MHC) 205 kd, β-galactosidase 116 kd, phosphorylase B 97.4 kd, bovine albumin 66 kd; (b) ▬ Type I bovine tendon standard; (c) perimysium precipitated with 1.2 M NaCl, without β-ME; (d) perimysium precipitated with 1.2 M NaCl, with β-ME; (e) perimysium precipitated with 2.5 M NaCl, without β-ME; (f) perimysium precipitated with 2.5 M NaCl, with β-ME. β-chains, large closed arrow; α1(III), small closed arrow; α1(I), large open arrow; α2(I) small open arrow.

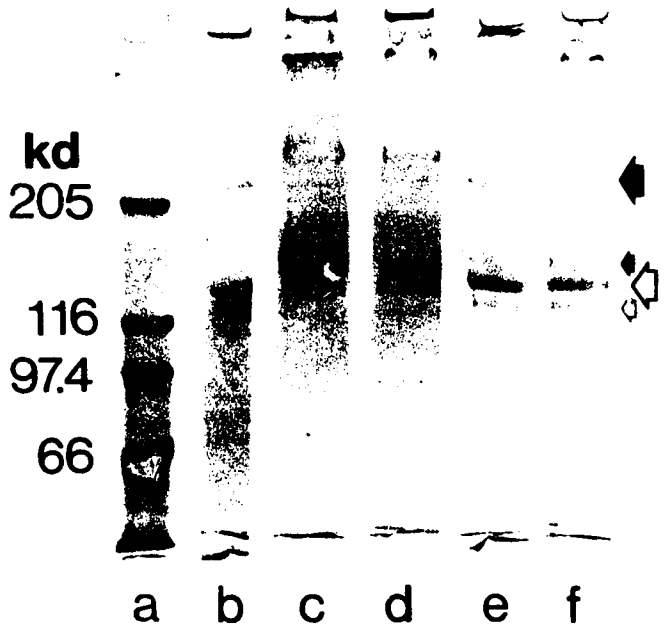

Plate 4. Electrophoresis profiles of endomysium from roasts stored 0 and 15 weeks: (a) high molecular weight standard, myosin heavy chain (MHC) 205 kd, β-galactosidase 116 kd, phosphorylase B 97.4 kd, bovine albumin 66 kd; (b) [redacted] Type I bovine collagen standard; (c) week 0 endomysium precipitated at 1.2 M NaCl; (d) week 15 endomysium precipitated at 1.2 M; (e) week 0 endomysium precipitated at 2.5 M NaCl; (f) week 15 endomysium precipitated at 2.5 M NaCl. β1,2(I), large closed arrow; α1(III), small closed arrow; α1(I), large open arrow; α2(I) small open arrow.

disulphide-bonded Type III collagen between weeks 0 and 15 storage, as evidenced by a decrease in the density of the α1(III) band (small closed arrow) in 4 of 6 samples.

Endomysial 2.5 M NaCl precipitate electrophoresis profiles showed it consisted of Type I (open arrows) with some disulphide-bonded Type III (small closed arrow) present in most samples (5 of 8)(Plate 4 (e) and (f)). Again, excessive pepsin digestion was evident as the α1(I) band was more than twice as dense as the α2(I) band, indicating the presence of α1(III) chains. There were no apparent differences in the electrophoresis profiles of the endomysial 2.5 M NaCl precipitates due to storage.

There was no change in the electrophoresis profiles of the perimysial 1.2 M NaCl precipitate due to storage (Plate 5 (c) and (d)). Again, the α1(III)-chains were digested excessively with pepsin and appeared in the α1(I) position (large open arrow). The faint α-2(I) band (small open arrow) indicated little Type I collagen was present.

The 2.5 M NaCl perimysial precipitates also showed signs of excessive pepsin digestion, with the α1(I) band (large open arrow) more than twice as dark as the α2(I) band (small open arrow)(Plate 5). There was an effect of storage time on the 2.5 M NaCl perimysial precipitate (Plate 5 (e) and (f)). The week 0 samples showed Type I α-chains (open arrows) and most (5 of 8 samples) contained disulphide-bonded Type III (small closed arrow)(Plate 5 (e)) that had been reduced following the delayed application of β-mercaptoethanol to the gels. The week 15 samples, however, displayed no α1(III) band (10 of 10)(Plate 5 (f)).

There was no effect of roast pH on the collagen constituents (Plate 6 and 7).

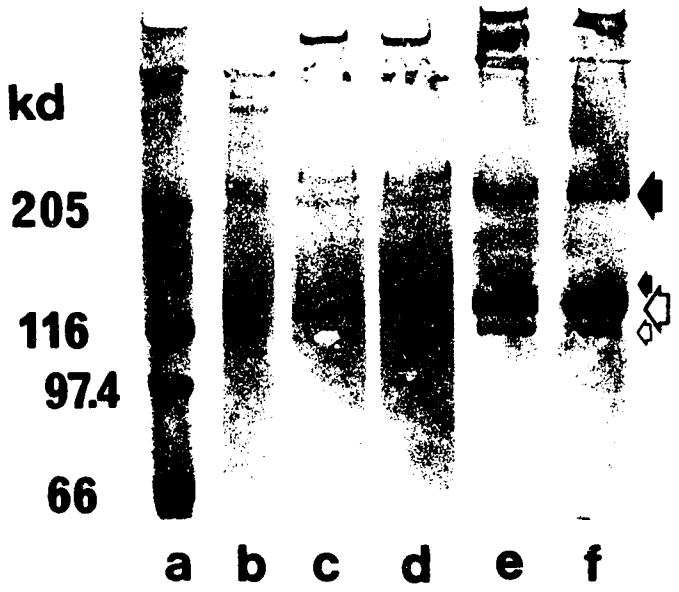

Plate 5. Electrophoresis profiles of perimysium from roasts stored 0 and 15 weeks: (a) high molecular weight standard, myosin heavy chain (MHC) 205 kd, β-galactosidase 116 kd, phosphorylase B 97.4 kd, bovine albumin 66 kd; (b) ▬▬ Type I bovine collagen standard; (c) week 0 perimysium precipitated with 1.2 M NaCl; (d) week 15 perimysium precipitated with 1.2 M NaCl; (e) week 0 precipitated with 2.5 M NaCl; (f) week 15 precipitated with 2.5 M NaCl. β1,2(I), large closed arrow; α1(III), small closed arrow; α1(I), large open arrow; α2(I) small open arrow.

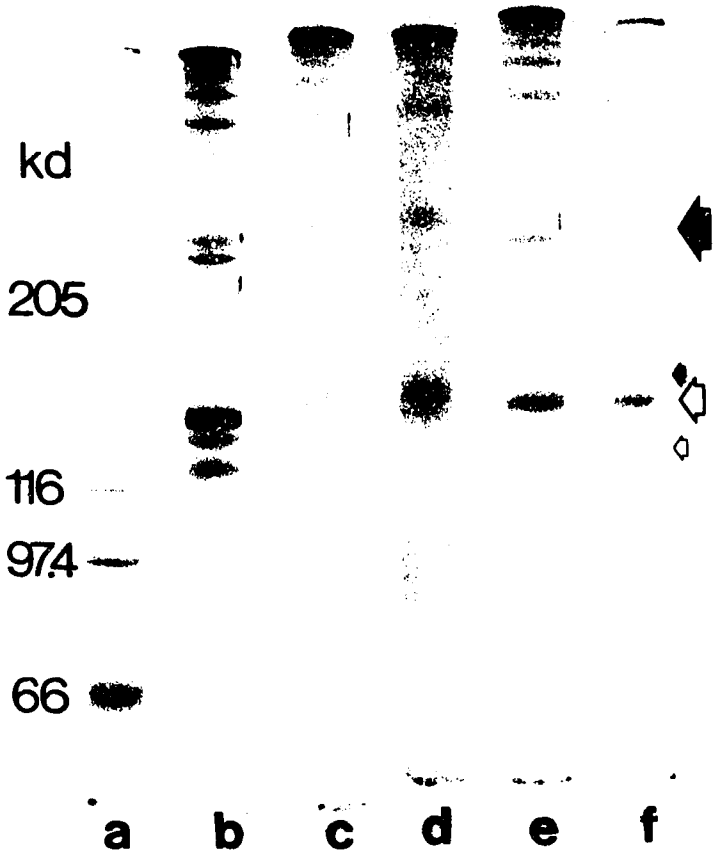

Plate 6. Electrophoresis profiles of endomysium extracted from roasts of normal and high pH: (a) high molecular weight standard, myosin heavy chain (MHC) 205 kd, β-galactosidase 116 kd, phosphorylase B 97.4 kd, bovine albumin 66 kd; (b) [redacted] Type I bovine collagen standard; (c) week 0, 1.2 M NaCl, normal pH; (d) week 0, 1.2 M NaCl, high pH; (e) week 0, 2.5 M NaCl, normal pH; (f) week 0, 2.5 M NaCl, high pH. β chains, large closed arrow; α1(III), small closed arrow; α1(I), large open arrow; α2(I) small open arrow.

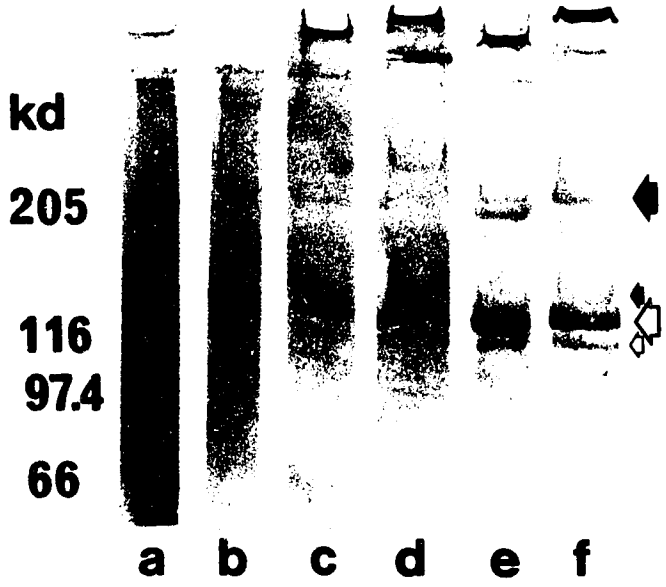

Plate 7. Electrophoresis profiles of perimysium extracted from roasts of normal and high pH: (a) high molecular weight standard, myosin heavy chain (MHC) 205 kd, β-galactosidase 116 kd, phosphorylase B 97.4 kd, bovine albumin 66 kd; (b) [redacted] Type I bovine collagen standard; (c) week 0, 1.2 M NaCl, normal pH; (d) week 0, 1.2 M NaCl, high pH; (e) week 0, 2.5 M NaCl, normal pH; (f) week 0, 2.5 M NaCl, high pH. β-chains, large closed arrow; α1(III), small closed arrow; α1(I), large open arrow; α2(I) small open arrow.

3.4.8. Proteoglycan Concentration

Incubation of endomysium and perimysium with 4 M GnHCl with 2% (w/v) Triton X-100 in 0.1 M sodium acetate, pH 5.8, failed to extract a detectable level of glycosaminoglycans. A second attempt was made to extract an increased concentration of glycosaminoglycans from the collagens by doubling the amount of collagen incubated in the extraction solution. The second attempt also yielded no detectable levels of glycosaminoglycans. Assay of dilutions of the heparin and chondroitin sulfate A standards did produce a linear response, verifying that the assay could detect glycosaminoglycans.

3.5. DISCUSSION

Estimation of the hydroxyproline concentration of the lyophilized endomysium and perimysium revealed an unexpected increase in the mean hydroxyproline content of the perimysium with CAP storage time (Figure 3.1). The increase in the hydroxyproline content of the perimysium was verified to be due to non-protein material loss by an analysis of the nitrogen content of randomly selected samples from the perimysium and endomysium (Table 3.2). The analysis showed that the mean nitrogen content of the perimysia increased significantly from week 0 to week 15 of storage, while that of the endomysia did not change with storage, paralleling the pattern shown by the hydroxyproline concentration of the residue. These data suggest that the non-collagen constituents associated with the perimysium were degraded or damaged during aging, facilitating their removal during collagen isolation with Hasselbach-Schneider solution. Glycoproteins and proteoglycans represent the bulk of non-collagenous constituents

associated with collagen. The increase in the mean hydroxyproline content of the perimysium with time of storage may have occurred because various non-collagen components that attach to collagen, such as glycoproteins and proteoglycans, may have been removed more easily from the perimysium during purification after they had been degraded or damaged proteolytically during aging.

Etherington (1977) found that the percentage of hydroxyproline in perimysium increased (0.8%) after extraction with 5 M GnHCl, which was employed to remove proteoglycans. This value corresponds to the accepted concentration of glycosaminoglycans in muscle, 0.1 to 1.0% (see Heinegard and Paulsson 1984). In the present experiment, however, an increase in hydroxyproline concentration of approximately 3% was observed between 0 to 8 weeks of storage, which is the equivalent of about 21% collagen (55% to 76% collagen from 0 to 8 weeks) if the increase is multiplied by 7.14 (Light and Champion 1984); consequently, although proteoglycans are present along with collagen, they are not in sufficient concentrations to account for the non-collagen substance associated with the collagen isolates of this experiment. Also, the extraction of proteoglycans with Hasselbach-Schneider buffer is doubtful, as extraction of proteoglycans from collagen requires strong denaturing solutions such as 5 M GnHCl (see Heinegard and Paulsson 1984).

Glycoproteins represent a larger portion of the non-collagen constituents capable of binding with collagen than do proteoglycans. Type III and Type IV collagens, which can be found in or near the basement membrane of muscle, are capable of binding to fibronectin and laminin, respectively (Jilck and Hormann 1979; Terranova et al. 1980).

As well, glycoproteins can be separated from collagen using high salt buffers (Timpl et al. 1982). Glycoproteins, therefore, are probably the non-collagen substance associated with the collagens isolated in this experiment. although no attempt was made to verify this. No research was found that showed a decrease in non-collagen components associated with the perimysium as an effect of storage.

No similar trend was noted in the endomysium, indicating that bonds between the endomysium and other constituents of the basement membrane were either stronger or more extensive than those found in the perimysium. The very high level of non-collagen constituents in the endomysium isolate may indicate that the endomysium forms extensive interactions with glycoproteins, which are the major non-collagen components of the basement membrane (Hakomori et al. 1984).

As well, the hydroxyproline concentration values showed that the perimysium and endomysium isolated at week 0 were approximately 55% and 29% collagen, respectively, when g hydroxyproline $\cdot$ g^{-1} lyophilized residue were converted to collagen using 7.14 (Light and Champion 1984). With the increase in hydroxyproline content observed in the perimysium after 8 weeks of aging, the difference in collagen concentration between the perimysium and endomysium was increased (76% and 31%, perimysium and endomysium, respectively). This data showed that the residue isolated and termed perimysium had approximately twice as much collagen as the residue termed endomysium. This may occur because the endomysium is more closely associated with ground substance than the perimysium, and may form more associations with other basement membrane constituents such as glycoproteins (Jilck and Hormann 1979; Terranova et al. 1980). The nitrogen

content analysis also showed that the differences in hydroxyproline content between the endomysium and perimysium were due in part to non-protein components, further substantiating the possibility of glycoprotein association. Light and Champion (1984) showed similar collagen content in their isolates, with the perimysium containing 95% collagen and the endomysium containing 42%, about half as much.

Assessment of the solubility of the perimysium and endomysium revealed a significant interaction between time of storage and roast 24 h post-mortem pH for the perimysium (Table 3.3). The differences between the means of the interaction showed that the perimysia from high pH roasts had higher mean heat solubility values than perimysia from normal roasts at 0 weeks of storage. Also, the perimysia from the normal roasts stored for 12 weeks were more soluble than perimysia from high pH roasts similarly stored after 30 min of cooking. Overall, the significant differences noted for this interaction did not present an identifiable trend.

Comparison of the mean hydroxyproline solubilities at the various cooking times throughout storage showed that the perimysia from roasts stored for 15 weeks released significantly more hydroxyproline after 15 min cooking than perimysia from the roasts stored for 0 weeks (Table 3.3). This result indicates an increase in the concentration of the very heat-labile collagen as this collagen would be released earliest during cooking. Collagen molecules or fibrils that are associated with the extracellular matrix via reducible crosslinks, such as those found in muscle that has undergone growth remodelling, are very heat-labile; therefore, these results suggest that the chemical structure of the perimysium was altered after 4 weeks of storage, possibly because of enzymatic degradation, rendering

it more heat labile (Goll et al. 1964; Hill 1966). No other discernable pattern of hydroxyproline release was found after 30 min of cooking, with significant differences between means occurring in a random pattern usually associated with sample variation.

Statistical analysis showed that time of storage and roast pH had no effect on the heat solubility of the endomysia. Nevertheless, there is a trend evident, with the endomysia from the roasts stored for 15 weeks consistently releasing more hydroxyproline into solution than those from roasts stored 0 weeks ($P = 0.26$)(Table 3.4). Stanton and Light (1990) found that the endomysium from aged beef was consistently more soluble than endomysium from beef that had not been aged; however, this trend was not significant. The data from the present study suggested that the endomysium may have sustained some proteolytic damage during CAP storage, although it was limited and did not exert a significant effect on the solubility of the endomysium.

Aging of beef is generally accepted to increase bovine collagen heat solubility. Herring et al. (1967) found no significant increase in bovine collagen heat solubility from 0 to 5 days of aging, but did find a significant increase in control samples from 0 to 10 days of aging (5.47% to 6.49% soluble collagen). Jeremiah and Martin (1981) found that the heat solubility of collagen from the longissimus muscle did not change significantly with 20 days of aging, although their data exhibited a trend in which collagen heat solubility increased with aging. Mills et al. (1989a,b) showed that collagen solubility increased during the first 24 h of aging, although these workers did not measure it beyond that time. The measurements of heat solubility in the present experiment indicate that changes are evident in the perimysium following 4 weeks of aging, and that aging appeared to exert

a greater effect on the heat lability of the perimysium than on the endomysium.

Comparison of the heat solubility values of the perimysium and endomysium, although not done statistically in this study, showed that the perimysium was substantially more soluble than the endomysium (Table 3.3 and 3.4). This observation agrees with that of Stanton and Light (1990), who noted that the endomysium was less soluble in urea than the perimysium, ever with aging. These data indicate that the endomysium may be the collagen determining beef toughness, a conclusion shared by Light (1987).

The reduced heat solubility of the endomysium may be due to a strong association of the endomysium with glycoproteins postulated earlier in the discussion. Glycoproteins are capable of modifying the physical properties of protein to which they are attached (see Hakomori et al. 1984). In particular, the oligosaccharides of the glycoprotein can reduce the susceptibility of a protein to denaturation (Chu et al. 1978).

The thermal labilities of the perimysium and endomysium in this experiment were investigated further using differential scanning calorimetry (DSC). In this experiment, DSC was used to assess the temperature and the heat of denaturation of the two sources of intramuscular collagen. The temperature and the heat of denaturation of most proteins are related through the equation $T_D = \Delta H_D/\Delta S_D$, where ΔH_D is the enthalpy of denaturation (endothermic interactions), ΔS_D is the entropy of denaturation (exothermic interactions) and T_D is the temperature at which denaturation occurs (Privalov and Tiktopulo 1970). Enthalpy is the heat required to denature the protein and entropy is related to the degree of randomness of the protein system achieved during denaturation (Stryer 1988).

In a preliminary experiment to assess the amount of water required for adequate

hydration of the perimysium, the mean T_m values for the perimysium stored for 0 weeks were observed to increase with the amount of hydration while those for the perimysium stored for 15 weeks did not (Table 3.5). The increase in T_m values for the week 0 sample with increasing water content was not accompanied by a change in the mean denaturation enthalpy, indicating that entropy was decreased in the week 0 sample. What caused the T_m values of the week 0 sample to increase with the water content is unclear. This phenomenon may have occurred because residual salt from purification may have been trapped in the week 0 collagen, despite dialysis against distilled water in conditions identical to those experienced by the week 15 sample. A possible explanation is that the perimysium from the roasts aged 0 weeks retained more salt from purification because it had a greater concentration of non-collagen substance than the week 15 sample that possibly may have affected its permeability. Findlay and Barbut (1993) stated in their review that the presence of salt will reduce the temperature of transition and the total heat required for denaturation. As well, salt ions interfere with the hydrogen bonding of collagen through water bridges, thus lowering the T_m and enthalpy of denaturation (Luescher et al. 1974). Judge and Aberle (1982) found that samples in a 0.05 M KH_2PO_4-NaOH buffer had a significantly lower mean T_m value (67.3 $\pm$ 0.3°C) than those in distilled water (69.4 $\pm$ 0.3°C), which concurs with Luescher et al. (1974). Possibly, in the present experiment, the T_m increased with the ratio of water to sample because the salt in the sample was diluted and the hydrogen bonds stabilized further. Judge and Aberle (1982), however, showed that the T_m of collagen decreased with decreasing salt concentration of analysis solution (5 to 1% NaCl), and that the mean T_m value of collagen

in a 1% NaCl solution was lower than that of similar collagen samples in 0.05 M KH_2PO_4-NaOH buffer (pH 7.1) or distilled water. The data from the present experiment suggest that at very low salt concentrations, dilution will raise T_m values to those of collagen analyzed in distilled water.

The denaturation enthalpy values of the perimysium were not affected by changes in hydration because samples were hydrated above the level of primary hydration (Luescher et al. 1974). Primary hydration water is water which is firmly bound to the collagen and is unfreezable above -70°C (Luescher et al. 1974).

Analysis of variance of the T_m values of the collagen showed a significant interaction between the source of collagen (perimysium or endomysium) and the time of storage (Figure 3.4). The mean T_m values of the perimysium were significantly greater at 0 and 4 weeks of CAP storage than those of the endomysium, but were significantly less than those of the endomysium at 12 and 15 weeks of CAP storage, indicating that the mean T_m values of the perimysium declined more rapidly throughout storage than those of the endomysium. The mean T_m values of the endomysium decreased between 0 and 4 weeks of aging, but changed little between 4 and 15 weeks of CAP storage. The mean T_m values of the perimysium, however, declined constantly throughout storage.

These results showed that both collagen fractions had a lower denaturation temperature after storage. McClain et al. (1970) noted no change in perimysial collagen T_m from beef aged 72 h, but this may have been due to short period of aging in their experiment because Judge and Aberle (1982) observed a significant decrease (8.2 $\pm$ 0.3°C) in the mean T_m value of perimysial collagen from beef aged 7 days. No research was found that examined

the T_m of the bovine endomysium.

A lowering of T_m as been shown to be indicative of a reduction in the strength of intermolecular bonds (Horgan et al. 1990; Smith and Judge 1991). Smith and Judge (1991) demonstrated that the onset temperature of denaturation increased with the concentration of pyridinoline (r = 0.34), a constituent of a trivalent, heat-stable intermolecular bond which increases in concentration with level of maturity (Eyre and Oguchi 1980). As well, Horgan et al. (1990) related T_m to pyridinoline concentration (r = 0.97) and to the concentration of Ehrlich's Chromogen (EC)(r = 0.9), an N-substituted pyrrole thought to participate in intermolecular bonding (Scott et al. 1983). Smith and Judge (1991), however, found that the denaturation enthalpy was not related to the concentration of pyridinoline.

In the perimysium, the T_m values decreased steadily during storage while denaturation enthalpy values remained the same (Figures 3.4 and 3.5). For the T_m to decrease while the denaturation enthalpy is constant, as in this system, the denaturation entropy must increase to satisfy the equation of Privalov and Tiktopulo (1970). The denaturation entropy does increase, from 0.414 at 0 weeks to 0.418 at 15 weeks. Hydrophobic interactions, which are exothermic, counter the enthalpy of the rupture of hydrogen and covalent bonds. Because hydrophobic interactions should not change post-mortem, covalent or hydrogen bonds must be weakened in order to cause the decrease in T_m. Collagen stability is maintained through hydrogen-bond water bridges connecting the hydroxyl groups of the hydroxyprolines with the peptide bonds in the triple helix, and any change in the hydrogen bonding has been found to affect not only the T_m but the

denaturation enthalpy as well (Luescher et al. 1974). As shown in Figure 3.5, the denaturation enthalpy of the perimysium did not change during storage; therefore, the reduction in T_m of the perimysium was probably due to post-mortem degradation of covalent bonds. Flandin et al. (1984) showed that an increase in reducible covalent bond concentration in collagen decreased its melting temperature without affecting its denaturation enthalpy.

There was a significant decrease in mean T_m values of the endomysium between 0 and 4 weeks of CAP storage. Concomitantly, there was a significant increase in denaturation enthalpy, which may indicate that a major decrease in hydrophobic bonding occurred (Finch et al. 1974). The simultaneous increase in enthalpy and decrease in T_m are typical of hydrophobic interactions being broken (Finch and Ledward 1972; Finch et al. 1974). Finch et al. (1974) used low concentrations of tetra-alkylammonium salts to selectively break hydrophobic bonds in collagen, and showed that the enthalpy increased while the T_D decreased. In the same study, the authors postulated that hydrophobic bonds were responsible for approximately 25% of the total enthalpy change in collagen denaturation.

From 4 to 12 weeks of CAP storage, the endomysial denaturation enthalpy and T_m did not change. Between 12 and 15 weeks of CAP storage, however, the endomysial denaturation enthalpy decreased significantly to a level similar to that recorded at 0 weeks of storage. This decrease in denaturation enthalpy, unaccompanied by a change in T_m, is difficult to explain. Flandin et al. (1984) observed a decrease in denaturation enthalpy when the stability of covalent bonds was increased through reduction of the collagen bonds with sodium borohydride. The authors found, using X-ray diffraction, that reduced

collagen heated to 80°C still had a large amount of triple helical structure remaining, which they concluded accounted for the reduced enthalpy. The authors suggested that the strengthened collagen resisted denaturation during the short heating period of DSC analysis, and that lengthy heating may have been necessary to completely denature the sample and attain the denaturation enthalpy observed prior to reduction. The reduced enthalpy of denaturation shown by the endomysium in the present experiment may indicate an increased number of heat stable bonds. The changes in denaturation enthalpy of the endomysium observed throughout storage could not be due to sample variation in hydroxyproline content (Privalov and Tiktopulo 1970) because denaturation enthalpies were adjusted for hydroxyproline content. A high content of Type IV collagen in the endomysium may have decreased denaturation enthalpy values because it consists mostly of heat-stable keto-amine crosslinks (Heathcote et al. 1980; Le Pape et al. 1981; Wu and Cohen 1982).

The reduction in T_m observed in both the perimysium and endomysium in the present experiment was suggestive of covalent bond disruption, with more covalent bonds ruptured in the perimysium than in the endomysium. This conclusion is supported by the DSC data from the Type I, III and IV collagen standards (Table 3.7). Types III and IV, which were acid-soluble standards, had mean T_m values that were significantly less than those of the insoluble Type I collagen. Acid solubility of collagen is determined by the type of intermolecular bonding, with collagen from immature vertebrates, which have a high proportion of reducible collagen crosslinks, often being more acid-soluble than collagen from mature vertebrates, which have fewer reducible collagen crosslinks (Bailey and Light

1989).

Comparison of the two collagen sources showed that the perimysium had a significantly greater mean denaturation enthalpy than the endomysium at the various storage times (Figure 3.5). As shown in Figure 3.5, the mean denaturation enthalpy of the perimysium over all the sampling times was 27.5 kJ · kg^{-1}, while that for the endomysium was 16.6 kJ · kg^{-1}. This result is not due to differences in hydroxyproline content, which determines the denaturation enthalpy (Privalov and Tiktopulo 1970), because the denaturation enthalpy values were adjusted for hydroxyproline content. As well, the T_m values for the perimysium and endomysium are similar; therefore, the increased denaturation enthalpy of the perimysium is indicative of increased entropy of denaturation. This can be clearly illustrated by using the equation of $\Delta S = T/\Delta H$ (Privalov and Tiktopulo 1970). The entropy of denaturation, after 0 weeks of storage, was 0.411 kj · $(kg \cdot °C)^{-1}$ for the perimysium and 0.171 kj · $(kg \cdot °C)^{-1}$ for the endomysium. This result indicates that there was a greater degree of structural randomness in the perimysium than in the endomysium after denaturation. This suggests that more bonds were broken in the perimysium during heating.

The observation that the perimysium is more heat labile than the endomysium was consistent with the data from the Type I, III, and IV collagen standards. The collagen standards Types III and IV, which have been rendered acid-soluble through digestion with pepsin, had no covalent bonding, which was confirmed with SDS-PAGE that showed the presence of only α-chains. These standards, therefore, had lower mean T_m values than the insoluble native Type I collagen standard, which contained covalently linked α-chains (β-

and γ-components) when profiled using SDS-PAGE. The enthalpies of the standards, however, were not significantly different (Table 3.7). This indicates that the entropy of the Type I collagen was less than that of the Type III and IV standards (ΔS = 0.25 and 0.35 kj · (kg · °C)$^{-1}$, Types I and III, respectively). This result is expected, because the standards with no covalent bonding would exhibit a higher level of random structure than the standard with covalent bonds and, with fewer bonds, require less heat for denaturation.

The results of the present experiment clearly indicate that perimysium was more degraded than the endomysium. The difference in the rate of T_m decline and thermal solubility between the perimysium and endomysium may have been due to the varying concentration of non-collagen substance associated with the two sources of collagen, which may have increased the resistance of the endomysium to denaturation, particularly if the non-collagen substance consisted of glycoproteins (Chu et al. 1978; Veragud and Christensen 1975). Etherington (1977) concluded that the associated proteoglycans and glycosaminoglycans of the ground substance were important in determining enzyme susceptibility by restricting the access of enzymes to the telopeptide ends of the collagen molecule where the intermolecular crosslinks are located. The possibility of proteoglycans interfering with post-mortem enzyme activity seems unlikely as too few present in muscle to pose this problem (see Heinegard and Paulsson 1984). Glycoproteins would only be capable of this if they were resistant to the enzymes that cleave collagen.

The perimysium may have been degraded more than the endomysium because it may have been more susceptible to post-mortem enzymes. The reason for this susceptibility is unclear, but may stem from the differences in collagen type (Light et al. 1985). Burson

and Hunt (1986) found that the proportion of Type I to III collagens was not correlated to toughness. Type IV collagen, however, which would be expected in the endomysial isolate, may affect meat toughness because of its extensive covalent crosslinking and insolubility (Glanville et al. 1979; Heathcote et al. 1980). The effect of Type IV collagen content on meat toughness has yet to be examined.

The decreases in T_m values exhibited by the endomysium and perimysium are small compared to those of Judge and Aberle (1982). This may be due to proteolysis slowing significantly at the storage temperature used in this experiment (-1.5 $\pm$ 0.5°C), compared to the conventional storage temperature of 4°C utilized by Judge and Aberle (1982). The results of the present study are in agreement with the in vitro studies of Kopp and Valin (1980-81) and Beltran et al. (1992). Kopp and Valin (1980-81) found that a crude extract of lysosomal enzymes caused a 3°C decrease in the T_m values of epimysium. Beltran et al. (1992) showed that cathepsin B decreased the T_m of collagen from steer carcasses approximately 3°C as well.

Comparison of the perimysial and endomysial T_m values also revealed a significant interaction between 24 h post-mortem roast pH and time of storage (Figure 3.6). Insertion of a regression line through the means indicated that the T_m values of the collagen from the high pH roasts declined slightly faster than those of collagen from the normal roasts. The high pH roasts had significantly higher mean T_m values at 0 and 4 weeks of storage than collagen from normal pH roasts. The difference in the roasts from the two 24 h post-mortem pH groups at week 0 was not due to animal variation, because day 0 of storage was 72 h post-mortem. The differences in T_m displayed between the two 24 h post-mortem

pH groups at week 0 were most likely a function of three days of aging. These data suggest that, after 3 days of storage, perimysium and endomysium from high pH roasts had significantly more covalent bonds than collagen from normal pH roasts. As can be seen in Figure 3.6, the variability of the mean T_m values for collagen from the normal roasts complicated interpretation of the interaction. No research was found during a search of the literature that reported similar results.

The high pH of the roasts appeared to reduce proteolytic activity during early storage, and may indicate that lysosomal enzymes are the primary enzymes acting on collagen during CAP storage. The T_m values for collagen from high and normal pH roasts are not different at 8, 12 and 15 weeks of storage, suggesting that the lysosomal enzyme system may have been more effective during the late post-mortem aging period.

Despite the different thermal labilities of endomysium and perimysium, SDS-PAGE mapping showed that both collagen fractions consisted of similar collagen types. Both collagen fractions consisted of Type I and III collagens (Plate 4 and 5). This is in agreement with other research that has shown co-existence of the Types I, III and IV in muscle (Duance et al. 1977). The results of the present study are similar to those of Stanton and Light (1990), who found the presence of both types of collagen in their CNBr digestion of endomysium. Light and Champion (1984) also found Type IV collagen in their endomysium preparations but SDS-PAGE profiles of the endomysium in the present experiment and in the results of Stanton and Light (1990) did not show bands characteristic of Type IV collagen. The Type IV collagen contains numerous covalent crosslinks and is very insoluble (Glanville et al. 1979; Heathcote et al. 1980).

The presence of Types I and III collagen in every fraction of the perimysium and endomysium may have occurred because of intermolecular bonds between the two types. Henkel and Glanville (1982) and Kuypers et al. (1994) have shown evidence for the presence of bonds between Type I and III molecules in the perimysium. Type III collagen was detected in the 1.2 M and the 2.5 M NaCl precipitates of the endomysium and the perimysium.

SDS-PAGE profiles of the 1.2 M NaCl precipitates of the endomysium showed that the α1(III) band density decreased with storage (Plate 4 (c) and (d)), and that there was no change in the 2.5 M NaCl precipitates of the endomysium (Plate 4 (e) and (f)). As well, the SDS-PAGE mapping showed that the α1(III) band in the perimysial 2.5 M NaCl precipitate decreased with CAP storage. In the perimysium, the α1(III) band was evident in 5 of 8 samples from roasts aged 0 weeks, but was absent in 10 of 10 samples from roasts aged 15 weeks. As was mentioned in the results, excessive pepsin digestion cleaved the telopeptides sequences containing the disulphide bonds of Type III; consequently, only the most resistant Type III collagen was found in the α1(III) band. The α1(III) band density decrease noted in the 1.2 M and 2.5 M NaCl fractions of the endomysium and perimysium, respectively, appears to have been due to storage. The decrease in the α1(III) band may be indicative of telopeptide proteolysis of Type III collagen, which would effectively break crosslinks derived from lysine as well as cysteine. This would increase the susceptibility of the Type III collagen to pepsin digestion; therefore, fewer α1(III)-chains would require reduction with β-mercaptoethanol and appear in the α1(III) band. Pfeiffer et al. (1972) did not notice degradation of a specific type of collagen using SDS-

PAGE, but did note that the bands containing the γ-components of collagen were lighter in gel profiles of collagen from aged beef than those from unaged beef. Kruggel and Field (1971) found a similar relationship using SDS-PAGE, with the collagen from aged beef having β-component bands that were less dense and α-component bands that were darker than those of collagen from unaged beef, which indicated that more bonds were broken with aging. The results presented in the present study may indicate Type III telopeptide proteolysis.

The α and β-component bands shown on the gel profiles of the perimysial samples were also more dense than those of the endomysium. The differences between the gel profiles of the endomysium and perimysium were not due to discrepancies in the quantities of collagen applied to the gels. Application of the same quantity of lyophilized collagen dissolved to the same concentration assured that the differences observed were real, and that the differences in band densities represent a difference in the penetration of the sample into the gel matrix. Contaminants in the lyophilized collagen, such as myofibrillar proteins, would have appeared on the gels as small peptides. The remainder of the endomysium samples can be seen at the origin on the gel plates. The decrease in band density shown by the endomysium indicated that it had fewer soluble α-chains than the perimysium (Plates 4 and 5). The reduced α-chain solubility of the endomysium may have arisen from the endomysium having more covalent crosslinks. The crosslinked aggregates must have been quite large to prevent penetration into the gel matrix, because the γ and β component bands of the endomysium are lighter than those of the perimysium. Post-mortem degradation of the endomysium may have been limited by covalent crosslinking,

but not by proteoglycan association.

In the present study, the enzyme system responsible for the degradation of collaegn was not investigated; however, the persistence of perimysium degradation late into the storage period and the enzymatic action on associated non-collagen substances does imply lysosomal enzyme action. Stanton and Light (1988) found that CNBr peptide damage to urea-insoluble perimysium from aged beef was similar to that exhibited by urea-insoluble perimysium incubated with a crude lysosomal slurry. Beltran et al. (1992) showed that cathepsin B decreased the T_m of collagen from steer carcasses, with no change in the enthalpy of denaturation.

Quantification of the concentration of proteoglycans associated with the endomysium and perimysium was unsuccessful using the extraction procedure outlined in Hascall et al. (1994) and quantitation method of Lammi and Tammi (1988). The inability to successful quantitate the proteoglycans in the present experiment suggested that they were not present in sufficient quantity to affect post-mortem proteolysis, although glycoproteins may be. The effect of glycoprotein-collagen interaction on the degradation of collagen during aging may warrant further investigation.

3.6. CONCLUSIONS

From the profile of the results, the data indicated that the perimysium was more heat soluble and heat labile than the endomysium as a result of CAP storage. The DSC data suggested that covalent bonds in the perimysium were degraded throughout CAP storage, whereas those of the endomysium degraded significantly only within the first 4 weeks of

storage, and were altered little after that. The SDS-PAGE electrophoresis profiles showed evidence of increased susceptibility of Type III collagen to pepsin digestion. These data suggest that the perimysium is affected most by proteolytic action during storage, possibly because of the types of collagens of which it consists. The enzyme system responsible for the increase in thermal lability of the perimysium and endomysium appears to be lysosomal, with cathepsins cleaving molecule telopeptides to weaken intermolecular crosslinks.

3.7. LITERATURE CITED

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4. GENERAL DISCUSSION AND CONCLUSIONS

The experiments presented in Chapters 2 and 3 of this thesis explored the effects of CAP storage on the connective tissue of beef. The series of experiments in the first study investigated the porous appearance of beef cooked after fresh storage in sealed, impermeable pouches filled with as atmosphere of pure CO_2 (O_2 < 200 ppm). This packaging and storage system has not been found to affect the taste of beef adversely; however, appearance has a major influence on the satisfaction of the consumer with a product, and the porous appearance is a decidedly negative factor in consumer acceptance. Gill and Penney (1990) postulated that this porous appearance of CAP-stored beef after it was cooked was a result of solubilization or degradation of muscle connective tissue proteins, due to the increased concentration of bicarbonate in the meat. Hence, to fully understand the state of collagen in beef that had been stored using CAP, the cause of the pores had to be examined.

The hypothesis tested in the first study (Chapter 2) was that the pore formation in beef stored using CAP was due to the rapid CO_2 gas evolution during cooking. In a series of experiments, the effect of sodium bicarbonate on beef was observed and was found to induce pores identical to those found in beef cooked following storage in CO_2 gas. To determine whether the pores remained following the removal of the sodium bicarbonate, the beef exposed to sodium bicarbonate solution was extensively dialyzed against water. No pores were observed after cooking of beef treated in this manner, indicating that there was no irreversible effect of bicarbonate on the connective tissue proteins. Finally, measurement of the gas produced during cooking supported the hypothesis by showing that

beef stored in CO_2 gas or in sodium bicarbonate solution produced almost twenty times more gas during cooking than beef packaged under vacuum.

The results of the first study strongly implied that the pores were indeed a result of CO_2 gas gathering along the perimysium, as the dissolved CO_2 in the aqueous portion of the meat became less soluble when the temperature of the meat increased during cooking. The pores tended to occur along the perimysium, most likely because the perimysium is one of the weakest structural components of cooked beef (Purslow 1985), and CO_2 would accumulate in the weak areas.

After verifying that bovine collagen was unaffected by increased levels of CO_2 in beef, a more thorough characterization of the modifications in collagen structure during CAP storage was undertaken. The CAP system was used to provide an extended period of storage so that the state of collagen during aging could be assessed. Detection of changes in bovine collagen during aging has eluded many researchers (Sharp 1963; Chizzolini et al. 1977; Wu et al. 1982). Only recently did Stanton and Light (1987; 1988; 1990) provide evidence of subtle changes in the two-dimensional SDS-PAGE CNBr peptide profiles of bovine perimysium and endomysium collagens due to aging. These researchers, however, did not characterize the thermal behaviour of the perimysium and endomysium collagens isolated, and were prevented from making strong conclusions with respect to the solubility of the endomysium collagen because of problems with sample purity. Clearly, further research was required to characterize the endomysium and perimysium collagen more completely.

The objectives of the second study were: to obtain an endomysium sample free of

myofibrillar protein; to investigate the effects of aging on the thermal solubility and lability of the endomysium and the perimysium; and to assess the types of collagen present. The first objective was achieved by isolating endomysium from beef with no myofibrillar protein contamination using the method of Helander (1957), who obtained a collagen residue free of myofibrillar proteins. The residue that was isolated as endomysium was approximately 30% collagen as estimated by measurement of its hydroxyproline content. Light and Champion (1984) found that the endomysium they isolated was 42.4% collagen, and the authors said it was heavily contaminated with myofibrillar proteins. In this thesis research, however, the substance associated with the endomysium was non-collagenous and, for the most part, non-protein as indicated by the results of nitrogen content analysis. The nitrogen analysis showed that approximately half the endomysium was proteinaceous, which indicated that not all the protein present was collagen. Extraction of the glycosaminoglycans from the endomysium showed that they were not present in detectable quantities. As well, the non-collagen protein and non-protein substances appeared strongly associated with the endomysium which suggested direct bonding of the substances to collagen. Glycoproteins are capable of close associations with collagen (Jilck and Hormann 1979; Terranova et al. 1980) and may have been the substance present in the endomysium isolated in this study.

Unexpectedly, the nitrogen and hydroxyproline contents of the perimysium increased with CAP storage. The collagen content of the perimysium increased from 55% to 76% from week 0 to week 8 of CAP storage. The protein content of the perimysium increased with storage as well, rising from 76% to 92%, at 0 and 15 weeks of storage, respectively.

The most probable contaminating substances which contain both protein and non-protein components are glycoproteins. The significant decrease in non-protein material exhibited by the perimysium may have been from oligosaccharides of the glycoproteins being degraded during CAP storage. There was no change in the composition of the endomysium during CAP storage, suggesting that it was resistant to the action of post-mortem lytic enzymes.

The heat solubility and denaturation temperature of the two sources of collagen were measured in order to assess how resistant each source was to the heat of cooking. This provided an estimate of how tough the collagen would have been in the cooked beef, with a high solubility and a low temperature of denaturation (T'_m) denoting collagen of reduced toughness. Measurement of the heat solubility of the perimysium showed that the perimysium from the high pH roasts had higher mean solubility values at 0 weeks of storage than the perimysium from the normal pH roasts. After 12 weeks of CAP storage, however, the perimysium samples from normal pH roasts were more heat soluble at 30 min of cooking than perimysium samples from high pH roasts. These significant differences did not present an identifiable trend and were attributed to sample variability made significant by the large number of samples included in the statistical analysis. Research has shown that high pH beef is not as tough as normal pH beef but this decrease in toughness was attributed to increased proteolysis of myofibrillar proteins and not collagen (Beltran et al. 1993).

Measurement of the heat solubility of the perimysium samples showed that the extremely heat-labile collagen, that which is soluble after 15 min of cooking, was

significantly greater at 15 weeks than at 0 weeks of storage. This result implies that the perimysium was altered in some way during storage, making some of it more heat-labile (Goll et al. 1964; Hill 1966). The changes in heat solubility were very small, however, and may not be detectable at a sensory level.

There was no significant effect of storage on the solubility of the endomysium samples; however, endomysium samples that had been stored for 15 weeks tended to be more soluble than those that were unstored ($P = 0.26$). These results are similar to those of Stanton and Light (1990), who found that bovine endomysium that had been aged was more soluble than that which had not been aged, although the trend in their data was not significant either. The consistency of the difference between the unstored and stored endomysium shown in this thesis strongly suggests that the endomysium did sustain limited proteolytic damage during CAP storage, although the changes in tenderness of cooked beef may be too small to detect.

The heat solubility data also showed that the perimysium was substantially more soluble than the endomysium at most stages of cooking (15, 30, 60 and 120 min of cooking). These results are also in agreement with Stanton and Light (1990), who found that the endomysium was less soluble in 6 M urea than the perimysium.

CAP storage affected the heat denaturation characteristics of the endomysium and perimysium differently, as denoted by the significant interaction between collagen source and time of storage for T_m and enthalpy. The mean T_m values of the perimysium were significantly greater at 0 and 4 weeks of storage than those of the endomysium, but were significantly less than those of the endomysium at 12 and 15 weeks of storage. The T_m

values of the endomysium decreased significantly between 0 and 4 weeks of storage, with no significant change after that time. There was no change in the mean enthalpy values of the perimysium during storage. The mean enthalpy values of the endomysium, however, increased between 0 and 4 weeks of storage, only to decrease from 12 to 15 weeks of storage. The fluctuations in the mean enthalpies of the endomysium during storage did not present an identifiable pattern, and the significant differences were attributed to the variability in the collagen content of the samples.

The decline in T_m values of the perimysium with storage shown in this thesis is in agreement with other researchers (Judge and Aberle 1982). No research on the characterization of the thermal behaviour of the endomysium can be found in the literature, possibly because the isolation of the endomysium has been accomplished only recently (Light and Champion 1984; Stanton and Light 1990).

The denaturation characteristics of the perimysium suggested that covalent bonds were hydrolyzed during storage. A decreased T_m and unchanged enthalpy has been associated with the reducibility of covalent bonds (Flandin et al. 1984; Horgan et al. 1990; Smith and Judge 1991). As well, the DSC measurements of the Type I, III and IV collagen standards supported the hypothesis that covalent bonds in the perimysium were broken during CAP storage. The acid-soluble collagens, Types III and IV, which had no covalent bonds, had significantly lower T_m values than the Type I collagen, which was covalently crosslinked to the point of insolubility in acetic acid. Covalent bonding had no effect on the denaturation enthalpy values because all three of the collagen standards had similar mean enthalpy values. Denaturation enthalpy of collagen is determined by the hydrogen bonds

disrupted in the triple helix (Luescher et al. 1974).

Overall, the perimysium and endomysium had similar mean T_m values, but the perimysium had significantly greater mean enthalpies at all storage times than the endomysium. Using the equation $T_D = \Delta H/\Delta S$ (Privalov and Tiktopulo 1970), the increase in denaturation enthalpy of the perimysium must be matched by an increase in its entropy in order to maintain similar T_m values between the two sources of collagen. The increased denaturation entropy of the perimysium is indicative of an increased number of bonds disrupted during denaturation, leading to a higher degree of structural randomness in the perimysium than in the endomysium. This supposition is supported by the data from the collagen standards, which showed clearly that the acid-soluble collagen standards Types III and IV had higher denaturation entropy values than the insoluble Type I standard.

The DSC data also showed that the collagen from the high pH roasts had significantly higher mean T_m values at 0 (3 days post-mortem) and 4 weeks of storage than collagen from normal pH roasts. This result suggests that collagen from high pH roasts had more covalent bonds early in storage than collagen from normal roasts. Covalent bonds may have been maintained in this muscle group because the high muscle pH may have reduced catheptic enzyme activity. Another explanation could be related to lysosomal integrity, with normal pH muscle having more disruption of lysosomal membranes than high pH muscle. Beltran et al. (1993) found that cathepsin B and L activity did not differ in bovine muscles of pH values less than 5.8 and greater than 6.0; however, large standard deviations in the results of their study indicated sample variability that may have masked

differences. The increased heat lability of the collagen from the normal pH roasts shown in this thesis may have been due to a reduction in the number of covalent bonds, which implies that lysosomal enzymes may be very important in collagen degradation during aging.

Profiles of collagen components on SDS-PAGE revealed that the endomysium and perimysium consisted of Types I and III collagen, results that agree with those of Stanton and Light (1990). Precipitation of the perimysium and endomysium with 1.2 M and 2.5 M NaCl following pepsin digestion crudely fractionated the collagen into Types III and I, respectively. Both Types I and III were detected in all fractions of the endomysium and in perimysium. The two types may have co-precipitated during salt fractionation because they were bonded covalently. Evidence exists that indicates the presence of bonds between Type I and III molecules (Henkel and Glanville 1982; Kuypers et al. 1994).

Excessive digestion of collagen from the two sources resulted in much of the α1(III) chains appearing with the α1(I) chains in the gels. The α1(III) chains that were separated from the α1(I) band using β-mercaptoethanol were from the Type III collagen that remained disulphide-bonded following pepsin digestion. The intensity of the α1(III) band decreased in the 1.2 M NaCl precipitate of the endomysium and was absent following 15 weeks of storage in the 2.5 M NaCl precipitate of the perimysium. The decrease in the density of the α1(III) band in these fractions may be evidence of an increased susceptibility of Type IiI collagen to enzyme digestion with storage. Stanton and Light (1990) found evidence that Type III collagen was degraded during aging as well, with the ratio of Type III relative to Type I decreasing in SDS-PAGE profiles of CNBr peptides..

Profiles of collagen components obtained using SDS-PAGE also showed that the perimysium had more soluble α and β-components than the endomysium. Most of the endomysial collagen remained at the origin of the gel, possibly because the applied sample contained crosslinked aggregates too large to penetrate the gel matrix.

To summarize, the results of the experiments combined in this thesis show that bovine collagen is unaffected by high levels of dissolved CO_2 during CAP storage, and that the heat lability of the perimysium is increased during storage possibly by the disruption of covalent bonds. As well, the results indicate that there is little change in the thermal lability and composition of the endomysium due to CAP storage. The endomysium may have been degraded less because it may contain a high proportion of Type IV collagen, which is densely crosslinked with heat-resistant keto-amine crosslinks (Heathcote et al. 1980; Le Pape et al. 1981; Wu and Cohen 1982), but this was not investigated in this thesis. Overall, the results of the research contained in this thesis show that collagen degradation occurs at the level of the perimysium, and this may contribute to the increased tenderness of beef during CAP storage.

The research in this thesis addressed the hypothesis that post-mortem proteolysis of covalent bonds increases heat lability of collagen. The data from the DSC analyses indicated that covalent bonds are hydrolyzed during CAP storage because the T_m declined while the enthalpy of denaturation remained unchanged (Flandin et al. 1984; Smith and Judge 1991). The T_m of collagen has been found to increase with increasing concentrations of covalent bonds that contain pyridinoline and Ehrlich's Chromogen (Horgan et al. 1990), with no change in enthalpy (Smith and Judge 1991).

Although the enzyme system responsible for the proteolysis of collagen during storage was not examined in this thesis, the continuance of the decline of the T_m values of the perimysium late into the CAP storage period, as well as the loss of non-protein material from the perimysium until 8 weeks of storage, imply lysosomal enzyme activity. Stanton and Light (1988) found that two-dimensional SDS-PAGE profiles of perimysium from aged beef were similar to profiles of perimysium exposed to crudely extracted lysosomal enzymes. Unfortunately, no research was found that compared two-dimensional SDS-PAGE profiles of aged perimysium to those of perimysium exposed to calpains or collagenases.

In the research performed for this thesis, proteoglycans could not be quantitated and probably did not exist in an amount sufficient to increase the resistance of the endomysium collagen to post-mortem proteolysis. The effect of glycoproteins on the resistance of collagen, particularly endomysium, to post-mortem proteolysis may warrant investigation because glycoproteins can increase the resistance to denaturation of a protein to which they are attached (Chu et al. 1978). The new method presented in this thesis for the isolation of endomysial collagen without the use of SDS will enable the endomysium to be studied in a more native state and should facilitate further characterization of the contribution of this collagen to meat tenderness.

4.1. LITERATURE CITED

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