

EXPLORING A ROLE IN TANNING FOR THE GAP REGION OF THE COLLAGEN FIBRIL: CATECHIN-COLLAGEN INTERACTIONS*

by

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ABSTRACT

Electron micrographs of stained collagen fibrils display a pattern of alternating light and dark bands perpendicular to the axis of the collagen fibril. Light bands correspond to regions of more dense lateral packing where adjacent collagen monomers overlap and dark bands correspond to 'gap' regions, domains of low-density molecular packing. Most studies of artificially stabilized collagen focus on crosslinks that utilize specific amino acid side chains, without considering whether these are more likely to be located in overlap or gap regions. The gap region with its lower molecular density, greater length, and available telopeptides appears promising as an area able to accommodate oligomeric compounds that are the typical tanning agents. This study uses the ERRC collagen microfibril model to explore the interactions of catechin, a model for vegetable tanning agents thought to interact with serine residues, in the collagen microfibril. Attempts at placing catechin near serine residues in the overlap region were not successful. Four catechin molecules were docked near serine residues in the gap region of the microfibril and subjected to conditions typical of tanning. Under these simulated conditions each catechin molecule moved into a position where intermolecular stabilizing interactions were likely, although not with serine. The method will be valuable in predicting the effectiveness of proposed new tanning agents.

RESUMEN

Electromicrografías de fibrilos colagénicos tintados demuestran bandas alternas oscuras y claras, perpendiculares al eje del fibrilo colagénico. Las bandas claras corresponden a regiones de paquetes lateralmente más densos en donde monómeros colagénicos adyacentes están sobre impuestos y las bandas oscuras corresponden a regiones de 'transición', o sea zonas de paquetes moleculares de baja densidad. La mayoría de los estudios sobre estabilización artificial de colágeno se enfocan en enlaces que utilicen cadenas de aminoácidos laterales específicas, sin considerar si estos se encuentran en las regiones de transición o traslape. Las regiones de transición con densidad molecular más baja, más longitud, y disponibilidad de telopéptidos, aparece promisorio como un área capaz de acomodar compuestos oligoméricos que son los típicos agentes curtientes. Este estudio utiliza el modelo microfibrilar colagénico ERRC para explorar las interacciones de catequina, un modelo para agentes vegetales curtientes que se creen reaccionar con residuos de serina, en el microfibrilo colagénico. Intentos de colocar catequina cerca de los grupos de serina en las zonas de traslape no tuvieron éxito. Cuatro moléculas de catequina fueron colocadas en la zona de traslape cerca de residuos serínicos y el microfibrilo fue sometido a condiciones típicas de curtido. Bajo estas condiciones simulatorias del curtido cada molécula de catequina se traslado a posiciones donde interacciones de estabilización intermolecular serían probables, pero no con serina. Este método será valioso en la predicción de la efectividad de nuevos agentes curtientes propuestos.

*Presented in part at the 29th IULTCS Congress/103rd Annual Meeting of the American Leather Chemists' Association, Washington, DC, June 21, 2007.

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Manuscript received February 15, 2008, and accepted for publication February 20, 2008

INTRODUCTION

The majority of proteins are roughly spherical in shape, with secondary structures comprised of α -helical, β -strand and unordered regions. The most abundant animal protein and the protein of importance to the leather community, collagen, is a notable exception. The collagen primary structure, a repeating tripeptide (Gly-Xxx-Yyy)_n where $n > 300$ and about 25% of X and Y residues are proline and hydroxyproline respectively, results in a secondary structure with characteristics of a lefthanded polyproline II helix. Three of these helices wind into the right-handed collagen triple helix. Supramolecular structures (microfibrils, fibrils, fibers) are formed by self-association of smaller units.

Molecular modeling of collagen has a long history. The highly organized supramolecular structure of collagen was observed in X-ray diffraction patterns in the 1950's and led to the conclusion that the structure was helical, but not α -helical. About the same time, knowledge of the unique features (33% glycine, high levels of imino acids, and post-translational hydroxylation of prolines and lysines) of the primary structure became available and encouraged early model builders to visualize the triple helix in ball and stick representations.¹ By the 1980's, computer-based molecular simulations had become feasible, and the Scheraga group at Cornell University explored the effects of various X and Y residues on the stability of the collagen triple helix.²

The ERRC leather research group began development of a collagen microfibril model in the early 1990's. For the initial studies, the conformational parameters developed by the Scheraga group² were used with data from X-ray diffraction³ and electron microscopy⁴ to begin the construction of a collagen model. First, chains of (Gly-Pro-Pro)_n, (Gly-Pro-Hyp)_n and (Gly-Ala-Ala)_n where n was 4 to 16, were constructed, each in the form of a left-handed helix. Three identical chains were then assembled into a right-handed triple-helix and energy-minimized to produce a triple helical template. Five of these triple helices were then docked to form a microfibril⁵ template based on the Smith⁶ model. Next, appropriate short segments of triple helix and microfibril were selected from the amino acid sequence of type I bovine collagen⁷ and substituted for Pro or Hyp residues in the models, based on the collagen packing analysis of Piez and Trus,⁸ to construct three-dimensional energy-minimized model fragments for calf-skin type I collagen triple helix and microfibril.^{9,10}

The direct substitution of other amino acid side chains for Pro and Hyp in a much longer (Gly-Pro-Hyp)_{n>100} microfibril model resulted in many bad contacts and general distortion of the backbone. A procedure was developed for growing new side chains in place, one bond at a time, with dynamics simulation and energy-minimization steps between additions to produce a model that when colored to represent a negative microscopy stain, showed a banding pattern typical of those seen in collagen micrographs.¹¹ This 15

chain, 315 residue long, model (Figure 1) which encompasses a single gap region, 159 residues long, with a 78 residue long overlap region at either end, is large enough to include all parts of the collagen monomer.

The initial model ignored the telopeptides because of the limited data then available for their secondary structures. The importance of telopeptides as anchors for the natural crosslinks that form in collagen as the animal ages was recognized, and as conformational data for isolated telopeptides^{12,13} became available, models of the N-terminal¹⁴ and C-terminal¹⁵ telopeptides were constructed to accommodate these natural crosslinks. The telopeptide conformations have now been modified in response to still more recent studies of their interactions with the triple helical domain of collagen.^{16,17} The impetus for construction of this model was to provide a basis for the study of those interactions between tanning materials and collagen that might be expected to contribute to the formation of a complex between collagen and the tanning agent or to the stabilization of the collagen fibril structure. An early attempt at predicting crosslinking sites for chromium salts¹⁸ focused on a fragment from the overlap region.

Recent developments, including a better understanding of the role of telopeptides in fibril stabilization, increased interest in tanning agents other than chromium, and a greater awareness of the roles of collagen modification and fibril coating have led to a closer examination of the gap region. A successful tanning agent is one that interacts with the collagen matrix of the hide in a way that provides stability. Under the conditions of tanning, most tanning agent molecules are of moderate size and may be in oligomeric form; thus one of the requirements may be adequate conformational space within the fiber structure to accommodate the oligomer. Metallic tanning agents, principally chromium, react with acidic residues (Asp and Glu) on collagen. Tanning agents with aldehyde functionality (glutaraldehyde, genipin, oxazolidine) react with amino groups of lysine (Lys) and hydroxylysine (Hly). Polyphenolic vegetable tannins generally interact with groups that are hydrophobic or have the potential for forming hydrogen bonds.

Catechin, a relatively small polyphenolic flavonoid molecule (MW = 290) isolated from a variety of natural sources including tea leaves, grape seeds, and tree bark, is a precursor of condensed tannins that has been used as a model for the study of tannin collagen interactions. In the early 1980's, Kuttan et al.¹⁹ observed that soluble collagen treated with catechin became less soluble, and more resistant to collagenase. Madhan et al.²⁰ in a recent study of the stabilization of collagen by catechin, reported on the interactions of a catechin model with a single 24 residue-long collagen triple helix. In this study, we have chosen to study some potential interactions of the catechin model with the fifteen-chain collagen microfibril.

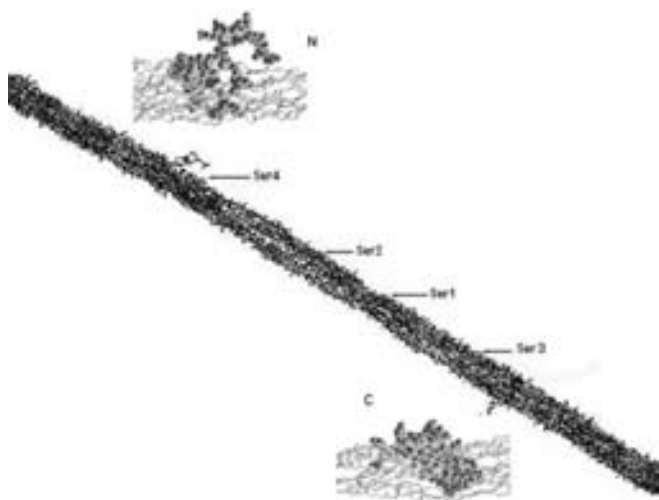


Figure 1: The ERRC collagen microfibril model labeled to indicate the positions of Ser1 - Ser4, with enlarged views of the N (top) and C (bottom) telopeptides extending from the overlap region into the gap region of the microfibril.

METHODS

As in previous studies from this laboratory, a Silicon Graphics workstation loaded with SYBYL 7.2²¹ software was used for constructing and manipulating the models. In a preliminary step, the C-telopeptide conformation¹⁵ was modified to be consistent with the conformation reported by Malone and Veis¹⁷ from a study of the interactions of the C-telopeptide with the collagen helix (Figure 1). The microfibril with telopeptides was separated into overlap and gap segments for analysis of the distribution of side chain functionality. The catechin model (Figure 2) was extracted from file 1JNQ of the Protein Data Bank (PDB) at <<http://www.pdb.org>>.²² The catechin model was used either without hydration or with a single hydration shell containing 18 water molecules.

The size of the model system, the inclusion of nonprotein molecules (catechin and water) and the limits of the computing system required modification to the protocol previously used by ERRC collagen modelers. To prepare for this study, the collagen microfibril model previously optimized with a Kollman force field²³ was energy-minimized using the Tripos force field.²⁴ All systems were prepared for calculations, either energy minimization or dynamics simulation as a function of temperature, by adding essential hydrogen atoms and loading Kollman charges onto the protein, and Gasteiger-Marsili charges onto nonprotein ligands.

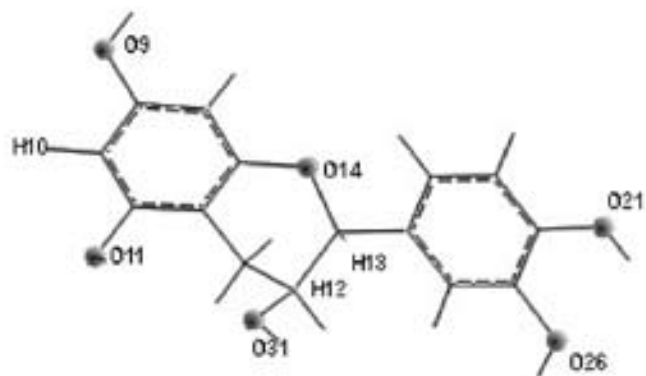


Figure 2: Catechin molecule with number labels on those atoms monitored in this study.

RESULTS

Although the distribution of amino acid residues with ionizable and hydrophobic side chain functionality is not uniform along the collagen peptide chain, the compositions of the gap and overlap regions of the microfibril are more uniform. To give a general picture of characteristics of the ERRC microfibril, the amino acid residue types, in order of decreasing hydrophobicity,²⁴ are listed in Table I, with the number of each type and the average atomic density in a sphere with a 12 Å radius around the C α -atoms of these residues in the gap and overlap regions.

To compare the accessibility of potential interaction sites in the gap and overlap regions of the microfibril structure to tanning agents, attention was focused on the atomic density around atoms most likely to participate in crosslinking or other stabilizing interactions. Regions around the ϵ -amino nitrogen of Lys or Hly, the carboxyl oxygen of Asp or Glu, or the carbon atom most distant from the peptide backbone of a hydrophobic group were examined. Although the total number of residues and the atomic density are greater in the overlap regions than in the gap region, 57% of the more hydrophobic residues (Phe, Ile, Leu, Val) are found in the gap region with a surrounding atomic density 21% lower than for similar groups in the overlap region. Of the acidic and basic residues, 52% are located in the gap region where the atomic density around the acidic groups is 12% less, and around the amino groups 17% less than in the overlap region. The 12 Å sphere for evaluating atomic density was chosen to be larger than the minimum size (~7 Å) of a site for a bi- or tri-nuclear chromium complex bridging two acid groups, or for a genipin²⁶ dimer bridging two amine groups.

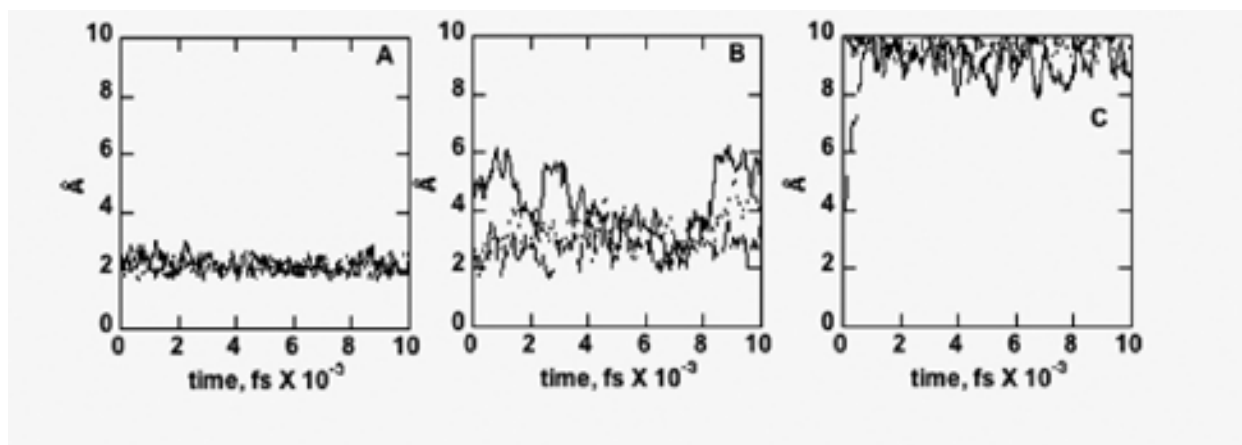


Figure 3: Dynamics analysis for a catechin molecule docked at Ser1. Dashed lines represent 300K, dotted lines 350K and solid lines 400K data. (A) O31 of catechin and a hydrogen atom at the end of a valine chain. (B) O11 of catechin and a hydrogen atom at the end of an alanine side chain. (C) H10 of catechin and a carboxyl oxygen of glutamic acid.

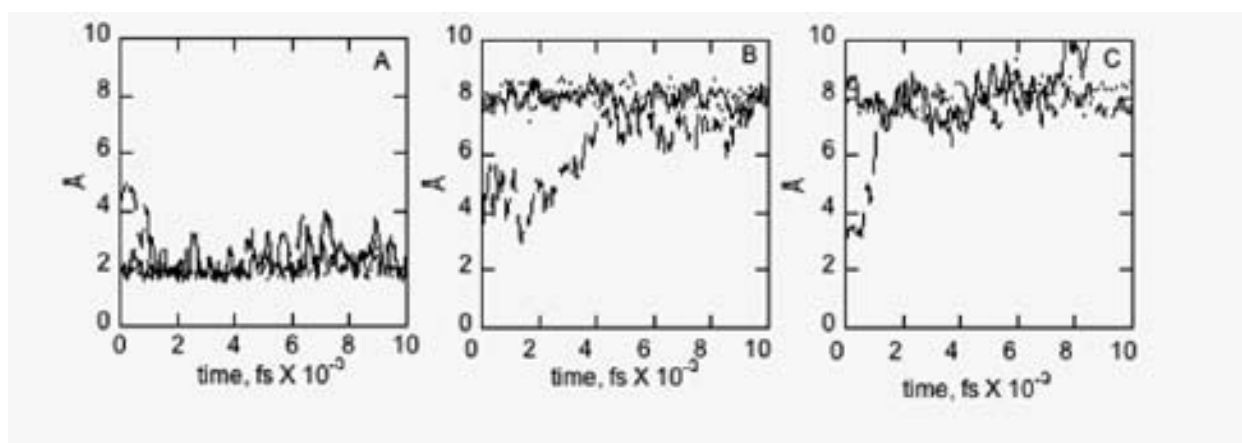


Figure 4: Dynamics analysis for a catechin molecule docked at Ser2. Dashed lines represent 300K, dotted lines 350K and solid lines 400K data. (A) H13 of catechin and the backbone oxygen of an alanine residue. (B) H12 of catechin and a carboxyl oxygen of aspartic acid. (C) H10 of catechin and the backbone oxygen of a lysine residue.

The research of Madhan et al.²⁰ suggested that interactions of catechin with hydroxyl groups on collagen side chains such as serine were likely. Thus, the microfibril model was examined to determine the relative atomic density around the hydroxyl groups of serine residues. Starting with the serine residue in the lowest atomic density region, attempts were made to dock a catechin model into a 15 Å pocket surrounding the serine hydroxyl. Four sites, all in the gap region, were located into which the catechin molecule could be docked without severely distorting the collagen structure. For the purposes of this report, the serine residues that define those sites will be referred to as Ser1 - Ser4 (Figure 1). Catechin molecules that were docked into the vicinity of serines were not attached to the collagen molecule, and were free to move in any direction. Once the docking operation was accomplished, the models were optimized by energy minimization. Next, the major portion of the microfibril was held motionless while a 15 Å sphere surrounding the catechin molecule was subjected to simulation of molecular motion at 300K, 350K and 400K.

Because hydrogen bond formation between the hydroxyl hydrogen of serine and an oxygen atom in the catechin molecule was an anticipated interaction, the docking pocket was examined for the presence of hydrogen bonds before and after the thermal simulation. Formation of essentially electrostatic hydrogen bonds between OH's or NH's of collagen and oxygen atoms of catechin occurs when the separation between atoms is in the 2.7 Å to 3.3 Å range. Shorter bond lengths, in the 1.9 Å range, are found when both atoms carry significant partial charges. Attractive forces between hydrophobic side chains on collagen and the polyphenol can shorten the distance between atoms into the 2 Å range, providing a second option for catechin-collagen interactions.

A catechin molecule (Figure 2) without hydration was first docked into a 15 Å sphere surrounding Ser1, located in the gap region about 2/3 of the distance from the N-terminal telopeptides to the C terminal telopeptides (Figure 1). This sphere contained all or parts of 74 amino acid residues with an average hydrophobicity of 0.43, and after energy

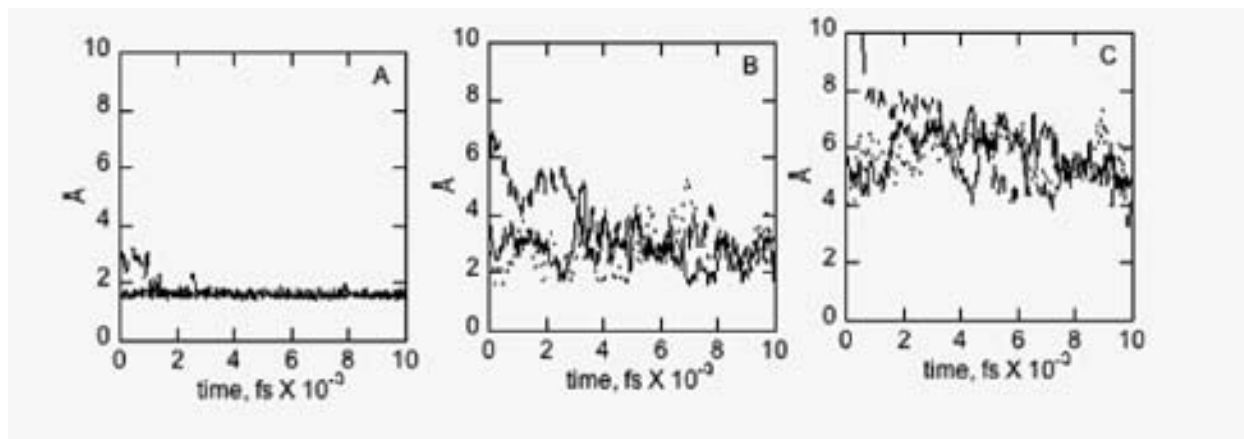


Figure 5: Dynamics analysis for a hydrated catechin molecule docked at Ser3. Dashed lines represent 300K, dotted lines 350K and solid lines 400K data. (A) O14 of catechin and NH of arginine. (B) O26 of catechin and the OH of the carboxyl group of glutamic acid. (C) O31 of catechin and an ϵ -amino hydrogen of lysine.

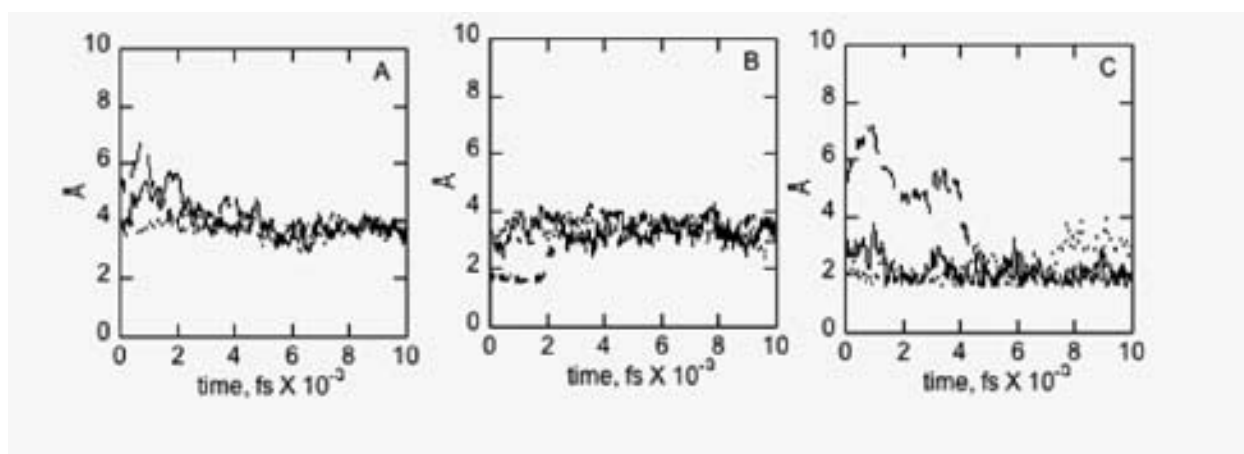


Figure 6: Dynamics analysis for a hydrated catechin molecule docked at Ser3. Dashed lines represent 300K, dotted lines 350K and solid lines 400K data. (A) O14 of catechin and NH of arginine. (B) O31 of catechin and OH of hydroxyproline. (C) O31 of catechin and a backbone NH of alanine.

minimization, several atoms were within 3 Å of the serine hydroxyl, suggesting that hydrogen bond formation might occur. Results of dynamic simulations at 300K - 400K were surprising, in that there was no observed motion of the catechin molecule toward Ser1 (data not shown). Upon the completion of the dynamics simulation, the model was examined to identify appropriate atom pairs, one in the catechin molecule and one in the collagen molecule, close enough to each other for hydrogen bond formation. Three pairs of atoms were selected and monitored throughout the simulation processes. The variation in distance between atoms, plotted in Figure 3, shows a variety of interaction patterns. In Figure 3A it can be seen that O31 of catechin, initially positioned 2.2 Å from the NH hydrogen of a valine residue, remained within the 2.2 ± 0.2 Å range throughout the simulations. While in Figure 3B, O11 of catechin, initially 2.5 Å from the NH hydrogen of an alanine residue, is seen to move away from this hydrogen to the 3 Å to 4 Å range during the thermal simulation. Figure 3C shows no interaction between H10 of catechin and the OH of a glutamate side chain. This glutamate side chain may be part

of an interaction with another residue in collagen that was not monitored in this study.

A second catechin molecule also without hydration was docked into a 15 Å sphere around Ser2, located near the center, end-to-end, of the gap region of the collagen microfibril. This 15 Å sphere contained parts of 97 amino acid residues with an average hydrophobicity of 0.50. Again, there was no observed motion of the catechin molecule toward Ser2 as a result of dynamic simulations. H13 of this catechin molecule approaches the backbone oxygen of an alanine from an initial distance of 4 Å to the 2.2 ± 0.5 Å range (Figure 4A) suggesting relatively strong hydrophobic interaction. The path of H12 of catechin relative to a carboxyl oxygen of an aspartic acid residue (Figure 4B) is similar to that seen in Figure 3C with the separation increasing from 5 Å to 9 Å as the temperature was increased. In Figure 4C, H10 of the catechin molecule, initially 2.8 Å from the collagen backbone oxygen of a lysine residue, is seen to move away from this atom to a distance of about 8 Å as the temperature simulation progressed from 300K to

TABLE I
Residue Comparison of the Overlap and Gap Regions

Res.	Hydrophobicity ^a	Gap		Overlap	
	value	No. res	density ^b	No. res	density ^b
Phe	1.00	33	109	17	218
Ile	0.94	21	197	26	192
Leu	0.94	57	111	48	140
Tyr	0.88	13	96	0	
Val	0.83	52	135	38	165
Met	0.74	12	159	12	132
Ala	0.62	255	48	213	67
Thr	0.45	34	157	46	156
Ser	0.36	64	117	94	121
Lys	0.28	56	117	64	131
Gln	0.25	45	128	67	133
Asn	0.24	32	165	34	183
His	0.17	11	163	6	204
Hly	0.10	16	161	2	328
Glu	0.04	94	94	120	96
Asp	0.03	67	117	46	135
Arg	0.00	105	90	119	93
All ^c		2010	115	2349	130

^aHydrophobicity of amino acids in type I collagen, other than the Gly,Pro,Hpr framework residues.²⁴

^bAverage number of non-hydrogen atoms in a 12 Å sphere around the C_α of this type residue.

^cThe total number of residues (including Gly,Pro,Hpr)and average atomic density in the overlap and gap regions of the ERRC model.

400K. At this site, the lysine and aspartic acid residues selected for possible hydrogen bonding are on different triple helices, and no significant interaction was observed between the catechin molecule and either of these residues. The apparent hydrophobic interaction is with an alanine on the same triple helix as the aspartic acid.

A third catechin molecule, hydrated with a single water layer (18 water molecules), was docked into a 15 Å sphere around Ser3, located in the gap region of the microfibril, near the C-terminal telopeptides. This sphere contains parts of 98 amino acid residues with an average hydrophobicity of 0.47. As the temperature was increased, water molecules escaped from the sphere, and there was no observed motion of the catechin molecule toward Ser3. O14 of this catechin is initially about 2 Å from a terminal hydrogen atom on an arginine side chain the distance between these two atoms decreased to 1.7 Å (Figure 5A) and remained constant throughout the dynamics simulations. The closeness of this approach combined with the relative charges (Table II) on these atoms strongly suggests hydrogen bond formation.

The path of H13, which is attached to O26 of the catechin molecule, relative to a carboxyl group of a glutamic acid residue (Figure 5B) begins at a distance of 6 Å and decreases into the hydrogen bonding range at about 3 Å. The path (Figure 5C) of O31 of catechin relative to the hydrogen at the ε-amino position on a lysine side chain shows an initial separation of about 10 Å, decreasing only to the 5 - 6 Å range, not close enough for hydrogen bonding.

The fourth catechin molecule, hydrated with a single layer of water, was docked into a 15 Å sphere around Ser4 located on an N-terminal telopeptide chain. This sphere contains parts of 74 amino acid residues with an average hydrophobicity of 0.44 and a significant amount of conformational space. This space is likely an artifact of the model because the telopeptides are partially extended for interactions with a neighboring microfibril that is not presented in this model. As at Ser3, water molecules migrated away from the microfibril during the dynamics simulations, and movement of the catechin molecule toward Ser4 was not observed. In Figure 6A the path of O14 of catechin relative to an arginine

TABLE II
Partial Charges on Atom Pairs Monitored

Ser1				
A	O31	-0.421	Val.H	0.248
B	O11	-0.359	Ala.H	0.248
C	H10	0.206	Asp.O	-0.706
Ser2				
A	H13	0.217	Ala.O	-0.500
B	H12	0.217	Asp.O	-0.500
C	H10	0.206	Lys.O	-0.500
Ser3				
A	O31	-0.518	Arg.H	0.361
B	H13	0.217	Glu.O	-0.500
C	O14	-0.421	Lys.H	0.311
Ser4				
A	O14	-0.518	Arg.NH	0.361
B	O31	-0.421	Hpr.OH	0.393
C	O31	-0.421	Ala.NH	0.248

NH group is seen. The separation stayed in the 4 Å to 6 Å range throughout the simulations. Figures 6B and 6C monitor the distance between O31 of catechin and (Figure 6B) the hydroxyl group of hydroxyproline where the initial separation of 3Å to 4 Å was maintained throughout the simulations, and (Figure 6C) the hydrogen atom on the backbone nitrogen of alanine where the initial separation was >5 Å, narrowing to the 2 Å to 3 Å range under dynamics at 400K, an interaction that is likely to be mainly hydrophobic.

DISCUSSION

A catechin molecule was docked into a relatively small pocket, a 15 Å sphere centered on the hydroxyl group of a serine residue in the gap region of a collagen microfibril. The system consisting of the collagen microfibril and catechin molecule with or without a hydration sphere was subjected to molecular dynamics simulations at 300K, 350K and 400K. These temperatures, relatively low for molecular dynamics calculations, were chosen to approximate leather tanning or testing processes. The catechin molecule and associated hydration layer were free to move in any direction during the simulations, with the restriction that only those atoms of the microfibril that were within the sphere could move. It was not surprising that water molecules migrated away from the hydration layer of catechin, in greater numbers as the temperature was raised. Catechin molecules remained in the docking pockets of the microfibril along with about half of the associated water molecules, a result that may reflect on the selection of docking sites. Serine residues were selected as targets because they had previously been implicated for

the potential to hydrogen bond with catechin.²⁰ The microfibril model contains 158 serine residues; favorable catechin docking sites were identified at several serine positions in the gap region, but not in the overlap region. It is possible that those characteristics of the environment of serines where docking was relatively easy could contribute to the stabilizing of catechin at those positions. This stabilization was such that no motion of the catechin molecule toward the serine hydroxyl was observed. Interactions leading to possible hydrophobic or hydrogen bonding with other residues in the docking pockets were observed.

Previously, Madhan et al.²⁰ reported a 4°C increase in the hydrothermal stability of rat-tail tendon collagen fibers when treated with as little as 0.5 mM catechin, and an additional 4°C increase with 10 mM catechin. They constructed a catechin model and determined that its size was consistent with a mechanism in which catechin would enter the pores in collagen fibrils as described by Smith⁶ and form both intra- and interchain crosslinks. They then built a 24-mer collagen triple helix containing five functional OH groups, docked catechin near these groups, energy-minimized the system, and observed hydrogen bonding at these sites. Their conclusion was that hydrogen bonding was the primary mode of interaction between catechin and collagen. In a study of the interactions of gallotannins with proteins, He et al.²⁷ concluded that the interactions between tannins and alkyl side chains of a protein were primarily hydrophobic in nature.

In this study, using the collagen microfibril model, both hydrogen bonding and hydrophobic interactions were observed. Four catechin molecules, appropriately hydrated where possible, were docked, each into a 15 Å pocket surrounding a serine residue, without causing major disruption to the collagen fibril structure. Although free to move in any direction, each of these catechin molecules remained within its docking pocket when the region was subjected to molecular dynamics simulations at temperatures up to 400K. By the midpoint of the dynamics simulation, each of catechin molecules had stabilized in a position at a 2 Å to 3 Å distance from a collagen side chain atom through interactions that could be described as hydrogen or hydrophobic bonding.

SUMMARY

The focus of this study is on the potential for hydrogen bond formation between a model vegetable tannin molecule and amino acid side chains located primarily in the gap region of collagen microfibril. The role of water in the stabilization of collagen and tannin-collagen structures is at least equally important,²⁸ and more difficult to evaluate in a model of this size. This research is a first step in the utilization of the collagen microfibril model to study tannin-collagen interactions. The results of this study provide a new window into potential tannin-collagen interactions that may contribute to an understanding of tanning effects of molecules that do not form covalent or electrostatic bonds with collagen.

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