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EFFECTS OF BEAMING AND TANNING ON COLLAGEN STABILITY, STUDIED BY DIFFERENTIAL SCANNING CALORIMETRY*

PAUL L. KRONICK AND PETER R. BUECHLER
Eastern Regional Research Center
U.S. Department of Agriculture
600 E. MERMAID LANE
PHILADELPHIA, PA 19118

Abstract

Differential scanning calorimetry shows two temperature regions of collagen denaturation in corium major of butt hide from mature cattle, a narrow region centered at 68°C and a wide region at 85°C. Both temperatures are sensitive to beaming and tanning processes, but the enthalpy of denaturation remains constant at 13 cal/g in all but tanned samples. It is concluded that there are two populations of collagen in the hide in commensurate amounts; only the heat sensitivity of the less stable population is indicated by the shrinkage temperature as usually measured.

Introduction

An important aim in leather processing is opening up the fibrous structure of hide so that noncollagenous components can be removed, so that reagents can be introduced, and to soften the leather. The methods for separating fibers include drastic treatments with acid and, particularly, alkali. These changes must be introduced without interfering too greatly with intermolecular attraction so that the structure will hold together. The cohesion depends on intermolecular secondary bonds. Stabilization also is provided by forces and restraints on the surfaces of fibrils, which confine molecular configurations. Stability of protein structures is commonly measured by the heat and temperature of denaturation. To determine the effects of leathermaking processes on the stability of the collagen in hide, we have applied differential scanning calorimetry to samples of a single hide as it proceeded from its fresh state through tanning.

Materials And Methods**BOVINE HIDE**

Butt swatches were taken from the hide of a mature steer just after slaughter and freezing, after unhairing, after liming, after bating, after pickling with and without recycled chromium liquor, and after chrome tanning. The procedures in this particular beamhouse process have been described earlier (1), and were carried out in the ERRC tannery. Samples were frozen at -35°C right after each step and stored for no more than three weeks before the thermal measurements.

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DIFFERENTIAL SCANNING CALORIMETRY (DSC)

Six to ten samples (0.02 cm^3) were cut from the treated volume of each hide swatch and, without alteration of the pH, were packed into hermetically sealed aluminum capsules fitting into the sample chamber of a Perkin-Elmer DSC1 scanning calorimeter. Thermal changes were measured with respect to a similar water sample as the temperatures of the samples were raised at the constant rate of $10^\circ\text{C}/\text{min}$ from 30°C to 120°C while flushing the chamber with dry nitrogen. This instrument records the rate at which heat must be applied to maintain the constant heating rate; when endothermic or exothermic reactions occur in the sample, the rate of heat input changes accordingly. Endotherms were calibrated against 99.99 percent benzoic acid (33.9 cal/g). The features observed in the endotherms were shifted downward by 5°C when heating was at $2.5^\circ\text{C}/\text{min}$. For every sample tested, on heating scans after the first, even if both scans were stopped below 120°C , the rate of heat input did not depart from the baseline; denaturation was irreversible.

After each temperature scan, the sample was removed from the capsule, dried, and weighed. In some cases the sample was instead hydrolyzed with 6 M hydrochloric acid and analyzed for 4-hydroxyproline with a Beckman amino-acid analyzer in order to refer the thermal data to the actually determined collagen content. The two methods gave identical average enthalpies (e.g., 13.6 vs. 13.1 cal/g), consistent with a collagen content in the samples of over 90 percent.

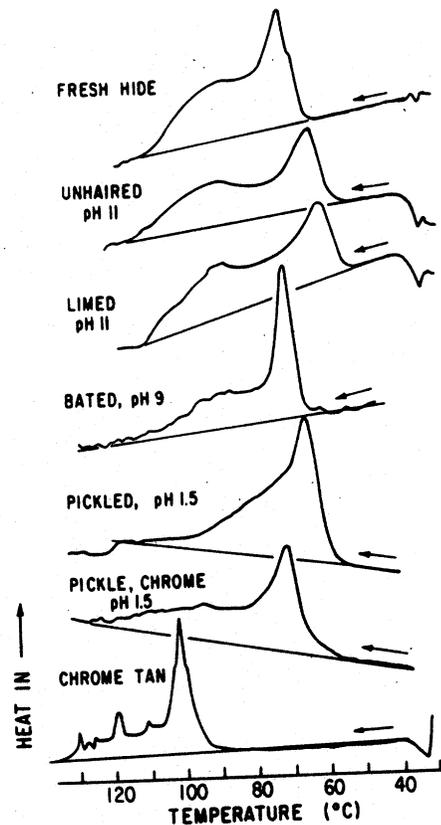


FIGURE 1. — Thermograms of cattle hide treated with sequential tannery steps. Arrows show direction of temperature scan.

RESULTS

FRESH HIDE

The denaturation endotherm (Figure 1) begins to depart from the baseline at 62°C. Naghski *et al.* (2) showed that this point, T_d , correlated with the shrinkage temperature of hide or tanned leather. This melting temperature is higher than that obtained by DSC of monomolecular collagen in solution, 38°C (3), as expected for T_d of medium-stabilized solid collagen. Nevertheless the heat of melting, 12.9 cal/g, calculated from the area under the endotherm, is only a little smaller than that of soluble collagen, 16 cal/g (3). Some lowering is expected as a result of crosslinks stabilizing some conformations of the native state at temperatures above the denaturation point.

Quite unexpected, however, was the display of two denaturation regions, one with an endothermic peak at 68°C and another, much broader, at 85°C. This indicates two populations of collagen in the sample, one that is much more stable than usual for untanned collagen in water. Some of this stable material is not denatured below 110°C, a degree of stability that was thought to require chrome tanning. From the relative areas of the two peaks, which we resolved graphically, we estimate the amount of this stable collagen to be 62 percent. The less stable collagen denatures over a temperature interval narrower than that of the stable material, but still twice the width of the 6°C interval of soluble collagen. The two denaturation regions were observed in a calf skin and two other mature bovine hides, one of which was run fresh from slaughter without freezing. The results are therefore general.

UNHAIRD HIDE

This material was prepared by treating the surfaces of the fresh hide with 2 percent NaHS and 1.5 percent lime in 100 percent float at 29.4°C for 2 hr. The pH of the flesh surface, the origin of the DSC sample, was 11.8. The heat of denaturation calculated from the total area under the complete endotherm is 13.0 cal/g, unchanged within experimental error from that of the fresh hide. Therefore the drastic treatment did not denature the collagen; the sample still contains the same total amount of native protein as did the fresh hide. The treatment did, however, diminish the stability, lowering the T_d to 52°C ($\pm 1.4^\circ\text{C}$), but this change is characteristic of the unstable portion only. It is seen from Figure 1 that the whole low-temperature peak of the endotherm is shifted to low temperature, and that it still retains its sharp character. But the relative areas of the two portions of the curve give a relative content of only 28 percent unstable portion, as though some of this material that had been in the sharp peak of the fresh hide were now being counted with the stable collagen. It is unlikely that the material has been rendered more stable; more likely it is more heterogeneous, causing the part of its endotherm that spreads into the temperature interval above 75°C, that we resolved into unstable collagen in the fresh hide, now to be counted as stable collagen.

RELIMED HIDE

The unhaired sample was relimed for 20 hr at 26.6°C in a float (200 percent) containing 2 percent lime, 0.5 percent Na_2S and 0.5 percent Triton X114 (Rohm and Haas Co. Philadelphia, PA*). The sample for DSC was taken from the center of the corium, where the pH was 11.8. The endotherm changed little because of the treatment, with a heat of denaturation of 12.9 cal/g and a T_d of 51°C ($\pm 0.5^\circ\text{C}$). The sample, taken from within the hide, probably had not reached the high pH of 11.8 during unhairing, so the two treatments appear to have equivalent effects on denaturation and probably can act independently.

*Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

BATED HIDE

After a ten-minute wash at 32.2°C the hide was delimed and bated for 1.5 hr at 32.2°C in a 100 percent float containing 1.5 percent ammonium sulfate and 4000 EU of bate, pH = 9. The endotherm of a sample of this hide, removed from the flesh side of the corium, is shown in Figure 1. The denaturation enthalpy is still 13.3 cal/g, but T_d is again raised to 59°C. The low-temperature peak is sharper than in the fresh hide, indicating greater homogeneity, but accounts for only 33 percent of the total heat absorbed.

Not apparent in the endotherm is any effect of the chemical alteration of collagen by hydrolysis of glutamine and asparagine during liming. The increased homogeneity of the less stable population, apparent from the sharpness of the low-temperature peak, may rather be due to removal of proteoglycans that interact with collagen on the surfaces of the fibrils. This is to speculate that the less stable population is located near the exterior of each fibril, where it is available to interact with interfibrillar matrix macromolecules, causing the stabilities of the collagen molecules in this population to become nonuniform. Removal of the proteoglycans by liming and bating would then cause these complexes to collapse to a uniform population of free collagen molecules. There is no obvious change in the high-temperature peak, which could be related to collagen molecules in the cores of the fibrils.

PICKLED HIDE

The bated hide was pickled in two different ways. In the laboratory (4) a 5 x 1 x 0.3 cm³ piece of bated hide was pickled by stirring for 17 hr at 22°C in 1 dm³ of a solution of 0.1 kg/dm³ sodium chloride and 4 x 10⁻³ kg/dm³ sulfuric acid (pH = 1.5). T_d in the endotherm in Figure 1 is again reduced to the value of the limed sample, 51°C. There is now in addition a longer low-temperature tail, the actual peak of the low-temperature transition being about 7°C higher than that of the limed sample. While this low-temperature peak seems as sharp as that of the bated material, it is here underlaid by the high-temperature peak, which has been shifted to lower temperature. This pickle is therefore the first condition which has affected the stable population, causing it to denature at lower temperature than in fresh hide. Its shape has not been changed, still being broad with a high-temperature tail. The enthalpy of the whole denaturation is unchanged at 12.7 cal/g.

In an alternative pickling process (1), the bated hide was washed for 20 min at 26.6°C and then pickled in a 50 percent float of recycled chrome from the tanning drum to which had been added 2 percent sulfuric acid and 6 percent sodium chloride, pH = 1.5, for 2 hr at 26.6°C. The resulting endotherm in Figure 1 resembles that of the fresh hide, but the heat of denaturation is somewhat reduced, to 10.2 cal/g. The increase of T_d to 58°C and decrease of ΔH_d from the corresponding thermal parameters of the simply pickled hide above (4) are likely effects of the chromium tanning reagent, which crosslinks the collagen chains and freezes them in native conformations above the melting point. By reducing ΔS_d , the entropy of the transition, this stabilization of conformation would reduce ΔH_d , since $\Delta H_d = T_d \Delta S_d$.

TANNED HIDE

The hide was chrome tanned for 3.5 hr at 43°C in 8 percent Tanolin R (Hamblet & Hayes Co. *) and 2 percent sodium formate (pH = 3 to 4) in an 80 percent float. The endotherm in Figure 1 is shifted to high temperature, showing a poorly defined T_d of about 77°C. While T_d is raised, the enthalpy of the whole transition is decreased to 7.5 cal/g. We cannot tell if this value is constant over the whole transition region from 77°C to 145°C or whether there

is a different value for each transition region, since we do not know what fraction of the total collagen is involved in each when the enthalpy changes. A decrease in enthalpy, however, is expected in crosslinked collagen, as explained above.

Discussion

As seen in Table I, the total enthalpies of denaturation, ΔH_{total} , remain constant throughout the manufacturing process until chrome is introduced, either in the drum pickling step with recycled chrome or in tanning. We might have expected to find two discrete collagen populations in the hide with different stabilities since they have been reported in rat skin (5). If we assume that the enthalpy changes are the same for the two populations, we can equate the ratios of the heats evolved in the two transitions to the ratios of the amounts of collagen in each population. Evidence for constancy of the enthalpy change is seen in the invariance of the average enthalpy for the total transition (which is what we calculate when we divide the total heat evolved by the mass of collagen) from one treatment to another except for tanning. For the enthalpy change to vary from one population to another, it would have to be always exactly compensated by changes in the sizes of the two populations to keep the average from varying, a coincidence that we have no reason to expect.

TABLE I
DENATURATION OF BEAMHOUSE-TREATED HIDE

TREATMENT	T_d (°C)	ΔH_{total} (cal/g)	$\Delta_{\text{less}}/\Delta H_{\text{total}}$
Fresh	63	12.9	0.3
Unhaired	52.3	13.0	0.28
Limed	50.6	12.9	0.23
Bated	59.0	13.3	0.46
Pickled	51.5	12.7	Not resolved
Pickled (Cr)	58.0	10.2	0.43
Tanned	94.7	7.5	0.46

The ratios of the enthalpies of denaturation of the less stable collagen, ΔH_{less} , to the total enthalpy changes, ΔH_{total} , remain at values below 0.3 in fresh, unhaired, and limed hide, then increase abruptly to above 0.4 after bating (Table I). The change may be due to an artifact of resolving the two peaks by drawing in underlying sections with splines, or it could be due to conversion of the more stable population to the less stable, perhaps by disruption of collagen fibrils by the bating enzyme.

The 5°C peak width reported for the endotherm of collagen in solution is considered to be atypically large for a melting transition. Its width is usually attributed to sequential melting of parts of the collagen molecule with different levels of stability, all the molecules being considered identical (3). The low-temperature peak of fresh hide is much wider, perhaps because of differences among molecules, some of which at the surfaces of the fibrils interact with proteoglycan. When these proteoglycans are removed the denaturation peak narrows, although not to the width of soluble collagen. Nevertheless, this peak is more like that of soluble collagen than like the peak due to the material which denatures at higher temperatures, up to 115°C. It is shifted to lower temperature by pH levels above and below that of fresh hide, 7.3.

The more stable collagen is sensitive to low, but not to high pH since its endotherm did not move after unhairing or liming but did shift to lower temperature at low pH. Apparently the collagen which is denaturing at these higher temperatures is accessible to acid but not to

calcium hydroxide. Since nearly all the material is native collagen, it is reasonable to conclude that the less accessible fraction is protected from reagents by the more accessible, by being contained within fibrils as densely packed cores within less tightly ordered collagen sheaths. Further, it is noticed in Figure 1 that the high-temperature component shifts downward in temperature by at least 15°C, while the low temperature peak moves downward only about 5°C in the chromeless pickle. Since the shift is limited by the 10 percent salt in the pickling reagent, this suggests that the less stable collagen is more accessible to the salt than the more stable, another result to be expected in a sheath-core structure. (More evidence for this effect of the structure on the accessibility of salt comes from the puzzling observation of Privalov (3) that, at pH = 3.5 in the presence of sodium chloride, the collagen aggregates that form also denature in two steps, the low-temperature step being reversible provided that the high-temperature denaturation is avoided. Sheath-core structure would explain this also if the core, containing hydrogen ion but not sodium, were denaturing at the lower temperature, the stable sheath forcing the restoration of native configuration after cooling.)

The manufacturing process which we have followed calorimetrically is usually divided into two main stages. The first is "opening up" the structure during liming and bating so that reagents can penetrate the hide and to soften it when it is dried. This stage affects the outside of the fibrils, removing proteoglycans and, from the DSC results, homogenizing the outside of the fibrils. The second step, tanning, affects all the collagen, including the cores of the fibrils, as the endotherms also show. In some samples, however, we have observed a long tail in the endotherm extending from 77°C to about 100°C, where the low-temperature peak proper actually rises. The temperature region of the tail coincides with that of the high-temperature peak of the original fresh hide. This tail might then indicate that chromium in tanning does not reach all the collagen in the cores of the fibrils.

The shrinkage temperature, which has been shown to be the same as the point on the endotherm that we call T_d (2), is characteristic only of the less stable population. After all treatments in the tannery, hide contains a large fraction of a more stable collagen that, while it does not limit the sensitivity of the hide or leather to thermal shrinkage, may be important in preserving the strength of the material under heating, since it remains intact up to very high temperatures.

Acknowledgements

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Discussion

MR. ROGER LANGLAIS (Blueside Co., Discussion Leader): Thank you Dr. Kronick for an interesting paper. I have one question. Are the shapes of the endotherms related to the rate of heating?

DR. KRONICK: No, the shapes are not related to the rate of heating, but the positions are. Differential scanning calorimetry, as we carry it out, with the precision that we have, requires heating at about 10 degrees per minute, and the system is not at thermal equilibrium. If we slow down the heating rate, the temperatures all shift to somewhat lower temperatures, but there is still a tail going up to or above 100 degrees Celcius.

MR. LANGLAIS: While the fresh hide shows two populations of collagen, do we see the same thing in the pickled and chrome tanned collagen?

DR. KRONICK: Yes. Actually the relative proportions of the two parts of the DSC curve, the sharp and the broad, remain fairly constant with respect to each other.

DR. K. T. W. ALEXANDER (BLMRA): I was very interested in this talk because we recently bought a new differential scanning calorimeter and that second peak has been puzzling us. Can you tell me, when you did your measurements, did you bring the hide to neutral pH for the run and do you think that is important?

DR. KRONICK: We did experiments readjusting the pH and yes, of course, it makes a difference. These curves were all run at the process pH's noted.

DR. ALEXANDER: In the scan for the fresh hide there is a little blip suggesting a third peak. Do you think there is a third peak?

DR. KRONICK: No. We did each sample six or eight times and the blip showed just on this one.

DR. WILLIAM PRENTISS (Rohm and Haas Co.): When you run a shrink temperature with a sample in liquid you actually see a change in dimension of the piece. Obviously you are not looking at that here. Have you been able to determine what the difference is between the two peaks that you observe? The lower one, in my experience, would be associated with the change in fiber structure. Do you have any ideas?

DR. KRONICK: We did some additional experiments. For one thing, we heated the sample on a hot stage between crossed polarizers under a microscope while measuring the birefringence quantitatively with a Babinet compensator, using the same heating rate. We find that as we go through the first peak at around 72 degrees Celcius for the bated hide, the birefringence disappears and most of the fibers shrink drastically but not all of them. We could find residual birefringence in thick fibers and some of them did not shrink. Therefore, we think that the first peak accompanies the mechanical forces which shrink the sample.

MR. LOWELL RASMUSSEN (Eagle Ottawa Leather Co.): Are there chemical or physical treatments, other than tanning or low pH, that alter the high temperature population?

DR. KRONICK: If the sample is treated with 2M calcium chloride it denatures the collagen and we observe a greatly lowered apparent heat of denaturation.

We did some experiments with some collagen prepared for us by Michael Komanowsky, which had gone through a harrowing life. It had been limed, treated with acid, chopped up in a Comitrol and finally presented to us in an aqueous suspension. This collagen denatured at a lower temperature than a fresh hide, in fact at a lower temperature than any of the others but it still had two peaks. We found that, when we treated it with guanidine hydrochloride, the material seemed to be transformed from the higher temperature type to the lower temperature type. We are still puzzled about that. In examining the literature on electron micrographs of guanidine treated material we see the breaking up of the banded fibrils into

little microfibrils. That is another reason why I think the high temperature peak is due to the cores of the fibrils.

DR. ALEXANDER: Why don't you see it in the liquid one of soluble collagen that you showed at the very beginning?

DR. KRONICK: The liquid one is a reproduction of a curve by Privalov from the Russian literature. This is probably the best curve for soluble collagen which is homogeneous, but we are not capable of running soluble collagen solutions. Our instrument is not sensitive enough.