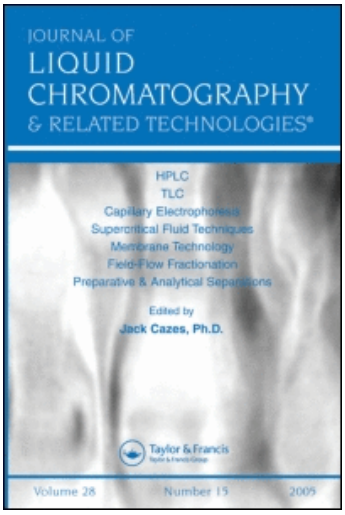

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Defining the Roles of Collagen and Collagen-Like Proteins Within the Proteome

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ABSTRACT

The collagen family is a diverse group of proteins distinguished by a native triple-helical structure. The collagen triple-helix is important for the integrity and workings of multiple connective tissues, including skin, bone, cartilage, tendon, and dentin. Most collagens assist in anchoring cells to the extracellular matrix and some function in cellular regulation. Multiple hereditary connective tissue diseases have been linked to collagen mutations. These mutations most often disrupt collagen folding. The very nature of the collagen family, one that is extensive, ubiquitous, abundant, well characterized, and responsible for various disease states, makes it a model protein family for intense proteomic studies. Proteomic analyses for triple-helical proteins may involve several different multidimensional approaches, such as two-dimensional liquid chromatography (2DLC),

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coupled liquid chromatography/capillary electrophoresis, and multidimensional protein identification technique (MudPIT) that couples 2DLC with mass spectrometry (MS). Following analysis, collagen and collagen-like proteins may be further characterized by expression of collagen and/or synthesis of triple-helical peptides (THPs) incorporating collagen sequences of interest. Collagen structure–function relationships have been well established by combined proteomic approaches.

Key Words: Collagen; Triple-helical peptides; Reversed-phase HPLC; Osteogenesis imperfecta; Proteome.

INTRODUCTION

The collagen family, made up of at least 25 members, is a diverse group of proteins.[1,2] Collagens are composed of three α chains of primarily repeating Gly-*X*-*Y* triplets, which induce each α chain to adopt a left-handed polyPro II helix. Three left handed chains then intertwine to form a right-handed superhelix (Fig. 1). The collagen triple-helix is important for the integrity and workings of multiple connective tissues, including skin, bone, cartilage, tendon, and dentin. The triple-helix also plays structural roles in the cardiovascular, urogenital, nervous, gastrointestinal, and respiratory systems. Most collagens assist in anchoring cells to the extracellular matrix and some function in cellular regulation.

Collagens have been classified according to their α chains. Homotrimeric collagens (i.e., types II and III) have three α chains of identical sequence. Heterotrimeric collagens have two α chains of identical sequence (designated α1) and one α chain of differing sequence (designated α2) (i.e., type I), or three α chains of differing sequence (designated α1, α2, and α3) (i.e., type VI).[3] Collagens are further classified into subfamilies, based on their quaternary structure. These subfamilies include fibrillar, fibril associated with interrupted triple-helices (FACIT), short chain, basement membrane, multiplexins, and membrane associated with interrupted triple-helices (MACIT).[3] The most common collagens (types I, II, III, V, and XI) have fibrillar structures.

There are atleast 38 collagen chain genes.[1] The collagen genes are found distributed throughout the genome, with a few instances of clustering (e.g., type VI collagen genes). The expression of the genes encoding the α chains for the various collagens must be coordinately regulated under normal conditions. Experiments performed in various laboratories have identified consensus regulatory sequences in the 5′ and 3′ untranslated regions of the collagen genes, including sequences involved in tissue-specific expression.[3]

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Figure 1. The representative structure of collagen, including post-translational modifications (disulfide bonds, hydroxylation of Pro and Lys, glycosylation of Hyl).

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Other important regulatory controls of the collagens are post-translational modification and protein folding steps. Before the collagen can be properly folded, a series of post-translational modifications on the central (Gly-*X*-*Y*)$_n$ domain must occur, including hydroxylation of most Pro and some Lys residues in the Y position, followed by glycosylation of certain hydroxylysines (Fig. 1). Glycosylation also occurs on some Asn residues in the C-terminal propeptides. Disulfide bonds between the propeptides are rearranged and isomerization of imino acids from *cis* to *trans* takes place.[4]

C-terminal propeptides mediate interaction between three α chains and hold these chains in place, nucleating triple-helical formation. Propagation of the triple-helix occurs in the C- to N-terminal direction. The triple-helical molecules are secreted from the cell and the N- and C-terminal propeptides that flank the central (Gly-*X*-*Y*)$_n$ domain are removed (in the fibrillar collagens). Finally, the collagens assemble and crosslink, forming supramolecular structures such as fibrils, filaments, or networks. These quaternary structures often contain more than one type of collagen.[5,6]

PROTEOMICS AND COLLAGEN

Proteomics is the study of all or part of the protein complement of genes in an organism or cell type, often involving the analysis of complex protein/peptide samples.[7] In the rapidly paced era of genomics, proteomics is emerging and changing the focus of protein chemistry. Chemists are moving away from the characterization of novel proteins and focusing more on examining protein domains. Total cellular protein is being analyzed for individual differences in protein levels between different normal tissues and diseased tissues. To a certain degree, chemists studying the collagen family of proteins have made huge contributions to the proteomic field already. The very nature of the collagen family, one that is extensive, ubiquitous, abundant, well characterized, and responsible for various disease states, makes it a model protein family for intense proteomic studies.

The triple-helical motif is found in a variety of non-collagenous proteins, such as macrophage scavenger receptors types I and II and bacteria-binding receptor MARCO, complement component C1q, pulmonary surfactant apoproteins A and D, acetylcholinesterase, bovine conglutinin, collectin-43, ficolins, aggretin, ectodysplasm, and mannose binding protein.[1,6,8] Any of the characterization methods performed on collagen domains are applicable to study of other triple-helical proteins.

Proteomic analyses for triple-helical proteins may involve several different multidimensional approaches. Traditionally, proteins are separated using 2-D gel electrophoresis (2D-GE), where the first dimension is isoelectrical

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focusing and the second dimension is mass resolution.[9] Two-dimensional gel electrophoresis has been used for analysis of collagen[10] Two-dimensional gel electrophoresis may be coupled to mass spectrometry (MS), allowing for protein identification. However, the resolution of GE is limited, and GE is time-consuming and labor-intensive. In addition, collagen is known to behave anomalously on SDS–PAGE due to conformation and low content of hydrophobic residues.[11] Recent improvements in separation methods have alleviated the need for 2D-GE.[9] Other methodologies, such as two-dimensional liquid chromatography (2DLC), coupled liquid chromatography/capillary electrophoresis, and multidimensional protein identification technique (MudPIT) that may couple 2DLC with MS, have become popular and are more amenable for the study of collagen. Following analysis, collagen and collagen-like proteins may be further characterized by expression of collagen and/or synthesis of triple-helical peptides (THPs) incorporating collagen sequences of interest. These studies, coupled with animal modeling and mutation analyses in human diseases, offer a comprehensive examination of a triple-helical protein structure and function.

COLLAGEN AND DISEASE

Collagens have been identified as the flawed factor in specific pathological conditions (Table 1). In particular, multiple hereditary connective tissue diseases have been linked to collagen mutations. These mutations most often disrupt collagen folding, resulting in its defective structure and function.[3–6,12] Both natural and induced mutations have been studied for many of the collagen genes.

Type I collagen, the most profuse and ubiquitous of the collagens, is found in most connective tissues and embryonic tissues (reviewed in Ref.[3]). Mutations of type I collagen genes have been identified in both osteogenesis imperfecta (OI) and Ehlers-Danlos syndrome (EDS).[1,3–6,12–14] Osteogenesis imperfecta is characterized by brittle bones, blue schlerae, and deformation of the skeleton. Over 90% of patients with OI have type I collagen mutations. The range of phenotypes for this syndrome runs from mild to lethal. Osteogenesis imperfecta is the result of one of two genetic mutational routes: dominant-negative mutations (the far more common route) or null mutations. Dominant-negative mutations can occur in either gene that encode the α chains of type I collagen and are typically missense mutations that change the Gly codons in the triple-helical motifs, or, less commonly, mutations that are responsible for splicing defects. Gly substitutions result in different effects on helix stability, depending on their location and the newly substituted amino acid. But the substitution of Gly in the collagen characteristic $(\text{Gly-}X\text{–}Y)_n$

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Table 1. Collagen types and role in disease.

Collagen subfamily	Representative associated disease(s)
Fibrillar (I, II, III, V, XI)	Osteogenesis imperfecta (OI)
	Ehlers-Danlos syndrome (EDS)
	Types I, II, IV, VII
	Bone chondrodysplasias
	Osteoarthritis (OA)
	Aortic aneurysms
	Suggested upregulation in cancer
Basement membrane (IV)	Alport syndromes, autosomal and X-linked
	Goodpasture syndrome
Short-chain (VIII, X)	Suggested upregulation in cancer
	Diabetes mellitus
	Schmid metaphyseal chondrodysplasia
	Osteoarthritis
	Chondrosarcomas
	Advanced disc degeneration
FACIT (IX, XII, XIV, XIX)	Multiple epiphyseal dysplasia
	Intervertebral disc disease
	Rheumatic diseases
	Fibrosis
	Suggested upregulation in cancer
	Possible congenital degenerative eye/brain diseases
Other (VI, VII, XVIII)	Congential heart defects and skin oedema in Trisomy 21
	Bethlem myopathy
	Hypertension
	Diabetes mellitus
	Rheumatic diseases
	Fibrosis
	Suggested upregulation in cancer
	Epidermolysis bullosa
	Systemic lupus erythematosus
	Some linear IgA bullous diseases
	Knoblach syndrome

repeat always has some pathological outcome. The importance of Gly in collagen structure is its small size (compared to the other amino acids), allowing for the proper folding of the triple-helix. Gly mutations often decrease the thermal stability of the triple-helix motif. The mutation's location within the protein will determine the probability of helix renucleation. Likewise, the seriousness of the OI phenotype will be prescribed by the nature of

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the substitution and its address, in terms of its resident α chain, its amino acid position, and the surrounding sequences on the α chain. Comprehensive surveys that examine multiple mutations of various collagens suggest that genotype–phenotype correlations are hard to establish.[12,13] The most severe cases of OI were caused by mutations resulting in α chains of abnormal structure but still capable of interaction with other type I collagen α chains, while the mutations that only decrease the expression of an α chain partially from one allele result in the milder forms of OI.[1,12,14]

Like Gly substitutions, point mutations and deletions/insertions resulting in splicing defects within the helical regions of collagens produce a range of OI phenotypes, depending on their location within the molecule. Mutations in the non-helical domains that reside within the carboxyl end of the collagens appear to be less frequent than those within the helical regions, but may alter the associations between the α chains and their triple-helical formation. Studies using site-directed mutagenesis to introduce specific collagen mutations into human cells or mice cells (resulting in transgenic or knock-out mice) have confirmed the importance of triple-helical structure formation in determining the degree of pathological phenotype of OI (reviewed in Refs.[3,6]).

The second mutational route causing OI are null mutations. Very little is known about the molecular mechanisms that underlie such mutations, although there have been some studies that point to inhibition of RNA transport to the cytoplasm, blocking its translation.[3,5]

There are multiple forms of EDS. The EDS type VII phenotype is characterized by major dislocations of the joints and fragility of the skin. The mutations causing this phenotype ultimately block N-terminal cleavage of type I collagen by either deleting the N-proteinase cleavage sites in type I procollagen or by resulting in N-proteinase enzyme deficiency. When propeptide cleavage is inhibited, packing into supramolecular structures is defective.[3,5,6,12]

Type II collagen is found in cartilage and the virteous humor. Its expression also occurs during embryogenesis. Mutations in type II collagen result in bone chondrodysplasias, including achondrogenesis-hypochondrogenesis, spondyloepiphyseal dysplasia congenita, Stickler syndrome, and Kniest syndrome.[1,3,5,6,12,15–17] Achondrogenesis-hypochondrogenesis is lethal short-limbed dwarfism. Mutations that cause this disorder have been identified as Gly substitutions in the triple-helical region of type II collagen. In at least one case, Gly substitution in collagen type II led to post-translational over-modification and dilated rough endoplasmic reticulum. Taken together, these observations suggest defective assembly and secretion of type II collagen.[3] Site-directed mutagenesis and introduction of mutant type II collagen constructs into human cells or transgenic mice, reaffirm the correlation between type II collagen mutations and the phenotype of chondroplasias (reviewed in Ref.[3]).

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There is evidence that increased synthesis of type II collagen accompanies osteoarthritis (OA), a common joint disease characterized by loss of cartilage and joint degradation.[18] The extracellular level of the C-propeptide of type II procollagen (CPII) is elevated in the middle and deep zones of OA cartilage. This CPII increase was not observed in OA patient serum, however.[18] More recently, a combined analysis of the rates of type II collagen synthesis and degradation from patients with knee OA was performed.[19] It was found that over time, the serum levels of the N-propeptide from type IIA procollagen decreased and the urine levels of the C-terminal crosslinking telopeptide of type II collagen increased, indicative of a decrease in type II collagen synthesis with an increase in degradation, respectively, in patients with progressive joint damage.[19]

Type III collagen is found in visceral and cardiovascular tissues, as well as in numerous tissues characterized by high type I collagen content. Mutations in type III collagen result in a range of phenotypes, from very severe EDS type IV (characterized by ruptured arteries, premature aging, and thin skin) to mild symptoms (typically joint hypermobility and thin skin).[1,3,6,12,20] An evaluation of 63 patients with type III collagen mutations found that over 50% were triple-helical Gly substitutions.[12]

Two additional fibrillar collagens are types V and XI, also known as type V/XI collagen, because of their similarities and their ability to form heterodimers.[5,6] These are quantitatively minor components compared to the other fibrillar collagens. Type V collagen is found in most connective tissue and type XI collagen is found in cartilage. In some tissues, type V collagen forms heterotypic fibrils with type I collagen.[5,6,21] Transgenic mouse experiments originally demonstrated that type V collagen mutations can lead to EDS; since then, some EDS patients have been identified with type V mutations.[22] Mutations in type XI collagen have been associated with dyplasias; these include triple-helical Gly substitutions.[12]

The non-fibrillar type IV collagen is the major collagen found in basement membranes, forming network-like structures. Mutations in type IV collagen have been identified in patients with X-linked or autosomal recessive Alport syndrome (a disease characterized by glomerulonephritis, and sometimes deafness), as well as people with Goodpasture syndrome (an autoimmune disorder causing glomerulonephritis and pulmonary haemorrhage) (reviewed in Refs.[1,3]).

In addition to structural and anchoring roles, collagen can modulate cellular responses. For example, cell adhesion to collagen regulates cellular gene expression and inhibits cell death via apoptosis. Thus, the relative levels of collagen expression have significant implications for cell homeostasis. Given this role in cellular behaviors, collagen expression has been implicated in pathological conditions. Gene expression of the α chains involved in the formation of types I and III collagen [more specifically, α2(I) and α1(III), respectively] is elevated in selected, highly metastatic pulmonary melanomas,

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as compared to poorly metastatic tumors.[23] It is presumed that these elevated collagen levels enhance melanoma survival. Conversely, fragments of types IV, XV, and XVIII collagen inhibit tumor cell growth and angiogenesis[1,24–28] Collagen modulation of metastasis is complex, but further understanding of the process offers significant therapeutic potential.

In addition to the collagens discussed above, the less abundant collagens have been implicated in numerous pathological conditions (Table 1 and reviewed in Refs.[1,29]). The mechanisms of disease relating to these collagens are, in many cases, similar to those outlined above.

PRODUCTION OF TRIPLE-HELICAL PROTEINS AND PEPTIDES

Synthesis of Triple-Helical Peptides

To fully understand the biological effects of collagen structure, THPs or "mini-collagens" incorporating collagen-like sequences have been constructed by several laboratories. Triple-helical peptides may range from 70 to 125 residues. Our laboratory developed a solid-phase THP synthetic method which features a C-terminal Lys covalent branch (Fig. 2).[30–32] Substantial stabilization of the triple-helical structure can be achieved with the introduction of a di-Lys or di-Glu template at the C- or N-terminal regions of the three peptide chains,[8,30,32–38] a double disulfide "knot" at the C-terminal region of the three peptide strands,[39,40] or a KTA,[41–44] cyclotriveratrylene,[45] or *tris*(2-aminoethyl)amine[46] template linked to the N-terminus of three peptide chains (Fig. 2). Conversely, we have described an approach by which the non-covalent association of lipophilic molecules, N-terminally linked to a peptide, can be used to form stable "peptide-amphiphile" triple-helices for similar studies.[47–49] The thermal stability of the triple-helical head group can be controlled based on the lipophilic tail length and extent of branching.[47,48,50] This is especially important for the function of bioactive sequences, where the maintenance of distinct three-dimensional structure may correlate to optimal activity.[35] In some cases, fluctuations in the triple-helical backbone are possible[51–53] and may be necessary for some collagen-mediated activities.[53–55]

Triple-helical peptides have been designed to house various Gly substitutions associated with diseases caused by different collagen mutations. Nuclear magnetic resonance (NMR) and circular dichroism (CD) spectroscopic studies of such peptides permit the folding upstream, downstream, and at the mutation site to be examined.[4] These tailor-made peptides allow the systematic study of the folding capabilities of different amino acid sequences. By using mutant "mini-collagens," steps in the normal nucleation and propagation of triple-helical

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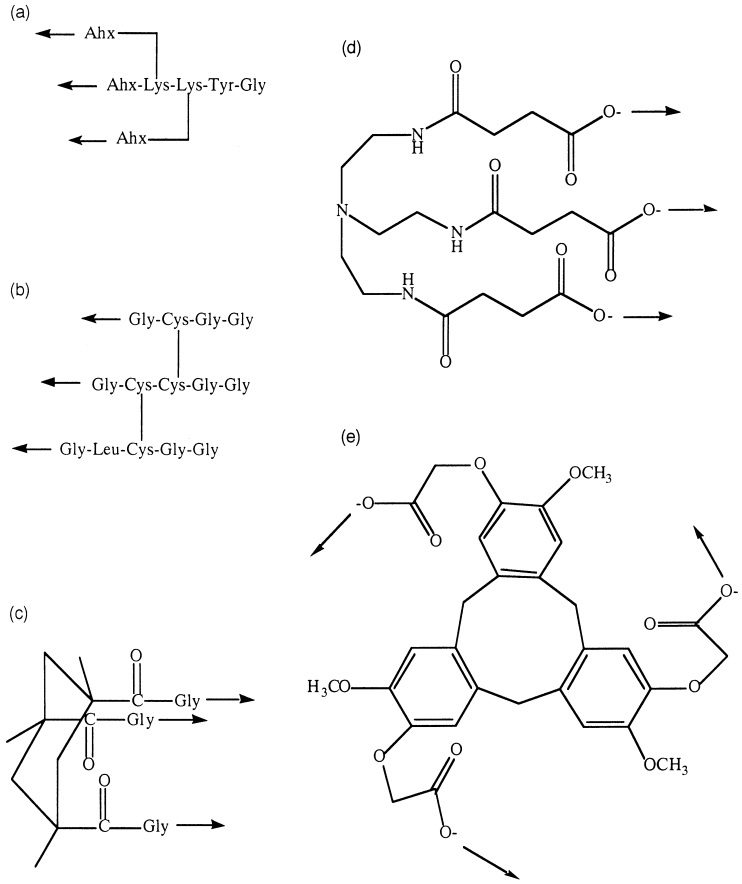

Figure 2. Templates used for the chemical synthesis of THPs: (a) di-Lys branch after coupling to 6-aminohexanoic acid (Ahx); (b) disulfide bridge (cystine-knot); (c) *cis*,*cis*-1,3,5-trimethylcyclo-hexane-1,3,5-tricarboxylic acid (KTA) after coupling to Gly; (d) *tris*(2-aminoethyl)amine (TREN) after coupling to succinic acid; and (e) cyclotriveratrylene (CTV) after coupling to α-bromoethanoic acid. The arrows indicate the direction of collagen-like sequence incorporation.

structures can be elucidated.[56–62] Nuclear magnetic resonance, CD, and x-ray crystallographic studies lend insight into the genotype–phenotype relationship of different collagen mutations with severity of associated disease.[63–66] opening new doors to prognostic and therapeutic interventions.

Triple-helical peptides have been used to identify cellular recognition sites within collagens. Discrete THP sequences have been described that bind to

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specific cell surface receptors, such as the $\alpha 1\beta 1$, $\alpha 2\beta 1$, and $\alpha 3\beta 1$ integrins[32,55,67–77] and CD44 chondroitin sulfate proteoglycans.[31,53] Triple-helical peptides containing Gly-Pro-Hyp repeats alone have been shown to bind platelet glycoprotein VI.[78,79] Cell signaling pathways have also been elucidated using THPs.[50,72,80–84]

Triple-helical peptides have been used to examine additional macromolecular interactions. The determinants for binding of the molecular chaperone HSP47 have been determined using THPs.[85] Our laboratory and the Moroder laboratory have developed THPs as substrates for members of the matrix metalloproteinase (MMP) family.[39,40,86–93] Goodman and colleagues, as well as our laboratory, have studied biomaterial applications of THPs.[50,94–96] Triple-helical peptide models of the macrophage scavenger receptor have been used to study binding interactions of acetylated low-density lipoproteins[36,97] and tetraplex nucleic acids.[98] Triple-helical peptides have also been used to identify and characterize glycosaminoglycan binding sites.[99,100]

Recombinant Expression of Collagen

Expression systems for recombinant homotrimeric types II, III, and XIII collagen and heterotrimeric types I and IV collagen have been described.[10,101–106] In addition, collagens with deleted domains[105,107,108] and biosynthetic collagen-like polymers (CLPs)[109–113] have been expressed. Collagen expression systems have been described in mammalian HT1080[101,107,108,114] and CHO[10] cells, yeast *Pichia pastoris*[106] and *Saccharomyces cerevisiae*,[115] and insect Sf9 cells.[102–105,116] However, expression of collagen chains can be difficult, due to degradation of individual α chains prior to triple-helix formation and/or inclusion body formation. Transgenic organisms, such as mice and tobacco plants, have been utilized to produce types I–III collagen.[117–119] Collagen-like polymers have been expressed in *Escherichia coli*.[109,111–113]

ANALYSIS AND/OR PURIFICATION OF TRIPLE-HELICAL PROTEINS AND PEPTIDES

Chromatographic Methods

There have been numerous studies describing the analysis and purification of collagen and collagen-derived fragments by RP-HPLC using cyanopropyl,[120–122] diphenyl,[123,124] C_4,[124] C_8,[123,125] and C_{18}[120,122,125–130] columns, unique pyridine–propanol gradients[121,123] or more "conventional"

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solvent systems such as water–acetonitrile,[120,124–127,130] and different counterions.[120,122,128] The earliest studies compared pore sizes (100 Å vs. 300 or 500 Å) and determined that large-pore columns reduced collagen chain peak widths[123] and improved collagen fragment resolution;[120] consequently, later collagen RP-HPLC studies used large-pore columns (300–500 Å) almost exclusively.[122,124,125,127,129] Large-pore (300 Å) columns have been utilized effectively for characterization of novel collagens.[131,132] However, switching from a large-pore (250 Å) silica-based column to a nonporous polymeric column improved resolution of type VI collagen chains.[133] Optimized RP-HPLC conditions were most often based on denatured collagen and collagen fragments. Unfortunately, triple-helices can appear heterogeneous upon partial denaturation[125] due to slow *cis–trans* isomerization of peptide bonds.[134] Triple-helical peptides and small proteins are especially susceptible to anomalous behaviors during RP-HPLC.

Given the documented thermal stabilities of some THPs,[30,135] it is more desirable to perform analytical RP-HPLC under non-denaturing conditions, as attempting to completely denature THPs could be difficult. The worst-case scenario would be the analysis of partially denatured species, as conformational effects would interfere with resolution of the desired species from other peptides. However, retaining native conformation is not trivial, as the thermal stability of triple-helices is decreased by RP-HPLC conditions.[125] For example, the T_m of type II collagen is lowered by almost 15°C in 50% acetonitrile, while 50% isopropanol lowers T_m by 7°C.[125] In addition, the RP stationary phase can dramatically destabilize triple-helices. Type II collagen, which has a $T_m = 30$–34°C in 10–20% 1-propanol, eluted as a very broad peak by C_8 RP-HPLC at 4°C over a gradient of 10–20% 1-propanol.[125]

Two studies have specifically examined the RP-HPLC behaviors of THPs.[136,137] The first[136] examined the effects of different stationary phases (diphenyl, C_4, and C_{18}), organic modifiers (acetonitrile and isopropanol), support pore sizes (nonporous, 120 Å, and 300 Å), and counterions (trifluoroacetic acid and heptafluorobutyric acid) for the RP-HPLC analysis of three homotrimeric branched THPs, one of 124 residues and two of 106 residues. HFBA increased retention times but did not improve resolution for C_{18} RP-HPLC analysis of THPs[136] and separation of denatured type I collagen-derived fragments.[122] None of the conditions provided satisfactory results for the analysis of THPs by large pore C_{18} or C_4 RP-HPLC. Subsequent studies have shown that the addition of salts, such as $NaClO_4$,[138] can enhance THP resolution on large pore C_{18} RP columns (Fig. 3).

Narrow-bore, small-pore C_{18} RP-HPLC can produce sharp peaks for some THPs.[30,31] Conversely, a branched THP was well resolved by nonporous C_{18} RP-HPLC using a gradient of 0–50% B in 30 min with an eluent system of $A = 0.1\%$ TFA in water and $B = 0.1\%$ TFA in acetonitrile.[139] The aforemen-

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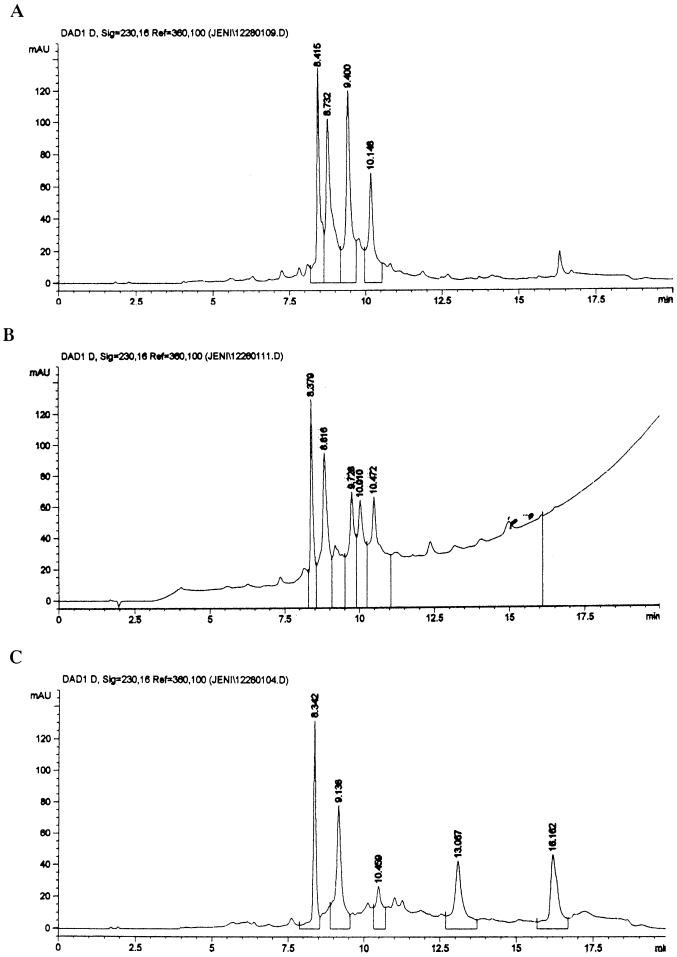

Figure 3. RP-HPLC analysis of a THP mixture using a Vydac C_{18} column (5 μm particle size, 300 Å pore size, 4.6 × 150 mm), a gradient of 0–100% B in 20 min, and detection at $\lambda = 230$ nm, where $A =$ water and $B =$ acetonitrile. The counterion and/or salt was (top) 0.1% TFA, (middle) 20 mM H_3PO_4, or (bottom) 20 mM $H_3PO_4 +$ 100 mM $NaClO_4$. The five THPs, in order of elution, are (Gly-Pro-Hyp)$_5$-Gly-Pro-Lys(Mca)-Gly-Pro-Pro-Gly ~ Val-Val-Gly-Glu-Lys-Gly-Glu-Gln-(Gly-Pro-Hyp)$_5$-NH_2, (Gly-Pro-Hyp)$_5$-Gly-Pro-Lys(Mca)-Gly-Pro-Pro-Gly ~ Val-Val-Gly-Glu-Lys(Dnp)-Gly-Glu-Gln-(Gly-Pro-Hyp)$_5$-NH_2, (Gly-Pro-Hyp)$_4$-Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly ~ Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-(Gly-Pro-Hyp)$_4$-NH_2, C_8-(Gly-Pro-Hyp)$_5$-Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly ~ Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-(Gly-Pro-Hyp)$_5$-NH_2, and C_{10}-(Gly-Pro-Hyp)$_5$-Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly ~ Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-(Gly-Pro-Hyp)$_5$-NH_2.

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tioned comprehensive study[136] found that the nonporous C_{18} and the diphenyl columns provided the best THP peak sharpness and resolution. However, one eluent system [$A = 10$ mM H_3PO_4 (pH 2.1) + 50 mM Na_2SO_4 in water and $B =$ acetonitrile] did create high back-pressure, particularly with the analytical diphenyl and small-pore columns. In light of this limitation, the eluent system produced sharp peaks by C_{18} RP-HPLC for $(Pro\text{-}Pro\text{-}Gly)_5$ and $(Pro\text{-}Pro\text{-}Gly)_{10}$,[140] and was effective for nonporous C_{18} and diphenyl RP-HPLC, but not for large-pore C_4 or C_{18} RP-HPLC, analysis of THPs.[136] An eluent system of $A = 0.1\%$ TFA in water and $B = 0.1\%$ TFA in acetonitrile worked well with either nonporous C_{18} or diphenyl RP-HPLC for a variety of THPs.[136]

The use of a diphenyl stationary phase resulted in considerably less denaturation of THPs than either C_4 or C_{18}.[136] Since THPs can be difficult to fully denature (see prior discussion), the most desirable RP-HPLC conditions are those which least effect the native conformation. Of the large-pore silica-based analytical columns (5 μm particle size, 300 Å pore size, 250 × 4.6 mm), the diphenyl stationary phase provided the best peak sharpness and resolution of THP products compared to C_4 or C_{18}.[136] C_{18} RP-HPLC could be used for THP analysis with a column containing a nonporous polymeric support. The purities of THPs were best evaluated by the combination of nonporous C_{18} and diphenyl RP-HPLC. The stability of THPs is affected by the nature of the stationary phase and support and/or the support pore size during RP-HPLC.[136,137]

The second study of RP-HPLC THP behavior[137] examined several Kemp triacid (*cis*,*cis*-1,3,5-trimethylcyclo-hexane-1,3,5-tricarboxylic acid, KTA) template-assembled models. Using large-pore C_{18} (5 μm particle size, 300 Å) RP-HPLC with $A = 0.1\%$ TFA in water and $B = 0.1$–0.15% TFA in acetonitrile, it was found that: (a) triple-helical conformations eluted at different times than non-triple-helical ones; (b) hydrophilic side-chains resulted in the triple-helical species eluting earlier than the non-triple-helical one; and (c) lack of hydrophilic side-chains resulted in the non-triple-helical species eluting earlier than the triple-helical one.

RP-HPLC does not represent the only HPLC technique that can be used to analyze collagen and/or THPs. There is a long history of collagen chain purification by either a combination of ion-exchange and size-exclusion (SE) chromatography or SE chromatography alone. These methods have been adapted for HPLC applications. Types I, III, and V collagen chains have been quantitated by a two-step HPLC approach, first by cation-exchange chromatography (Mono S HR 5/5) followed by SE chromatography (Bio-Sil SEC-125).[141] Separon HEMA 1000 Glc (a copolymer of 2-hydroxyethyl methacrylate with ethylene dimethacrylate covalently coated with glucose) SE chromatography has been used to separate types I, III, IV, and V collagen chains.[142,143] Bio-Gel TSK 60XL SE chromatography has proven particularly useful for the analysis of type I collagen monomers and oligomers, as well as for the separation of native vs.

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denatured collagen.[144] Molecular weight analysis of denatured collagen chains following SE chromatography can be achieved by multiangle light scattering.[145] Sephadex G50 SE chromatography has been used previously to analyze THPs,[30,31] as THPs did not suffer from the anomalous behaviors sometimes exhibited by collagen chains during SE chromatography.[143]

Hydroxyapatite (R-type crystals) has been used for HPLC separation of collagen chains.[146] Hydrophobic-interaction (HI) HPLC has also been shown to be useful for analysis of THPs.[30]

The isolation and purification of collagen on a large scale often requires a combination of traditional column and HPLC chromatographic techniques. As an example, we can consider type V collagen, which exists in $[\alpha1(V)]_2\alpha2(V)$ and $\alpha1(V)\alpha2(V)\alpha3(V)$ forms. The individual sub-molecular species of type V collagen could be purified from bovine or porcine crude preparations using Fractogel EMD SO_3^- chromatography[147] or filter paper-based (FPB) DEAE-cellulose chromatography followed by cation exchange (Bakerbond WP-CSX) chromatography.[148] The subunit chains could be further isolated using anion-exchange (Bakerbond WP-PEI) and, if necessary, C_{18} RP-, HPLC.[147,148]

Capillary Electrophoretic Methods

Initial studies on the separation of collagen chains (types I, II, V, IX, and XI) and CNBr-derived peptides were performed using a 50 cm × 100 μm I.D. fused-silica capillary and a buffer system of 2.5 mM sodium tetraborate, pH 9.2 or 10.5.[149,150] A linear relationship was found for the relative retention time vs. molecular mass of collagen chain polymers.[150] CNBr-derived collagen peptides obeyed the relationship of relative retention time $= -3.25 \times M^{2/3}/Z + 28.2$, where M is molecular mass and Z is the number of acidic residues.[149,150] Improved resolution of CNBr-derived peptides was obtained using a 50 cm × 50 μm I.D. or 70 cm × 75 μm I.D. capillary and a buffer system of 100 mM phosphate, pH 2.5.[151,152] Peptide separation appeared to involve sorption to the capillary wall, as modification of the inner capillary surface or addition of organic modifier ruined the separation.[152] Later studies on separation of intact collagen chains found that good resolution was achieved using a 60 cm × 50 μm I.D. fused-silica capillary coated with α-dodecyl-ω-hydroxy poly(oxyethylene) (Brij 35) to reduce protein adsorption.[153] The buffer system was 20 mM sodium phosphate, pH 5.6–6.5.

Mass Spectrometric Methods

Mass spectrometric analysis of intact collagen chains has not been reported, most likely due to the large molecular mass of the protein.

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MALDI-TOF MS was successfully used to analyze the mass of a 73,274 Da biosynthetic CLP using a sinapinic acid matrix.[113] Triple-helical peptides that are formed by association may be analyzed by a variety of MS techniques, which usually dissociate the peptides into individual strands.[47,48,53,90] Mass spectrometric analysis of branched THPs is more challenging, as highly branched peptides ionize poorly, especially if the number of charged residues is low. Data for branched THPs has been obtained using ion-spray/electrospray triple quadrupole MS,[36,45,86,154,155] but is more readily achieved by MALDI-TOF using α-cyano-4-hydroxycinnamic acid or 2,5-dihydroxybenzoic acid/2-hydroxy-5-methoxy-benzoic acid (9 : 1, v/v) as a matrix.[32,37–39,86–89,156]

SUMMARY

The collagen family encompasses a diverse group of proteins that have important structural and regulatory roles in normal physiology. The production of mutant collagens has been correlated to numerous pathological conditions.

Table 2. Analysis of collagen and THPs.

Analytical method	Advantages	Disadvantages
GE	Resolution of individual collagen chains	Anamolous behavior due to collagen conformation and low content of hydrophobic residues; relatively poor resolving power
RP-HPLC	Nonporous polymeric C_{18} and diphenyl columns produce good triple-helical protein peak sharpness and resolution	Anamolous behavior due to slow *cis-trans* isomerization of collagen backbone
SE-HPLC	Smaller triple-helical proteins resolve well	Anamolous behavior of longer triple-helical proteins due to conformation
CE	Resolution not affected by triple-helical conformation	Charge distribution may not be greatly varied between certain collagen chains; reproducibility; not a robust technology
MS	Determine exact molecular mass and sequence for triple-helical proteins	Molecular weight limitation as most intact collagens are too large

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For many years, attempts to analyze collagens and other triple-helical proteins had proven difficult, based on the inherent conformation and distribution of charged residues. Recent advances in chromatographic, electrophoretic, and mass spectrometric techniques now allow for effective analysis of collagenous proteins (Table 2). Once the identity of a collagen is established, structure–function relationships can be explored by (a) either recombinant expression or chemical synthesis of triple-helical proteins and (b) knock-out and knock-in animal models. This combination of approaches allows for the determination of the roles of collagens within the proteome.

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