โมเลคิวลาร์โลไซและไกลโคซิเลชั่นของดีไฮโดรฮิสติดิโนไฮดรอกซีเมโรเดสโมซีน ในเส้นใยคอลลาเจนแบบที่หนึ่งของเนื้อเยื่อที่ไม่มินเนราลไลซ์

ศิริวิมล ศรีสวัสดิ์

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MOLECULAR LOCI AND GLYCOSYLATION OF DEHYDRO-HISTIDINOHYDROXYMERODESMOSINE IN NON-MINERALIZED TYPE I COLLAGEN FIBRILS

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ศิริวิมล ศรีสวัสดิ์ : โมเลคิวลาร์โลไซและไกลโคซิเลชั่นของดีไฮโดรฮิสติดิโนไฮดรอกซีเมโรเดสโมซีนในเส้นใยคอลลาเจนแบบที่หนึ่งของเนื้อเยื่อที่ไม่มินเนราลไลซ์ (MOLECULAR LOCI AND GLYCOSYLATION OF DEHYDRO-HISTIDINOHYDROXYMERODESMOSINE IN NON-MINERALIZED TYPE I COLLAGEN FIBRILS) อ. ที่ปรึกษา : ผศ.ทพ.ดร.ประสิทธิ์ ภวสันต์, อ.ที่ปรึกษาร่วม : Prof.Dr.Mitsuo Yamauchi 116 หน้า. ISBN 974-13-0601-6.

การเชื่อมต่อกันระหว่างโมเลกุลของคอลลาเจนโดยครอสลิงค์แบบพันธะโคเวเลนต์เป็นสิ่งสำคัญที่ทำให้เครือข่ายของคอลลาเจนมีคุณสมบัติทางชีวกายภาพที่เหมาะสม ดีไฮโดรฮิสติดิโนไฮดรอกซีเมโรเดสโมซีนเป็นสารครอสลิงค์ชนิดเตตราเวเลนต์ที่พบในเนื้อเยื่อที่ไม่มินเนราลไลซ์ทั่วไป อย่างไรก็ดีถึงแม้จะมีปริมาณมาก สารครอสลิงค์ตัวนี้ก็ไม่ได้รับการศึกษาอย่างละเอียดถ่องแท้ วัตถุ ประสงค์ของการศึกษานี้คือ เพื่อทำการแยกสารครอสลิงค์นี้ และศึกษาถึงตำแหน่งในโมเลกุล ตลอดจนภาวะไกลโคซิเลชั่นของครอสลิงค์นี้โดยศึกษาในเนื้อเยื่อคอลลาเจนส่วนที่ไม่มินเนราลไลซ์ของเอ็นขาไก่งวง โดยนำเนื้อเยื่อคอลลาเจนดังกล่าวมาทำการรีดิวซ์โดยใช้สารโซเดียมโบโรไฮไดรด์ที่มี กัมมันตภาพรังสี และนำไปทำการย่อยเป็นลำดับขั้นโดยใช้เอนไซม์ชนิดต่างๆ สารครอสลิงค์ถูกแยกออกจากกันโดยขบวนการโครมาโตกราฟฟีชนิดแรงดันสูง ผลที่ได้นำมาหาลำดับของกรดอะมิโนและการวิเคราะห์หามวล พบว่าครอสลิงค์นี้อาจเกิดได้ที่สามตำแหน่งในโมเลกุล ได้แก่ 1) ไลซิล อัลดีไฮด์ (อัลฟา1 9N) X ไลซิล อัลดีไฮด์ (อัลฟา1 9N) X ไฮดรอกซีไลซีน (อัลฟา1 930) X ฮิสติดีน (อัลฟา2 935) 2) ไลซิล อัลดีไฮด์ (อัลฟา1 9N) X ไลซิล อัลดีไฮด์ (อัลฟา 2 6N) X ไฮดรอกซีไลซีน (อัลฟา1 930) X ฮิสติดีน (อัลฟา 2 935) 3) ไลซิล อัลดีไฮด์ (อัลฟา1 9N) X ไลซิล อัลดีไฮด์ (อัลฟา2 6N) X ไฮดรอกซีไลซีน (อัลฟา1 930) X ฮิสติดีน (อัลฟา1 932) ผลการทดลองที่ได้แสดงให้เห็นว่าตำแหน่งในโมเลกุลที่มักเกิดครอสลิงค์ชนิดนี้มากกว่าเกี่ยวข้องกับอัลดีไฮด์ที่มาจากปลายทางด้านอะมิโน ไฮดรอกซีไลซีนจากตำแหน่ง 930 บนสายอัลฟา1 และฮิสติดีนจากตำแหน่ง 935 บนสายอัลฟา 2 นอกจากนี้ยังพบว่าไฮดรอกซีไลซีนบนสายอัลฟา 1ตำแหน่ง930ซึ่งเกี่ยวข้องกับการเกิดครอสลิงค์ชนิดนี้ไม่มีไกลโคซิเลชั่น แสดงว่ากลุ่มคาร์โบไฮเดรตที่เกาะที่ไฮดรอกซีไลซีน 930 อาจเป็นตัวกำหนดทิศทางการเกิดครอสลิงค์

หลักสูตร ชีววิทยาช่องปาก ลายมือชื่อนิสิต.....

สาขาวิชา ชีววิทยาช่ องปาก ลายมือชื่ออาจารย์ที่ปรึกษา.....

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KEY WORD: TYPE I COLLAGEN / TURKEY TENDON / DEHYDRO-HISTIDINOHYDROXYMERODESMOSINE

SIRIVIMOL SRISAWASDI : MOLECULAR LOCI AND GLYCOSYLATION OF DEHYDRO-HISTIDINOHYDROXYMERODESMOSINE IN NON-MINERALIZED TYPE I COLLAGEN FIBRILS. THESIS ADVISOR : ASST. PROF. DR.PRASIT PAVASANT, THESIS COADVISOR : PROF. DR.MITSUO YAMAUCHI, 116 pp. ISBN 974-13-0601-6.

The covalent intermolecular cross-linking is essential in providing collagen matrices with physicochemical properties. Dehydro-histidinohydroxymerodesmosine is a major tetravalent collagen cross-link present in most non-mineralized tissues. Despite its abundance, this cross-link has been poorly characterized. The objectives of this study were to isolate the cross-linked peptides, determine the molecular loci and evaluate the state of glycosylation of the cross-link. The peripheral layer of the turkey leg tendon (never-mineralized collagenous tissue) was reduced with tritiated sodium borohydride, subjected to sequential enzymatic/chemical digestion and several cross-linked peptides were isolated by means of HPLC. Based on the amino-terminal sequence, amino acid composition and mass spectrometric analyses of the purified peptides, three molecular loci were identified. They are lysyl aldehyde (α1-9N) X lysyl aldehyde (α1-9N) X hydroxylysine α1-930) X histidine (α2-935), lysyl aldehyde (α1-9N) X lysyl aldehyde (α2-6N) X hydroxylysine (α1-930) X histidine (α2-935) and lysyl aldehyde (α1-9N) X lysyl aldehyde (α2-6N) X hydroxylysine (α1-930) X histidine (α1-932). The data indicate that the aldol involved in the cross-link is derived from the amino-terminal telopeptidyl aldehydes, and that the hydroxylysine on an α1 chain (residue 930) and histidine from α2-935 is the preferred cross-linking site. The latter suggests a stereospecific packing of the type I collagen molecules in the fibril. In addition, chemical and mass spectroscopic analyses of the peptides and their acid/base hydrolysates revealed that the hydroxylysine residues (α1-930) that are involved in the formation of this complex cross-link were not glycosylated. These results suggest that the carbohydrate moiety on the α1-930 hydroxylysine may have a role in regulating the cross-link formation.

Department Oral Biology Student's signature.....

Field of study Oral Biology Advisor's signature.....

Academic year 2000 Co-advisor's signature.....

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List of Abbreviations

Asn	= asparagine
CNBr	= cyanogen bromide
DDW	= deionized distilled water
DeH-DHLNL	= dehydro-dihydroxylysinonorleucine
DeH-HLNL	= dehydro-hydroxylysinonorleucine
DeH-HHMD	= dehydro-histidinohydroxymerodesmosine
DeH-LNL	= dehydro-lysinonorleucine
DHLNL	= dihydroxylysinonorleucine
d-Pyr	= deoxypyridinoline
DTT	= dithiothreitol
ECM	= extracellular matrix
EDTA	= ethylenediamine tetra-acetic acid
ESI	= Electrospray Ionization
FPLC	= Fast Protein Liquid Chromatography
Gly	= glycine
HHL	= histidinohydroxylysinonorleucine
HHMD	= histidinohydroxymerodesmosine
His	= histidine
HLKNL	= hydroxylysino-5-ketonorleucine
HLNL	= hydroxylysinonorleucine
HL-Pyr	= hydroxylysyl pyridinoline
HPLC	= High Performance (or Pressure) Liquid Chromatography
Hyl	= hydroxylysine
Hylald	= hydroxylysyl aldehyde
Hyp	= hydroxyproline
IPA	= isopropanol
LH	= lysyl hydroxylase
LHNL	= lysinohydroxynorleucine
LKNL	= lysino-5-ketonorleucine

LNL	= lysinonorleucine
L-Pyr	= lysyl- pyridinoline
Lysald	= lysyl aldehyde
MALDI-MS	= Matrix-assisted Laser Dessorption /Ionization Mass Spectrometry
MS/MS	= Tandem Mass Spectrometry
PBS	= phosphate buffered saline
PDL	= periodontal ligament
Pyr	= pyridinoline

Chapter I

Background and Significance

Chemistry of Type I Collagen Cross-links

Type I Collagen : Definition, Classification and Biosynthesis

The most abundant proteins of the extracellular matrix (ECM), of the vertebrate body, are member of the collagen superfamily. The name “collagen” is generically used to cover a wide range of protein molecules which form supramolecular ECM structures and share the basic structural motif of three polypeptide chains wound in a characteristic triple helical configuration. Collagen, by definition, is a structural protein that contains one or more domains of a triple helix comprised of three polypeptides, α chains, and assembled into an aggregate in the extracellular space which gives connective tissues their physicochemical properties (Van Der Rest et al., 1993, Yamauchi, 1995). Each of the three polypeptide chains forms a left-handed helix, stabilized by high imino acid content (i.e. proline and hydroxyproline), and the three chains are supercoiled around a common axis in the right-handed manner to form a triple helix. Collagens contain large domains comprised of repeating -Gly-X-Y- sequences where the residues in the X and Y positions are often proline and hydroxyproline respectively (Piez, 1984, Linsenmayer, 1991, Van Der Rest et al., 1993). The presence of glycine as every third amino acid in the repeating Gly-X-Y- sequence is essential because a larger amino acid will not fit in the center of the triple helix, and only glycine which is the smallest amino acid with a side chain limited to a single hydrogen atom

can fit into this position providing flexibilty and conformation of the triple helix. The triple helix is relatively rigid giving the molecule resistance to extension and compression which is important for the biological function of the protein. The positions -X-Y- can be occupied by any amino acid without having any effect on the triple helical structure, however, proline and 4-hydroxyproline are often found in the -X-Y- positions respectively, which helps stabilizing the triple helical structure (Yamauchi, 1995, Bateman et al., 1996). Another important chracteristic of collagen is its extensive post-translational modifications many of which are unique to collagen. The modifications include the enzymatic hydroxylation of specific proline and lysine residues, and the further modification by glycosylation of some specific hydroxylysines, and finally the formation of covalent intra/intermolecular cross-links. The formation of hydroxyproline is important in stabilizing the structure of triple helix by the formation of extra hydrogen bonds (Miles and Bailey, 2001). Lysyl residues in the –Y- position of the Gly-X-Y triplets can be converted to hydroxylysines by lysyl hydroxylases. Specific hydroxylysine residues then undergo further post-translational enzymatic modifications including glycosylation and oxidation. Both hydroxyproline and hydroxylysine are nearly unique to collagen. The ability of collagen to form highly stable supramolecular assemblies in the ECM is due to the formation of covalent intermolecular cross-links which involve specific lysine, hydroxylysine and histidine residues. Side chains of -X-Y- residues point outward from the helix and are available for lateral interactions between adjacent triple helices leading to fibril formation and for interactions with other matrix proteins and cells.

Collagen Superfamily (Van Der Rest et al., 1993, Yamauchi, 1995, Bateman et al., 1996)

Collagens can be classified into three major groups (Figure 1.1) :

1. Fibril-forming collagens , such as types I, II, III, V, XI

2. Fibril Associated Collagen with Interrupted Triple Helices (FACIT) , such as types IX, XII, XIV, XIX.

3. Others or non-fibrillar collagen, such as types IV, VI, VII, VIII, X, XIII

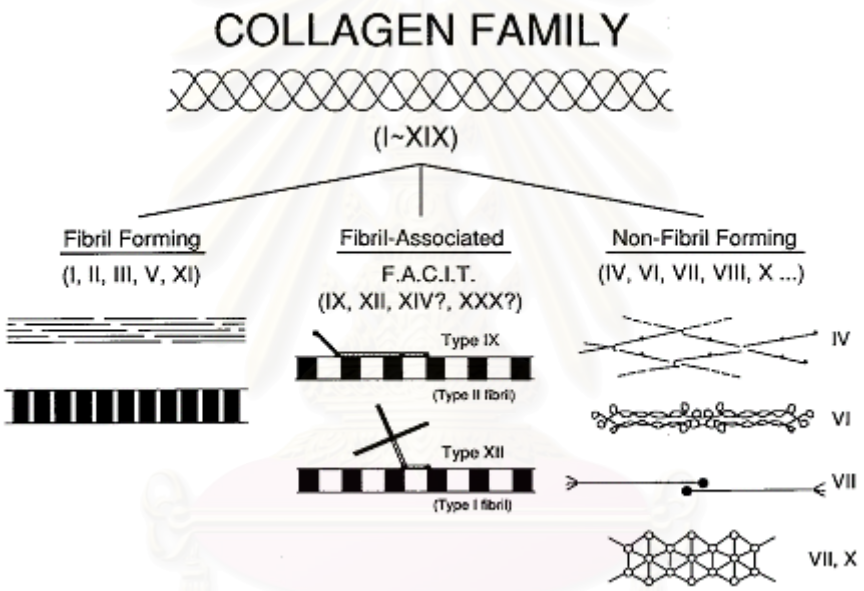

Figure. 1.1 Types of collagens (Reprinted with permission from Yamauchi,

Fibril-forming Collagens (Linsenmayer, 1991, Van Der Rest et al., 1993, Yamauchi 1995, Bateman et al., 1996)

Collagen types I, II, III, V and XI belong to the fibril-forming group. Of all these collagen types, type I collagen is the most abundant and represents the major extracellular matrix component in most tissues and organs of mammals. Type I collagen, using an electron microscopy with negative staining, appears as a characteristic banding pattern along the fibril axis of about 65-67 nm periodicity called the D-period (Figure 1.2). This striation of the fibril (D period) corresponds to about 1 / 4.4 the length of an individual molecule. The D-period results from the packing of individual collagen molecules, which are about 300 nm or 4.4 D long, into a staggered overlap arrangement with respect to one another by the axial repeat distance of 65-67 nm or 1.0 D resulting in a gap of about 0.6 D between the end of one molecule and the beginning of the next. Such molecular arrangement creates alternate zones of high and low packing densities as alternate clear (overlap zone = 0.4 D) and dark (gap or hole zone = 0.6 D) bands, when viewed using an electron microscopy with negative staining. It should be noted that the striated patterns of fibril-forming type of collagen have been found to be slightly different among tissues and also can be varied depending on sample processing techniques when viewing with electron microscope. However, studies of x-ray diffraction using tendon, bone, dura mater and cartilage, where potential sample preparation artefacts are avoided, confirm the presence of 67 nm D-period. In other tissues including skin, periodontal ligament, gingival tissue, heart valves and granuloma tissue, a shorter D-period of about 65-66 nm has been observed. A

common feature of all these tissues exhibiting a shorter D-period is that they are more elastic and flexible, and contain a significant amount of type III collagen suggesting a possible role of this collagen in regulating the packing arrangement of type I collagen. The packing difference may also be due to the effect of collagen-binding matrix molecules (e.g. certain classes of small leucine-rich proteoglycans) that have been shown to regulate the collagen fibrillogenesis. Evidences have also shown that collagen fibrils are comprised of several collagen types, heterotypic fibrils, and this copolymerization may play an important role in regulating fibril architecture.

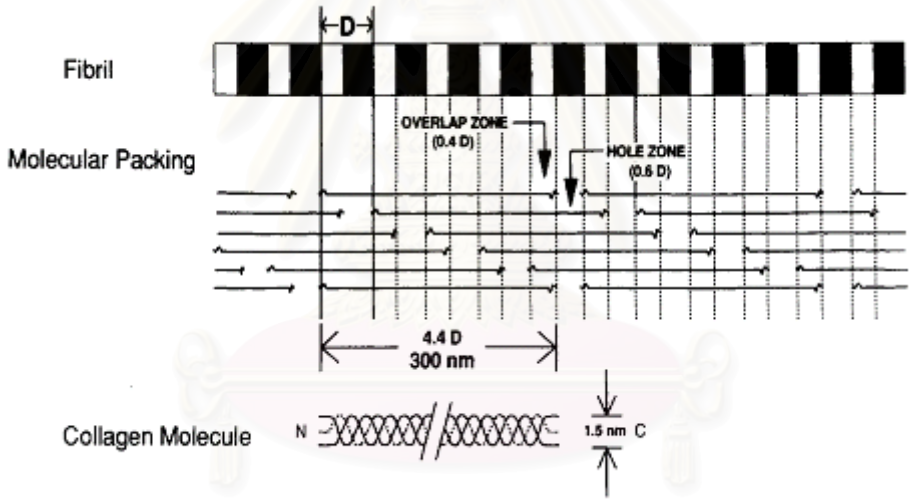

Figure 1.2 Banding pattern of type I collagen
(Reprinted with permission , from Yamauchi , 1995)

Molecular Structure of Type I Collagen

Type I collagen is a heterotrimer comprised of two identical α□1 (I) chains and one α 2 (I) chain, $[\alpha 1\ (I)]_2\ \alpha$ 2 (I), and is the major fibrillar component of a wide variety of tissues. Type I collagen can exist as a homotrimer of $[\alpha\ 1\ (I)]_3$, however, the functional significance of this minor molecular form has not been elucidated. A collagen molecule consists of three domains which are the short NH_2-terminal and COOH-terminal non-triple helical and the long triple helical domains. The α chains of the fibrillar collagen are synthesised as large precursors, pro α chains with N- and C- terminal globular extensions called propeptides. The N- and C-propeptides then are proteolytically removed by enzymes N- and C-proteinases producing the collagen monomers consisting of the long triple helix and short non-helical N- and C-telopeptides at the ends of the molecule. The amino acid sequences of both domains vary between chain types and species. The N- and C-telopeptidyl lysines are important cross-linking sites for collagen types I, II and III suggesting that the main function of the telopeptides is to stabilize the collagen fibrils.

Fibril-Associated Collagens with Interrupted Triple Helices (FACIT) : Types IX, XII, XIV, XVI and XIX (Yamauchi, 1995, Bateman et al., 1996)

The Fibril-Associated Collagens with Interrupted Triple helices or FACIT collagens contain small triple helical domains interrupted by short non-helical domains. They alone do not form fibrils but interact with the fibrillar groups of collagen, such as types I and II, and project their amino-terminal globular domains out into the matrix. For example, type IX

collagen, which is a heterotrimer of α1(IX) α2(IX) α3(IX), locates periodically along the surface of type II collagen. Types XII and XIV, homotrimeric molecules, are present mainly in tissues that contain type I collagen as a major fibril-forming type, and both types have recently been identified in developing cartilage. These FACIT collagens may link fibrils together or modulate interactions between fibrils and other matrix molecules and/or cells.

Other Types of Collagen or Non-Fibrillar Collagens (Van Der Rest et al., 1993, Bateman et al., 1996)

Types IV, VI, VII, VIII and X collagens belong to the non-fibril forming group. Type IV collagen is present exclusively in basement membrane and associated structures as a very long, 400 nm, and flexible molecule. It exists predominantly as a heterotrimer of $[\alpha 1(IV)]_2$ α2(IV), and each molecule consists of a central triple helix containing several short interruptions to the Gly-X-Y sequence. Individual type IV collagen molecules assemble to form a sheet-like network which acts as the basic framework of basement membrane providing the basement membrane a molecular sieving role and regulating cell migration, growth and differentiation, in addition to providing a mechanical support for cells. Type VI collagen forms an extensive network of microfilaments in the ECM of all connective tissues. Type VI collagen has a short triple helical domain and a very large terminal globular domain. The secreted tetramers aggregate to form filaments. This type of collagen has been proposed to modulate the organization of the microfilaments and interactions with cells and other matrix molecules. However, the precise function of type VI collagen has not yet been

determined. Type VII collagen is the major component of anchoring fibrils. It is a large homotrimeric molecule consisting of a small C-terminal globular domain, a central interrupted triple helical domain and a very large N-terminal globular domain. It is secreted as dimers which subsequently aggregate to form fibers. Types VIII and X collagens are structurally related short-chain collagens. Type VIII collagen is the major structural protein of Descemet's membrane, a specialized basement membrane synthesized by corneal endothelial cells, where it forms hexagonal lattices. However, the packing arrangement of type VIII collagen in other tissues has not been identified. Type X collagen is a homotrimer of $[\alpha 1(X)]_3$ exhibiting a similar hexagonal lattice pattern when forming aggregates in vitro shown by rotary shadowing. Type X collagen, however, has been localized in fine mat-like filaments and in association with type II fibrils in cartilage.

Several types of collagen, such as types XIII, XV, XVI, XVIII and XIX are unique collagen chains known only from cDNA and genomic sequencing. Type XIII is expressed in a wide range of tissues, however physiologic function and macromolecular structure have not been elucidated. Types XVI and XIX show the characteristic of the FACIT group and also have been found to be associated with major collagen fibrils and modulate matrix interactions. Types XV and XVIII have been identified recently as two homologous collagens having multiple triple helical cassettes flanked and separated by non-collagenous sequences. Type XV mRNA is expressed predominantly in internal organs, such as the adrenal gland, kidney and pancreas. Type XVIII has highest mRNA levels in liver, kidney and placenta.

Collagen Characteristics in Other Proteins (Bateman et al., 1996)

The collagen motif is also found in other proteins such as in complement component C1q, serum mannan binding protein, in integral membrane components of acetylcholinesterase, the macrophage scavenger receptor, pulmonary surfactant proteins and conglutinin D. The ability to fold into a triple helix and to form a semi-rigid elongated structure, the two characteristics of the collagen motif, allows these proteins to promote subunit oligomerization and to provide a rigid spacing element. In some of these proteins the collagen motif may play a functional role, such as platelet adhesion or fibronectin binding, in addition to being a structural element.

Biosynthesis of Type I Collagen (Kivirikko and Myllyla, 1984, Olsen, 1991, Yamauchi, 1995, Bateman et al., 1996)

The pathway of collagen biosynthesis from gene transcription to secretion and aggregation of collagen monomers into functional fibrils is complex (Figure 1.3). The biosynthesis pathway of the fibril-forming group of collagen, especially type I, has been extensively studied. Collagen gene transcription and mRNA processing within the nucleus follow the general pathway described for other eukaryotic genes. Mature mRNAs are transported into the cytoplasm where translation occurs. Type I collagen translation products are initially directed into the endoplasmic reticulum by the N-terminal signal peptide which subsequently is cleaved from the nascent polypeptide chain by signal peptidase.

The nascent polypeptides then require a number of co- and post-translational modifications both intracellularly and extracellularly.

Intracellular Post-translational Modification

Individual procollagen chains require a number of enzyme-catalysed post-translational modification prior to the completion of chain assembly and the folding of the triple helix (Yamauchi, 1995). Prolines in the –Y- position of the Gly-X-Y sequence are hydroxylated by prolyl-4-hydroxylase. Ascorbate, in addition to ferrous ion, O_2 and α-ketoglutarate, is a co-factor for the enzymatic activity of prolyl hydroxylase. Ascorbate in low concentration is essential for the production of collagen, because a minimum of 35% of the prolyl residues need to be hydroxylated for the collagen molecule to maintain its triple helical conformation at the physiologic condition (Davidson et al., 1997). Ascorbate is also a co-factor for lysyl hydroxylase which hydroxylates lysyl residues into hydroxylysine. Hydroxylation of specific lysyl residues in the Y position of the Gly-X-Y triplet sequence and in the telopeptides is crucial for collagen fibrillogenesis, formation of covalent intermolecular cross-links, and glycosylation (Bateman et al., 1996, Notbohm et al., 1999). To date, three isoforms of lysyl hydroxylase (LH) enzymes, LH1, LH2 and LH3, have been identified and proposed to modify lysyl residues in different domains of the collagen molecule (Uzawa et al., 1999, Yeowell and Walker, 1999). Hydroxylysines then are further modified by addition of monomeric sugar, galactose, or dimeric sugar, glucosylgalactose catalysed by enzymes galactosyltransferase and galactosylhydroxylysyl glucosyltransferase respectively.

Recently, Heikkinen et al. (2000) demonstrated that lysyl hydroxylase 3 possesses both lysyl hydroxylase and galactosylhydroxylysyl glucosyltransferase activities indicating that this is a multifunctional enzyme. The precise functions of glycosylated hydroxylysines are not clear, but it has been proposed that, due to their bulky structure and hydrophilicity, they regulate the interactions between collagen molecules thus controlling the molecular packing pattern of the fibrils, and also regulate the cross-linking pattern/maturation. Procollagen chains then align with their C-propeptides in close proximity, the assembly proceeds via the folding of C-terminal propeptide domains followed by association and alignment of the chains and finally the formation of interchain disulphide bonds stabilizing the propeptide assembly. After C-propeptides association a nucleus of triple helix is formed in the C-terminal region, and rapidly propagates from C- to N-terminus of the protein in a zipper-like manner. It is now clear that intracellular protein folding, including procollagen association and folding, involves the interactions with enzymes, polypeptide chain binding proteins and several molecular chaperones. Once formed, the Procollagen molecules are packed and transformed into secretory granules and secreted through the Golgi complex.

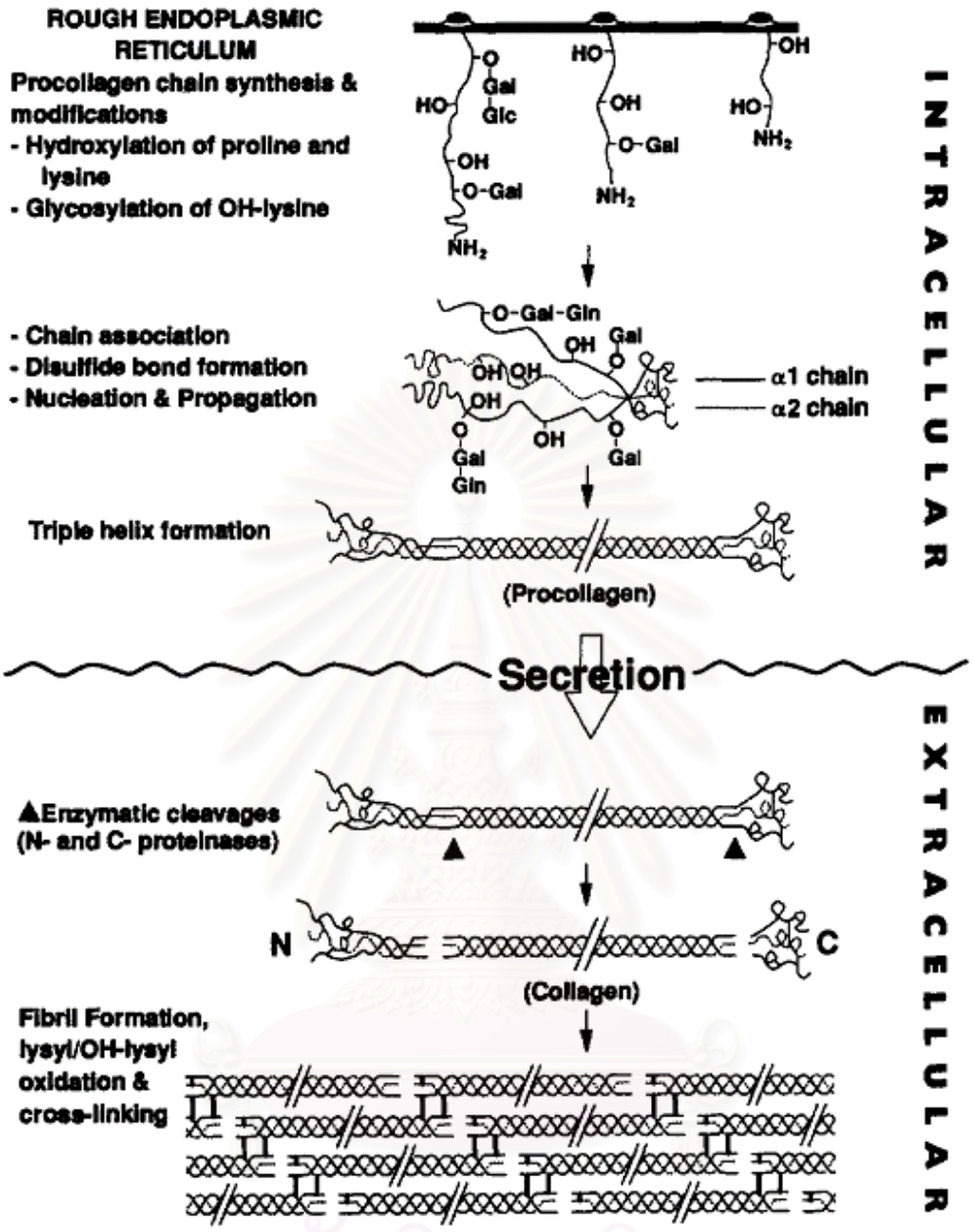

Figure 1.3 Biosynthesis of type I collagen (Reprinted with permission , from Yamauchi , 1995)

Extracellular Post-translational Modifications

After secretion, N-propeptides are cleaved by procollagen N-proteinase and C-propeptides by procollagen C-proteinase, the proteins then self-assemble into fibrils via both hydrophobic and electrostatic interactions between adjacent chains. Subsequently, lysyl oxidase, which is the only enzyme known to be required for cross-link formation, converts some lysines and hydroxylysines present in the carboxy- and amino-termini of the molecule into aldehydes and a series of covalent inter- and intra-molecular cross-links are formed to further stabilize the collagen fibrils.

Basic Chemistry of Type I Collagen Cross-linking

The enzymatic cross-linking of type I collagen is initiated by aldehyde formation of the telopeptidyl lysine or hydroxylysine residue through the action of enzyme lysyl oxidase (Yamauchi and Mechanic, 1988, Bateman et al., 1996). Lysyl oxidase (protein-lysine 6-oxidase, EC 1.4.3.13), a copper dependent enzyme, is the enzyme which oxidatively deaminates ε-amino groups on these residues (Figure 1.4). Inhibition of this enzyme activity occured in cases such as during copper deficiency and lathyrism have profound effects on collagenous tissues leading to the impairment of cross-linking formation and subsequent tissue fragility and damage (Rucker et al., 1998). After the initial enzymatic step, the subsequent reactions are spontaneous and the chemistries are controlled by a number of specific factors (see below).

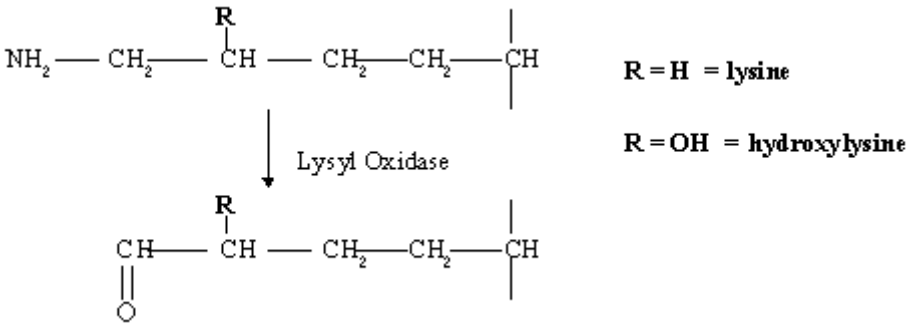

Figure 1.4 Formation of aldehyde of specific lysine and hydroxylysine residues by the action of lysyl oxidase (Yamauchi, 1995).

There are two major pathways of type I collagen cross-linkings, lysyl aldehyde pathway and hydroxylysyl aldehyde pathway (Yamauchi and Mechanic, 1988, Yamauchi, 1995). There are two routes in lysyl aldehyde pathway, one leads to the formation of the reducible dehydro-hydroxylysinonorleucine (deH-HLNL) bifunctional cross-link which further matures into a more stable non-reducible histidino-hydroxylysinonorleucine (HHL) cross-link predominantly found in skin collagen, another leads to the formation of the reducible deH-HHMD iminium cross-link via aldol which is predominant in soft connective tissues. Hydroxylysyl aldehyde pathway, predominant in skeletal tissues such as bone, cartilage, tendon and dentin, produces the reducible dehydro-dihydroxylysinonorleucine (deH-DHLNL) bifunctional cross-link which subsequently undergoes Amadori arrangement and forms the mature non-reducible naturally fluorescent pyridinoline cross-links and, possibly, a pyrrole.

The pattern of cross-links, their concentration and molecular distribution seem to be tissue-specific, which can be due to many factors such as tissue turnover rate, degree of lysyl hydroxylation, degree of hydroxylysyl glycosylation and modality of molecular packing, all of which are related to tissues' physiological function (Yamauchi,1995). Distinctive differences in the cross-linking chemistry between mineralized and non-mineralized tissues have been found. Firstly, there is a scarcity of deH-HHMD cross-link in mineralized connective tissue while it is a major one in soft connective tissues. Secondly, the free aldehyde derived from the C-telopeptide region is present in the mineralized tissue collagens, i.e. bone, dentin, mineralizing turkey tendon, (Otsubo et al., 1992, Yamauchi et al., 1992, Yamauchi et al., 1996, Yamauchi et al., 1997), but it is absent from predentin, periodontal ligament and non-mineralized part of turkey tendon (Yamauchi et al.,1992, Yamauchi et al., 1996, Yamauchi et al., 1986a). The free aldehyde has been speculated to be derived from dissociation of iminium cross-links brought about by mineralization. However, Knott et al. (1997) proposed that the free aldehydes were resulted from a newly formed special matrix required for mineralization rather than the result of mineralization.

Although cross-linking occurs extracellularly, it is apparent that the type of cross-link formed clearly depends on the previous intracellular post-translational modification of the collagen molecule, particularly hydroxylation of lysines (Yamauchi, 1995, Bailey et al., 1998, Wassen et al., 2000). The degree of lysyl hydroxylation both in the telopeptide and helical domains of the molecule varies greatly between collagen types and tissues. The specific hydroxylation of lysines governs the type of cross-link formed, and consequently the

biomechanical properties of the tissue. The hydroxylation of telopeptide lysines lead to the predominant formation of ketoimine type of cross-links giving the tissue its insolubility and stability.

Collagen Cross-linking in Mineralized Tissues

Hydroxylysyl aldehyde pathway is the major collagen cross-linking pathway in mineralized tissues (Yamauchi et al., 1988, Yamauchi, 1995, Bailey et al., 1998). Study in fetal bovine bone shows that most of 16 ^{C} lysyl residues are hydroxylated. Cross-linking is then initiated with the conversion of these specific telopeptidyl Hyl into hydroxylysyl aldehydes soon after the molecule is synthesized. These aldehydes are located near the "gap zones" and react with the juxtaposed Hyl on a neighboring molecule to form the Schiff base bifunctional cross-link deH-DHLNL. DeH-DHLNL is formed between α1 16 ^{C} Hyl ^{ald} and Hyl-87 in α1 or α2 chain on adjacent molecule. (Figure 1.5 shows an example of the molecular locations of residues involving in the cross-linking which have been identified.) The Schiff base then spontaneously undergoes an Amadori rearrangement to form a ketoimine HLKNL. The ketoimine is stable to acid and heat accounting for the insolubility of bone and cartilage collagens. These compounds can be stabilized by reduction with borohydride to form DHLNL. Another cross-link, deH-LHNL, may also form involving the reaction between a Hyl^{ald} and a helical lysine, which subsequently can undergo an Amadori rearrangement to form a ketoimine LKNL. This cross-link can be reduced to form LHNL

which is a structural isomer of HLNL, therefore rarely mentioned in the literatures, and has been found to always co-elute with the aldimine cross-link upon analysis (see Figure 1.6).

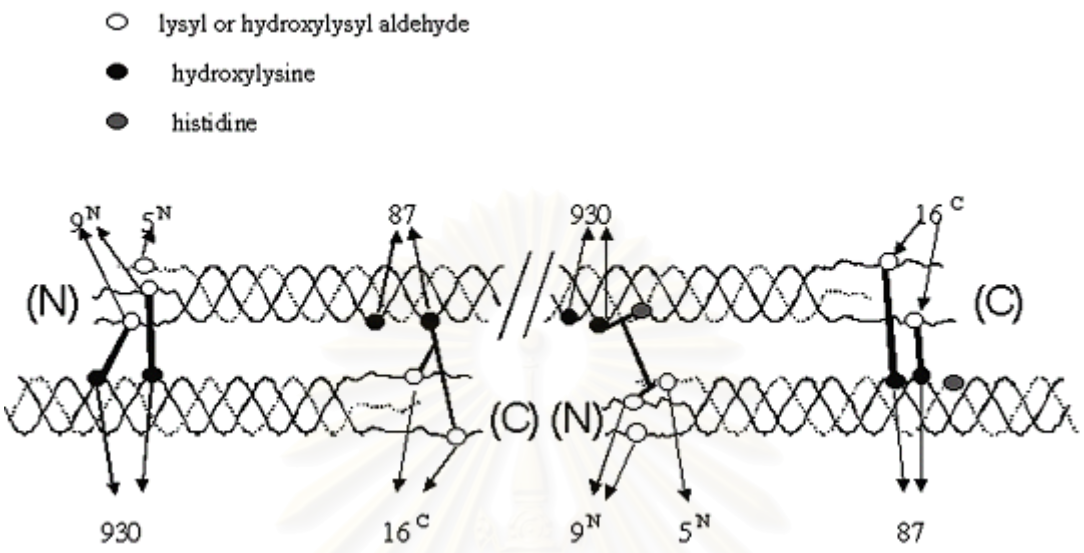

Figure 1.5 Example of locations in the molecule of residues involving in the formation of collagen cross-linking showing Lys^{ald} and Hyl^{ald} at positions 9N in α 1 chains , 5N in α2 chain and 16C in α1 chains, and helical Hyl-87, Hyl-930 in α1 chains and His 92, His-935 in α2 cahin (reprinted with permission from Prof.Mitsuo Yamauchi, personal communication).

The Formation of Mature Stable Cross-links in Mineralized Tissues

A more stable trivalent cross-link, pyridinoline, subsequently forms when two deH-DHLNL cross-links or its keto form (HLKNL) comes into contact to each other. If the reaction involves two hydroxylysyl aldehydes and one helical hydroxylysine, a pyridinoline (or hydroxylysylpyridinoline) cross-link would be formed (Yamauchi, 1995, Bailey et al., 1998). Deoxypyridinoline (d-Pyr), or lysylpyridinoline, is likely formed by the reaction involving two hydroxylysyl aldehydes and a helical lysine, and this cross-link has been found mainly in

mineralized tissues (see Figure 1.7). Studies have shown that not all deH-DHLNL cross-links mature into pyridinolines. The pyridinoline content in bone collagen has been found to be relatively low, representing about 0.2 - 0.5 residues per collagen molecule (Knott and Bailey, 1998, Bailey et al., 1998), despite the fact that bone collagen is insoluble and more resistant against acid swelling when compared to other tissue, such as cartilage which has much higher level of pyridinoline, i.e. 1.2-2.0 residues per collagen molecule. These observations led investigators to speculate that there must be other mature cross-links in bone collagen. Due to the stereospecific molecular packing it has been found that Hyl on α1 chain involves in reacting with C-telopeptidyl aldehyde more than Hyl on α2 chain, resulting in the ratio of 3.3 : 1 (Yamauchi et al., 1989, Yamauchi and Katz, 1993).

Pyrrole has been identified as another trivalent non-reducible cross-link in several connective tissues. Scott et al. (1981) first identified a pyrrolic compound in several bovine connective tissues such as skin, bone, tendon, cartilage and periosteum. The compound was termed Ehrlich chromogen (EC) since it gave pink color when reacted with Ehrlich's reagent. Evidences from several studies later (Henkel et al., 1987, Kemp and Scott, 1988, Horgan et al., 1990) showed that EC was a trifunctional cross-link located at the molecular sites analogous to those of pyridinoline cross-link. Kuypers et al., 1992, proposed that collagen-associated EC was 1,3,4-trisubstituted pyrrole formed by condensation between a bifunctional ketoamine cross-link (or a Schiff base deH-DHLNL cross- link) and a Lys^{ald} (see Figure 1.8). Hanson and Eyre (1996) using a bacterial collagenase digest obtained from demineralized human bone matrix, found that pyrrole was an alternative cross-link at the

same sites to that of pyridinoline, N-telopeptide to helix and C- telopeptide to helix, but concentrated at the N-telopeptide domain at the locus of $\alpha 1(I)^{N}$ x $\alpha 2(I)^{N}$ x $\alpha 2(I)^{helix}$. The relevance of pyrrole in mineralized tissues, such as bone and dentin, has been of interest due to the fact that the contents of the trivalent stable pyridinoline cross-links (about 0.2 - 0.5 mole/mole of collagen) in these tissues are relatively low. Therefore, it has been speculated that pyrrole might play an important role in providing the proper functional strength to the tissues (Knott et al., 1995). Isolation and characterization of pyrrole cross-link has been difficult due to its inherent instability, and the quantity in tissues varies from one report to another (Kemp and Scott, 1988, Horgan et al., 1990, Risteli et al., 1994, Knott et al., 1994, Knott et al., 1997).

The result of cross-linking is connection of groups of molecules laterally into a sheet-like structure giving a three-dimensional template which facilitates the deposition and packing of mineral crystals resulting in the formation of a strong rigid biomaterial. The connectivity established has been found to decrease by dissociation of the iminium bond of deH-DHLNL when collagen is mineralized, which is possibly due to deformation

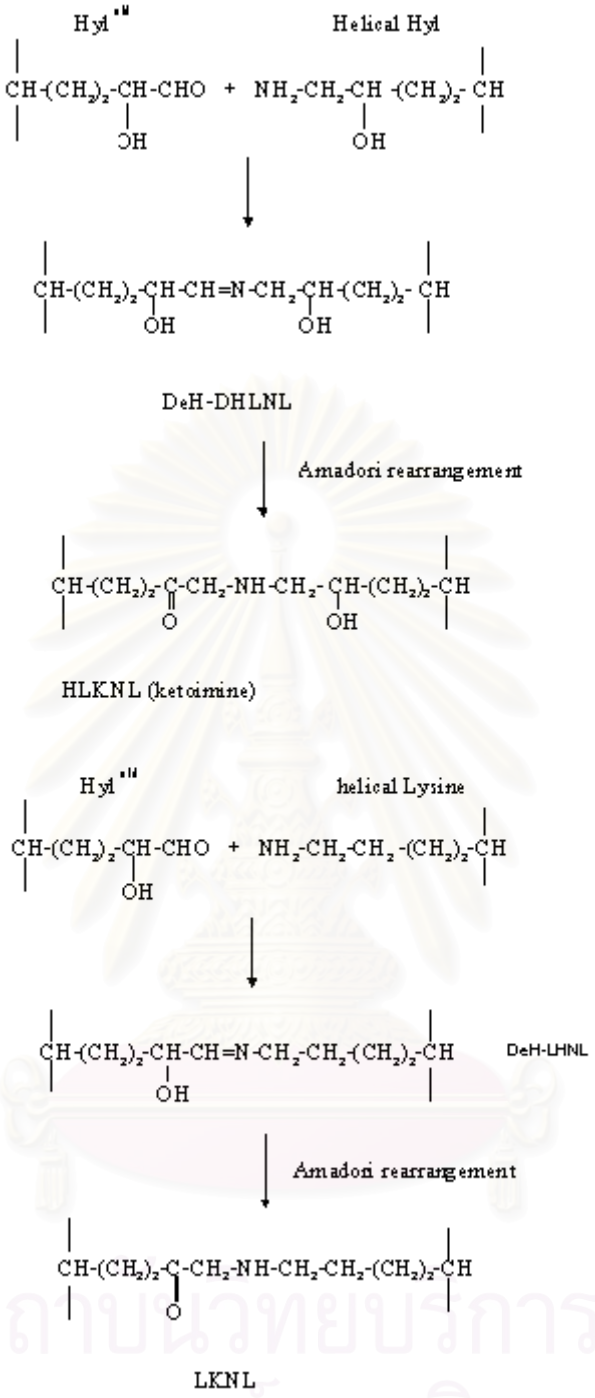

Figure1.6 The formations and structures of deH-DHLNL and deH-LHNL, and their keto forms (Bailey et al., 1998).

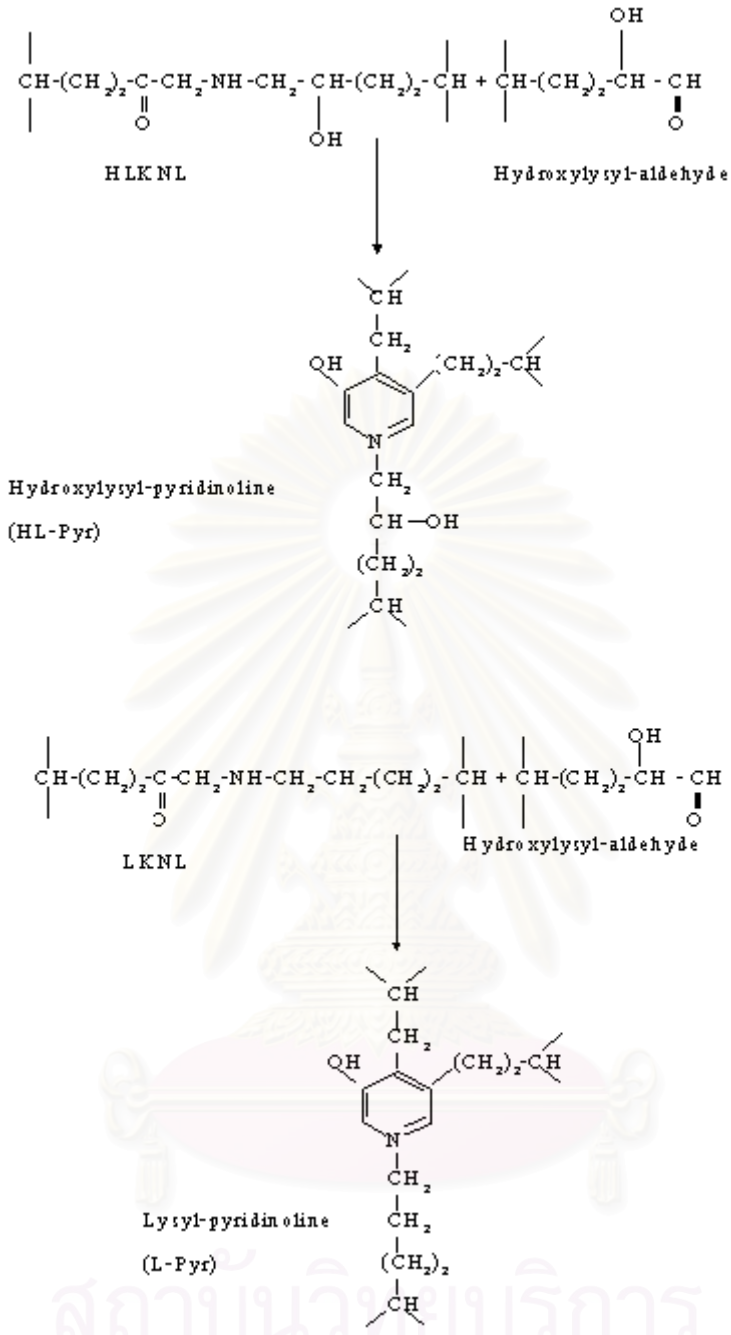

Figure 1.7 The formations and structures of HL-Pyr and L-Pyr (Bailey et al., 1998)

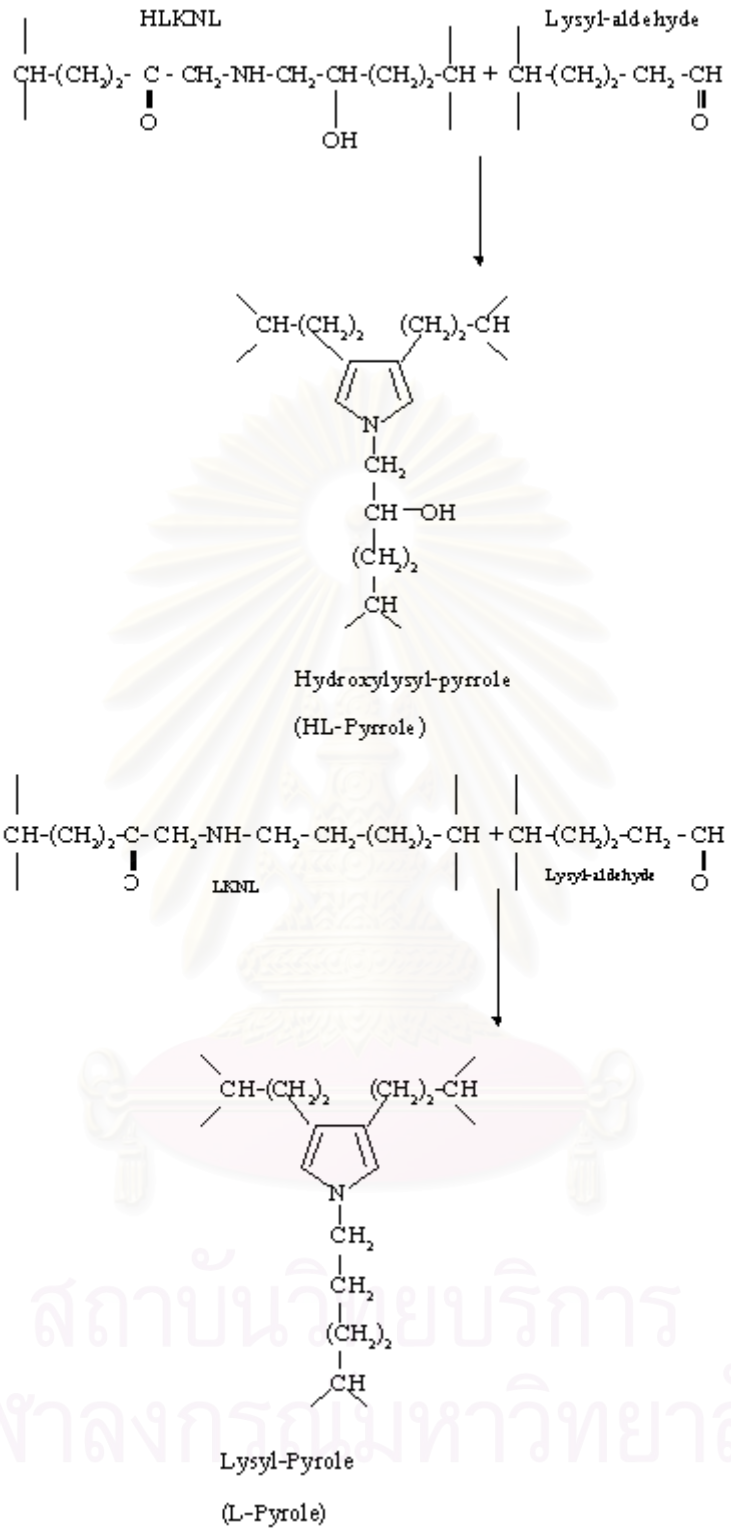

Figure 1.8 The formations and structures of HL-Pyrrole and L-Pyrrole (Bailey et al., 1998)

of the fibrils caused by growth of mineral packing. The presence of free aldehydes at position 16^{C} has been thought as the result of the dissociation of the bifunctional cross-links both in bone and dentin (Otsubo et al., 1992, Yamauchi et al., 1992). On the other hand no free aldehyde was found in predentin collagen which is nonmineralized part of dentin (Yamauchi et al., 1992). Study in turkey leg tendons has shown that mineralization is initiated near the N-terminal telopeptides suggesting that they might have a role in regulating mineralization by providing some conformation that might initiate deposition of mineral crystals.

Collagen Cross-linking in Non-mineralized Tissues

The Formation of Aldimine Cross-links (Yamauchi and Mechanic, 1988, Yamauchi, 1995, Bailey et al., 1998)

Collagen of non-mineralized tissues, such as skin, tendon, periodontal ligament and cornea etc., form major intermolecular cross-links via the lysyl aldehyde pathway. Lysyl aldehydes formed in the regions of C- and N-telopeptides, α1- 16^{C}, α1- 9^{N}, α2- 5^{N}, can condense with helical hydroxylysyl residues on the neighboring α1 and α2 chains to form a reducible Schiff base, deH-HLNL (see Figure 1.9). DeH-LNL (Figure 1.9) might form by condensation of Lys^{ald} with helical lysine on a neighboring molecule as a minor species. Both α1 Hyl–87 and –930 have been found to be involved in the formation of deH-HLNL and often found to be the major glycosylation sites.

In soft connective tissues such as skin and cornea with a low level of lysyl hydroxylation, the aldimine cross-links predominate. These aldimine cross-links are readily cleaved at acid pH and elevated temperature, responsible for the high solubility of young collagenous tissues. These Schiff bases can be stabilized by reduction with borohydride to form HLNL and LNL respectively. DeH-HLNL when comes into contact with the imidazole C-2 carbon atom of the helical α2 H-92 will further form a stable non-reducible trivalent cross-link histidinohydroxylysinononorleucine (HHL, see Figure 1.10) (Yamauchi et al., 1987, Mechanic et al., 1987). HHL is the major stable cross-link in skin and cornea collagen while pyridinoline, which is a well-characterized fluorescent compound, presents in various tissues and is abundant in mineralized tissues but absent from skin collagen. HHL has been found to be an age-related cross-link because studies show that the content of deH-HLNL decreases with aging and HHL content shows a continuous increase throughout chronological aging without diminishing (Yamauchi et al., 1988). Its abundance has also been found to be related to relative insolubility of skin collagen .

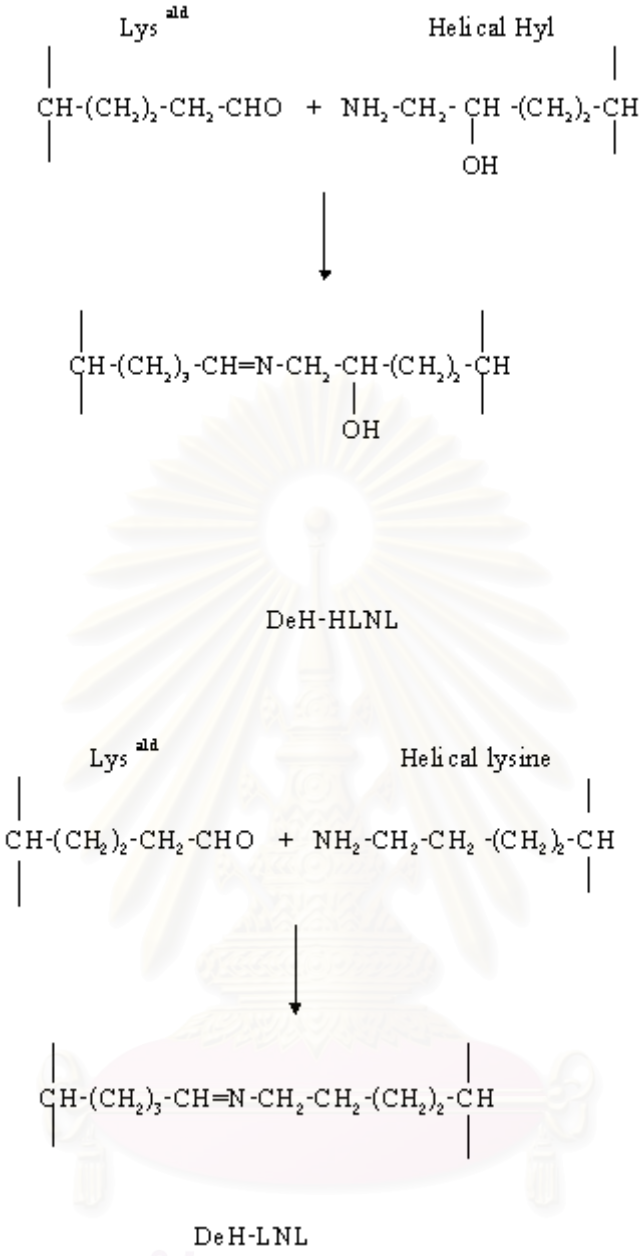

Figure 1.9 The formations and structures of deH-HLNL and deH-LNL (Bailey et al., 1998)

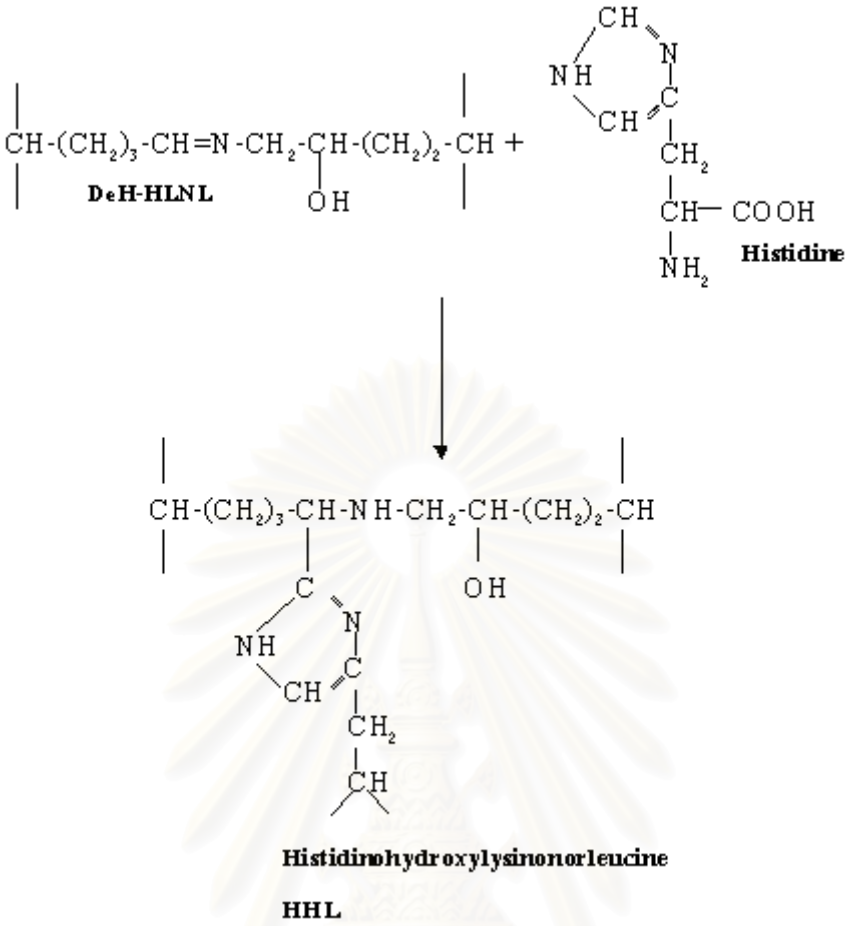

Figure 1.10 The formations and structures of HHL (Bailey et al., 1998)

Aldol-derived Cross-links

Lysyl aldehyde, formed in the telopeptides regions condenses with another lysyl aldehyde of α1- 9^{N} or α2- 5^{N} to form Aldol Condensation Product (ACP) which is an intramolecular cross-link (Yamauchi and Mechanic , 1988). ACP then can react with Hyl and His on the staggered neighboring molecules to form a reducible tetravalent cross-link deH-HHMD (see Figure 1.11).

Dehydro-Histidinohydroxymerodesmosine (DeH-HHMD)

Tanzer et al. (1973) were the first to isolate and characterize this histidine-containing, tritium-labeled polyfunctional cross-link from NaB^{3}H$_4$-reduced collagen. They demonstrated

that histidine, which is sparse presenting less than 1% of total amino acids in collagen and specifically distributed, participates in the formation of collagen cross-links. Additionally, they also demonstrated that a large compound capable of cross-linking several polypeptides are present in type I collagen. They proposed that the formation of the polyfunctional compound deH-HHMD occurred by Michael addition of the N of histidine to the β carbon of the α, β unsaturated aldehyde, and then condensation of the resulting new aldehyde, aldol histidine, with hydroxylysyl residue to form the iminium compound deH-HHMD. Reduction of the Schiff base, deH-HHMD, with NaB^3H_4 results in the formation of secondary amine, HHMD, which also stabilizes the cross-link. Robins and Bailey (1973 and 1977) claimed that the polyfunctional cross-links aldol-histidine and deH-HHMD did not exist *in vivo*, but rather they were products of base-catalyzed condensations caused by reducing agent anions BH_4^- and BH_3CN^-, and the resulting compounds were artifacts. Bernstein and Mechanic, 1980, however, later confirmed the existence of deH-HHMD as a natural cross-link by demonstrating the reformation of the cross-link using acid-cleaved collagen fibril and that the reaction was time-dependent and slow, and in concordant with a Michael condensation which was the reaction proposed by Tanzer et al. (1973). They also speculated, based upon gathering experimental evidence, that His-89 of the α1 chain participated in the formation of deH-HHMD by condensation with juxtaposed C-terminal non-helical aldol to form the Michael adduct aldehyde, aldol histidine, which would then condense with the ε-amino group of Hyl on a neighboring molecule to form the imminium Michael adduct deH-HHMD. However, the molecular locus proposed is highly speculative and has never been confirmed. These

preliminary data (Tanzer et al., 1973, Bernstein and Mechanic, 1980) suggested that this cross-link tied together four polypeptide chains. Two of the polypeptides involved the telopeptides of an $\alpha 1$ and possibly an $\alpha 2$ chain. The relative involvement of $\alpha 2-5^{N}$ Lys^{ald} and the origins of Hyl and histidine are still unknown.

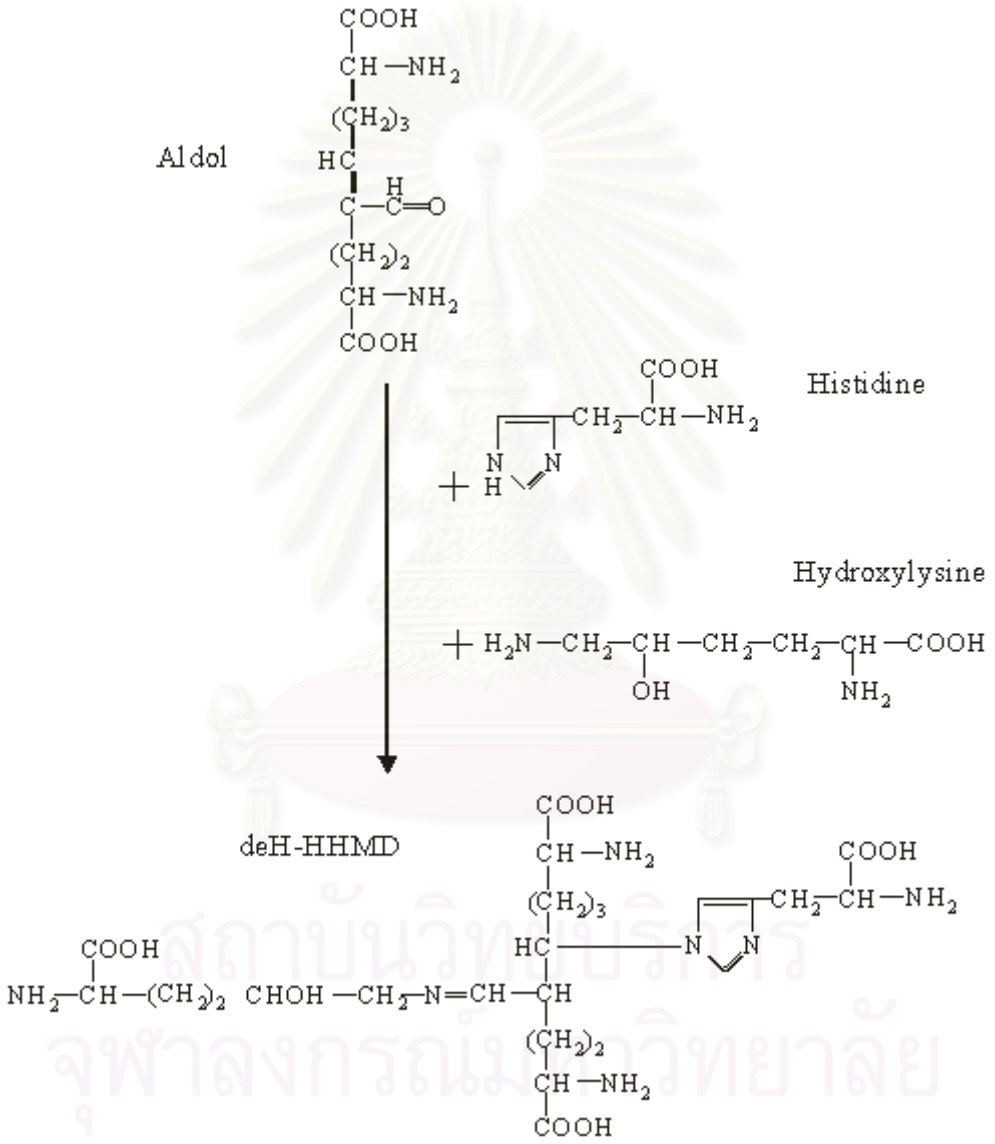

Figure 1.11 Proposed formatin and structure of deH-HHMD by Tanzer et al. (1973)

Glycosylation of Type I Collagen

Enzymatic glycosylation of hydroxylysyl residues, occurs intracellualrly prior to the formation of triple helix formation, results in covalent linkage of either galactose or glucosylgalactose (Yamauchi, 1995, Bateman et al., 1996). It was found that an increase in the amount of glycosylated hydroxylysine in pro α chain decreased the diameter of the fibrils formed due to the steric hindrance of the packing monomers introduced by the glycosylated hydroxylysyl residues (Torre-Blanco et al., 1992). Yamauchi et al. (1982) speculated that glycosylation of specific helical hydroxylysyl residues inhibited their subsequent involvement in the formation of more complex stable cross-link . It was found later (Yamauchi et al., 1986a) in the study using periodontal ligament type I collagen fibrils that this tissue, in contrast to other soft connective tissues, contained relatively abundant amounts of reducible cross-links such as DHLNL, HLNL and HHMD and only small amount of mature non-reducible cross-links. This profile may be due to the exceptionally high rate of collagen turnover in the tissue. Hydroxylysyl residues of the C-terminal cross-links were found to be completely glycosylated, mostly glucosylgalactosyl hydroxylysine, which might sterically hinder the reactive sites, therefore, preventing maturation of cross-links. Henkel et al. (1976) found that the degree of glycosylation was tissue-dependent and did not depend on the specific sites in the molecule. Galactosyl hydroxylysyl residues were found to be abundant in rabbit bone collagen, whereas more glucosylgalactosyl hydroxylysyl residues were found in skin and only slight glycosylation was found in tendon peptide of rabbit type I

collagen. The functional role of glycosylation of type I collagen, however, has not been clearly established. Its location in the molecule would place it in the “hole regions” of the fibrils suggesting its possible involvement in directing of molecular assembly and modulating cross-link formation .

Chapter 2

Statement of the Problem, Specific Aims and Hypothesis

Rationale of the Present Investigation

Collagen is a class of proteins in the extracellular matrix providing the form, mechanical support and structural organization. The most abundant form of collagen is type I collagen, which is a product of two single genes, one for α1(I) chain and one for α2(I) chain. However, chemistry of type I collagen varies among tissues due to post-translational modifications. The structural resilience of fibrillar collagen depends greatly on the correct molecular registration, which is stabilized by intermolecular cross-linking. Therefore, when intermolecular cross-linking is impaired, the integrity of the connective tissues is severely affected. Using cross-linking chemistry at the specific molecular loci, it has been found that there is more than one modality of molecular packing in the type I collagen fibrils (Yamauchi et al., 1986, Mechanic et al., 1987, Otsubo et al., 1992, Yamauchi and Katz, 1993). In order to obtain insights into the tissue specific molecular packing structure and its function, therefore, it is critical to determine the molecular distribution, the frequency of its occurrence and microenvironment of the cross-links. Studies have shown that periodontal ligament collagen and bone collagen share very similar chemistries regarding cross-linking pattern in the region of C-telopeptides, Hyl and Lys in the C-terminal telopeptides in both tissues were quantitatively converted to

aldehydes which then cross-linked to Hyl-87 on both α1 and α2 chains of neighboring molecules, and the α1 to α2 cross-linked ratio was approximately 3.3 : 1 indicating the stereospecificity of the cross-linking reaction.. However, the situation at the N-termini areas of the molecules were quite different (Yamauchi et al., 1989, Yamauchi et al., 1986). While the Lys^{ald} in the N-telopeptides are fully utilized for deH-HLNL and possible deH-HHMD in PDL, the cross-linking in this region of bone/dentin collagen is relatively scarce and the chemistry is apparently different (i.e. deH-HHMD is essentially absent). The scarcity of the tetrafunctional cross-link, deH-HHMD at N-terminal domains of mineralized tissue collagen may favor the accommodation mineral crystal in the process of mineralization, whereas the more abundant presence of these cross-links in non-mineralized tissues may provide a more constrained environment, thus not allowing mineralization to occur. Despite the abundance in the soft connective tissue collagen, molecular nature, molecular loci and glycosylation, of the deH-HHMD cross-link has been poorly investigated. The objectives of this study were to elucidate the molecular distribution of this cross-link and to characterize its glycosylation state in order to obtain insights into the tissue specific structure and function of type I collagen fibrils.

Specific Aims

1. To isolate the major histidinohydroxymerodesmosine (HHMD) cross-linked peptides from reduced (never-mineralized) peripheral layer of turkey leg tendon collagen fibrils
2. To determine the major molecular loci of the cross-link
3. To characterize the state of glycosylation of hydroxylysine that is involved in the formation of deH-HHMD

Hypothesis

Dehydro–histidinohydroxymerodesmosine (deH-HHMD), a complex tetravalent intermolecular cross-link found mostly in non-mineralized tissues, is formed by the interaction between an aldol formed in the amino terminal non-triple helical domains of a collagen molecule, and histidine and hydroxylysine that are located in the helical domains of neighboring collagen molecule(s). The hydroxylysyl residue involved in the formation of this cross-link is not glycosylated since the bulky carbohydrate moiety may hinder the condensation reaction between an aldol/histidine and hydroxylysine.

Chapter 3

Experimental Procedures

Preparation of Turkey Tendon Collagen

The peripheral non-mineralized layer of the 32 week old male turkey leg tendon, obtained from a commercial source (Aries Scientific, Texas), was manually dissected, cut into small pieces and pulverized into a fine powder in liquid nitrogen using a Spex Freezer Mill (Spex Industries Inc., U.S.A.). Tissue powder then was washed with cold phosphate buffered saline (PBS), and lyophilized using Freeze Dry/ Shell FreezeSystem (Labconco Corporation, Kansas City, MO) and kept at –80 ^{o}C until used.

Reduction of Tendon Collagen with Tritiated Sodium Borohydride (NaB^3H_4)

Lyophilized tendon collagen, about 2 grams dry weight, was suspended in 100 ml. of 0.15 M N-trismethyl-2-aminoethanesulfonic acid (TES) buffer pH 7.5 with a few drops of antifoam (Antifoam A Compound, Dow Corning), and reduced with standardized tritiated sodium borohydride (NaB^3H_4, Amersham, #TRK 838), with 3 intervals of 10, 10 and 30 minutes. The amount of NaB^3H_4 used was determined based on the assumption that there are maximum of five reducible groups per collagen molecule. A total of 50-100 fold molar excess of NaB^3H_4 was used for reduction. The pH of the solution was then lowered to 4 by adding several drops of 50% acetic acid to terminate the reaction. The reduced sample was washed with cold deionized distilled

water (DDW) thoroughly by repeated high speed centrifugation, and dialysed exhaustively using a Spectra/por membrane with 6,000-8,000 dalton molecular weight cut off (Spectrum Medical Industries Inc., U.S.A.), against cold distilled water to remove excess NaB^3H_4. The sample was then lyophilized.

Determination of the Specific Activity of NaB^3H_4

Specific activity of NaB^3H_4 was determined using demineralized dentin collagens prepared in our laboratory. The demineralized dentin collagen was reduced with the NaB^3H_4 used for the study, and hydrolyzed with 6 N HCl for 22 hours at 105 ^{o}C. The hydrolysate was then dried using a SpeedVac speed vacuum concentrator (Savant Instruments Inc.), dissolved in DDW, filtered and applied onto a P2 (P2 Bio-Gel resin, BioRad) molecular sieve column equilibrated with 0.1 N acetic acid. The eluent was continuously monitored by absorbance at 230 nm using a photodiode array detector (HP1100, Hewlett Packard) and the fractions were collected. Radioactivity of each fraction was counted in order to identify and isolate DHLNL and HLNL cross-links. It has been known that DHLNL is the major cross-link found in dentin collagen (Yamauchi et al., 1992, Yamauchi, 1995). The fractions containing DHLNL were pooled, dried by the SpeedVac speed vacuum concentrator and dissolved in DDW. Equal amounts of the hydrolysate were then applied to the standardized amino acid and cross-link analyzers. From the former the concentrations of DHLNL was determined as nmole, and the latter

its count as DPM. The specific activity of NaB^3H_4 used for this study was found to be 4.64×10^4 DPM/nmole.

Cross-link Analyses

All reducible cross-links were identified and quantified as their reduced forms (i.e. deH-DHLNL-->DHLNL, deH-HLNL-->HLNL, deH-HHMD-->HHMD). Approximately 2-3 mg of each reduced sample was hydrolyzed with 6 N distilled HCl (Pierce, IL) in vacuo, after flushing with N_2, for 22 hours at 105 ^{o}C. The hydrolysates were dried using the SpeedVac speed vacuum concentrator, and the residues were dissolved in 500 μl of DDW and filtered with a 0.22 μm cellulose acetate membrane (Biomar, Marsh Biomedical Products, Inc.) by centrifugation. An aliquot of the hydrolysate was subjected to amino acid analysis to determine the amount of hydroxyproline on a Varian 9050 liquid chromatography (Varian Inc., USA) configured as an amino acid analyzer using ninhydrin for color development. Hydrolysate in amounts containing 300 nmole of hydroxyproline (i.e. 1 nM of collagen, see below) was then analyzed for cross-links on a Waters 600E HPLC fitted with an ion-exchange column (AA911, Transgenomic) which is linked to an on-line fluorescence flow monitor (Jasco Instrument Co.) to detect non-reducible fluorescent cross-links (Excitation at 330 nm, Emission at 390 nm), and liquid scintillation monitor (Flo-one Beta, Radiomatic Instruments and Chemical Co.) using Ecoscint H liquid scintillant (National Diagnostics, # LS-275) to detect reducible cross-links. Using this system, non-reducible fluorescent cross-links, pyridinoline and

deoxypyridinoline, and reducible cross-links, HHMD, HLNL and DHLNL, were analyzed. All cross-links were identified by their chromatographic elution positions on the analyzer in comparison with the respective standards, which are extant in this laboratory. Quantitation of the various reducible cross-links was performed by integrating the respective areas and converting the numbers into a residues per mole of collagen basis using the specific activity of NaB^3H_4 and a hydroxyproline value (i.e. 300 residues of Hyp per collagen molecule). Likewise the fluorescent cross-links, pyridinoline and deoxypyridinoline, were also calculated by converting the peak areas into the numbers (Yamauchi and Katz, 1993) and a Hyp value.

Trypsin Digestion of Tendon Collagen

In order to isolate the cross-linked peptides, the reduced collagen was first digested with trypsin by the method modified from Kuboki et al. (1981). Typical cleavage sites of trypsin are the carboxyl side of R and K (Kellner and Houthaeve,1999). See Figures 3.1 and 3.2 for amino acid sequences of chicken which have been assumed to be similar to those of turkey. Approximately one gram of reduced sample was suspended in 100 ml of 0.2 M ammonium bicarbonate (NH_4HCO_3) buffer pH 7.9, heated to 65°C for about 15 minutes and cooled down to 37°C. The amount of 1% w/w of L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin (EC 3.4.21.4, Worthington) was added and the sample solution was left at 37°C with continuous stirring for 4 hours. Then the sample was reheated to 65°C for 15 minutes,

cooled to 37°C and the amount of 0.5 % w/w TPCK-treated trypsin was added. The sample was then left at 37°C for additional another 2 hours with continuous stirring. The trypsinized sample was dialyzed against cold distilled water using a 1,000 dalton molecular weight cut off Spectra/por membrane to remove ammonium salt and uncrosslinked low molecular weight peptides. The tryptic digest was then lyophilized.

Initial Separation by Molecular Sieve Column Chromatography

The tryptic digest, 25 mg dry weight, was dissolved in 500 μl of 0.025 M ammonium bicarbonate containing 5% isopropanol (IPA), pH 7.0, filtered by centrifugation using a 5.0 μm Ultrafree-MC Millipore membrane (Millipore Corporation, U.S.A.), and then subjected to initial separation by a standardized molecular sieve Superdex 75 (Pharmacia LKB Biotechnology) column (10 mm X 300 mm) chromatography. The column was equilibrated with 0.025 M ammonium bicarbonate containing 5% IPA at a flow rate of 0.6 ml/minute at room temperature on an HP 1100 HPLC system (Hewlett Packard, CA) with constant monitoring of absorbance at 230 nm using a photodiode array detector (Hewlett Packard, CA). The eluent was collected into 2 minutes fractions and an aliquot of each fraction was counted for radioactivity. The HHMD peptides were identified by their radioactivity, elution positions on the chromatograph and by cross-link analysis. Fractions containing the HHMD-crosslinked peptides were pooled and lyophilized.

Digestion with Bacterial Collagenase

In order to further truncate and purify the cross-linked peptides into smaller peptides, a bacterial collagenase digestion was performed. The specific cleavage site of bacterial collagenase is at the carboxyl side of X in the sequence –P-X-G-P- where often X is found to be hydroxyproline (Peterkofsky, 1982). HHMD-containing peptides were suspended in 0.1 M calcium chloride ($CaCl_2$), 0.25 M Tris and 0.05% N-ethylmaleimide (NEM) pH 7.4, and heated to 65 ^{o}C for 15 minutes, then cooled down to 37 ^{o}C. A purified CLSPA class of bacterial collagenase (EC 3.4.24.3, Worthington) with the amount of 1/20 w/w was added, and samples were incubated at 37^{o}C overnight with continuous stirring (Hanson and Eyre, 1996). The digest was then lyophilized.

Further Purification Using Series of HPLC/FPLC

The truncated HHMD peptides were subjected to further purification using a series of HPLC/FPLC by a C4 (Vydac, USA) reversed phase column using a gradient of 0-40 % acetonitrile in 0.1 % trifluoroacetic acid (TFA) with a flow rate of 1.2 ml/minute (Stone and Williams, 1996), a standardized Superdex 75 molecular sieve column chromatography using 0.025 M ammonium bicarbonate containing 5 % IPA with the flow rate of 0.4 ml/minute (a slower flow rate was used in order to improve the separation). The presence of HHMD-crosslinked peptides was confirmed by radioactivity and cross-link analyses. A C18 reversed phase column (ResElut, Varian Inc., USA) chromatography using a gradient of 0-40 % acetonitrile in 0.1% TFA was used prior to

analytical procedures (e.g. Mass spectrometry, N-terminal sequence analysis) when necessary, in order to remove small molecular weight contaminants and improve purity of the fractions.

Amino Acid Sequence Analyses

The purified cross-linked peptides were subjected to N-terminal amino acid sequence analyses. Preliminary data obtained from this laboratory indicated that the two lysyl aldehydes involved in the formation of deH-HHMD were mainly located at the N-telopeptides, α1-9 ^{N} and possibly α2- 5 ^{N}. If this is the case, two aldehyde-containing peptides are N-terminally blocked by pyroglutamate, therefore, the helical portions of the other 2 peptide chains were sequenced first by Edman degradation to determine the locations of Hyl and His using a Procise Protein Sequencer (Perkin-Elmer Corporation).

Mass Spectrometry Analyses

The molecular weights of the HHMD-containing peptides were determined by Matrix-assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS) in collaboration with Dr.Kenneth Tomer (leader, Mass Spectrometer Unit, NIEHS). The lyophilized cross-linked peptide was rechromatographed using a 5 micron C4 column (Vydac), 4.6 x 250 mm., with a gradient of 10 - 60 % acetonitrile in water over 70 minutes then to 90% for 10 minutes on an HP1100 HPLC system. Fractions were

collected over 1 minute interval, and MALDI/TOF (Time-of-Flight) mass spectra were obtained for each fraction on a PE Biosystems Voyager DE STR MALDI /TOF mass spectrometer (Framingham, MA) equipped with a nitrogen laser. The instrument was operated in the linear mode at an accelerating voltage of 20 kV. Internal calibration was performed by external standards in adjacent to sample spots. The matrix was α-cyano-4-hydroxycinnamic acid. Electrospray Ionization (ESI) mass spectra and Tandem Mass Spectrometry (MS/MS) spectra were obtained on a hybrid quadrupole-time-of-flight tandem mass spectrometer (QTof, Micromass, Altrincham, UK) using a nanoflow ESI source with pressurized flow injection (Borchers et al., 1999).

Chymotrypsin Digestion

In order to determine the involvement of the α1 and α2 chain N-terminal telopeptides and to further characterize the peptide structure, a chymotrypsin digestion was performed. The typical cleavage sites of chymotrypsin are at the carboxyl side of hydrophobic residues, such as Y and F (Kellner and Houthaeve, 1999) which have been shown to be present in the N-telopeptide domains of both α1 and α2 chains (see Figures 3.1 and 3.2). Lyophilized collagenase digest of HHMD-containing peptides were suspended in 0.1 M ammonium bicarbonate. Chymotrypsin (EC 3.4.21.1, Worthington), 5% w/w, was added and left at room temperature with continuous stirring for 6 hours. The digested samples were then lyophilized and applied to the

standardized Superdex 75 molecular sieve column using the condition described earlier.

Deblocking of N-terminal Amino Acids Using Pyroglutamate Aminopeptidase (Walker and Sweeney, 1996, The Perkin-Elmer Corporation, 1995, Tsunasawa et al., 1998)

N-terminal amino acid deblocking was performed by treatment with a *Pfu* pyroglutamate aminopeptidase (Takara Biomedicals, Japan) in order to elucidate the involvement of N-telopeptide amino acid residues in the formation of the cross-link. The lyophilized samples were dissolved in a deblocking buffer, 250 mM sodium phosphate pH 7.0 containing 50 mM dithiothreitol (DTT), and 5 mM ethylenediamine tetra-acetic acid (EDTA), with the concentration of 1 mg/ml. Pyroglutamate aminopeptidase was added with the substrate : enzyme ratio of 25 : 1, after flushing with nitrogen stream for several minutes, the samples were incubated, in a screw-capped vial, at 70 ^{o}C for 4 hours. The digested samples were diluted 5 times with DDW and lyophilized. The lyophilized peptides were then subjected to the Superdex 75 molecular sieve column chromatography to remove the enzyme and salt. An additional amino acid sequencing was then performed.

Cyanogen Bromide Cleavage (Yamauchi and Katz, 1993)

Cyanogen bromide cleavage was performed in order to determine the locations of Hyl and His involved in the formation of the HHMD cross-link. Cyanogen bromide

cleaves the carboxyl side of methionine, and it has been used extensively in the studies of collagen chemistry. Lyophilized collagenase digest of the HHMD-containing peptide was suspended in 0.2 M ammonium bicarbonate at the concentration of 0.5 mg/ml. The sample was then reduced, to convert methionine sulfoxide to methionine, using 5% of 2-mercaptoethanol for 24 hours at room temperature, after flushing with nitrogen, with continuous stirring in a light-protected screw capped vial. The sample then was diluted with 5 volumes of DDW and lyophilized. The lyophilized sample then was dissolved in fresh 70% formic acid and flushed with nitrogen. Equal weight of cyanogen bromide white crystal (Sigma, USA) was added, flushed with nitrogen stream again for several minutes, screw-cap sealed and left in darkness for 4 hours at room temperature with continuous stirring (Epstein et al., 1971, Yamauchi et al., 1986b). The digested sample then was diluted with 10 volumes of DDW, lyophilized, and subjected to the Superdex 75 molecular sieve column chromatography using the same conditions described above. The HHMD-containing fractions were determined by their radioactivity, pooled, lyophilized and further purified by the C4 reversed phase column chromatography using the condition as described above.

Alkali Hydrolysis to Determine the Involvement of Glycosylation in the Formation of the HHMD Cross-link

The involvement of glycosylation of hydroxylysyl residues in the formation of deH-HHMD was determined by 3 methods.

1. Acid/alkali hydrolysis to determine the glycosylation state by HPLC - The isolated HHMD cross-linked peptides (collagenase digest of the tryptic peptide) were subjected to both acid and alkali hydrolyses (Yamauchi et al.,1982, Henkel et al., 1976). For alkali hydrolysis, the peptide was hydrolyzed using 2 N sodium hydroxide at 105 ^{o}C in vacuo for 22 hours after flushing with nitrogen, neutralized with 2 N hydrochloric acid and filtered. Acid hydrolysis was performed as described above. Equal amounts of both alkali and acid hydrolysates were subjected to cross-link analyses to determine the glycosylated forms of the cross-link. Both acid and alkali hydrolysates, with equal amounts, were also subjected to the amino acid analyses using ninhydrin color development to determine the di-, mono- and non-glycosylated forms of HHMD cross-links.

2. To determine the cross-link structure and its glycosylation by mass spectrometry - In order to further confirm the structure of HHMD cross-link and its glycosyaltion state by mass spectrometric analysis, both acid and alkali hydrolysates were then subjected to the P 2 molecular sieve column chromatography equilibrated with 0.1 N acetic acid and the HHMD cross-link was isolated. The isolated cross-link was applied to the C18 reversed phase column (ResElut, Varian Inc.) using a gradient of 0-40 % acetonitrile in 0.1 % TFA to be further purified and to remove any possible contaminants. The purified cross-link was then subjected to ESI and MS/MS mass spectrometry to confirm the structure of the cross-link.

3. Mass analysis of the HHMD peptide before and after enzymatic deglycosylation - The purified HHMD cross-linked peptide was treated with alpha-glycosidase (Roche Molecular Biochemicals) and subjected to mass spectrometry (MALDI-MS) analysis to determine the involvement of glycosylation.

P02457

COLLAGEN ALPHA 1(I) CHAIN

PRECURSOR

LOCUS CA11_CHICK 1453 aa VRT 15-JUL-1999

DEFINITION COLLAGEN ALPHA 1(I) CHAIN PRECURSOR.

ACCESSION P02457

DBSOURCE swissprot: locus CA11_CHICK, accession P02457;

class: standard.

created: Jul 21, 1986.

sequence updated: Oct 1, 1989.

KEYWORDS Extracellular matrix; Connective tissue; Repeat; Hydroxylation;

Glycoprotein; Collagen; Signal.

SOURCE chicken.

ORGANISM *Gallus gallus*

Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Archosauria;

Aves; Neognathae; Galliformes; Phasianidae; Phasianinae; Gallus.

^{1N}qmsygyde^{9N}ksagvavp^{1}gpmgpagprglpgppgapgpqgfqgppgepgepgasgpmgprgpagppg
kngddgeagkpgrpgqrgppgpqgarglpgtaglpgm^{87}kghrgfsgldgakgqpgpagpkgepgspgenga
pgqmgprglpgergrpgpsgpagargndgapgaagppgptgpagppgfpgaagakgetgpqgarsegpqgs
rgepgppgpagaagpagnpgadgqpgakgatgapgiagapgfpgargpsgpqgpsgapgpkgnsgepga
pgnkgdtgakgepgpagvqgppgpageegkrgargepgpaglpgpagergapgsrgfpgadgiagpkgppg
ergspgavgpkgspgeagrpgeaglpgakgltgspgspgpdgktgppgpagqdgrpgpagppgargqagvm
gfpgpkgaagepgkpgergapgppgavgaagkdgeagaqgppgptgpagergeqgpagapgfqglpgpag
ppgeagkpgeqgvpgnagapgpagargergfpgergvqgppgpqgprgangapgndgakgdagapgapg
negppglegmpgergaaglpgakgdrgdpgpkgadgapgkdglrgltgpigppgpagapgdkgeagppgpa
gptgargapgdrgepgppgpagfagppgadgqpgakgetgdagakgdagppgpagptgapgpagzvgapg
pkgargsagppgatgfpgaagrvgppgpsgniglpgppgpagkzgskgprgetgpagrpgepgpagppgppg
ekgspgadgpigapgtpgpqgiagqrgvvglpgqrgergfpglpgpsgepgkqgpsgasgergppgpmgppg
lagppgeagregapgaegapgrdgaagpkgdrgetgpagppgapgapgapgpvgpagkngdrgetgpagp
agppgpargpagpqgprgdkgetgeqgdr^{928}gm^{930}kghrgfsglqgppgppgapgeqgpsgasgpagpr
gppgsagaagkdglnglpgpigppgprgrtgevgpvgppgppgppgppgpp^{1C}**sggfdfsflpqppqe^{16C}kahd**
ggryy

Figure 3.1 Amino acid sequence of chicken type I collagen α 1 chain modified from Pubmed database, swissprot accession number P02457. Bold letters at the beginning and the end of the sequence represent the amino acid residues in the N- and C-telopeptide regions respectively.

P02467

COLLAGEN ALPHA 2(I) CHAIN

PRECURSOR

LOCUS CA21_CHICK 1362 aa VRT 15-JUL-1999

DEFINITION COLLAGEN ALPHA 2(I) CHAIN PRECURSOR.

ACCESSION P02467

VERSION P02467 GI:5921192

DBSOURCE swissprot: locus CA21_CHICK, accession P02467;

class: standard.

extra accessions:Q90795,Q90797,P87492,Q90758,Q92014,P87491,Q90792,

created: Jul 21, 1986.

sequence updated: Dec 15, 1998.

annotation updated: Jul 15, 1999.

KEYWORDS Extracellular matrix; Connective tissue; Repeat; Hydroxylation;

Glycoprotein; Collagen; Signal.

SOURCE chicken.

ORGANISM *Gallus gallus*

Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Archosauria;

Aves; Neognathae; Galliformes; Phasianidae; Phasianinae; Gallus.

^{1N}qydps^{6N}kaadfgp^{1}gpmglmgprgppgasgppgppgfqgvpgepgepgqtgpqgprgppgppgkaged
ghpgkpgrpgergvqgargfpgtpgppgf^{87}kgirg^{92}hngldgltgqpgapgtkgepgapgengtpgqpgarglp
gergrigapgpagargsdgsagptgpaxxxxxxxxxxxxxxxxxgeigpagnegptgpagprgeiglpgssgpv
gppgnpganglpgakgaaglpgvagapglpgprgipgpgpagpsgarglvgepgpagakgesgnkgepgaa
gppgppgpsgeegkrgsngepgsagppgpaglrgepgsrglpgadgrgvmgpagnrgasgpvgakgpngd
agrpgepglmgprxxxxxxxxxxxxxxxxxgfpgadgrvgpigpagnrgepgnigfpgpkptgepgkpgekgn
vglagprgapgpegnngaqgppgvtgnqgakgetgpagppgfqglpgpsgpageagkpgerglhgefgvgpa
gprgerglpgesgavgpagpigsrgpsgppgpdgnkgepgnvgpagapgpagpggipgergvagvpggkge
kgapglrgdtgatgrdgarglpgaigapgpaggagdrgeggpagpagpagargipgergepgpvgpsgfagpp
gaagqpgakgergpkgpkgetgptgaigpigasgppgpvgaagpagprgdagppgmtgfpgaagrvgppgp
agitgppgppgpagkdgprglrgdvgpvgeqgiagppgfagekgpsgeagaagppgtpgpqgilgapgilglpg
srgerglpgiagatgepgplgvsgppgargpsgpvgspgpgapgeagrdgnpgndgppgrdgapgfkgergap
gnpgpsgalgapgphgqvgpsgkpgnrgdpgpvgpvgpagafgprglagpqgprgekgepgdkghrglp^{931}
gl^{933}kg^{935}hnglqglpglagqhgdqgppgnngpagprgppgpsgppgkdgrnglpgpigpagvrgshgsqgp
agppgppgppgppg

Figure 3.2 Amino acid sequence of chicken type I collagen α 2 chain modified from Pubmed database, swissprot accession number P02467. Bold letters at the beginning of the sequence represent the amino acid residues in the N-telopeptide region.

Chapter 4

Results

Cross-link Profiles of the Turkey Tendon Collagen

Figures 4.1 and 4.2 show the typical collagen cross-link profiles of the reduced never-mineralized peripheral layer and the reduced inner mineralized portion of turkey leg tendon, respectively. Figures 4.1 A and B are the chromatographic patterns of radioactivity showing reducible cross-links, and fluorescence detecting non-reducible cross-link, pyridinoline, respectively, of the never-mineralized peripheral layer. Figures 4.2 A and B are the chromatographic patterns of radioactivity showing raducible cross-links, and fluorescence detecting non-reducible cross-links, pyridinoline and deoxypyridinoline, respectively, of the mineralized portion. The cross-link pattern of these two portions of the same tendon was distinct from each other. In the peripheral never-mineralized layer, the HHMD was the major cross-link, and DHLNL and HLNL minor. Significant amount of pyridinoline was identified in this portion of tendon but its lysyl analog, deoxypyridinoline, was neglegible. In the mineralized portion, however, HHMD was minimal while DHLNL and HLNL represented the major cross-link species. Significant amount of deoxypyridinoline was also found in this portion.

The following is a summary of the quantitative cross-link analyses of these two portions of tendon expressed as mole/mole of collagen.

	DHLNL	HLNL	HHMD	Pyr	d-Pyr
Never-mineralized	0.30	0.18	1.05	0.4	-
Mineralized	0.49	0.36	0.13	0.24	0.11

Thus, the peripheral layer of the tendon was prepared for this study.

Isolation and Purification of HHMD Cross-linked Peptide by Enzymatic Digestions and A Series of Column Chromatography

Reduced peripheral non-mineralized layer of turkey leg tendon collagen fibrils were trypsin digested and subjected to initial separation using the standardized Superdex 75 molecular sieve column equilibrated with 0.025 M ammonium bicarbonate containing 5 % IPA, and absorbance at 230 nm was monitored simultaneously. Figure 4.3 shows the chromatographic profile of a tryptic digest of turkey tendon collagen fractionated by the standardized molecular sieve column. All peptide fractions were determined for radioactivity, and five major radioactive peptide peaks were identified, denoted as peaks I-V respectively. Radioactive fractions of each peak were collected, lyophilized, and redissolved in deionized distilled water. An aliquot from each peak, with equal amount, was then subjected to acid hydrolysis and cross-link analysis to determine the cross-link profile of each peak. Figures 4.4 A to E show the cross-link profiles of peaks I to V, respectively. Peak I (Figure 4.4A) showed no collagen cross-links. The radioactivity of this peak is, therefore, most likely not associated with collagen cross-links. The radioactive peaks II-V, from Figure 4.3, were found to contain various

amounts of HHMD cross-links. Peak II (from Figure 4.3), shown as a small peak between peaks I and III, was found to contain HHMD-crosslinked peptide with the slightest contamination of other cross-links, as shown in Figure 4.4 B, the ratio of HHMD : pyridinoline was 10 : 1. Peak III, Figure 4.4 C, contained large amount of HHMD but also contained significant amount of pyridinoline cross-link with the ratio of HHMD : pyridinoline of 3 : 1. Although peaks IV and V also contained significant amounts of HHMD, the majority of DHLNL and HLNL, and some pyridinoline cross-links were recovered in these fractions (Figures 4.4 D and E). Therefore, for the present study, only peaks II and III were chosen for further characterization of HHMD cross-linked peptides. Peaks II and III were subjected to further purification by bacterial collagenase digestion followed by a series of HPLC/FPLC. The bacterial collagenase digests of peaks II and III were subjected to the standardized Superdex 75 molecular sieve column with simultaneous monitoring of absorbance at 230 nm. An aliquot of each fraction was counted for radioactivity and the count was plotted on the graph. Two major radioactive peaks were identified from the digest of peak II, denoted as peaks IIa and IIb, as shown in Figure 4.5. Each radioactive peak was subjected to cross-link analysis, and it was found that peak IIa was the major HHMD-containing peptide with a negligible amount of pyridinoline, shown in Figure 4.5. Most of the pyridinoline in peak II, on the other hand, was recovered in peak IIb. Relatively small amount of HHMD-peptide found in peak IIb might be a portion of the major HHMD peptides in peak III since the collagenase-digested HHMD peptides in peak III eluted at the same position

as peak IIb (see Figures 4.5 and 4.7) on the same chromatography. Two major radioactive peaks were also identified from the collagenase digest of peak III, denoted as peaks IIIa and IIIb, as shown in Figure 4.7. Each radioactive peak was subjected to cross-link analysis, and it was found that peak IIIa was the major HHMD-containing peptide, although some pyridinoline-peptides were also persent as shown in Figure 4.8. The ratio of HHMD to pyridinoline was about 3:1 at this stage. Other reducible cross-links, DHLNL and HLNL, were present in peak IIIb indicating that these cross-linked peptides were separated from the HHMD peptides by this purification step.

Confirmation of the HHMD structure by Mass Spectrometric Analysis

In order to confirm the structure and molecular mass of the HHMD cross-link itself, the HHMD peptide was subjected to acid hydrolysis, and the hydrolysate applied onto the P2 molecular sieve column chromatography to isolate the HHMD cross-link. The HHMD cross-link, obtained from the P2 molecular sieve column chromatography, was subjected to a C18 reversed phase column (Res Elut, Varian) using a gradient of 0-40% acetonitrile in 0.1 % TFA, the pure HHMD fractions were obtained and subjected to ESI and MS/MS mass spectrometry. Figure 4.9 shows the ESI MS (A) and MS/MS (B) mass spectra of the cross-link which shows a protonated molecular ion of mass 574.3 daltons corresponding to the mass and the structure, a tetravalent cross-link, of the reduced deH-HHMD proposed by Tanzer et al. in 1973 (see figure 1.11). The color factor of HHMD cross-link was determined by subjecting equal amounts of the acid

hydrolysate to the cross-link and amino acid analyses. The color factor of HHMD was found to be about 2.5 times of leucine.

Characterization and Determination of the Molecular Loci of the HHMD cross-linked Peptide

Pooled fractions of peaks IIa and IIIa were lyophilized and subjected to further purification by a C4 reversed phase column using a gradient of 0-40% acetonitrile in 0.1 % TFA. The major HHMD peptides were then rechromatographed using the same condition. The cross-link profiles of the radioactive peak collected from this HPLC showed only HHMD cross-link. The pure peptides were rechromatographed on the Superdex 75 molecular sieve column chromatography in order to evaluate the elution positions and patterns using absorbance at 230 nm and radioactivity of the respective HHMD peptides before subjecting to further characterizations. Figures 4.10 A and B show the final chromatographic patterns of peaks IIa and IIIa on the Superdex 75 molecular sieve column, respectively. The pattern demonstrated that each absorbance peak corresponded well with that of radioactivity, and that the molecular size of peak IIa is slightly larger than that of peak IIIa.The purified fractions were then subjected to mass spectrometry analyses using a MALDI-TOF mass spectrometry and amino acid sequence analyses.

Table 1 shows the results of amino acid sequence analysis of peak IIa (collagenase digest of peak II) and its chymotryptic digest. The sequence data of the former (collagenase digest) showed a single amino acid (G) in the first cycle but two amino acids in the second cycle (M and L). In the third cycle, a peak of Hyl was identified and a single G in the fourth cycle, then H, R, G, F, S and G in the following cycles with relatively low quantities when compared with those of the first 4 cycles. The results are consistent with the sequences of an α1 (G 928-G 937) and an α2 (G 931-G 934). The third and fifth amino acids of both α1 (G 928-->G 937) and α2 (G 931-->G 934) peptides are Hyl and H. The small peak area of Hyl in the third sequence and the significantly low yield of H in the fifth cycle indicate that one residue of Hyl in the third cycle and one H in the fifth cycle are utilized for the HHMD cross-link.

In order to determine the origins of Hyl and H the peptide was treated with CNBr (M is present at 929 of an α1 peptide and at 2N of an α1 N-telopeptide). After the cleavage, the peptide was subjected to amino-terminal sequence analysis. Table 2 summarizes the results. The first cycle of α1 helical sequence was blank indicating the cross-linking site which was α1 Hyl 930, showing that the cleavage by cyanogen bromide occurred between M 929 and Hyl 930. In the third cycle, Hyl and H were identified. This is possible, only when Hyl 930 on an α1-derived peptide and H 935 on an α2 are not available for the sequence (likely by being involved in the HHMD cross-link), but H 932 and Hyl 933 on an α1 and α2, respectively, are. The sequence of α1

helical –G 934–F 935–S 936–G 937- found indicates that trypsin was unable to cleave the R 933–G 934 bond, which most likely is due to its proximity to the complex HHMD cross-link structure, as found in a previous study (Mechanic et al., 1987). A small amount of α1 N telopeptide sequence starting from α1-G 5N was also observed in the collagenase as well as CNBr peptides (data not shown) which is due to the chymotrypic activity present in the trypsin used. The sequence data from chymotryptic peptide (the right section of Table 1) show similar sequences indicating the involvement of α1 and α2 helical residues and α1 N telopeptide in the formation of the HHMD cross-link at this location. The small amount of Hyl and H found in cycles 3 and 5 indicated the possible cross-linking sites. Chymotrypsin digestion was performed in order to determine whether or not N-telopeptides were involved in the formation of the HHMD cross-link based on the knowledge that there are two tyrosine residues at the α1 N-telopeptide, α1-Y 4N and -Y 6N, and one at the α2 N-telopeptide, α2-Y 2N (amino acid sequence numbering based on Swissprot accession P02457 , locus CA11_CHICK, and Swissprot accession P02467 , locus CA21_CHICK, see figures 3.1 and 3.2). It was found that chymotrypsin cleaved between α1-Y 4N and α1-G 5N but not between Y 6N and D 7N, therefore, at this point it was still not known whether or not α2 N-telopeptide involved in the formation of the cross-link. The sequencing of the α2-derived peptide did not go beyond H 935. This is most likely due to the cyclization of Asn-Gly bond that is known to block the Edman degradation (Bornstein and Balian, 1977, Rexrodt et al.,

1975). In order to clearly determine the involvement of N-telopeptides, especially α2N-telopeptide, in the formation of the HHMD coss-link, peak IIa was subjected to N-terminal amino acid deblocking using pyroglutamate aminopeptidase. Table 3 shows the amino acid sequence data of the pyroglutamate aminopeptidase treated of peak IIa. Both α1 and α2 N-telopeptide residues were found with the amount of α1 N telopeptide residues higher than those of α2 N. The amount of the amino acid residues, shown in picomoles, of the helical residues indicated the ratio of α1 helical residues : α2 helical residues was approximately 1 : 1. The lower yields of amino acid residues of α2 N-telopeptide found suggesting another species of HHMD cross-linked peptide .

The MALDI mass spectra obtained from purified peak IIa (collagenase digest) contained an abundant ion of mass 8,778, shown in Figure 4.11. Treatment of this peptide with alpha-glycosidase (Roche Molecular Biochemicals) did not result in any changes in the MALDI spectra, indicating that no glycosylation was involved in this peptide. The ESI/MS spectrum of this peptide was obtained and deconvoluted using MaxEnt software. A series of ions separated by 22 Dalton, sodium (Na), was observed with the highest molecular-ion species corresponding to the M+2Na ion of the 8,778 species observed in the MALDI mass spectrum. This mass is about 0.3 % different from the mass calculated from the proposed structure of the cross-linked peptide, α1 (1N- 9^{helix}) X α1 (1N- 9^{helix}) X α1 (928^{helix}- 932^{helix}) X α2 (931^{helix}- 963^{helix}). Figure

4.12 shows the MALDI/MS of a chymotryptic digestion of the HPLC purified peptide for 6 hours at 35 °C identifying ion of m/z 7,774, loss of 1,004 which may correspond to cleavage of the α1 N telopeptide chain between Y 4N and G 5N plus loss of one oxygen (possibly from oxidized methionine-2N).

Table 4 shows the results of the N-terminal amino acid sequence data, amounts shown in picomoles in parentheses, of peak IIIa (purified collagenase digest of peak III, see figure 4.7), and chymotryptic digest of IIIa. The data showed the residues of helical α1 starting with G-928 and helical α2 starting with G-931, and also the residues of α1 N-telopepide starting with G-5N (chymotryptic activity of typsin used), which are similar to what were found from peak IIa. The cleavage between α1 R933-G934 was complete since no G934-F-S-G- was observed from the sequence analysis. The action of chymotrypsin shows the involvement of α1 N-telopeptide domain in the formation of the cross-link. However, due to the the reason mentioned earlier, it could not be determined at this point the involvement of α2 N-telopeptide residues, therefore deblocking of the N-terminal amino acids was performed. Table 5 shows the amino acid residues found after digestion with pyroglutamate aminopeptidase of peak IIIa. The sequence data of the pyroglutamate aminopeptidase treated of peak IIIa showed the sequence of α1 N and α2 N-telopeptide residues along with α1 and α2 helical sequence and the sequence resulting from chymotryptic action of trypsin on α1 N telopeptide. The amount of α1 N telopeptide amino acid residues including the peptide

derived from chymotryptic activity of the enzyme was higher than those of α2 N telopeptide suggesting the presence of mixed population of peptides derived from α1 N X α1 N and α1 N X α2 N. Mass spectrometric analysis of this peptide was performed using MALDI/MS. The MALDI mass spectra of HPLC purified peak IIIa contained abundant ions of masses 3,669 and 4,604 (Figure 4.13). The spectra of other fractions showed other molecular ions and/or a broad non-resolved hump indicating the heterogeneity of this peak. The mass of 3,669 suggested the peptide structure of α1 5N –16N X α2 1N- 10N X α1 928 ^{helix} –933 ^{helix} X α1 928 ^{helix} – 933 ^{helix}. The mass of 4,604 suggested the peptide structure of α1 (1N-14N) X α2 (1N X 12N) X α1 (928-933) X α2 (931-942), which might be the truncated version of one of the structures identified in peak IIa.

Based on the information of amino acid sequence and mass analyses gathered from the present study, three possible molecular locations of HHMD cross-link were proposed as shown in figure 4.14. We proposed the peptide structure in A as a species of HHMD at the location of peak IIa based on the amino acid sequence and mass data (mass of 8,778). The relatively high yield of α1N sequence compared to α2N indicated the presence of α1N X α1N at this location. The peptide structure in B was proposed to be at the locations of both peaks IIa and IIIa, possibly the major species which is derived from N-telopeptide, based on the amino acid sequence and mass data (mass of 4,604 from peak IIIa). The structure in C was proposed to be a possibility of

another species of HHMD found at the location of peak IIIa. All sequence data indicate the presence of structures B and A, except the lack of the involvement of α2 helical sequence indicated by mass data, 3,669 dalton, suggested the presence of C.

Determination of the State of Glycosylation of Hydroxylysines Involving in the Formation of HHMD Cross-linked Peptide

The purified HHMD cross-linked peptides of peaks IIa and IIIa were subjected to both alkali and acid hydrolyses to determine the state of glycosylation of hydroxylysine residues involving the formation of the cross-link. The elution position and quantity of the HHMD cross-link of both acid and alkali hydrolysates were identical on the cross-link analyzer indicating that there was no glycosylation involved in the formation of these cross-linked peptides. An aliquot with equal amounts of both alkali and acid hydrolysates was also subjected to the amino acid analyses. The elution positions and amounts of Hyl residues obtained from the amino acid analyses of both hydrolysates were unchanged, suggesting that both the α1 Hyl930 and α2 Hyl933 were not glycosyalted. Both of the hydrolysates were subjected to the P2 molecular sieve column chromatography equilibrated with 0.1 N acetic acid to separate the HHMD cross-link from other amino acids and cross-links. An aliquot of the HHMD-containing radioactive peak was then lyophilized and subjected to the amino acid analyses. Again, it was found that the elution positions and quantities of the HHMD prepared from both hydrolysates were identical, as shown in Figure 4.15, confirming the purity of the

crosslink. This purified cross-link was subjected to mass spectrometric analysis to determine the structure as described earlier in this chapter. Treatment of the cross-linked peptide with alpha-glycosidase did not give any change in the mass spectra, as mentioned earlier in this chapter, and The mass spectra obtained was similar to spectra shown in Figure 4.11 indicating that there was no glycosylation involved in the formation of the cross-link.

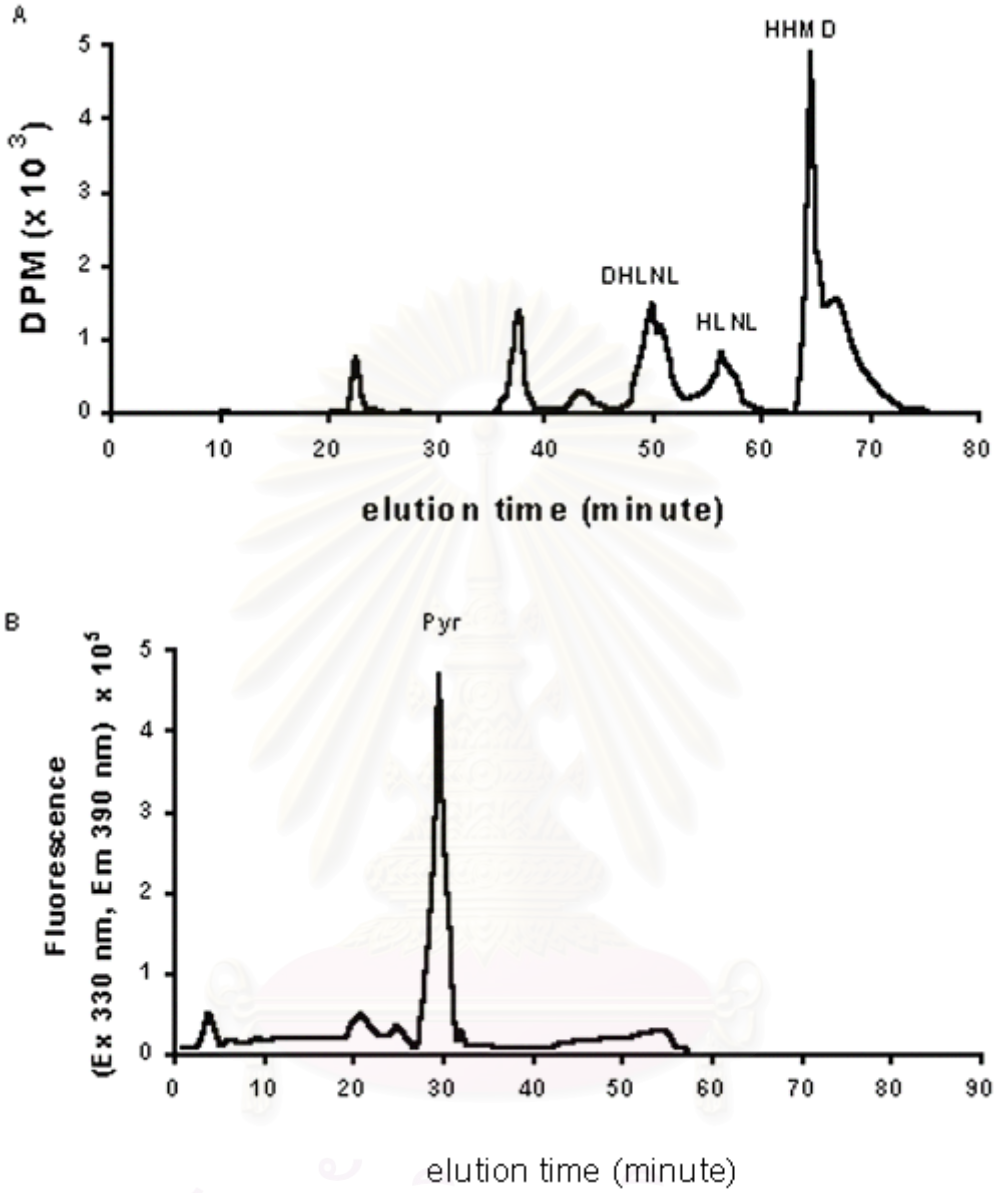

Figure 4.1 The cross-link profiles of reduced never-mineralized peripheral layer of turkey tendon acid hydrolysates. A) shows the reducible cross-link profile of never-mineralized layer indicating HHMD as a major cross-link whereas B) shows the pyridinoline profile obtained from the same analysis as in A.

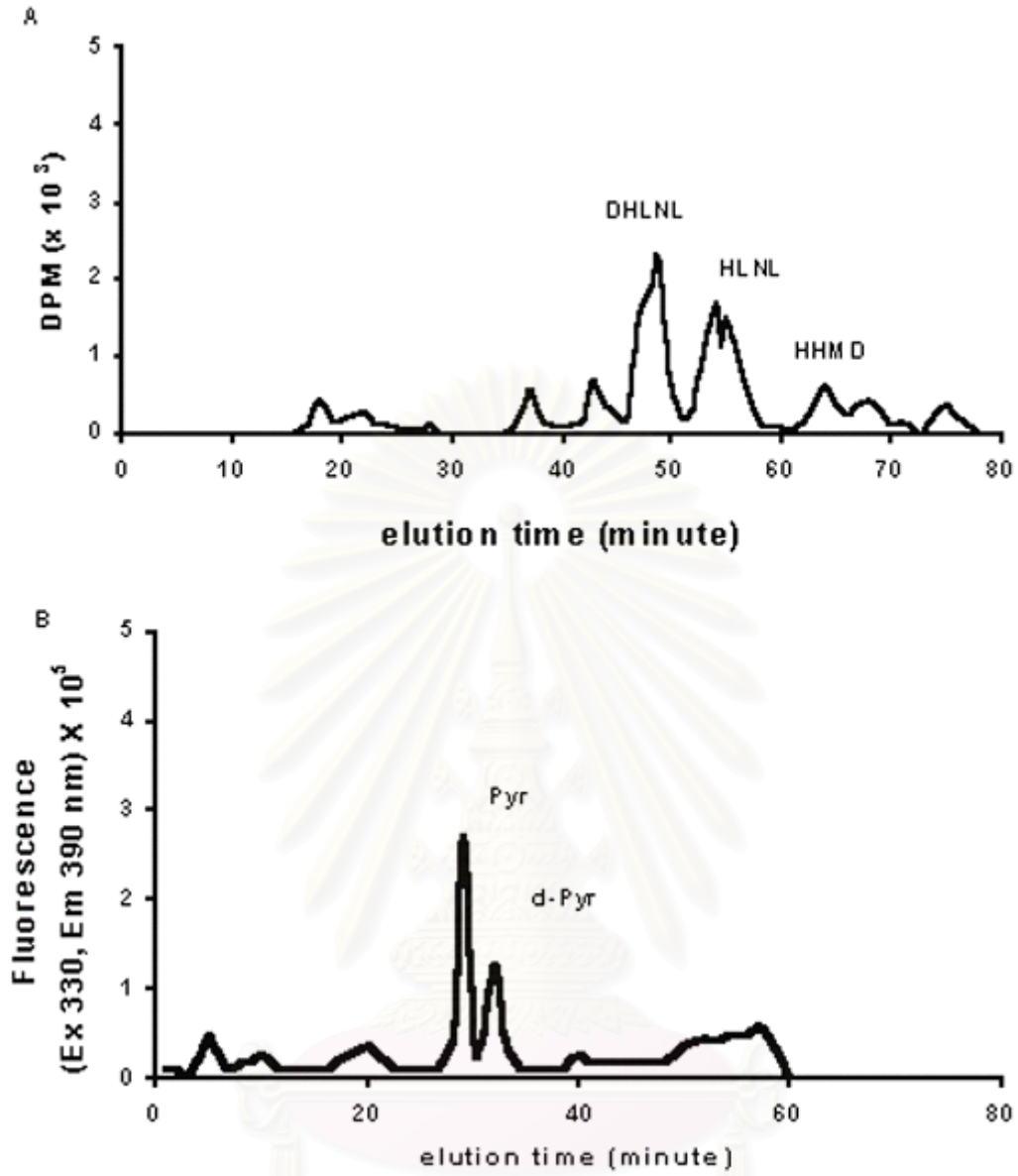

Figure 4.2 The cross-link profiles of reduced mineralized layer of turkey tendon acid hydrolysates. A) shows the reducible cross-link profile of the mineralized layer of the tendon indicating that DHLNL and HLNL are the major populations whereas in B) both pyridinoline and deoxy-pyridinoline are identified.

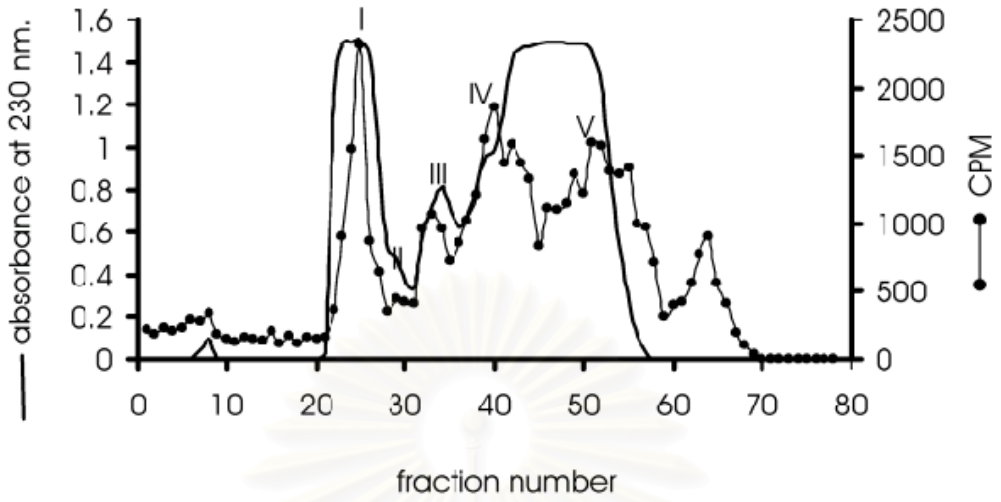

Figure 4.3 Chromatographic profile of the tryptic peptide of peripheral non-mineralized layer of turkey tendon collagen fractionated by a standardized molecular sieve, 10 mm X 300 mm, column chromatography using 0.025 M ammonium bicarbonate containing 5% IPA with a flow rate of 0.6 ml/minute, with continuous monitoring of absorbance at 230 nm. An aliquot from each fraction was determined for radioactivity. Five major radioactive peaks were identified, denoted as peaks I-V. Each peak was collected and subjected to further characterization.

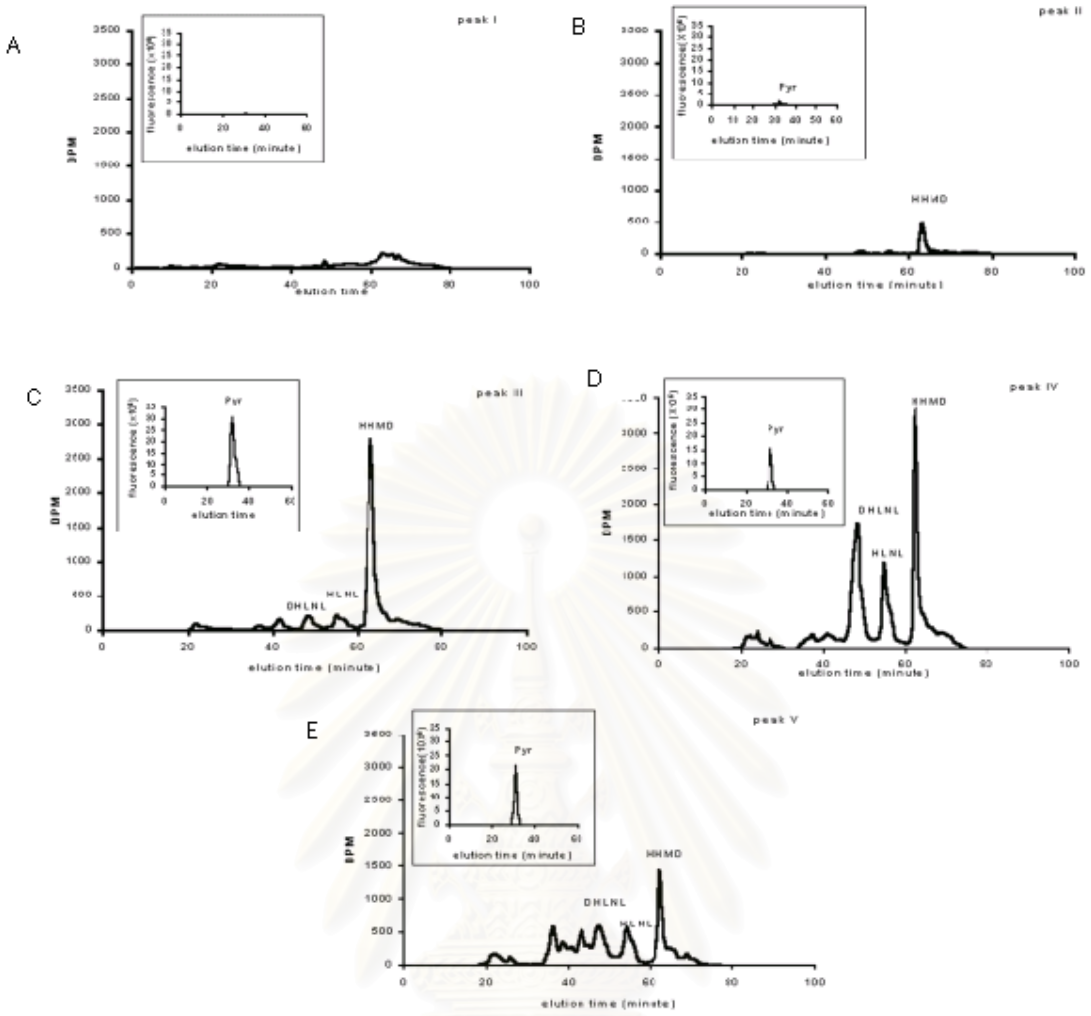

Figure 4.4 A-E show the cross-link profiles of peaks I - V (from Figure 4.3) respectively. Radioactive fractions across each peptide peak on the Superdex 75 chromatograph were pooled and lyophilized, and an aliquot from each peak was subjected to acid hydrolyses. The hydrolysates were dried and dissolved in DDW, and an aliquot was then subjected to cross-link analyses. Peak I does not contain any cross-link. Peaks II and III were subjected to further purification and characterization because they contain the least of contamination from other cross-links when compared to peaks IV and V.

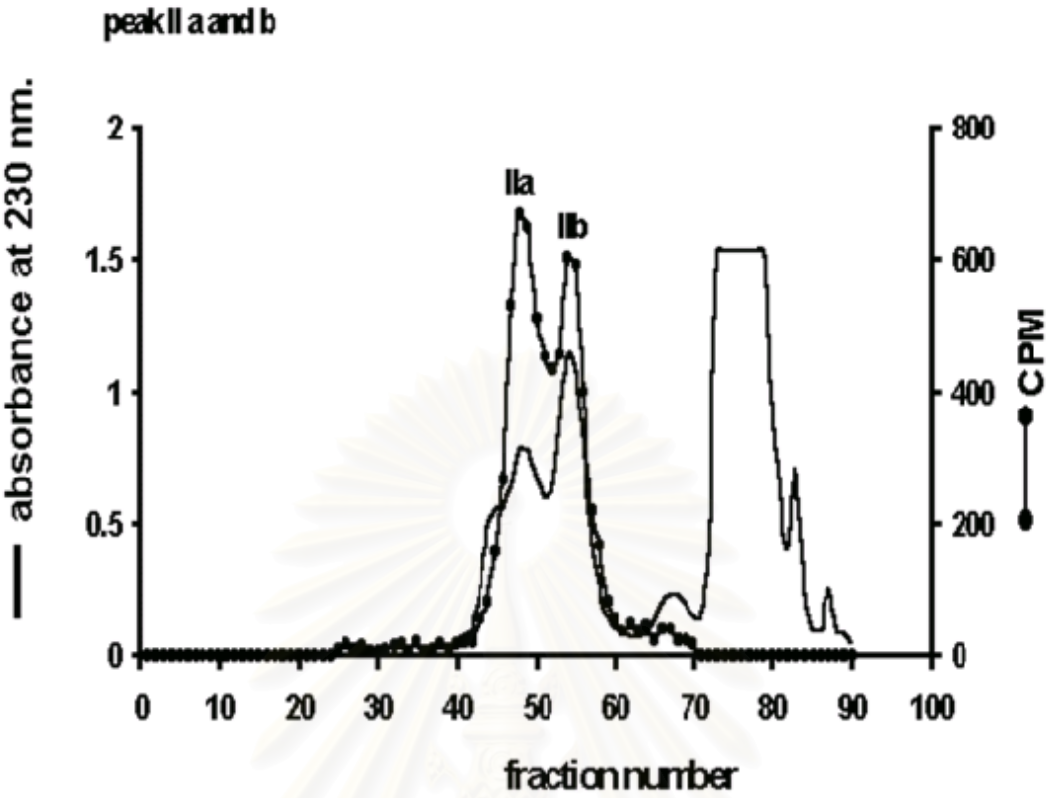

Figure 4.5 The chromatographic profile of the collagenase digest of peak II (from Figure 4.3) The collagenase digest of peak II was fractionated on the standardized Superdex 75 molecular sieve column chromatography using 0.025 M ammonium bicarbonate containing 5% IPA with a flow rate of 0.4 ml/minute. An aliquot from each fraction was determined for radioactivity, and two radioactive peaks were identified from the digest, denoted as peaks II a and II b, respectively.

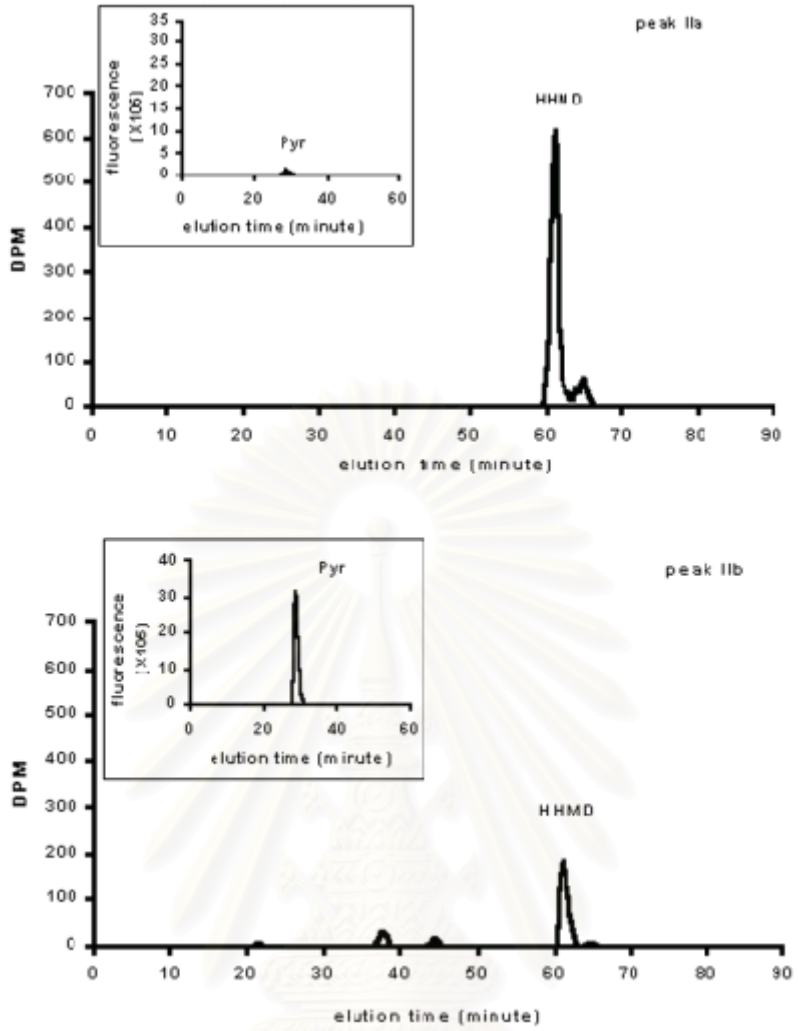

Figure 4.6 The cross-link profiles of peaks IIa and IIb (from figure 4.5). An aliquot from each radioactive peak, IIa and IIb, was subjected to acid hydrolysis and cross-link analysis. Both peaks IIa and IIb contained HHMD cross-linked peptides, with the former containing the higher amount of HHMD and less of pyridinoline.

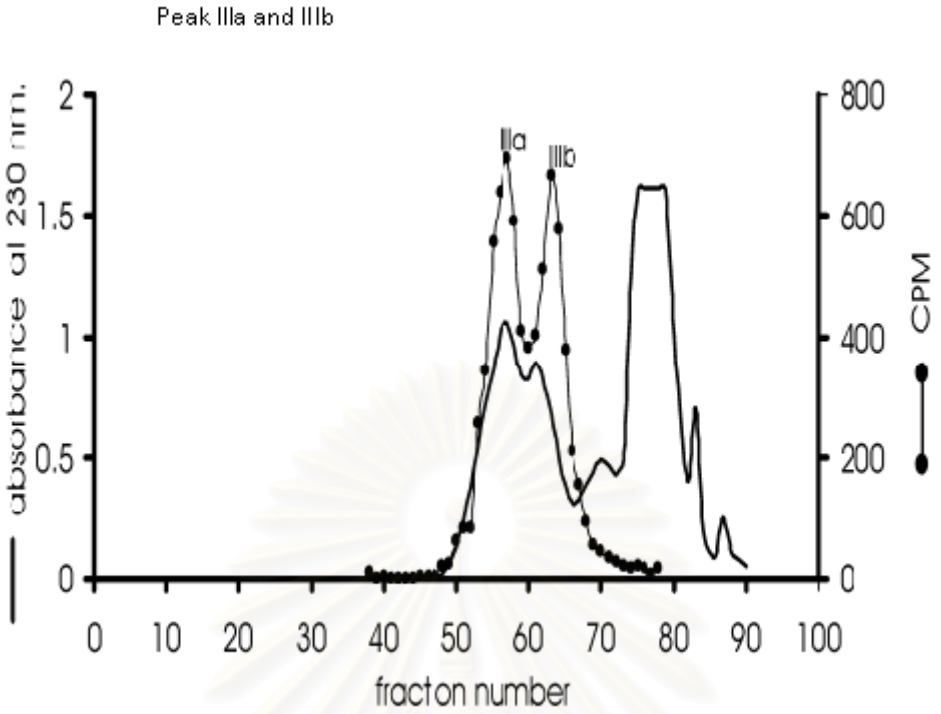

Figure 4.7 The chromatographic profile of the collagenase digest of peak III (from Figure 4.3). The collagenase digest of peak III was fractionated on the standardized molecular sieve column chromatography using 0.025 M ammonium bicarbonate containing 5% IPA with a flow rate of 0.4 ml/minute. An aliquot from each fraction was determined for radioactivity and two radioactive peaks were identified from the digest, denoted as peaks IIIa and IIIb respectively.

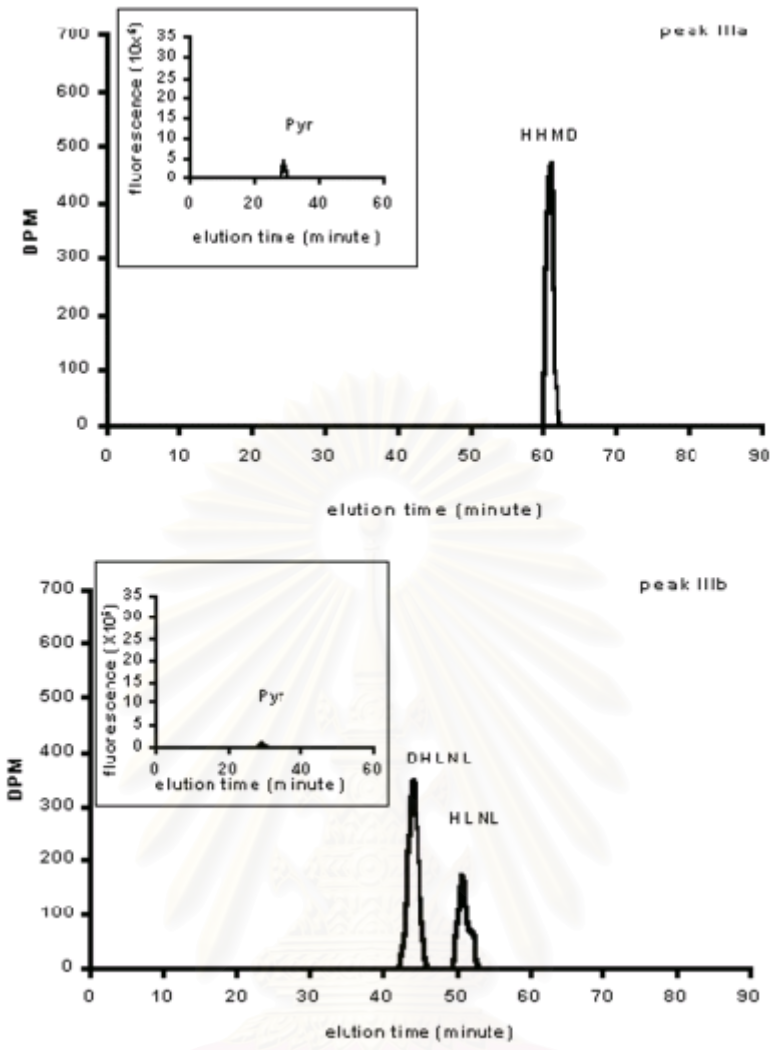

Figure 4.8 The cross-link profiles of peaks IIIa and IIIb (from Figure 4.7). An aliquot from each radioactive peak, IIIa and IIIb, was subjected to acid hydrolysis and cross-link analysis. Only peak IIIa was found to contain a significant amount of HHMD cross-link along with some pyridinoline cross-link. Peak IIIb was found to contain other reducible cross-links, DHLNL and HLNL.

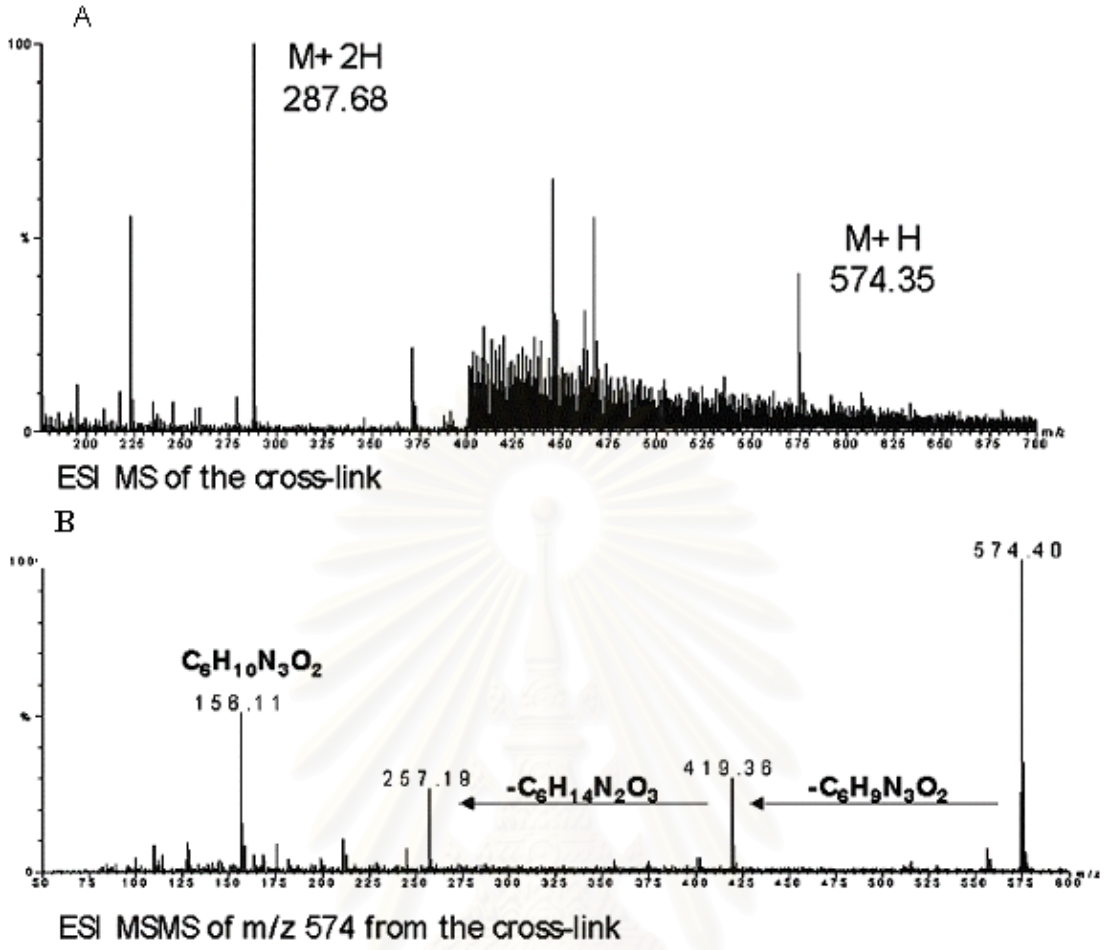

Figure 4.9 The ESI MS (A) and MS/MS (B) spectra of the purified HHMD cross-link show a protonated molecular ion of mass 574.3 daltons which corresponds to the mass and the structure of the reduced deH-HHMD proposed by Tanzer et al. (1973).

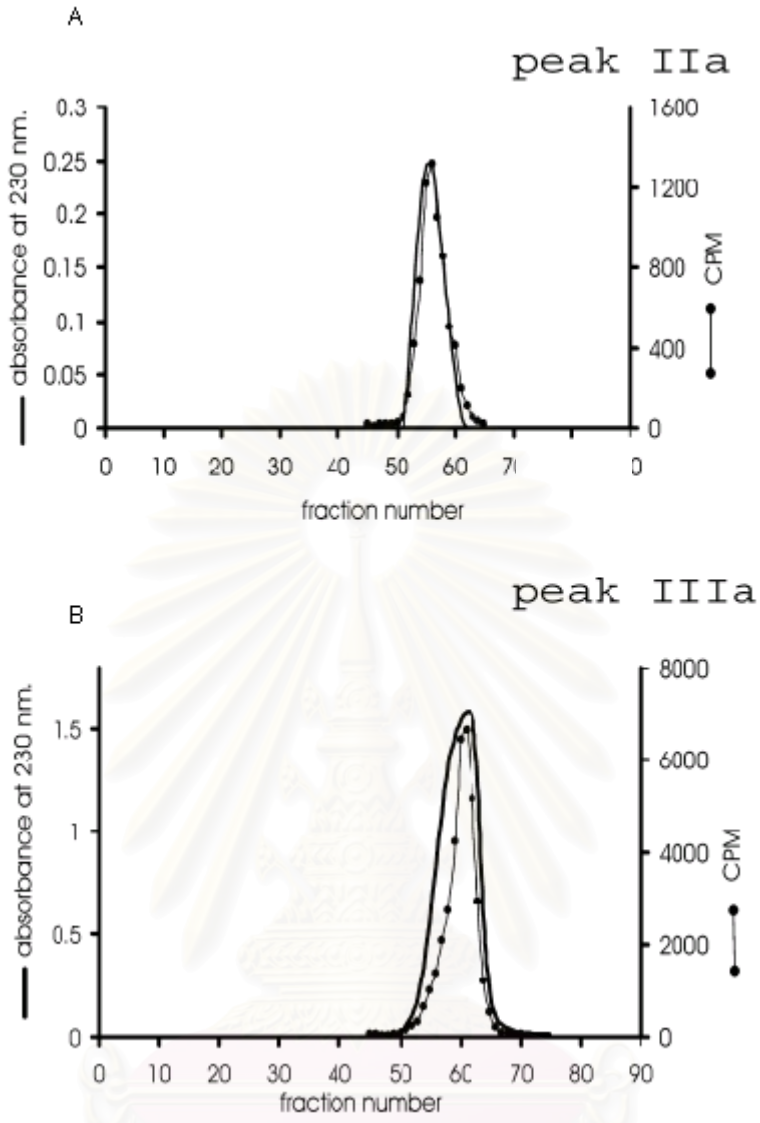

Figure 4.10 The final chromatographic and radioactive profiles of A) peak IIa and B) peak IIIa, respectively, on the Superdex 75 molecular sieve column chromatography. The cross-linked peptides were subjected to a series of HPLC/FPLC, with monitoring of absorbance at 230 nm. The fractions were determined for radioactivity to identify the presence of HHMD-peptide. The final chromatography was performed using a C4 reversed phase column, 4.6 mm X 250 mm with 300 A^{O} pore size, (Vydac) with a gradient of 0-40% acetonitrile in 0.1 % TFA, flow rate of 1.2 ml/minute, and HHMD cross-linked peptides were rechromatographed on the Superdex 75 molecular sieve column chromatography. At this point the cross-link profile showed only the presence of HHMD (data not shown). The pure fractions were pooled and dried, and subjected to further analyses.

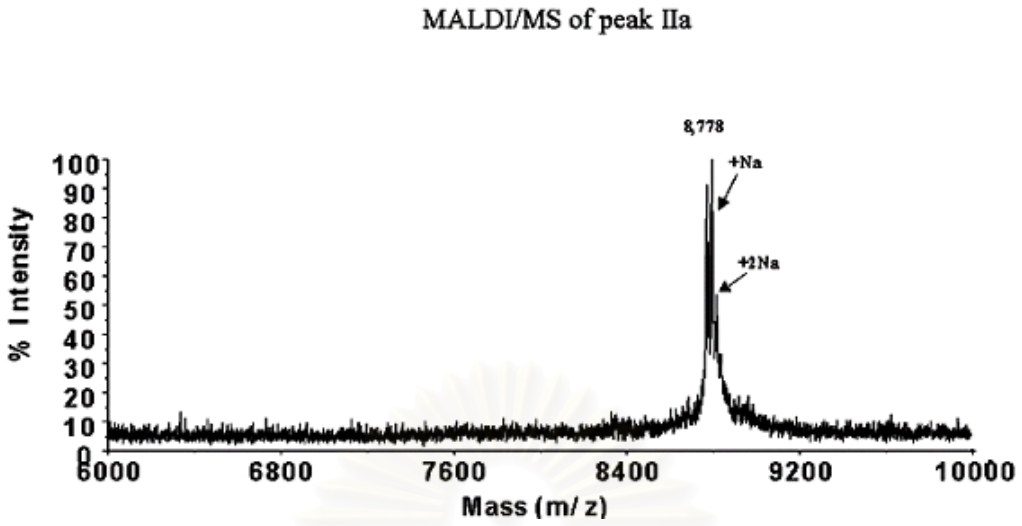

Figure 4.11 MALDI/MS spectra of peak IIa, collagenase digest of turkey tendon tryptic peptide, show an abundant molecular ion of 8,778. A series of ions separated by 22 dalton, sodium (Na), was also observed with the highest molecular ion corresponding to the M+2Na ion of the 8,778 species.

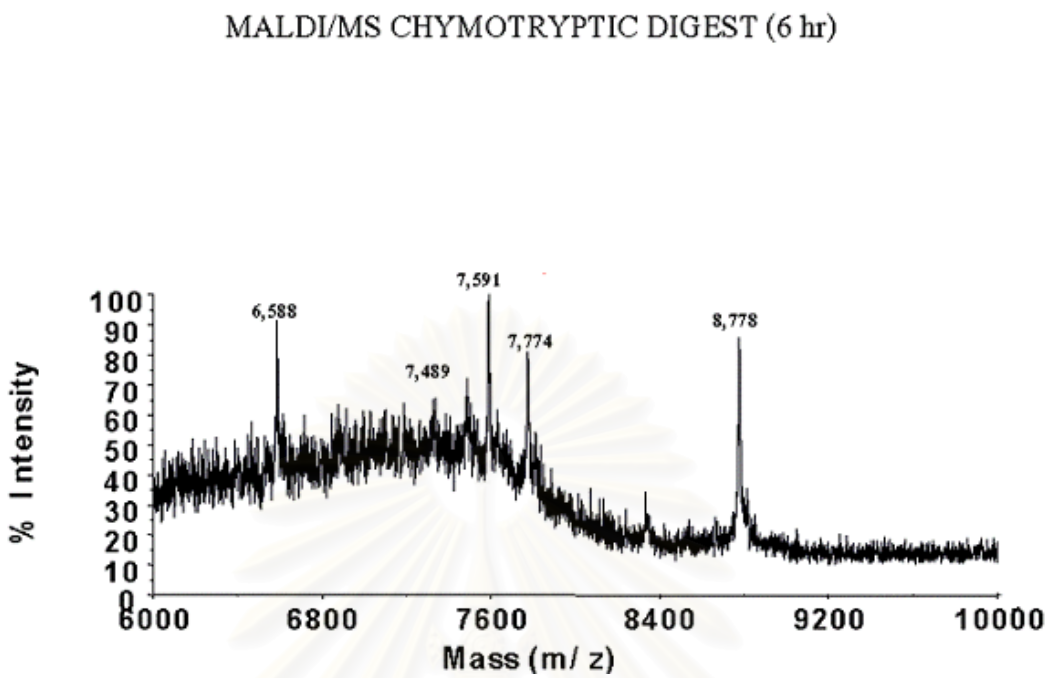

Figure 4.12 MALDI/MS spectra of chymotryptic digest of peak IIa. The MALDI/MS of a chymotryptic digestion of the HPLC fraction of peak IIa for 6 hours at 35 ^{o}C shows ion of m/z 7,774 dalton. The loss of 1,004 dalton, compared to the 8,778 ion may correspond to cleavage of the α 1 N telopeptide chain between Y 4N and G 5N plus loss of one oxygen.

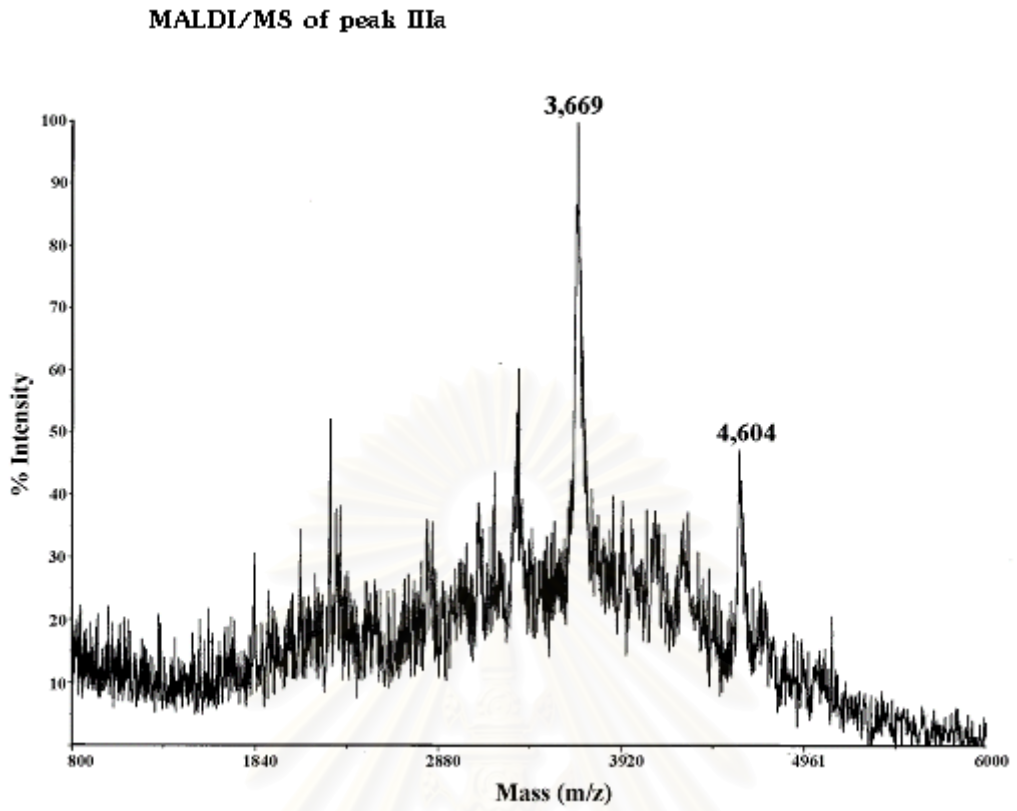

Figure 4.13 MALDI/MS mass spetra of peak IIIa. The spectra contain abundant ions of masses 3,669 and 4,604 dalton. The spectra also show other unidentified molecular ions indicating the heterogeneity of this peak.

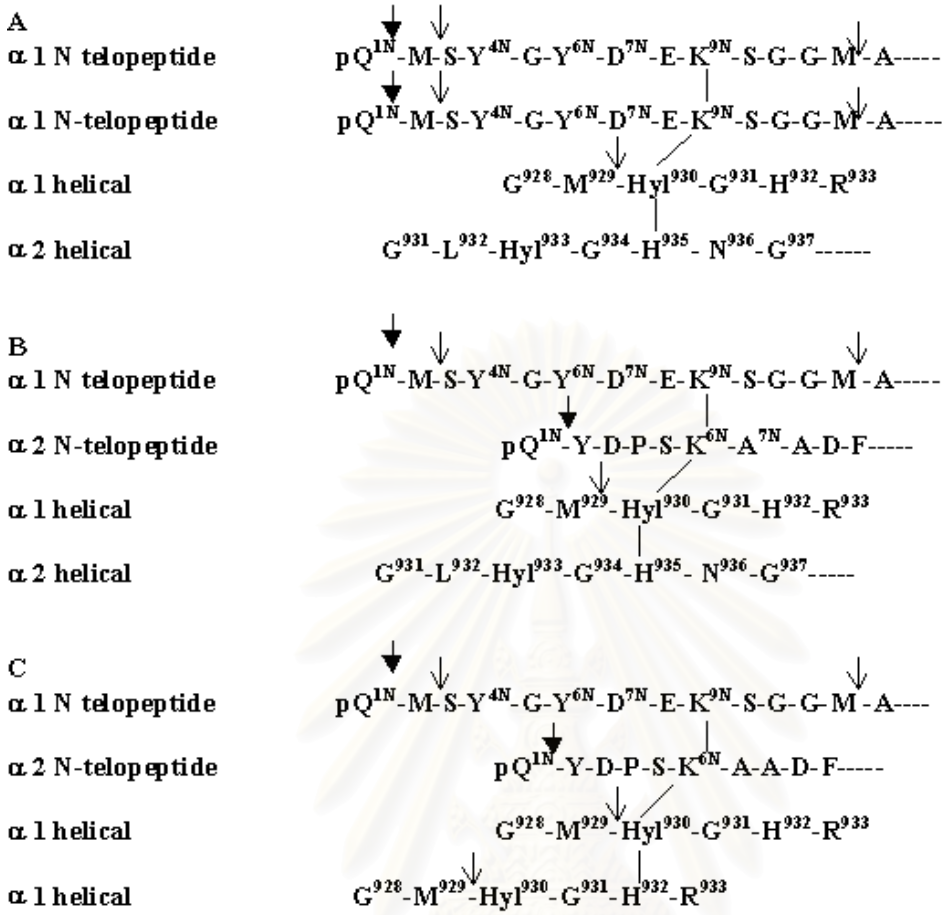

Figure 4.14. Based on the gathered experimental evidence in the present study, three possible locations (A-C) of residues involving in the formation of HHMD cross-linked peptides were proposed. (numbering of amino acid sequences was based on the Pubmed database, swissprot accession numbers PO2457 and PO2467)

pQ = pyroglutamate

↓ indicates the typical cleavage site of pyroglutamate aminopeptidase and

↓ indicates the typical cleavage site of CNBr

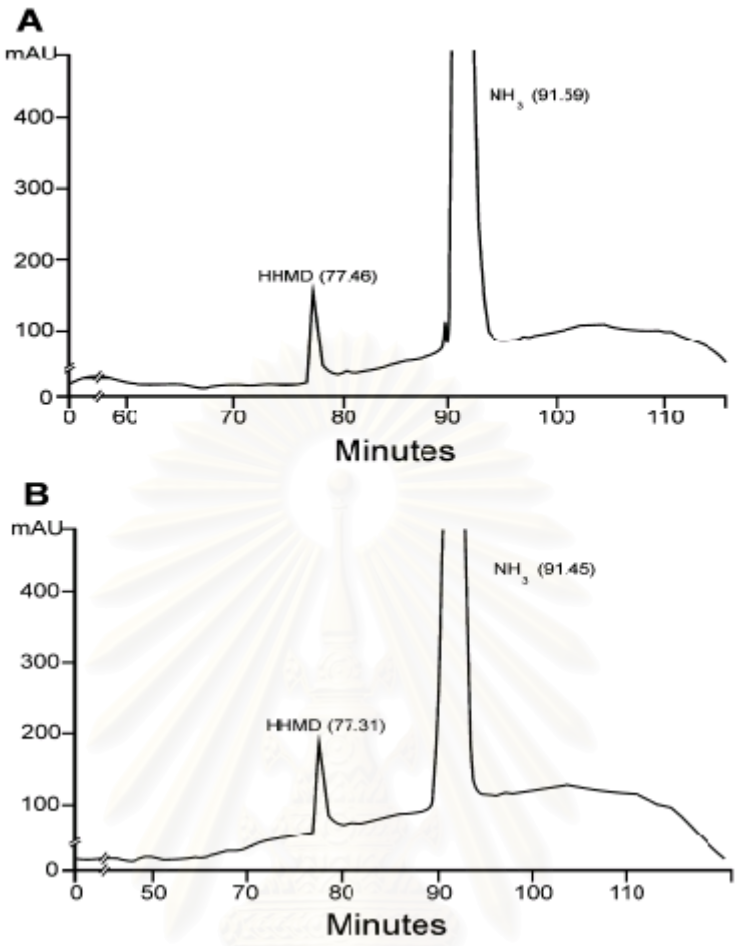

Figure 4.15 The chromatographic profiles of the purified HHMD cross-link on a strong cation exchange column chromatography configured as an amino acid analyzer using ninhydrin for color development. Acid and alkali hydrolysates of HHMD cross-linked peptide were fractionated on a P2 molecular sieve column. The purified HHMD cross-link was identified by its radioactivity and by cross-link analysis. The cross-links from both hydrolysates, A) acid and B) alkali hydrolysates, were then subjected to amino acid analyses, which showed that their elution positions were unchanged indicating no glycosylation involved in the formation of the cross-link.

Table 1 Amino acid sequence data of peak IIa (collagenase digest of peak II), and chymotrypsin digest of peak IIa. Ten cycles of N-teminal amino acid sequencing were performed for each sample.

cycle	Amino acid residues found from collagenase digest (peak IIa), amount shown in picomoles in parentheses		Amino acid residues found from chymotryptic digest of peak IIa , amount shown in picomoles in parentheses		
	α1	α2	α1	α2	α1
1	G -928 (287)	G –931 (287)	G-928 (38)	G-931 (38)	G-5N (38)
2	M-929 (437)	L-932 (282)	M-929 (40)	L-932 (24)	Y-6N (65)
3	-	Hyl -933(nq)	-	Hyl-933 (nq)	D-7N (84)
4	G-931 (206)	G-934 (206)	G-931 (39)	G-934	E-8N (50)
5	H-932 (126)	-	H-932 (8)	-	-
6	R-933 (186)		R-933(22)		S-10N (25)
7	G-934 (231)				G-11N (50)
8	F-935 (100)				G-12N (40)
9	S-936 (84)				
10	G-937 (138)				

Table 2 Amino acid sequence data of cyanogen bromide cleavage product of peak IIa. Ten cycles of N-terminal amni acid sequencing were performed, but amino acid residues were identified from only 8 cycles.

cycle	Amino acid residues from cyanogen bromide digest of peak IIa, amount shown in picomoles in parentheses		
	α1	α1	α2
1	S-3N (19)	-	G-931 (30)
2	Y-4N (29)	G-931 (31)	L-932 (16)
3	G-5N (22)	H-932 (9)	Hyl-933 (nq)
4	Y-6N (22)	R-933 (38)	G-934 (22)
5	D-7N (25)		
6	E-8N (20)		
7	-		
8	S–10N(16)		

Table 3 Amino acid sequence data of pyroglutamate aminopeptidase digest of peak IIa. Fifteen cycles of N-teminal amino acid sequencing was performed, but amino acid residues were identified from only thirteen cycles.

cycle	Amino acid residues from pyroglutamate aminopeptidase digest of peak IIa, amount shown in picomoles in parentheses			
	α1	α2	α1	α2
1	M-2N (53)	Y-2N (9)	G-928 (15)	G-931 (16)
2	S-3N (35)	D-3N (11)	M-929 (11)	L–932 (12)
3	Y-4N (36)	P-4N (8)	-	Hyl-933 (nq)
4	G-5N (17)	S-5N (8)	G-931 (17)	G-934 (17)
5	Y-6N (40)	-	H-932 (2)	-
6	D-7N(28)	A-7N (9)	R-933 (2)	
7	E-8N (29)	A-8N (11)		
8	-	D-9N (12)		
9	S-10N(20)	F-10N (6)		
10	G-11N(32)			
11	G-12N (32)			
12	M-13N(17)			
13	A-14N(10)			

Table 4 Amino acid sequence data of peak IIIa (collagenase digest of peak III), and chymotryptic digest of peak IIIa. Ten cycles of Edman degradation amino acid sequencing were performed.

Cycle	Amino acid residues found from collagenase digest (peak IIIa) , amount shown in picomoles in parentheses		Amino acid residues found from chymotryptic digest of peak IIIa , amount shown in picomoles in parentheses		
	α1	α2	α1	α2	α1
1	G-928 (25)	G-931 (25)	G-928 (94)	G-931 (94)	G-5N (94)
2	M-929 (19)	L-932 (13)	M-929 (177)	L-932(149)	Y-6N (279)
3	-	Hyl-933 (nq)	-	Hyl-933(nq)	D-7N (218)
4	G-931 (22)	G-934 (22)	G-931 (85)	G-934 (85)	E-8N (229)
5	H-932 (3)	-	H-932 (75)		-
6	R-933 (4)		R-933 (100)		S –10N(119)
7					G-11N (168)
8					G-12N(142)
9					M-13N(101)
10					A-14N(32)

Table 5 Amino acid sequence data of pyroglutamate aminopeptidase digest of peak IIIa. Ten cycles of N-terminal amino acid sequencing were performed.

cycle	Amino acid residues from pyroglutamate aminopeptidase digest of peak IIIa, amount shown in picomoles in parentheses				
	α1	α2	α1	α2	α1
1	M-2N(13)	Y-2N(8)	G-928 (36)	G-931 (36)	G-5N (36)
2	S-3N(19)	D-3N(15)	M-929 (61)	L-932 (50)	Y-6N (21)
3	Y-4N(21)	P-4N(18)	-	Hyl-933 (nq)	D-7N(29)
4	G-5N (35)	S-5N(13)	G-931 (35)	G-934 (35)	E-8N (33)
5	Y-6N(18)	-	H-932 (13)	-	-
6	D-7N(44)	A-7N(22)	R-933 (20)		S-10N (27)
7	E-8N (31)	A-8N (22)			G-11N (66)
8	-	D-9N (35)			G-12N (54)
9	S-10 (20)	F-10N (14)			M-13N(17)
10	G-11 (49)				A-14N(21)

Chapter 5

Discussion and Conclusion

The mineralizing turkey leg tendon has been widely used for studies on collagen structure, spatial relationship between collagen and mineral crystals, and post-translational phenotypes of collagen (Landis et al.,1993, Traub et al., 1992, Yamauchi and Katz, 1993, Knott et al., 1997). This is due to the fact that this tissue has a relatively simple arrangement of continuous collagen fibrils, and the tissue has three distinct compartments that can be manually dissected out for the chemical and structural analysis. The three compartments include mineralizing (inner portion), non-mineralizing but eventually will be mineralizing (both ends of the tendon) and never-mineralizing (most peripheral layer) compartments. Yamauchi and Katz, 1993, analyzed collagen in these compartments by using CNBr peptide mapping and amino acid analysis, and found out that collagen in all three compartments were essentially type I collagen (Yamauchi and Katz 1993, Yamauchi et al, unpublished). Distinct difference in type I collagen among these compartments was found, however, in their post-translational chemistries. It was found, in the present investigation as well as another's (Yamauchi and Katz, 1993), that never-mineralized compartment (peripheral layer of tendon) contained relatively significant level of pyridinoline cross-link (0.4 mole/mole of collagen), however, by using sequential enzymatic digestion and a series of HPLC, most of the pyridinoline cross-linked peptides could be separated from other cross-linked peptides. It has been a long term goal of this laboratory to elucidate the post-translational chemistries and molecular packing structure of type I collagen in order to further establish the structure-function relationship of this protein especially in the field of biomineralization. Evidences have shown that only trace amount of deH-HHMD was found in the mineralized tissue collagen, and preliminary data gathered by our laboratory has shown that the formation of deH-HHMD might involve two lysyl aldehydes originated from N-telopeptides. Studies

have suggested that the cross-linking chemistries in the N-terminal region are more complicated than those of the C-terminal region in non-mineralized tissues (Yamauchi et al., 1989). Therefore, it has been speculated that the N-terminal regions of collagen, a putative nucleation site (Berthet-Colominas et al., 1979), in non-mineralized tissues are spatially constrained thus downregulating the mineralization process. It was the objective of this study to determine the molecular loci of deH-HHMD and to characterize its glycosylation state in the never-mineralizing portion of tendon collagen to obtain insights into the potential structural role of the cross-link in non-mineralized tissues.

Tanzer et al., 1973, were the first to identify a tetravalent cross-link deH-HHMD, a large compound capable of cross-linking several peptides, in collagen and to demonstrate that histidine participated in the formation of the cross-link. They found that though this polyfunctional compound was different from the pyridinium cross-links found in elastin, they were related because they may arise from the same precursor molecules. It is of interest to note that only small amount of histidine is present in collagen, less than 1% of total amino acids, but they appear to be specifically distributed and their participation in the formation of natural cross-links in collagen is of biological significance. Although lysyl aldehydes have been found to locate in both the C- and N-termini of the collagen molecule, the aldol condensation product which is formed between two lysyl aldehyde residues has been reported to occur at the N-terminal portion of the molecule (Kang et al.,1969). Tanzer et al. (1973) noted that the specific location of an aldol and its condensation with a histidine, a sparse amino acid in the collagen molecule, on a neighboring molecule are a strong indication of stereospecificity of cross-linking reaction. Robins and Bailey in 1973 and 1977

questioned the existence of deH-HHMD as a natural cross-link under physiologic condition. Based on their experimental data, they found that fibers cleaved with acid phosphate failed to form deH-HHMD upon borohydride reduction resulted in a concomittent rise in the amount of intramolecular aldol. They claimed that Michael addition did not occur under physiologic condition but was caused by the base-catalyzed reaction by reduction with borohydride, therefore, HHMD did not exist as a natural intermolecular cross-link but rather an artifact. However, Bernstein and Mechanic in 1980 confirmed the existence of deH-HHMD as a natural collagen cross-link. They acid-cleaved insoluble steer skin collagen fibrils at pH 4.3 to break the cross-link into its precursors and allowed reformation of the cross-link to occur. They found that the reformation of the HHMD cross-link was time-dependent and reached the control values at 24 hours. The findings were consistent with the slow reaction of Michael addition of the N of histidine to the ethylene double bond of the aldol condensation product which was in concordant with the reaction proposed by Tanzer et al. (1973). Bernstein and Mechanic (1980) also found that the N of histidine that participated in the cross-link formation was protected against modification by alkylation as the Michael adduct. They also proposed that His-89 of α 1 chain participated in the formation of the cross-link. They concluded that deH-HHMD was a natural occurring cross-link and not an artifactual compound formed by the reduction process as claimed by Robins and Bailey. Although the scope of the present study does not include isolation and characterization of unreduced form of HHMD, it was suggested by Tanzer

et al. (1973) that unreduced aldol-histidine could be formed by an α Michael addition of the imidazole of histidine to the β-carbon of the α,β unsaturated bond of aldol condensation product (see figure 1.11). Furthermore, an active carbonyl moiety remains in unreduced aldol-histidine, Schiff base formation can occur yielding an unreduced form of histidinohydroxymerodesmosine. Tanzer et al. (1973) also suggested that alternatively dehydrohydroxymerodesmosine may initially form, similar to dehydromerodesmosine observed in elastin, and then Michael addition may occur. The latter pathway was suggested by the finding that a small amount of a compound having the mass spectral pattern consistent with hydroxymerodesmosine could be isolated from the NaB^3H_4-reduced collagen.

In this study, in order to purify and characterize the HHMD peptides, several enzymatic digestions were performed. The use of trypsin to truncate collagen molecules into small peptides followed by column chromatography has been widely used to isolate and characterize collagen cross-linked peptides (Aguilar et al., 1973, Kuboki et al., 1981, Yamauchi et al., 1986b, Yamauchi et al., 1989). The cleavage site specificity of trypsin at carboxyl side of arginine or lysine made the enzyme suitable for the study of collagen. However, trypsin used has been found to contain chymotryptic activity even though it has been treated with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK) as shown in our findings that both peaks IIa and IIIa contained the cleavage products resulting from chymotryptic action of trypsin, which were residues starting with G 5N- Y 6N- D 7N- E 8N ----from α1 N telopeptide domain. The sequence

of helical α1 GMKGHRGFSG was also found indicating the incomplete cleavage of R-G bond by trypsin which might be due to the steric hinder effect of the cross-link that made it difficult for trypsin to cleave between R and G. The use of bacterial collagenase was expected to be useful for cleaving the large structure of collagen into smaller peptides. The specific cleavage site of bacterial collagenase is at the carboxyl side of X in the sequence –P-X-G-P- where often X is found to be hydroxyproline (Peterkofsky, 1982). Specific cleavage sites of bacterial collagenase have been studied extensively, and at least 16 cleavage sites have been identified for classes I and II of bacterial collagenase by several investigators (Mookhtiar et al., 1985, Mallya et al., 1992, Mookhtiar and Van Wart, 1992, Lin et al., 1999). Bacterial collagenase used in the present study was the CLSPA class purified collagenase (Worthington), a mixture of two separate but very similar classes of collagenase namely class I and class II collagenases, which theoretically are able to cleave the collagen tryptic peptide into several smaller peptides (Lin et al., 1999). In the present study we could clearly show that bacterial collagenase cleaved between P-930^{helix} and G-931^{helix} of α2 chain as shown in the sequencing data of peak IIa (see tables 1-3). It was also found that collagenase cleaved between P-16N and G-1^{helix} of α1 chain as indicated by mass data of peak IIIa (see Results chapter).

Chymotrypsin cleavage sites have also been found to be slightly different from that of theoretical sites, which usually are the carboxyl side of hydrophobic residues such as Y, F, and W (Kellner and Houthaeve, 1999). In the present study we found that

chymotrypsin cleaved between α1 Y-4N and G-5N as shown by amino acid sequencing and mass data, but not at the carboxyl side of α 1 Y6N or α2 Y2N which both had aspartic acids in the P1' sites. Although the amino acids at P1' are relatively nonspecific, it has been recognized that if the residue at P1' is an acidic amino acid (D or E), the cleavage is significantly reduced, and if it is proline, no hydrolysis takes place (from"Practical Protein Chemistry-A Hand Book", 1986, A. Darbre (ed) p 349). α

The shortcomings of the N-terminal sequencing by Edman degradation may involve the cumulative left-over of the residues from previous cycle, an internal cleavage due to the labile peptide bonds as the sequencing proceeds because of the harsh condition of TFA and high temperature used, the loss of some amino acids such as serine, up to 80%, and threonine, up to 50 %, due to dehydration, the low yield of histidine and arginine, and the high background of some amino acid such as aspartic acid (Lottspeich et al., 1999). The presence of the complex HHMD cross-link in the peptide may also contribute to the difficulty in sequencing. These factors should be taken into consideration when interpreting the sequence data.

From the results of sequence after pyroglutamate aminopeptidase treatment, the complete sequences of an α1 and α2 N telopeptides were confirmed in the HHMD peptides isolated. It was clearly shown from the present study that the amino acid sequence found in an α1 N telopeptide is slightly different from those of chicken collagen those shown in the database (see figures 3.1 and 3.2). In the present study, the residues of α1 A-11N was substituted by G-11N , and α1 V-13N by M-13N. It is

likely that the sequence of turkey collagen is slightly different from that of chicken. Slight difference in amino acid compositions of type I collagen among animals in the same genus has been shown (Mechanic et al., 1987, Kuypers et al., 1992). The 0.3% difference between calculated mass based on the chicken collagen sequence and the mass observed (see Results chapter) could be due to the potential differences in the sequence between the two species. In addition, a significant number of amino acids of α2 helical peptide were unable to be sequenced because of the cyclization of N-G bond. The amino acid composition obtained from the amino acid analyses of peaks IIa and IIIa did not completely correspond to those of the theoretical values (data not shown). This also suggests minor differences in sequence between the two species. Further investigation is warranted to support this speculation.

The major locus appears to be α1-9N (Lys^{ald}) X α2-6N (Lys^{ald}) X α1-930 (Hyl) X α2-935 (His) based on the sequence results. This structure is present in both peak II and peak III. The possible explanation why they were separated on the Superdex 75 might be due to the partial cleavage of trypsin between α1 R933-G934. These specific residues are involved in the cross-link , not α2-933 (Hyl) or α1-932 (His) which are located very close to those residues, indicating that the packing of collagen molecules and the cross-linking reactions are stereospecific. This stereospecificity is probably required to form this complex cross-link, thus to stabilize the collagen fibrils as a whole. From the current study, we can not conclude if Hyl and His are from the same molecule or different molecules. If the cross-link ties three molecules, two of them that

provide His and Hyl, respectively, are almost in register and an aldol-containing molecule is axially staggered to both of these by 4 X 65 nm as in the case of skin collagen (Mechanic et al., 1987, Yamauchi and Katz, 1993). Absolute frequency of occurrence of deH-HHMD at several loci could not be established from this study because of the lack of information on HHMD in peaks IV and V. The characterization of HHMD in these peaks is needed to establish the relative participation of α1 and α2 chains in this cross-link.

Considering the complex structure of the HHMD cross-link we propose that the possible functions of this cross-link is to stabilize the collagen molecules in the fibril because it is an intermolecular cross-link, providing the tissue its resistance to mechanical tension. The role of collagen matrix to provide mechanical support especially tensile strength has been reported (Knott et al., 1995, Misof et al., 1997, Bank et al., 2000), and alteration in the nature and quality of collagen biochemistry has been shown to lead to impairment of tissues. The investigation regarding the relationship between the amount of deH-HHMD and biomechaincal strength of the tissue is needed. Additionally, this cross-link may have a role in the fixation of the N-terminal telopeptides and stabilization of the relatively flexible domains of a collagen molecule due to the lack of GPP sequence in the helical domain near the N-termini (Miles and Bailey, 2001). Studies using turkey tendon collagen fibrils showed that mineralization of the fibrils started in the gap zone close to N-telopeptide domain. The abundantly presence of the deH-HHMD cross-link, which is a strong tetravalent structure, in the non-mineralized

layer of the tendon involving the N-telopeptide domains of the molecules may cause spatial constraint for mineralization. In addition, a small leucine-rich proteoglycan, decorin, binding site has been reported to be close to the α1 Hyl-930, not α2 (Keene et al., 2000). Whether or not the cross-link would act as a receptor or have any communication with decorin needs further investigation.

The roles of selective glycosylation of specific hydroxylysyl residues in type I collagen molecules have been of interest (Morgan et al., 1970, Aguilar et al., 1973, Yamauchi et al., 1982). Henkel et al. (1976) pointed out that glycosylation was tissue-dependent and did not seem to be dependent on the localization of the cross-link in the molecule. The role of tissue-dependent glycosylation has not yet been well understood, however, studies have shown a possible relationship between glycosylation and fibril diameter and cross-linking formation (Yamauchi et al., 1982, Morgan et al., 1970, Eyre and Glimcher, 1973, Notbohm et al., 1999). It has been suggested that glycosylation might be involved in molecular assembly because their locations in the molecule would place them in the “hole” zones (Morgan et al., 1970). Morgan et al. (1970) speculated that the projecting carbohydrate groups may prevent or seriously impair the molecular packing of collagen, therefore the degree of glycosylation would be related to the order of fibril formation. Notbohm et al. (1999) studied the potential roles of hydroxylation/glycosylation of collagen using recombinant type II collagen that contained various degrees of hydroxylation/glycosylation and found that collagen with high contents of hydroxylysine/glycosylation compared to that with low

hydroxylysine/glycosylation formed thinner fibrils. Their data indicated that the extent of lysine hydroxylation and glycosylation may play a role in the regulation of collagen fibril formation and morphology. Eyre and Glimcher (1973) found from their study that a specific hydroxylysine in the sequence –G-M-Hyl ^{87}-G-H-R- which cross-linked to 16^{C}-Hyl^{ald} in the C-telopeptide forming the bifunctional deH-DHLNL cross-link was the major glycosylation site, and they concluded that glycosylation may direct the formation of the cross-link. Yamauchi et al. (1982) found that Hyl-87 involved in the complex trifunctional cross-link, histidinohydroxylysinonorleucine, was not glycosylated. Based on this finding, they speculated that the role of glycosylation was to prevent the formation of complex stable cross-link. In addition to these potential roles, i.e. regulation of fibrillogenesis, regulation of cross-link maturation, carbohydrate group attached to the G-X-Y sequence of collagen might play a role as a receptor site for cell signaling (Vogel et al., 1997, Shrivastava et al., 1997).

The present study clearly demonstrated that the hydroxylysine residue (α1 Hyl-930) involved in the deH-HHMD cross-link was not glycosylated. This was confirmed by cross-link, amino acid and mass spectrometric analyses using the alkali and acid hydrolysates of the purified HHMD peptides. The α2 Hyl-933 which is the second amino acid from the cross-link involved His toward N-terminus was also not glycosylated. However, both Hyl residues have been proposed as glycosylation sites (Kuboki et al., 1993, Kuypers et al., 1992).

It has been shown that Hyl-930 on an α1 chain and Hyl 933 on an α2 chain of type I collagen molecule, which involved in the formation of N-telopeptide derived pyridinoline and pyrrole cross-links, are not glycosylated (Hanson and Eyre, 1996). Preliminary (unpublished) data obtained from our laboratory showed that about 40% of Hyl residues (likely α1-930) involving in the formation of N-telopeptide derived bifunctional cross-links (deH-HLNL, deH-DHLNL) were glycosylated. These results suggest that the Hyl residue at 930 on an α1 chain, when glycosylated, participates in the formation of a simple bifunctional cross-links, but not in the multivalent cross-links. This could be due to the steric hindrance by the presence of carbohydrate moiety as suggested by Yang et al. (1993) that galactosylglucosyl group attached to a hydroxylysyl residue may laterally shield 3-4 amino acids. The absence of carbohydrate, on the contrary, may allow contact between an aldol and His, then with Hyl to form this complex cross-link. This is consistent with a previous hypothesis that glycosylation of Hyl prevents the formation of mature/complex cross-links (Yamauchi et al., 1982). Robins (1983) also noted the extreme lability of glycosylated pyridinoline found in his study compared to the glycosylated bifunctional cross-links, and offered the same explanation to others' findings regarding the absence of glycosylated products of complex cross-link.

Conclusion

Under the condition of the present study, the HHMD-crosslinked peptides were isolated and characterized. The present study demonstrates that, in never-mineralized

portion of turkey leg tendon, an aldol involved in deH-HHMD cross-link is derived from the N-terminal telopeptide portion (α1-9N-Lys^{ald} and α2-6N-Lys^{ald}). The possible molecular loci of the tetravalent cross-link HHMD in the never-mineralized part of turkey leg tendon collagen fibrils are proposed to be N-telopeptide-derived. Lysyl adehydes involved in the formation of the cross-link are from α1 9-N X α2 6-N or α1 9-N X α1 9-N. The helical Hyl involved is from α1 Hyl-930, and the helical His involved is from either α2 His-935 or α1 His-932. The helical hydroxylysines involved, α1Hyl-930 and α2 Hyl-933, in the formation of the cross-linked peptide are not glycosylated.

Based on the information of amino acid sequence and mass analyses gathered from the present study three possible molecular loci of HHMD cross-link are proposed, which are α1 Lys ^{ald} 9N X α1 Lys ^{ald} 9N X α1 Hyl 930 X α2 His 935 , α1 Lys ^{ald} 9N X α2 Lys ^{ald} 6N X α1 Hyl 930 X α2 His 935 , and α1 Lys ^{ald} 9 N X α2 Lys ^{ald} 6 N X α1 Hyl 930 X α1 His 932. The complex structure of deH-HHMD leads us to propose its function as to provide tensile strength and stability to the tissue, however, further investigations regarding the functional significance of this cross-link are definitely needed. Furthermore, the molecular loci proposed, resulted from the present findings, are from only two locations on the column chromatographs. The other two HHMD cross-linked peptide peaks await further characterization.

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