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# DSC investigation of bovine hide collagen at varying degrees of crosslinking and humidities

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ABSTRACT

Bovine hide collagen (nonCLC; non-**C**ross**L**inked **C**ollagen) was analysed by differential scanning calorimetry (DSC) at different hydration degrees and compared with hide collagen samples crosslinked with glutaraldehyde (CLC-GA) and chromium(III) ions (CLC-Cr), respectively. Crosslinking and drying were confirmed to increase the denaturation temperature. Different regions were assigned, that reflect the variation of the influence of water on the denaturation temperature. Furthermore, at moderate hydration degrees, the enthalpies of non-crosslinked collagen increase compared to the fully hydrated state. This reflects a glue-like action of water in the range of 25% hydration. Crosslinking of bovine hide collagen decreases the enthalpy by 25% in the fully hydrated state, even at very low levels of crosslinking This can be explained by intensive effects of the crosslinking agent on the hydration network of the collagen molecules, assuming that the enthalpies are principally a result of hydrogen bonding. At very low water contents DSC peaks of CLC-Cr completely disappear. This could be explained by competition between hydroxosulfochromate(III) complexes and collagen for water.

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## 1. Introduction

Collagenic materials utilised as hemostyptic sponges or matrices for cell culture and leather are known to physically shrink at elevated temperatures. This thermal denaturation is irreversible and caused by an uncoupling of the collagen triple helices. The thermal stability of collagen has been investigated intensively for several decades but it is still a relatively basic field of research and not fully understood, despite a high level of practical relevance [1–4]. Notably, tissue and process-dependent differences in the thermal stability of collagen and collagenic materials are of significant interest.

*Collagen structure and denaturation*

Fibril-forming collagen molecules consist of three similar or identical protein α-chains, with a left-handed twist, that are wound to form a supercoiled right-handed triple helical structure. In tissues such as skin and tendon, these molecules are further arranged to form a fibrillar superstructure. For soluble collagen, heating causes the triple helices of the molecules to disintegrate into single strands. The rod-like structure of the collagen molecule changes into coil shaped structure and, the corresponding temperature is called denaturation temperature $T_D$ [2,5].

However, fibrillar collagen in tissue does not allow this transition into a coil because the triple helices are restricted by neighbouring collagen molecules [3,6,7]. While $T_D$ of soluble collagen is close to body temperature [8], that of fully hydrated pelt (tissue) without “synthetic” crosslinking is approximately 60 °C. “Synthetic” Crosslinking with glutaraldehyde increases $T_D$ up to 85 °C. Stabilisation with chromium(III) sulfate, which is commonly used in the leather industry, increases $T_D$ up to 120 °C [9]. It is distinguished between crosslinking by glutaraldehyde and stabilization by chromium ions, because the chemical reaction of glutaraldehyde is justified [10] while the kind of stabilisation by chromium is still an object of discussion [11,12].

To explain the different $T_D$ of soluble collagen molecules, fibres, crosslinked fibres and the increase in $T_D$ caused by dehydration, Miles and Gelashvili [7] applied the polymer-in-a-box model to collagen. This hypothesis was first formulated for synthetic polymers by Doi and Edwards [13] who explained the entropy of a polymer by a box which constrains the mobility of the polymer chain. A small box decreases the entropy and in the case of collagen $T_D$ increases. A large box correlates with low restrictions and low $T_D$. This model conclusively explains the different $T_D$ but not the mechanism of denaturation.

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The mathematical description of the denaturation mechanism of complete collagen triple helices had been a subject of much controversy. $T_D$ reflects the thermodynamic stability of the collagen triple helix and it consists of an enthalpic ($\Delta H_D$) and an entropic part. While Miles [14] proposed a pure rate mechanism of denaturation, others interpreted the results of denaturation experiments as an equilibrium between an activated and a non-activated triple helical form of the molecules [6,15,16]. The group of Kobayashi [17] investigated collagen-like model peptides by DSC. They could show that the enthalpic and the entropic part of denaturation may vary depending on the sequence.

The thermal stability of collagen is markedly higher than that of globular proteins and the reasons for this have been discussed for many years [6,7,15,18,19]. Though many different ideas were discussed about the stabilising principles of the triple helix, such as hydrogen bonds, electrostatic interactions, van der Waals interactions but also hydrophobic interactions and stereoelectronic effects, it is now mostly accepted that a ladder of hydrogen bonds internally stabilize the collagen triple helix. X-ray diffraction studies in the middle of the 20th century and further biochemical and physicochemical investigations showed, that these stabilizing bonds are located between N—H of Glycine (Gly) and the C═O of other amino acids in X-position of the following strand. Doubtless the hydroxyl group of hydroxyproline (Hyp) has an important influence on the thermal stability, because a species dependent increase of Hyp can be correlated to an increase in $T_D$ and $\Delta H_D$ [6].

Furthermore, the triple helices are surrounded by a network of water molecules that increase the thermal stability of the triple helices that support their high $\Delta H_D$ [20–22]. While most investigations only rendered indirect hints regarding this water network [23–26], Bella et al. [27] could model and calculate a surrounding water structure for collagen-like peptides. To sum up, hydrogen bonds determine the enthalpies with and without the contribution of water. The entropic part, reflected by $T_D$, correlates with the chain mobility, which directly depends on the hydration degree of the triple helices [24,28] and which is also affected by crosslinking.

Most authors only measured the thermal stability of rat tail tendon collagen or of soluble collagen extracted with acids [29–31]. Only one paper compared tissue specific differences of the thermal stability of tendon, bone, and skin collagen but without considering synthetic crosslinking [32].

Because skin is the most important collagen source for medical products (e.g. membranes and sponges), food (casings) and technical applications (e.g. leather) this contribution aimed to measure the thermal stability of skin by differential scanning calorimetry (DSC) before and after treatment with organic (glutaraldehyde) and mineral (chromium(III) sulfate) stabilising agents. Furthermore, DSC investigations were performed at different degrees of hydration.

This investigation confirms previous research, which showed increased enthalpies at moderate hydration degrees of collagen without any crosslinking or chemical stabilisation [33,34]. Markedly lower enthalpies were observed when the samples were chemically stabilised. At low hydration levels the enthalpies of chromium treated collagen completely disappeared but reappeared after rehydration.

## 2. Material and methods

### 2.1. Collagen materials

#### 2.1.1. Material preparation

Chemically unhaired bovine hides (pelt) were used as supplied from the FILK pilot tannery (nonCLC – non-**C**ross**L**inked **C**ollagen), and was stabilized either by glutaraldehyde (CLC-GA) or by chromium(III) sulfate (CLC-Cr), respectively. The technologies were performed using conventional industrial processes (recipes in the supplements section). The quantity of water and chemicals applied were calculated based on percent of the wet pelt. The pH of the pelts was first adjusted to 9 by the addition of 4% ammonium sulfate (Carl Roth, Germany, technical grade) in 150% water; they were washed twice with 300% tap water, treated with 0.6 % of an enzymatic preparation (Oropon OO, TFL, Germany) and washed again twice with water. The wet pelts were soaked in 300% water, treated with 6% NaCl (Brenntag, Germany, technical grade), and the pH was decreased to 3 with a mixture of 0,5 % formic acid (85%, technical grade) and 0,7% sulphuric acid (10%, analytical grade) (both Brenntag) which were diluted 1:10 with water prior to addition. The prepared materials were stabilised with basic chromium(III) sulfate, and glutaraldehyde, respectively.

#### 2.1.2. Stabilization with chromium and crosslinking with glutaraldehyde

Stabilization by chromium was performed with the addition of 12% of Chromitan B (basic chromium(III) sulfate) (BASF), the hides were drummed overnight and the pH was finally adjusted to 3,8 by addition on MgO (Applichem, Germany) and the material was washed 2 times with 300% water.

The hides were crosslinked with glutaraldehyde by addition of 12% Relugan GT (BASF), and the pH was adjusted to 7 in three steps by the addition of 0.4% sodium hydrogen carbonate each. Finally, the materials were washed twice with 300% water and air dried (see supplements for exact recipes).

### 2.2. Analytics

#### 2.2.1. Differential scanning calorimetry (DSC)

Calorimetric measurements were performed using a DSC 1 (Mettler –Toledo). The samples (~10 mg each) were placed in DSC pans (Al-pan; medium pressure) and different humidities were adjusted by subjecting the open pans for different times to saturated water vapour, drying agent (storage in a desiccator over phosphorus pentoxide, Roth, p.a.), or deionised water, which was directly added. The pans were then tightly sealed, weighed and DSC scans were performed at a scanning rate of 5 °C/min between 1 °C and 220 °C to be sure to cover all relevant peaks. After each scan, the pan lids were pierced, dried at 105 °C at 100 mbar overnight, cooled in a desiccator, reweighed, and the material humidity was calculated.

The temperature at the beginning of the denaturation ($T_{onset}$) was used for the final evaluation. In most cases $T_{max}$, which is the maximum temperature of a DSC scan, correlated well with $T_{onset}$. In some cases the DSC peaks were very broad or markedly asymmetric. Under these circumstances, $T_{max}$ did not reflect the denaturation temperature of the complete scan and $T_{onset}$ was used to describe the denaturation temperature more accurately (see also Figs. 1, 3, 4).

#### 2.2.2. Hydroxyproline and chromium content

The DSC peak area, which reflects the denaturation enthalpy $\Delta H_D$, was estimated in relation to the collagen content of the samples. To calculate collagen content the Hyp content of the samples was measured by photometric determination and multiplied by the factor 7.46 [35].

The chromium oxide content of the leather samples was measured according to DIN EN ISO 5398-1.

#### 2.2.3. Determination of lysine and hydroxylysine

Glutaraldehyde mainly reacts with lysine (Lys) and hydroxylysine (Hyl) side chains [10]. To estimate the degree of crosslinking

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