Link-Lock: the mechanism of stabilising collagen by chemical reactions.

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Abstract

An analysis of chemistries known to confer high hydrothermal stability to collagen has arrived at a series of conditions that must be met. These include the formation of a stable supramolecular matrix, which must be firmly bound to the collagen triple helices. In most stabilising reactions, the chemical reactions are limited to linking elements of the collagen structure to a relatively unstable matrix. Typically, this linking step confers only moderate hydrothermal stability because the matrix is readily displaced by shrinking. In those chemical processes which result in high hydrothermal stability, the linking step is combined with an additional step that locks the components of the matrix together. In this way, the matrix acts like a single chemical compound, which is much less easily displaced.

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Introduction

The hydrothermal stability of collagen has received much attention, regarding the origin of its high stability relative to other proteins. The stability of intact, native collagen can be attributed in the first instance to its hierarchy of structure, within which hydrogen bonding (1) and inductive effects (2) are likely to operate. In addition, it has been postulated that the packing of the triple helices constitute a 'polymer in a box' model (3), contributing to stability. Recent studies on unmodified and chemically modified collagen indicate that the observed hydrothermal stability is dependent on the moisture content: reducing the water content causes the fibres to approach more closely, preventing them from collapsing into the interstices and which is correlated with elevated denaturation temperature (4). Therefore, a reduced ability to shrink is the same as increased hydrothermal stability. Consequently, reducing the ability of collagen to shrink by chemical modification results in higher observed denaturation temperature.

The hydrothermal stability of collagen can be altered by many different chemical reactions, well known in the fields of histology and leather tanning (5). The effects of some of these chemical modifications can be summarised as follows, where the denaturation temperature is typically measured by the perceptible onset of shrinking.

Table I. Typically observed effects of some chemical modifications on denaturation temperature ranges of collagen.

Chemical modification	Denaturation temperature (^{o}C)
None	65
Metal salts: eg Al(III), Ti(IV), Zr(IV) etc.	70-80
Plant polyphenol: gallotannin, ellagitannin, or flavonoid	75-85
Aldehyde: formaldehyde or glutaraldehyde	80-85
Basic chromium(III) sulfate	105-115
Combination: gallotannin + Al(III)	105-115
Combination: flavonoid polyphenol + oxazolidine	105-115

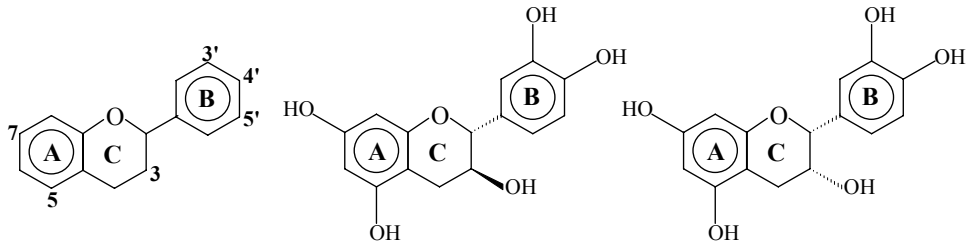
From these data, which do not represent the complete extent of the options available, it appears that the stabilising effects fall into two groups: moderate increase or large increase in hydrothermal stability. This has been rationalised in terms of the entropic and enthalpic contributions to the modified collagen structure (6), when the shrinkage kinetics are controlled by the enthalpy of activation, moderated by the entropy of activation (5). In this way, the majority of chemical reaction are limited to the moderate shrinkage temperature rise observed in the majority of cases.

This begs the question: what is the mechanism by which collagen can achieve high hydrothermal stability?

Discussion

The combination reactions in Table 1 have some features in common. The primary reaction between hydrolysable plant polyphenol and collagen is multiple hydrogen bonding. The subsequent reaction is to crosslink the tannin molecules together, via the pyrogallol moieties (7,8). There is a similar primary reaction between the flavonoid tannins, which is weaker in terms of the availability of phenolic hydroxyl reaction sites on the tannin, but which also includes some covalent bonding, via quinoid reactions.

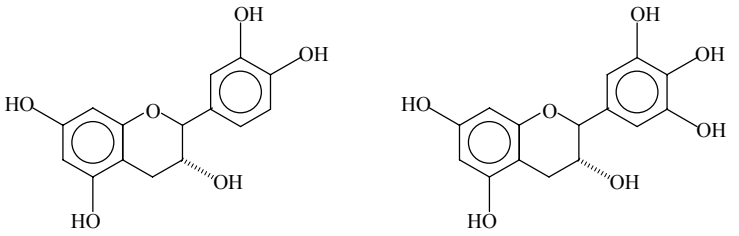
Fig. 1. The flavonoid ring system



(+)-catechin **(-)-epicatechin**

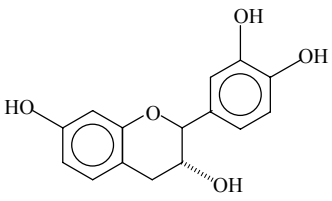
The next reaction is crosslinking the tannin molecules by oxazolidine, an aldehydic reactant: this occurs at the 6- and 8-positions on the A-rings of procyanidin or prorobinetinidin polyphenols (9), additionally at the 2'- and 6'-positions of the B-rings of prodelphinidin or profisetinidin polyphenols (10), where the latter polyphenolic reactions yield higher denaturation temperatures.

Fig. 2. Hydroxylation patterns in condensed tannins, illustrated by the monomeric precursors

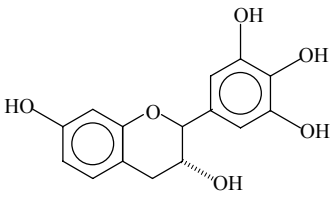


Procyanidin (I)

Prodelphinidin (II)



Profisetinidin (III)



Prorobinetinidin (IV)

In each case, the synergistic combination reactions create a matrix of cross-linked polyphenolic species, which act in concert, effectively working as a single chemical moiety.

In the case of flavonoid combination stabilisation reaction, it has been shown that an important feature of the reaction is the linking of the polyphenol species to collagen via the aldehydic cross-linking reaction (10). Similar high stability combination reactions have been observed with melamine-formaldehyde polymer as primary reactant, with tetrakis hydroxymethyl phosphonium sulfate as aldehydic cross-linker (11) and with low molecular weight phenolic compound cross-linked with aldehydic compounds or laccase, polyphenol oxidase (12).

In the group of high stability reactions, the process involving chromium(III) salt appears to be a chemical exception, having little in common with the combination reactions. This is deceptive. It is known that the chromium(III) species are covalently bound at carboxyl sidechains. Extended X-ray absorption fine structure (EXAFS) studies (13) of chromium(III) bound to collagen showed that the dominating species are linear tetrachromium compounds, but the counterion, in this case sulfate, is not directly bound to the chromium as a ligand. Furthermore, if the counterion is different, for example chloride or perchlorate (14,15), the effect of the stabilisation is only moderate, producing a denaturation temperature of about 85^{o}C. It has been demonstrated that the triple helix is surrounded by a supramolecular water sheath nucleated at the hydroxyproline sidechains (16), so the chromium(III) species and the counterion must create a matrix with this structure, in an analogous system to the combination reactions. In the case of the inorganic reaction, the effect is a combination of the metal ions and the counterions with water.

It is also a feature of these high hydrothermal stability tannages that they exhibit some covalent reaction with collagen. In this way, a proportion of the tanning matrix is stable to hydrogen bond breaking. It has been proposed that this aspect of the combination reaction is an important element in the overall requirement for matrix stabilisation of collagen that leads to high shrinkage temperature (5).

The results of combination reactions involving flavonoid compounds with oxazolidine are presented in Table II (10). The reaction is illustrated in Fig. 3, showing the structure of the crosslinker and the sites of reaction on the flavonoid.

A simple calculation on the additive effect of the two components of the reaction reveals the extent of the synergy of the reaction, measuring the influence of the matrix:

$$\text{Synergy} = \Delta T_{observed} - \Delta T_{polyphenol} - \Delta T_{oxazolidine}$$

Fig. 3. Illustration of the reaction between oxazolidine and catechin (17).

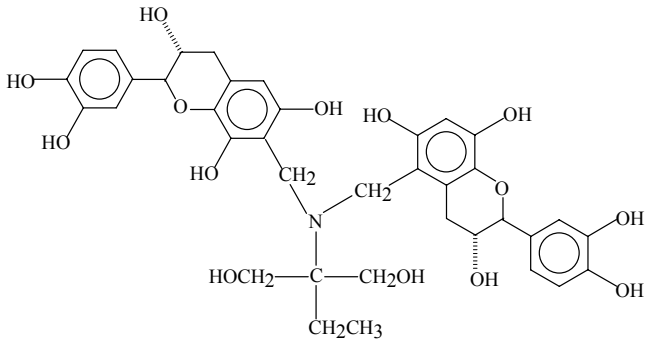


Table II. The effects of crosslinking polyphenol with oxazolidine on the denaturation temperature, the synergy of the reaction and the effect on the hydrothermal stability of acetone washing to break hydrogen bonding (ΔTs) (°C).

Polyphenol offer	Polyphenol alone	Oxazolidine crosslinked	Synergy	ΔTs
Control: no treatment	60			
Control: 8% oxazolidine		83		0
5% phloroglucinol	60	95	+12	-2
5% tea polyphenol	68	101	+10	-5
20% pecan	83	112	+6	-8
20% myrica	85	113	+5	-8
20% mimosa	82	110	+5	-6
20% quebracho	80	98	-5	-5

A useful method for gaining insight into the structure of modified collagen is hydrothermal isometric tension, when samples are constrained against shrinking and the forces generated during transitions are recorded. The HIT curves of skin and different leathers are shown in Figs. 4-6. They can be divided into three parts: the first is the tension increasing process from zero to maximum tension, followed by a relatively constant tension process, if present; finally, the tension will be constant or a relaxation process will occur, due to the gradual destruction of collagen structure or rupture of some cross-linking bonds. The slope of the curve in the tension increasing process accounts for the collagen fibre rigidity, caused by cross-links: the steeper the slope of the contraction curve, the more cross-links should be present in the collagen materials. Relaxation represents the stability of these connecting elements (cross-linking bond): the steeper the rate of the relaxation curve, the more unstable is the cross-linking.

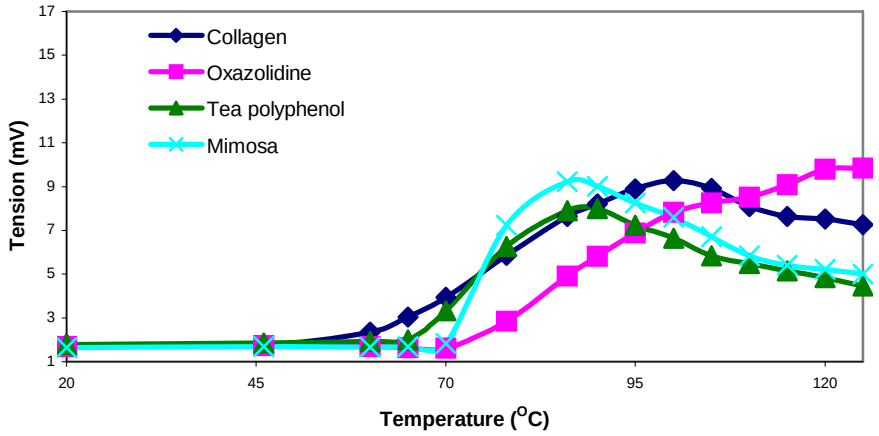


Fig. 4. HIT of leathers tanned by organic tanning methods

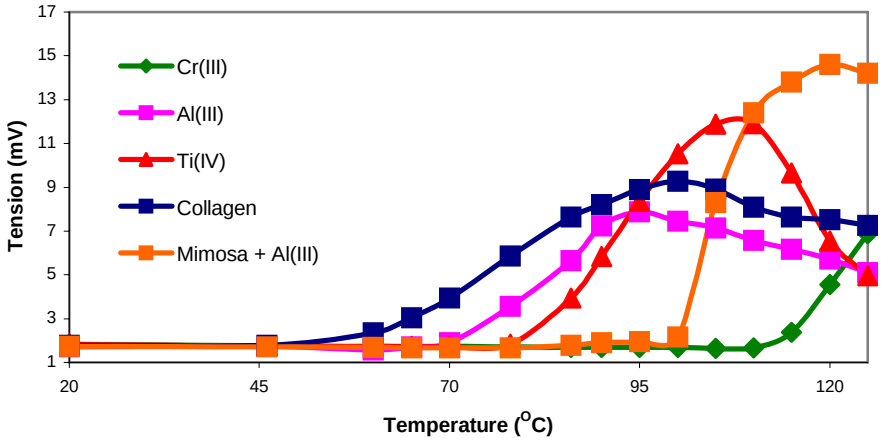


Fig. 5. HIT of leathers tanned by mineral tanning methods

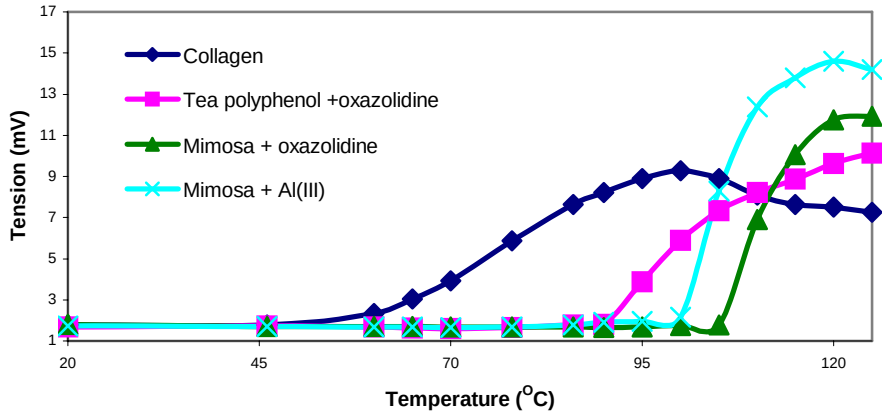


Fig. 6. HIT of leathers tanned by combination tanning methods

To date, no mathematical or physical model has been proposed for a full analysis of hydrothermal isometric tension curves obtained under linear heating conditions (18). In 1987, J. Kopp and M. Bonnet proposed a tentative model for the development of isometric tension in collagen (19). Unfortunately, these equations are probably only suited to very limited conditions (medium, pH, ionic strength *et al.*) and cannot be applied to these experiments. But we still can get some useful information about the relative crosslink density and stability from the shapes of the HIT curves. The calculated results are shown in Table III (20).

Table III. Relative rates of increase and decrease in tension at the shrinking transition

Tannage	Slope of contraction *	Slope of relaxation
None (raw collagen)	0.25	-0.13
Aluminium(III)	0.27	-0.20
Oxazolidine	0.28	0.00
Chromium(III))	0.29	Assumed zero
Green tea polyphenol	0.37	-0.13
Titanium(IV)	0.50	-0.52
Mimosa	0.67	-0.20
Mimosa + oxazolidine	0.91	Assumed zero
Mimosa +aluminium(III)	1.23	-0.05

* measured once shrinking transition is initiated

Stabilisation by polyphenol reactions yields results that can be understood in terms of the availability of hydrogen bonding and subsequent localised cross-linking. However, the rate of tension increase during the shrinking transition of chromium(III) stabilised collagen exhibits a result similar to that of raw collagen. The natural conclusion is that chromium does not cross-link collagen by joining adjacent sidechains. Therefore, the traditional view of cross-linking does not have to be invoked in explaining these stabilisation reaction. The explanation lies in the creation of a matrix, securely bound to the collagen. Supporting evidence of the part played by fixation of the matrix to collagen comes from the work of Holmes (21). He showed that modifying collagen by binding chelating pairs of carboxyl groups to the amino groups of lysine could enhance the weak stabilising effect of aluminium(III) salts, to the extent of increasing the denaturation temperature from 75 to 95^{o}C.

Bone is an example of a situation where the stability of collagen clearly comes from the presence of a matrix. Here, the matrix is polymeric hydroxyapatite, not thought of as a typical cross-linker, but which can raise the shrinkage temperature of the constrained collagen to 155^{o}C (22).

Conclusion

The stability of unmodified or chemically modified collagen must depend on its ability to collapse or shrink by unravelling its chains into the available space between the chains. The ease with which this can happen depends on the constraints applied to the chains or the ease with which the intervening molecules can be displaced as the collagen shrinks. If the molecules are water, this matrix can be displaced relatively easily. If the matrix is stabilised by the inclusion of species bound to collagen, but also by substituting some of the supramolecular water and interacting with the remaining water, the matrix is less easily displaced, observed as a rise in denaturation temperature. However, merely loading the structure with molecular species is not sufficient to confer high hydrothermal stability. For example, plant polyphenols confer only moderate stability, limited to about 85^{o}C, even when present at 30% on dry weight of collagen. Like most other stabilising reactions, the chemical reactions are limited to linking elements of the collagen structure to a relatively unstable matrix. In this case, a packed array of unlinked molecules. Typically, the linking step of collagen stabilisation confers only moderate hydrothermal stability because the matrix is readily displaced by shrinking.

In those chemical processes which result in high hydrothermal stability, the linking step is combined with an additional step that locks the components of the matrix together. In this way, the matrix acts more like a single chemical compound, which is much less easily displaced. The higher energy required to achieve breakdown of the structure is observed as higher temperature transition. It is an important aspect of the matrix stabilising mechanism that the matrix should be bound the collagen in a stable way, so that displacement of the interaction, which might lead to allowing shrinking, is prevented.

This new understanding of how high hydrothermal stability is conferred to collagen, link-lock, has profound implications for the development of alternative processes for industries exploiting the properties of collagen. Hitherto, it has been assumed that high hydrothermal stability of modified collagen is the property of a few unrelated chemical reactions. We have demonstrated that the mechanism is in fact general and high hydrothermal stability may be achieved merely by virtue of the reaction conforming to the requirements of a stable matrix, as defined here.

The global leather industry currently relies on chromium(III) salts to confer the properties required for modern applications. Other reactions capable of achieving high hydrothermal stability can offer alternative routes to modern leathers and their many high performance applications . More generally, the development of new chemistries for stabilising collagen to different degrees, based on a clearer understanding of the origin of hydrothermal stability, will be important in the field of new biomaterials.

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