PE7.16 Efficacy of sodium and ammonium hydroxide as connective tissue tenderizers 169.00

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***Abstract*— This study investigated the prospects of using sodium and ammonium hydroxide as a tenderizing agent for connective tissue. Bovine *longissimus* epimysium from mature cows was used as the connective tissue source. Samples were pre-equilibrated (0, 90, 180 min) with three concentrations of alkali and heated at 55 and 70 ºC for 15 min. Treatments were applied in a split-plot design. Alkali-induced changes to epimysial shear stress, thermal denaturation temperatures and amide bands from FTIR spectroscopy were compared with samples subjected to aqueous heating at 55 and 70 ºC. The melting temperature of collagen was increased from 63 ºC (in water) to 66 ºC following pre-equilibration in 0.25 M ammonium hydroxide, but no change was observed due to 0.05 M sodium hydroxide treatment. At 55 ºC, 0.05 M sodium hydroxide was a superior connective tissue tenderizer than water. However, epimysial shear stress values following 0.1-0.5 M ammonium hydroxide treatment did not decrease with heating at 55 °C as was observed for samples heated in water, but were similar to that of the raw tissue. Within the concentrations used, neither of the alkali had a connective tissue tenderizing effect at 70 ºC beyond that resulting from aqueous heating alone.**

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***Index Terms*—connective tissue, collagen, shear stress, tenderizer, ammonium hydroxide, sodium hydroxide, heating**

I. Introduction

MEAT tenderization treatments are generally applied onto the meat matrix in which connective tissue and muscle fibres are interconnected together. As a result, the effect of tenderizing agents on the contributors to toughness and particularly on connective tissue itself is not elucidated.

Alkalis are not widely used as meat tenderizers except for some attempts to use sodium bicarbonate [8] and ammonium hydroxide [3]. Sodium hydroxide is widely used in the production of gelatin from animal sources but not as a meat tenderizer. Also, there is evidence that toughness (shear stress) of epimysium from young cattle (heifers) can be substantially decreased by aqueous heating, but the epimysium from mature cattle (cows) remains tougher before and after aqueous heating [7].

In this study, cow epimysium was used as a model to assess the potential to utilize sodium hydroxide and ammonium hydroxide as connective tissue tenderizers and to understand the impact of alkali on connective tissue properties.

II. Materials and Methods

Six *longissimus* muscles from cows of utility grade were purchased and stored at 4 °C. On the 10th day post-mortem, dorsal epimysium (45% crude protein; 4% fat; 55% moisture; 38 N/mm^2 shear stress) was excised and stored at -20 °C. Prior to use, visible fat and muscle fibres were removed and the epimysium was cut into two strips (2 cm width) along the length. From the strips, pieces measuring 2 cm wide by 2.5 cm in length were prepared. Treatments were applied in a split-plot design with temperature as the main plot and concentration of alkali and pre-equilibration time as sub-plots. This experiment was repeated with epimysium from three different animals. Epimysium pieces were pre-equilibrated (0, 90, 180 min) in aqueous sodium hydroxide (0.01, 0.025, 0.05 M; pH 12.0-12.7) and aqueous ammonium hydroxide (0.1, 0.25, 0.5 M; pH 11.1-11.4) prior to heating at 55 °C and 70 °C for 15 min. As a control, epimysium pieces from the same animals were heated in de-ionized water (10 mL) for 15 min at 70 °C.

Protein released from the epimysium was measured using the bicinchoninic acid (BCA) assay [9]. Briefly, the aqueous phase collected after heating was centrifuged at 31,000x*g* and the volume adjusted to 10 mL. BCA assay reagents A (sodium carbonate, sodium bicarbonate, sodium tartrate and BCA detection reagent in 0.1 N sodium hydroxide) and B (4% cupric sulphate pentahydrate) were mixed in the ratio 100:2 (v/v), respectively. An aliquot of 100 µL of appropriately diluted aqueous phase was mixed with 2 mL of the reagent mixture. Samples were immediately vortexed for 30 s and heated at 60 ºC for 30 min. Contents were cooled and absorbance measured at 562 nm. Gelatin has a lower absorbance than the commonly used standard BSA and also the absorbance curve of gelatin at 562 nm is more curvilinear [9]. Thus, calibration standards were prepared from bovine skin gelatine type B (Sigma-Aldrich, St. Louis, MO, USA).

Epimysial shear stress before and after heating was measured. Three strips of 0.5 cm width were cut from each epimysium sample parallel to the visible fibre direction. The length of the strips were not controlled as epimysium pieces shrank after heating. Strips at room temperature were sheared perpendicular to the fibre direction using a TMS-Pro texture system (Food Technology Corp., Sterling, VA, USA) equipped with a Warner-Bratzler shear attachment and a 1000 N load cell. The maximum shear stress generated was expressed as the force applied on a unit cross sectional area of sample and also corrected for raw thickness (N/mm^2).

Thermal denaturation temperatures of epimysial proteins were determined. Briefly, raw epimysium from 6 cows was first cut into small pieces and then ground after freezing in liquid N_2. About 0.5 g of ground epimysium was suspended in 10 mL of de-ionized water (control), 0.25 M NH_4OH and 0.025 M NaOH. Samples were equilibrated overnight at 4 ºC. Excess liquid was drained out and epimysium samples were briefly blotted on filter paper. Then, samples (7-8 mg) were weighed into coated aluminum pans and hermetically sealed with lids. Pans were heated from 20 to 140 ºC using a differential scanning calorimeter (DSC Q 2000, TA Instruments, USA) at a rate of 5 ºC/min. A sealed, empty pan was used as a reference. Data analysis was carried out using curve integration software (TA Universal Analysis 2000).

IR spectra of amide bands of epimysial proteins were determined. Epimysium pieces (2 cm wide x 2.5 cm long) were suspended in 10 mL of de-ionized water (control) and 0.5 M NH_4OH (180 min pre-equilibration). In a pre-heated water bath, the internal temperatures of samples were brought to 70 ºC and heating continued for 15 min. Samples were stored at -20 ºC. Selected samples were sectioned, placed in plastic moulds and filled with embedding compound OCT cryomatrix® (Shandon Company, USA). The preparation was frozen in liquid nitrogen and stored at -20 ºC. Serial sections (6 µm) were cut from cryo-preserved samples using a cryo-microtome. Sections were placed on reflective-coated Low-e® microscope slides (Kevley Technologies, USA) for spectroscopic analysis. FTIR imaging was conducted using the 01B1-1 Mid IR beam line (800-2000 cm^{-1}) at the Canadian Light Source (University of Saskatchewan). Spectroscopic mapping was performed in reflection mode with diffraction-limited spatial resolution. For each pixel, 64 scans, with a 4 cm^{-1} spectral resolution, were co-added. Data analysis was conducted using OPUS software (Bruker Optics, Germany).

PROC-GLM of SAS 9.1 programme was used for the statistical analysis of data.

III. RESULTS AND DISCUSSION

After alkali treatments, the maximum amount of protein released was <2% of the weight of epimysium or <5% of epimysial proteins. Following 180 min pre-equilibration in sodium hydroxide (0.01-0.025 M) and subsequent heating to 55 °C, single (α) and double (β) strands of collagen were liberated (Figure 1a). As the concentration of sodium hydroxide (0.05 M) and temperature (70 °C) were increased, collagen peptides were hydrolysed. Ammonium hydroxide also had liberated single and double strands of collagen at the two temperatures investigated (Figure 1b). Some hydrolysis of collagen peptides was noted after 0.5 M ammonium hydroxide treatment (180 min pre-equilibration) followed by heating to 70 °C. At the concentrations used, with pre-equilibration treatments (90-180 min) and subsequent heating to 55 and 70 °C, sodium and ammonium hydroxide were ineffective in liberating proteins from epimysium.

Shear stress of treated epimysium samples (raw shear stress was 38 N/mm^2) was decreased to ~25-29 N/ mm^2 as samples were pre-equilibrated (0-180 min) in sodium hydroxide (0.01-0.025 M) and subsequently heated to 55 °C (Figure 2a and b). The decrease in shear stress is attributed to the effect of temperature (55 °C) because samples heated in water had reached similar values (~23 N/mm^2). As the concentration of sodium hydroxide was increased to 0.05 M, shear stress was further decreased by 10 N/mm^2. Ammonium hydroxide concentration (0.1-0.5 M) and pre-equilibration (0-180 min) did not decrease shear stress (~39 N/mm^2) at 55 °C (Figure 3a and b). The effect of temperature (55 °C) in decreasing shear stress was eliminated by ammonium hydroxide treatment because samples heated in water reached 24 N/mm^2. Heating at 70 °C had a significant effect on epimysial shear stress reduction. Shear stress of sodium hydroxide and ammonium hydroxide treated epimysium was decreased to 3-6 N/mm^2 after heating at 70 °C and was similar to that heated in water (~5 N/mm^2). The epimysium stabilizing effect of ammonium hydroxide was eliminated at 70 °C.

Epimysium samples saturated in water prior to heating had a thermal denaturation temperature of ~63 °C (Table 1). At the alkali concentration used (0.025 M), sodium hydroxide did not change the melting temperature of epimysial proteins (~64 °C). Following ammonium hydroxide treatment (0.25 M), the denaturation temperature was significantly increased to ~66 °C with a narrower denaturation curve compared to other treatments. This observation supports the hypothesis that ammonium hydroxide has a structure stabilization effect on collagen because ~91% of the epimysial proteins consisted of collagen [7].

In a related work from our laboratory, after aqueous heating of epimysium (from animals <30 months and 3-5 years of age) at 70-95 °C, 14-16 g amorphous proteins per 100 g epimysium (or ~30% on a protein basis) was extracted with pronase [7] indicating a helix→coil transition. However, aqueous heating at 70 °C (15 min) did not introduce changes to the typical amide bands I (1675-1658 cm^{-1}) and II (1550 cm^{-1}) of epimysial proteins (Figure 4a). Therefore, C=O and N-H bond stretching [6] might not have occurred in collagen peptides during heating at 70 °C for 15 min, even though some of the epimysial collagen was converted to an amorphous state under similar conditions of heating [7]. Or else, significant recoiling of collagen (coil→helix transition) [4] might have happened during cooling. A new peak emerged (1640-1620 cm^{-1}) on the shoulder of the amide I band of ammonium hydroxide-treated and subsequently heated epimysium (Figure 4b). This band may represent random coils [5] or β sheets [1]. Because glycine comprises one-third of the amino acids in collagen [2], the main epimysial protein, it is thought this new peak represents random coils [10]. Perhaps, ammonium hydroxide has prevented or decreased the random coil→helix transition of thermally denatured collagen at 55 °C.

IV. Conclusion

Sodium hydroxide (0.05 M) tenderizes connective tissue more than water following heating at 55 °C but when epimysium is heated at 70 °C, water is an equally good tenderizer as alkali. Ammonium hydroxide (0.1- 0.5 M) had no useful tenderizing effect on connective tissues. Instead, ammonium hydroxide had a collagen stabilization effect and raised the melting temperature of collagen.

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Table 1. Melting temperatures (mean ± SE) of epimysial proteins
after aqueous and alkali heating

	Melting temperature peak (ºC)	Onset temperature (ºC)
Water	63.0±0.5	53.3±0.6
0.025 M NaOH	64.0±0.3	55.9±0.6
0.25 M NH_4OH	66.2±0.1	55.9±0.5
P-value	0.05	0.05
LSD	2.00	2.18

Heating carried out at 5 ºC/min from 20-140 ºC. Each treatment had 6 replicates and three sub samples.

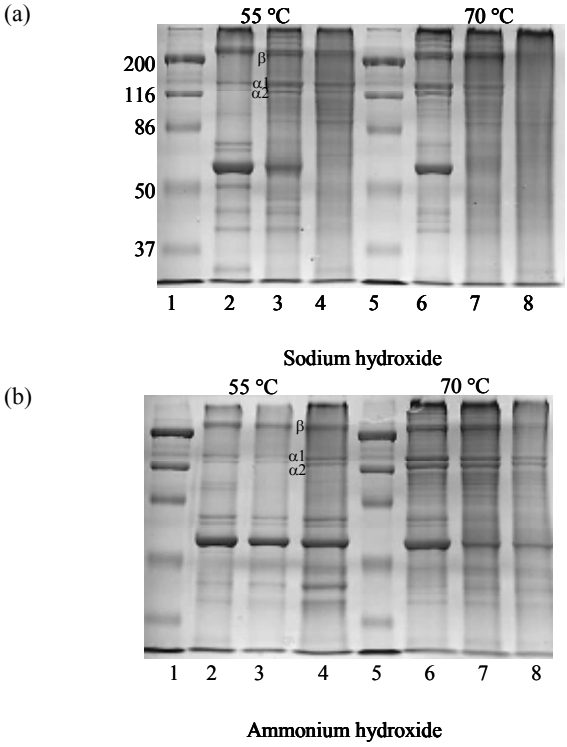
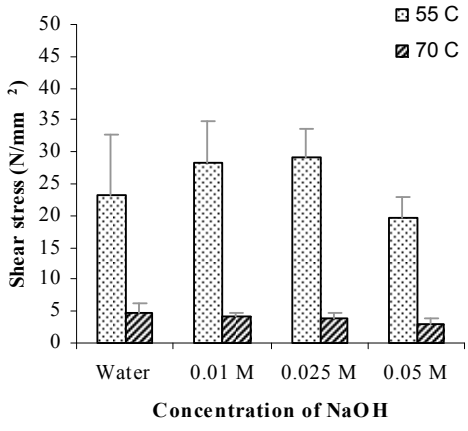


Figure 1. Peptides released from epimysium following 180 min pre-equilibration in alkali and heating at 55 and 70 °C for 15 min. (a) Sodium hydroxide; Lane 2, 3, 4 (55 °C) and 6, 7, 8 (70 °C) show concentrations 0.01, 0.025 and 0.05 M, respectively. (b) Ammonium hydroxide; Lane 2, 3, 4 (55 °C) and also 6, 7, 8 (70 °C) show concentrations 0.1, 0.25 and 0.5 M, respectively. Lane 1 and 5 are molecular weight standards (kDa). α1 and α2 represent single strands of collagen and β represents double strands of collagen.

(a)



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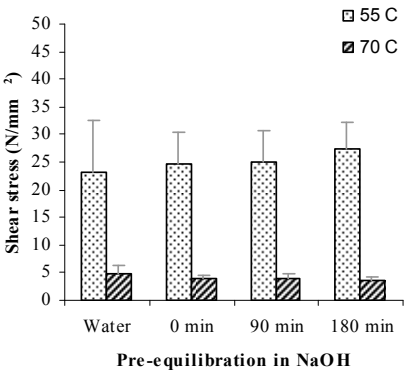
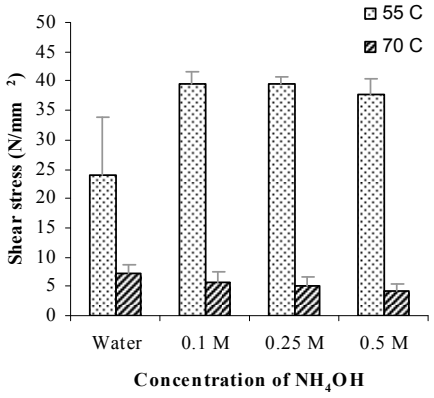


Figure 2. Shear stress of epimysium (means ± SE) following (a) concentration of sodium hydroxide and temperature and (b) pre-equilibration time in sodium hydroxide and temperature.

(a)



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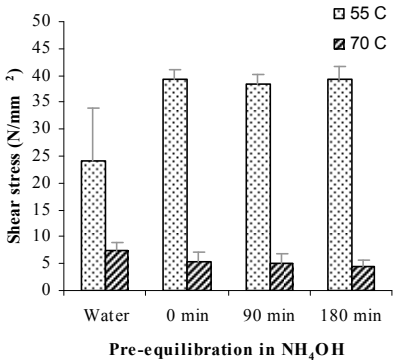
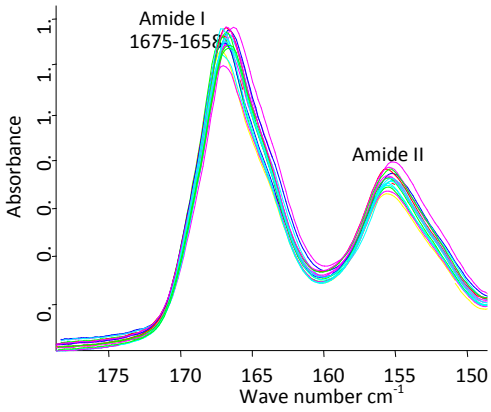


Figure 3. Shear stress of epimysium (means ± SE) following (a) concentration of ammonium hydroxide and temperature (b) pre-equilibration time in ammonium hydroxide and temperature.

(a)



(b)

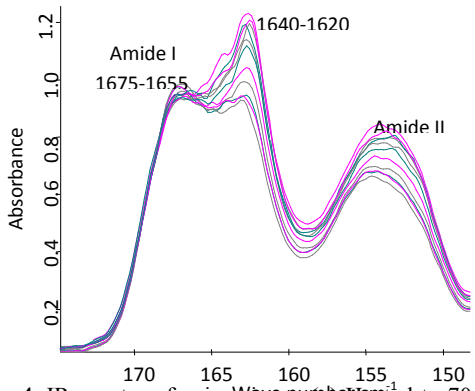


Figure 4. IR spectra of epimysium (a) Heated to 70 ºC with de-ionized water with no pre-equilibration. Amide bands I (1675-1658 cm^{-1}) and II (1550 cm^{-1}) typical to proteins are observed despite the exposure to high temperature. (b) Pre-equilibrated for 180 min in 0.25 M NH_4OH and heated to 70 ºC for 15 min. On the shoulder of the characteristic amide I band (1675-1655 cm^{-1}) a new peak emerged (1640-1620 cm^{-1}).

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