EFFECT OF A CLOSTRIDIUM HISTOLYTICUM COLLAGENASE ON THE DENATURATION CHARACTERISTICS OF BEEF INTRAMUSCULAR COLLAGEN.

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SUMMARY

A commercial *Clostridium histolyticum* collagenase was used to investigate the main denaturation characteristics of intramuscular collagen with different degree of reticulation obtained from bovine animals of ages ranging from 3 months to 8 years. Collagenase treatment caused a decrease in enthalpy of denaturation in calf and steer collagens, but no change was observed in cow collagen. In all cases we have demonstrated a collagen breakdown evidentiated by the appearance of a new peak of denaturation at a lower temperature (55-57°C) than the denaturation peak of intact collagen (62-63°C). Intensity of peak shift appeared to be inversely related to animal age.

INTRODUCTION

The toughness is probably the most critical quality factor of the meat to the consumer. Meat toughness is a complex property which depends mainly on the two protein systems which give the muscle its mechanical strentgh: the connective tissue and the myofibrils.

Each of the structural domains of connective tissue (endomysium, perimysium and epimysium) makes a distinct contribution to the back-ground toughness of meat (Purslow, 1985; Light, 1985). The basic collagen structure, known as tropocollagen, consists of three polypeptide chains each twisted in a left handed helix, coiled around each other to form a right handed triple super helix (Aberle and Mills, 1983). As temperature is increased, collagen's regular structure breaks (denatures) and the chains separate and fold into random structures without any residual native structure.

Collagen is polymerized through the formation of covalent crosslinks. There are reducible crosslinks (heat labile aldimine and heat stable keto-imine) involved in head-to-tail longitudinal crosslinking confering considerable tensile strength to the collagen fibers and additional transverse non reducible interfibrilar crosslinks, of yet unknown nature, whose prevent microfibril slippage during mechanical stress (Bailey, 1984).

Changes in thermal solubility of collagen take place as the animal ages (Cross, 1972; Reagan, 1973 and Berry, 1984) have shown a significant relationship between meat tenderness and the degree of connective tissue solubility. Thermal solubility must be understood as the resistance of the protein molecule against unfolding as a result of heat treatment, two direct objetive measurements of this resistance as the amount of energy required to accomplish the unfolding (enthalpy of denaturation) and the temperature at which unfolding takes place (denaturation temperature); both parameters can be determined using differential scanning calorimetry (DSC), this technique offers a direct method for studying the collagen denaturation with increasing temperature.

The degradation of collagen by Clostridium histolyticum collagenase is well known since 1960 by Heyns and Legler. Clostridial collagenase extensively degrades the collagen molecule in the helical regions predominantly the bond Y-Gly in sequences of the type -Pro-Y-Gly-Pro-, where Y is most frequently a neutral amino acid (Nagai, 1961). *Clostridium histolyticum* collagenases are less effective in degrading the more highly crosslinked collagens from older animals (Bailey and Etherington, 1985).

In this work we have studied the effect of a commercial *Clostridium histolyticum* collagenase on the collagen of different reticulation degree by DSC in order to relate the denaturation characteristics with collagen crosslinking state.

MATERIAL & METHODS

Intramuscular collagen of *M. Pectoralis profundis* from bovine carcasses of different ages (from 3 months to 8 years) was used in the experiments. *Clostridium histolyticum* collagenase (type 1A, EC 3.4.24.3) was obtained from Sigma Chemical Co. (St. Louis, MO).

Extraction of intramuscular collagen.-

Intramuscular collagens were extracted according to Kopp et al. (1989). The muscles were ground with a slide grinder and homogenized in tap water for 30s at medium speed in a Waring Blendor. The homogenate was filtered through a graded grid with 1mm square holes and rinsed, the materials not passing through the filter were re-homogenized and refiltered; this process was repeated a further two times, at which point non-filtrate was recupered, air dryed and lipid-free with acetone. The final product comprises endomysial and perimysial collagen.

Thermal solubility determination.-

According to Kopp and Bonnet (1982) samples (50mg of intramuscular collagen) were heated for 6h at 90°C in Tris-ClH buffer pH 7.5 (Tris-ClH 0.02M, ClNa 0.23M) and filtered, the hydroxyproline content of the residue insoluble was determinated according to the method of Bergman and Loxley

(1963) after hydrolisis of the sample in perchloric acid (Bonnet & Kopp, 1984). Collagen content was calculated as the product of hydroxyproline content times 7.5.

Parallelment total collagen concentration was determinated with the same method described for thermal insoluble collagen. Thermal solubility was defined as the difference between total and insoluble collagen concentrations expressed in percentage.

Differential Scanning Calorimetry (DSC).-

Calorimetry experiments were performed in a DSC 111 SETARAM and according to the method of Kopp et al. (1989). Quadruplicate collagen samples (10-20mg) were covered with approximately 100µl of Tris-ClH buffer pH 7 (50mM Tris, 0.15M NaCl, 20mM $CaCl_2$) and sealed into stainless pans. They were incubated 24h at 30°C and after that they were heated from 25°C to 100°C at a heating rate of 3°K/min. A sealed pan containing an equivalent weight of Tris-ClH buffer was used as reference.

The thermodynamic parameters (enthalpy and temperatures of denaturation) were calculated with a program elaborated in the "Meat Research Station INRA-Theix" with a Hewlett-Packard HP-85 Computer.

RESULTS AND DISCUSSION

The main characteristics of isolated intramuscular collagen are shown in table 1. Thermal solubility decreased with age, being differences between younger (3 months old) and older animals (20 months to 8 years old) highly outstanding, thus confirming the well known close relationship between age and thermal solubility. Denaturation temperature in calf was lower than that in steer and cow and we have confirmed that changes in collagen thermal solubility taking place with animal age seem to be accompanied by changes in thermal stability, in agreement with Bernal and Stanley (1986). Figure 1 shows the relationship between thermal solubility and initial temperature of denaturation. A very high positive correlation between both parameters (r= 0.99) has been calculated.

Table 2 summarizes the main denaturation features of intramuscular collagens studied. Collagenase treatment caused a decrease in the enthalpy of denaturation in calf and steer, which constituted an indicative of collagen breakdown. These results are in agreement with those obtained, in insoluble endomysial and perymisial connective tissue, by Tunick (1988). In contrast with those results no change was observed in the enthalpy of cow collagen denaturation. Cow intramuscular collagen, a more resistant tissue, showed not only a lower thermal solubility but also a higher resistance to collagenase treatment.

Table 1.- Denaturation characteristics and thermal solubility of intramuscular collagen from *M. Pectoralis profundis* obtained from bovine animals of different age.

SAMPLE	COLLAGEN (%)	SOLUBILITY (%)	(ΔH) J/g collagen	Øt (°C)
calf (3 months)	42.6±0.6	30.3	58.9±2.8	61.9±0.2
calf (3 months)	46±1.1	28.2	54.5±2.9	62.7±0.4
steer (20 months)	52.9±4.8	17.2	42.6±2.8	64.3±0.3
steer (30 months)	48.4±1.1	12.6	46.5±0.7	64.7±0.6
cow (5 years)	52.6±0.6	10.6	37.6±4.7	64.8±0.3
cow (8 years)	63.6±5.3	7.8	41±2.8	65.5±0.3

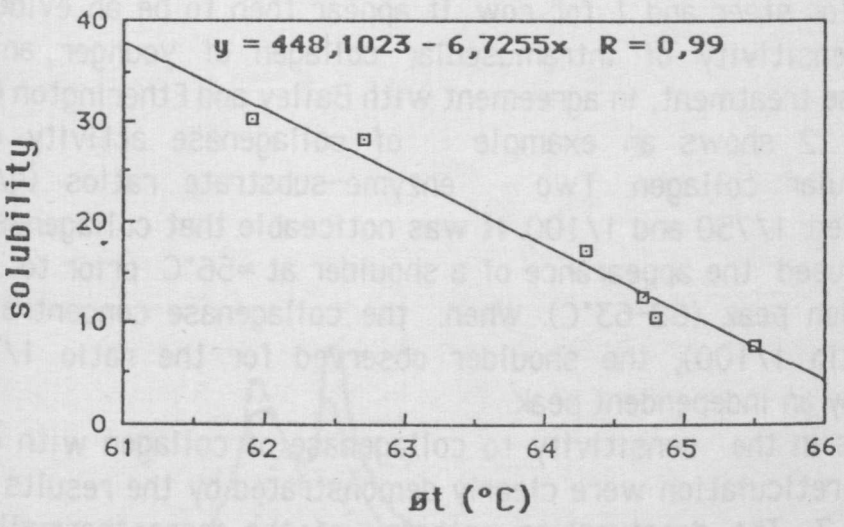

Figure 1.- Relationship between thermal solubility and initial denaturation temperature of bovine intramuscular collagen.

Table 2.- Temperature and enthalpy of denaturation of intact and collagenase treated intramuscular collagen from *M. Pectoralis profundis* of calf, steer and cow in pH 7 Tris-ClH buffer (enzyme/substrate ratio 1/100) determined by differential scanning calorimetry.
a. Øt initial denaturation temperature.

SAMPLE	(ΔH) J/g			Øt (°C)a	
	peak 1	peak 2	Σ	peak 1	peak 2
Intact calf collagen	---	67.1±6.1	67.1±6.1	---	57.1±0.6
Collagenase treated calf collagen	10.8±1.9	4.9±0.7	15.7±2.6	53.9±0.4	---
Intact steer collagen	---	42.4±1.9	42.4±1.9	---	59.8±0.7
Collagenase treated steer collagen	18.6±2.5	13.2±3.5	31.8±5.6	50.8±0.3	59.0±0.4
Intact cow collagen	---	36.1±2.7	36.1±2.7	---	60.0±0.6
Collagenase treated cow collagen	20±1.5	20±3.6	40.0±4.9	49.5±0.4	60.2±0.4

It must be pointed out the appearance of a new peak of denaturation after collagenase treatment in all cases. The ratio between the two peaks (peak at 55-57°C/ peak at 62-63°C) clearly decreased with age: 2.2 for calf, 1.4 for steer and 1 for cow. It appear then to be an evidence of a greater sensitivity of intramuscular collagen of younger animals to collagenase treatment, in agreement with Bailey and Etherington (1985).

Figure 2 shows an example of collagenase activity on steer intramuscular collagen. Two enzyme-substrate ratios (g/g) were investigated: 1/750 and 1/100. It was noticeable that collagenase (ratio 1/750) caused the appearance of a shoulder at ≈56°C prior to the main denaturation peak (62-63°C). When the collagenase concentration was rised (ratio 1/100), the shoulder observed for the ratio 1/750 was replaced by an independent peak.

Changes in the sensitivity to collagenase of collagen with different degree of reticulation were clearly demonstrated by the results depicted in figure 3. The denaturation enthalpy of the more thermally stable fraction (Øt= 62-63°C) decreased as a function of animal age, inversely the one of the less stable fraction (Øt= 55-57°C) increased with ageing. This shift in denaturation temperature was found to be further more intense the younger were the animals used for collagen isolation.

The greater susceptibility to heat denaturation of the less reticulate form of collagen after collagenase treatment could then serve as an indirect method to characterize the degree of reticulation of a collagen.

Denaturation characteristics combined with thermal solubility are avalaible methods to investigate the degree of reticulation. A possible amelioration of these usual methods could be the study of denaturation characteristics after histolyticum collagenase treatment.

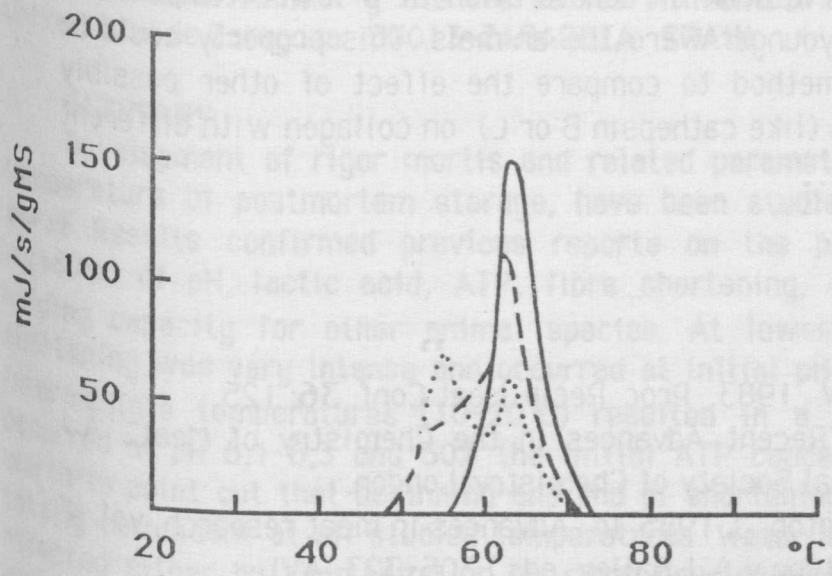

Figure 2.- Thermal denaturation curves of untreated (———) and collagenase treated steer collagen: enzyme/substrate ratio 1/750 (– – –), enzyme/substrate ratio 1/100 (------).

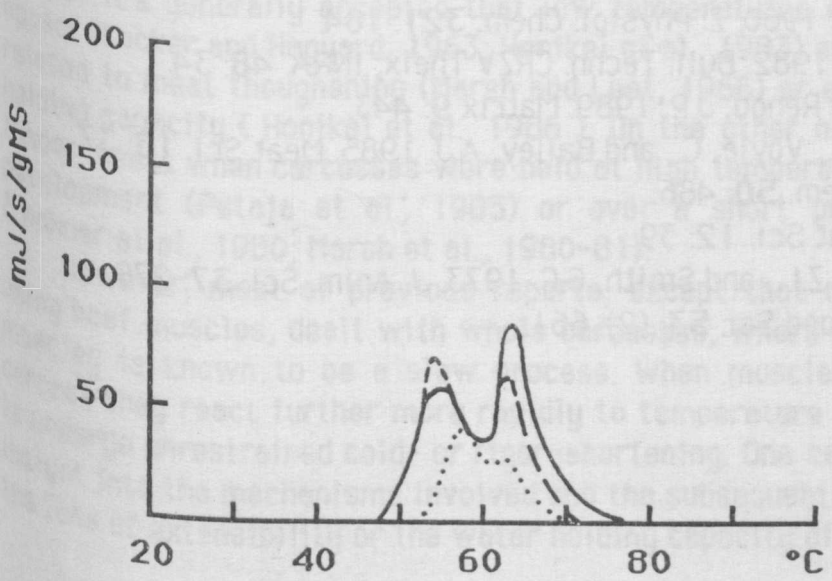

Figure 3.- Thermal denaturation curves of collagenase treated intramuscular collagen, enzyme/substrate ratio 1/100: (-------) calf collagen, (– – –) steer collagen and (——) cow collagen.

CONCLUSIONS

Collagenase of *Clostridium histolyticum* produced a decrease in enthalpy of denaturation in calf and steer intramuscular collagens, but no change was observed in cow collagen. Collagenase treatment caused in all cases the appearance of a new peak of denaturation at a lower temperature (55-57°C), larger the younger were the animals. This property could be used as a reference method to compare the effect of other possibly collagenolityc enzymes (like cathepsin B or L) on collagen with different degree of reticulation.

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