Available online at www.sciencedirect.com
SCIENCE DIRECT®
Thermochimica Acta 407 (2003) 105–112
thermochimica acta
www.elsevier.com/locate/tca

The effects of pH, NaCl and $CaCl_2$ on thermal denaturation characteristics of intramuscular connective tissue

N. Aktaş*

Food Engineering Department, Agricultural Collage, Atatürk University, 25240 Erzurum, Turkey

Received 30 July 2002; received in revised form 10 January 2003; accepted 16 May 2003

Abstract

The research was conducted with two different experiments on intramuscular connective tissue obtained from *Longissimus dorsi* muscle of 4-year-old beef carcasses. Differential scanning calorimetry (DSC) was used to determine the denaturation onset temperature (T_o), denaturation peak temperature (T_p), and denaturation enthalpy (ΔH_D) of intramuscular connective tissue. In the first experiment, equilibration of the collagen in citrate buffers in the pH range 2.9–6.5 resulted in the decline of T_o and T_p with decreasing pH, but statistically significant differences were not recorded for ΔH_D. In the second experiment, NaCl and $CaCl_2$ solutions at 0.34, 0.68 and 1.02 ionic strength at pH 3.7–5.7 were studied. Increasing NaCl resulted in the increase of T_o and T_p, whilst T_o and T_p was decreased with increasing $CaCl_2$.

Keywords: Intramuscular connective tissue; pH; NaCl; $CaCl_2$; Differential scanning calorimetry

1. Introduction

Collagen is the major structural protein of connective tissue. It is fibrous, rigid and shear resistant protein. Toughness of a muscle is proportional to its intramuscular collagen content. When collagen is heated, the breakage of hydrogen links induces shrinkage of the collagen fibres, followed by solubilisation and gelatinisation. Covalent crosslinks are responsible for continuity between collagen molecules. These crosslinks become increasingly thermostable with collagen ageing, while at the same time collagen thermal stability increases [1]. Introduction of crosslinks leads to a further increase in the transition temperature by an extent that depends on number, location and stability of the crosslinks [2].

The transition temperature of collagen in solution depends on numerous factors including the hydroxyproline content, the presence of mucopolysaccharides, pH and ionic composition of the aqueous environment. Above certain minimum levels, the transition temperature increases with collagen concentration. Aggregation of collagen into fibrils is accompanied by a relatively large increase in melting temperature (approximately 10 °C) and this change has been attributed to the local increase of collagen concentration in the fibril.

Several methods are available to examine the physical state of collagen in solution [3–6]. But many of them are not applicable for measuring the changes in the fibrous tissue. DSC provides a better understanding of collagen triple helix denaturation. Denaturation of collagen is an endothermic reaction which occurs at a very slow rate. Several authors [7] have analysed the endothermic helix to coil transition (collagen to

* Tel.: +90-442-2311625; fax: +90-442-2360958.
E-mail address: aktasnesimi@hotmail.com (N. Aktaş).

0040-6031/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S0040-6031(03)00306-X

gelatin transition) in soluble collagen or in the fibrous form with DSC investigations and have determined the corresponding enthalpy and temperature.

Numerous studies have described the effects of salt solutions on the properties of collagen and gelatin solutions, collagen fibres and gelatin gels [8,9]. The anion and cations of various salts appear to operate independently and additively on the stability of collagen structure in both gelatin gels and collagen fibres [10]. King [2], McClain and Wiley [11], Judge and Aberle [12] obtained contradictory results when they examined the effects of postmortem ageing on intramuscular collagen by measuring its transition temperature.

Our study was undertaken to determine the effects of pH, NaCl and $CaCl_2$ on the transition temperature of collagen, including postmortem changes to check whether a reduction in pH may be responsible for some of the contradictory observations reported by other workers.

2. Materials and methods

2.1. Materials

The meat samples used in this research were obtained from *Longissimus dorsi* muscle of 4-year-old beef carcasses obtained from a major slaughter-house in Erzurum, Turkey. The carcasses were chilled for 24 h at 5 ± 1 °C. Following chilling, all trimmable fat and connective tissue (epimysium) were removed from the muscle.

2.2. Methods

2.2.1. Preparation of intramuscular connective tissue

After removal of any overlying layers of connective tissue, epimysium and/or tendons were removed from the *L. dorsi* muscle surfaces by dissection. Any adhering muscle tissue was scraped away with a scalpel. Samples of intramuscular connective tissue were prepared from the diced muscle (free of tendon, and epimysium) using a mixer-emulsifier [2]. The tissue was homogenised for 10 s at maximum speed, in 0.1 M potassium chloride and 0.02 M potassium dihydrogen phosphate pH 6.1 buffer and the connective tissue adhering to the homogeniser blades and the homogenate were filtered through a 25 mesh sieve. The connective tissue was re-suspended in buffer and re-homogenised for 10 s. This procedure was repeated three times. The samples were defatted with acetone, and dialysed against distilled water for 9 h at 4 °C in seamless cellular tubing (Sigma, D-0405), lyophilised and stored.

2.2.2. pH and salt equilibration

The intramuscular connective tissue was soaked for 24 h at 5 ± 1 °C in citrate buffer (1:4 intramuscular connective tissue: buffer) of the desired pH in the range 2.9–3.3–3.7–4.1–4.5–4.9–5.3–5.7–6.1 and 6.5. Moreover, the intramuscular connective tissue was treated with NaCl and $CaCl_2$ solutions at 0.34, 0.68 and 1.02 ionic strength of the 3.7 and 5.7 pH.

2.2.3. Thermal transition measurement with DSC

The endothermal transition of intramuscular connective tissue was measured as described by Arganosa and Marriot [13]. Using a Shimadzu DSC-50, a circa 10 mg sample of the intramuscular connective tissue was blotted between layers of paper towel weighed into an aluminium hermetic cell which was sealed with a crimper. Samples were heated from 20 to 90 °C at 5 °C/min using an empty cell as a reference. For ΔH_D after DSC analysis, the sample cells were punctured and the dry weight of the samples determined after drying at 105 °C for 24 h. For temperature and heat flow calibration was used indium (T, 156.4 °C; ΔH, 28.47 J/g).

2.2.4. Statistical analysis

This experiment was conducted according to a completely randomised block design with three replicates. Analysis of variance of all data was conducted by using the general linear models procedure [14].

3. Results and discussions

Typical curves of intramuscular connective tissue at different pHs are shown in Fig. 1. As can be seen curves, lower pH values were at lower temperatures than the higher pH values. Fig. 2 shows the effect of pH on denaturation onset temperature (T_o) and denaturation peak temperature (T_p) of intramuscular connective tissue. At the lower pH value, the T_o and T_p of intramuscular connective tissue were significantly lower

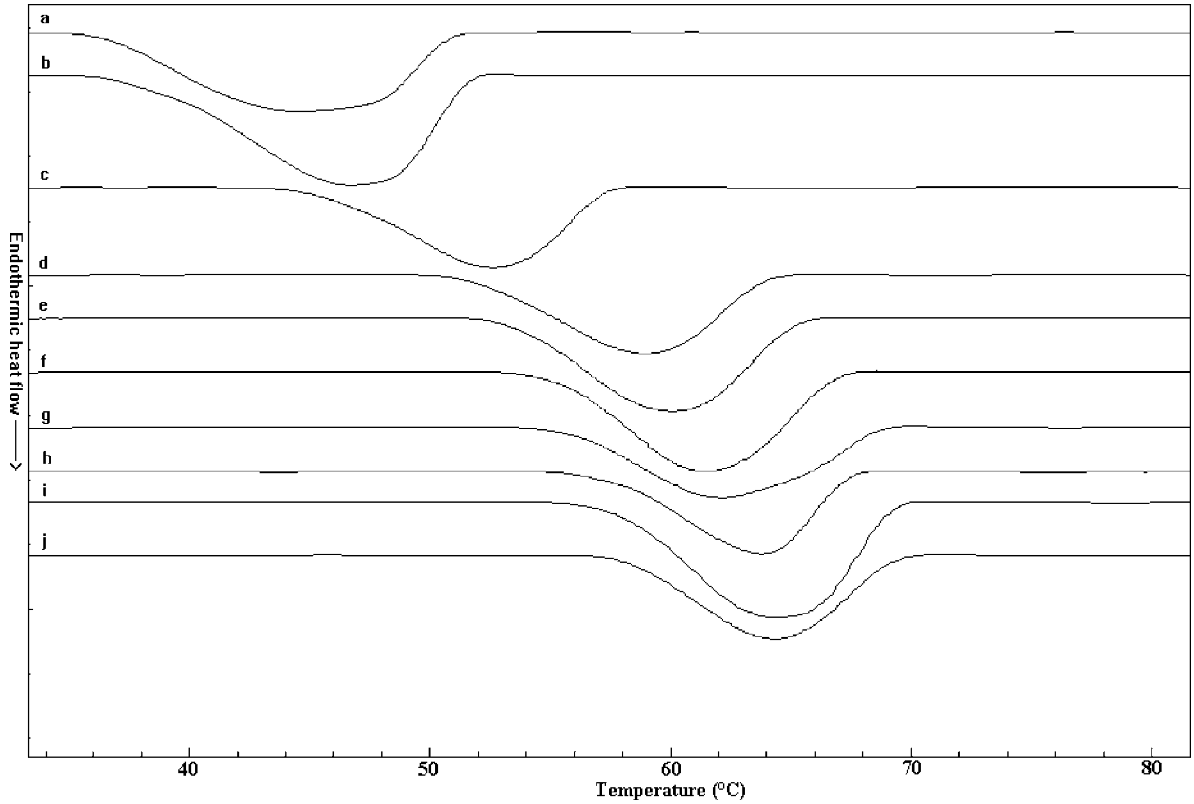

Fig. 1. Thermal transition curves of intramuscular connective tissue at different pHs: (a) 2.9, (b) 3.3, (c) 3.7, (d) 4.1, (e) 4.5, (f) 4.9, (g) 5.3, (h) 5.7, (i) 6.1, (j) 6.5.

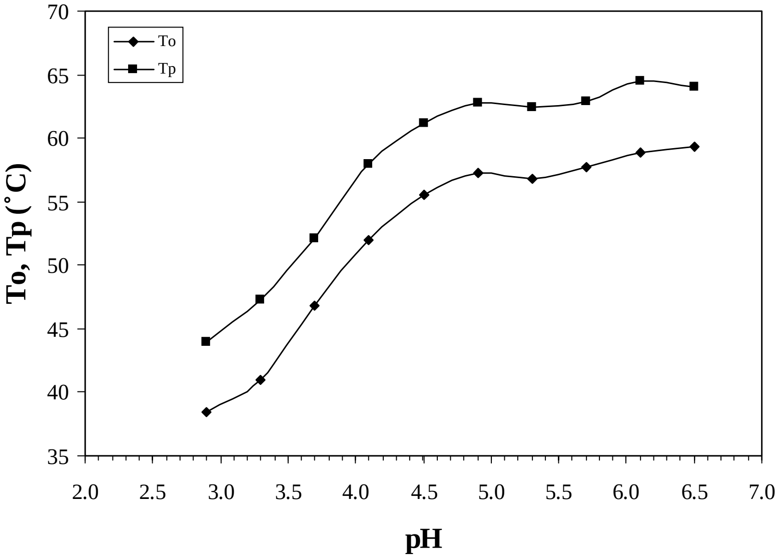

Fig. 2. The effect of pH on T_o and T_p of intramuscular connective tissue.

Table 1
Effect of pH on thermal transition of intramuscular connective tissue[a]

pH	T_o (°C)	T_p (°C)	ΔH_D (J/g)
2.9	38.47 ± 1.20 h	43.99 ± 0.76 g	2.25 ± 1.39 a
3.3	41.02 ± 1.10 g	47.29 ± 0.54 f	2.60 ± 1.14 a
3.7	46.80 ± 0.91 f	52.11 ± 0.61 e	2.34 ± 0.44 a
4.1	52.04 ± 1.08 e	57.96 ± 0.85 d	2.43 ± 0.40 a
4.5	55.58 ± 0.45 d	61.21 ± 1.69 c	2.74 ± 0.68 a
4.9	57.23 ± 0.55 c	62.80 ± 1.19 b	3.47 ± 0.45 a
5.3	56.75 ± 1.37 cd	62.44 ± 0.34 bc	2.41 ± 0.91 a
5.7	57.74 ± 0.44 bc	62.92 ± 0.94 b	2.13 ± 0.08 a
6.1	58.87 ± 0.16 ab	64.51 ± 0.43 a	2.57 ± 1.01 a
6.5	59.33 ± 0.71 a	64.03 ± 0.49 ab	2.31 ± 0.40 a

[a] ± represents standard deviation three of replicate. Values in a column with the same letters are not significantly different by Duncan's multiple comparison test ($P < 0.05$).

than those of the higher pH value. Even though T_o was more sensitive to pH decline than T_p parallel trends were observed (Fig. 2). Because T_o measures the thermal stability of less stable components than T_p which is considered a measure of average thermal stability. This result was in accord with those of King [2], Ledward et al. [15], and Horgan et al. [16]. They found that T_o is related to the least stable collagen crosslinks, that is, the aldimine bonds. These crosslinks were particularly sensitive to pH decline, and cleavage and reformation of aldimine crosslinks were pH-dependent.

Collagen from samples treated with low pH exhibited a decrease ($P < 0.05$) in T_o and T_p (Table 1). These results indicated that low pH treatment resulted in denaturation of the collagen as exhibited by the lower T_o and T_p. The low pH appeared to enhance alterations in the structural stability of the collagen, with the effect being least pronounced in samples treated with citrate buffers. Similar effects were observed by Arganosa and Marriot [13]. It is known that some of lysine, hydroxylysine, and histidine residues are nontitratable because of the participation of these groups in Schiff-base formation, aldol condensation, and their rearrangement products, the extent and pattern of which is tissue and age specific [17]. The hydrolysis of the Schiff base occurs readily in acid solution and the kinetics has been studied in detail [18–20]. The precise mechanism is a function of the reactant structure and pH.

The temperature range of thermal transitions of all products of collagen decompositions due to the pH was 39–65 °C. According to Bailey [21], the collapse of the triple helix occurs around 39 °C for mammalian collagen. Based on this information, and the data obtained from our experiments, we presume that weak acid treatments promote the disruption of noncovalent intermolecular bonds that reinforce the collagen fibril structure, enhancing the swelling effect (random water–protein interaction) and decreasing the temperature of denaturation. Noncovalent, intermolecular interactions (hydrogen bonds, dipole or ion–pair interactions and intermolecular bridges) are more important for thermal stability of collagen than the covalent crosslinks [22,23].

The effect of swelling, for 24 h, at different pH values on values of denaturation enthalpy (ΔH_D), T_o and T_p are summarised in Table 1. It is seen that in the pH range 2.9–6.5 there is negligible effect on ΔH_D. This is consistent with the data of Privalov and Tiktopulo [24] who showed that ΔH_D for the denaturation of tropocollagen is only slightly affected by pH in the range 3–6. Thus, the ΔH_D can be taken to be virtually independent of pH, and it is possible to consider denaturation heat effects of different tropocollagens at any certain pH value. It is concluded that electrostatic interactions, to judge from the insignificant change in the denaturation enthalpy with pH variation from 6.5 to 2.9, evidently do not play any determining role in the stabilisation of the specific collagen structure.

Ions other than hydrogen ions in the variable-pH buffer solutions have effectively changed the collagen T_o and T_p (Table 2, Fig. 3). The pH, salt and ionic strength caused significant changes in T_o and T_p (Table 3). Also, the pH × salt (Table 4), ionic strength × salt (Fig. 4) and ionic strength × pH (Fig. 5) interactions had significant changes in T_o and T_p. It is seen that swelling in all ionic strength of NaCl at constant pH (pH 3.7–5.7) causes significant increases in both T_o and T_p (Table 2), which is in agreement with the results of Judge and Aberle [12]. This increase in the thermal stability must represent a change in the nature of the collagen or its environment as the swelling agent is the same in all cases. It has been suggested that both hydrophobic bonds (which break exothermally) and hydrogen bonds (which break endothermally) are important in the stabilisation of collagen fibres and such a postulate may help to explain the results obtained.

Table 2
T_o, T_p and ΔH_D values determined at different pHs, salt and ionic strength and the results of Duncan's multiple comparisons test[a]

pH	Salt	Ionic strength	T_o (°C)	T_p (°C)	ΔH_D (J/g)
3.7	NaCl	0	46.80 ± 0.91 d	52.11 ± 0.61 d	2.34 ± 0.44 a
		0.34	51.29 ± 0.76 c	58.19 ± 0.67 c	3.31 ± 1.36 a
		0.68	57.91 ± 0.44 b	63.69 ± 1.02 b	3.17 ± 1.59 a
		1.02	64.48 ± 0.59 a	68.10 ± 0.53 a	1.94 ± 0.45 a
	$CaCl_2$	0	46.80 ± 0.91 b	52.11 ± 0.61 b	2.34 ± 0.44 a
		0.34	43.39 ± 0.98 d	48.83 ± 0.77 c	2.30 ± 0.78 a
		0.68	45.38 ± 0.24 c	51.37 ± 0.47 b	2.36 ± 0.42 a
		1.02	50.58 ± 0.24 a	56.49 ± 0.97 a	1.63 ± 0.81 a
5.7	NaCl	0	57.74 ± 0.44 d	62.92 ± 0.94 d	2.13 ± 0.08 a
		0.34	60.54 ± 0.48 c	66.41 ± 0.73 c	2.64 ± 0.84 a
		0.68	63.28 ± 0.15 b	68.54 ± 0.80 b	2.48 ± 0.58 a
		1.02	66.52 ± 0.48 a	70.55 ± 0.31 a	1.89 ± 0.58 a
	$CaCl_2$	0	57.74 ± 0.44 a	62.92 ± 0.94 a	2.13 ± 0.08 a
		0.34	57.64 ± 1.11 a	63.81 ± 1.48 a	3.88 ± 2.39 a
		0.68	55.17 ± 1.52 b	62.65 ± 0.37 a	3.77 ± 0.21 a
		1.02	56.10 ± 1.53 ab	62.04 ± 0.90 a	3.22 ± 0.37 a

[a] ± represents standard deviation three of replicate. Values in a column with the same superscript are not significantly different by Duncan's multiple comparison test ($P < 0.05$).

Rosenblatt et al. [4,5] and Wallace [25] reported that the isoelectric point of collagen is pH 7.0–7.5, but the charge varies little from pH 5 to 9. The pH values of treated intramuscular connective tissue were lower than the isoelectric pH values. The pH-dependent salt effects below the isoelectric point indicate that charge interactions are involved in collagen aggregation and stabilisation. In acid media at low salt concentration, mutual repulsion between collagen molecules carrying an excess of positive charge would be expected to minimise aggregation. As the NaCl concentration is increased, however, preferential anion binding may decrease the cationic character of the protein resulting in progressive aggregation of the collagen at rates governed by the Cl^- ion concentration. Electrophoretic evidence for such preferential binding for Cl^- ions on collagen has been presented by Aktaş and Kaya [26]. With increasing NaCl concentration, additional structural stabilisation in the fibrils may arise from an increase in polypeptide-chain rigidity in aggregated molecules (Fig. 4). Such mechanism of charge interaction may thus account for the effects of NaCl

Table 3
The general effects of pH, salt and ionic strength on T_o, T_p and ΔH_D[a]

		T_o (°C)	T_p (°C)	ΔH_D (J/g)
pH	3.7	50.83 ± 6.82 b	56.36 ± 6.41 b	2.43 ± 0.92 a
	5.7	59.34 ± 3.77 a	64.98 ± 3.10 a	2.77 ± 1.08 a
Salt	NaCl	58.57 ± 6.44 a	63.82 ± 5.87 a	2.49 ± 0.89 a
	$CaCl_2$	51.60 ± 5.62 b	57.53 ± 5.85 b	2.70 ± 1.12 a
Ionic strength	0	52.27 ± 5.74 d	57.52 ± 5.68 d	2.23 ± 0.29 ab
	0.34	53.21 ± 6.91 c	59.31 ± 7.08 c	3.03 ± 1.42 a
	0.68	55.44 ± 6.82 b	61.56 ± 6.59 b	2.94 ± 0.95 ab
	1.02	59.42 ± 6.75 a	64.30 ± 5.74 a	2.17 ± 0.81 b

[a] ± represents standard deviation three of replicate. Values in a column with the same superscript are not significantly different by Duncan's multiple comparison test ($P < 0.05$).

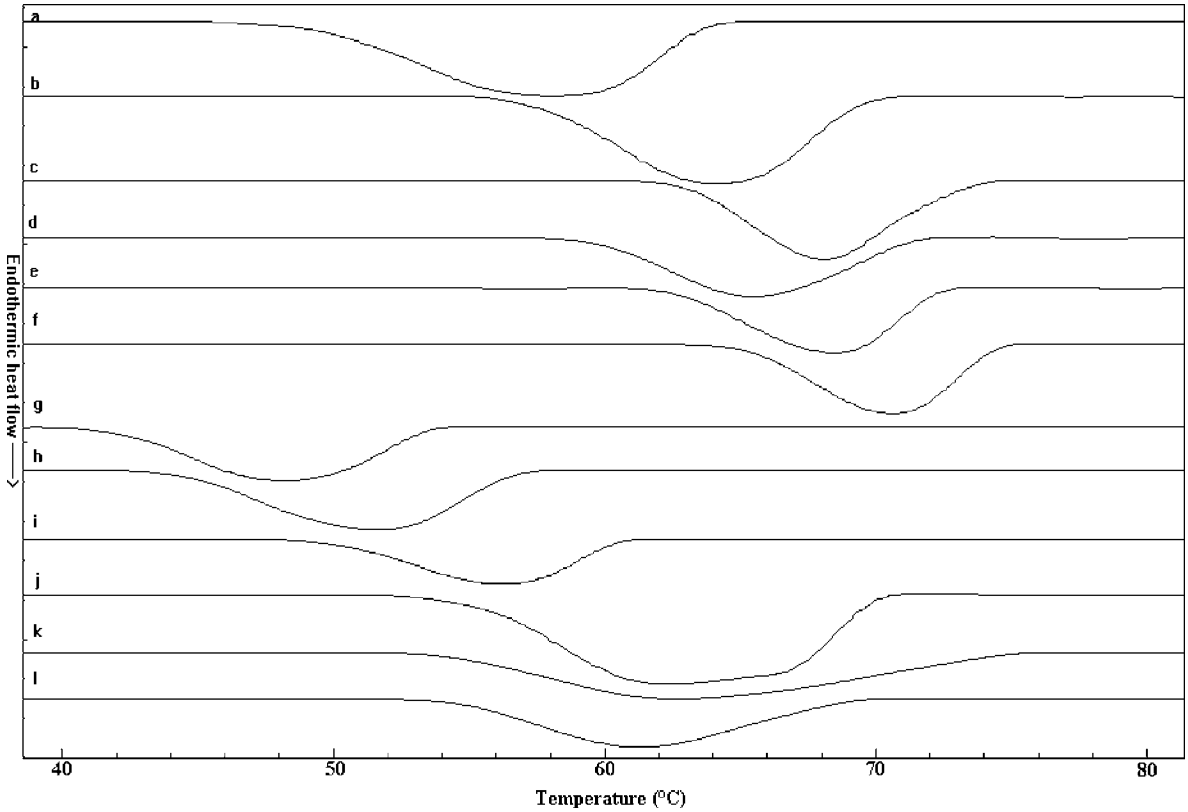

Fig. 3. Thermal transition curves of intramuscular connective tissue in NaCl, $CaCl_2$ at pH 3.7 and 5.7: (a) pH 3.7 at 0.34 ionic strength of NaCl, (b) pH 3.7 at 0.68 ionic strength of NaCl, (c) pH 3.7 at 1.02 ionic strength of NaCl, (d) pH 3.7 at 0.34 ionic strength of $CaCl_2$, (e) pH 3.7 at 0.68 ionic strength of $CaCl_2$, (f) pH 3.7 at 1.02 ionic strength of $CaCl_2$, (g) pH 5.7 at 0.34 ionic strength of NaCl, (h) pH 5.7 at 0.68 ionic strength of NaCl, (i) pH 5.7 at 1.02 ionic strength of NaCl, (j) pH 5.7 at 0.34 ionic strength of $CaCl_2$, (k) pH 5.7 at 0.68 ionic strength of $CaCl_2$, (l) pH 5.7 at 1.02 ionic strength of $CaCl_2$.

on the thermal stability of collagen, in which dissolution is prevented by the presence of the covalent crosslinks. Variations in thermal stability with different salt concentration were qualitatively similar to that noted for fibrils precipitated in 4 and 6% NaCl [27].

Table 4
The effect of pH × salt interaction on T_o and T_p[a]

pH	Salt	T_o (°C)	T_p (°C)
3.7	NaCl	55.12 ± 7.01 a	60.53 ± 6.29 a
	$CaCl_2$	46.54 ± 2.81 b	52.20 ± 2.94 b
5.7	NaCl	62.02 ± 3.41 a	67.10 ± 3.01 a
	$CaCl_2$	56.66 ± 1.54 b	62.85 ± 1.08 b

[a] ± represents standard deviation three of replicate. Values in a column with the same superscript are not significantly different by Duncan's multiple comparison test ($P < 0.05$).

The effects of salts are currently considered to involve ion–dipole association or hydrogen bonding of a decrease in double-bond character and consequent loss of polypeptide-chain rigidity. Evidence for such interaction stems from salt effects on the conformational changes in proline, which lacks internal hydrogen bonding [28]. Alternatively, for proteins, modification of the water structure by ions may favour exposure of internal hydrophobic groups to the solvent, resulting in unfolding of the native structure. The ionic radius of the chloride ion is 1.89 Å, which is comparable to the size of water molecule, and the ionic radii of Na^+ and Ca^{++} are even smaller, being about 1 Å. Therefore, there would be no steric barrier preventing these ions from penetrating into all of the spaces in the fibrils including the helical groove of the molecule.

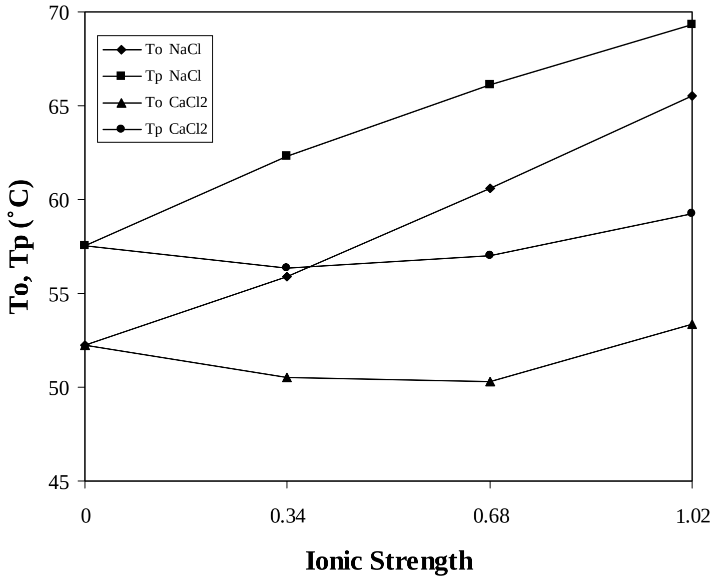

Fig. 4. The effect of pH × salt interaction on T_o and T_p.

Indeed, nuclear magnetic resonance studies indicate that these ions can either occupy the sites for the structural water of collagen, or that this water can enter into the hydration shells of these ions [17].

Unlike NaCl, increasing ionic strength of $CaCl_2$ caused a decrease in T_o and T_p (Figs. 3 and 4). This effect can be account for by the fact that as the thickness of the hydrate layer on the protein gets narrower,

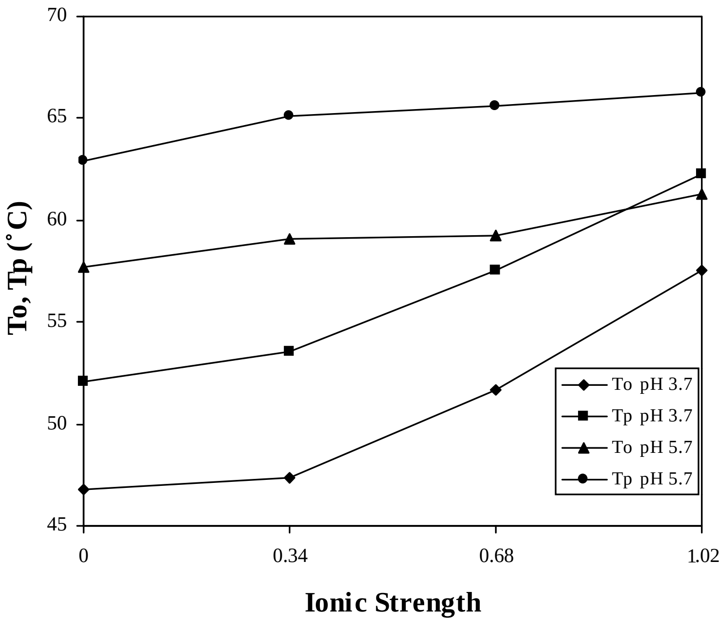

Fig. 5. The effect of ionic strength × pH interaction on T_o and T_p.

denaturation occurs at lower temperatures. In this case, aggregation of proteins may have caused a narrower hydrate layer on the protein and thus lower T_o and T_p. Covalent crosslinks contribute to the strength and rigidity of the collagen fibres [28]. Collagens contain a double-bond formed between an amine nitrogen and an aldehydic carbon. The effects of $CaCl_2$ on such a –N=CH– Schiff base has been suggested as a reason for its effect on both T_o and T_p compared to NaCl [29].

4. Conclusions

Our results showed that the T_o and T_p were strongly influenced by pH. These results suggested that intramuscular connective tissue isolated at different time postmortem should be equilibrated to a common pH before measurement of its thermal transition temperature. Different pH values were not effect on ΔH_D. However, different salt and salt concentration affected the thermal stability of intramuscular connective tissue. This situation showed that thermal stability of collagen was much affected by hydrogen bonds and hydrophobic interaction types than electrostatic interaction.

References

[1] J. Kopp, M. Bonnet, J.P. Renou, Matrix 9 (1989) 443 450.
[2] N.L. King, Meat Sci. 20 (1987) 25–37.
[3] M.D. Ranganayaki, A. Asghar, R.L. Henrickson, J. Food Sci. 47 (1982) 705–710.
[4] J. Rosenblatt, B. Devereux, D.G. Wallace, Biomaterials 13 (1992) 878–886.
[5] J. Rosenblatt, B. Devereux, D.G. Wallace, J. Appl. Polym. Sci. 50 (1993) 953–963.
[6] S. Matsushita, S. Deki, M. Mizuhata, M. Sugita, Y. Kanaji, J. Appl. Polym. Sci. 54 (1994) 1561–1566.
[7] R. Usha, T. Ramasami, Thermochim. Acta 338 (1999) 17–25.
[8] A.T. Miller, E. Karmas, M. Fu Lu, J. Food Sci. 48 (1983) 681–685.
[9] P.B. Kenney, R.L. Henrickson, P.L. Claypool, B.R. Rao, J. Food Sci. 15 (1986) 277–287.
[10] A. Finch, D.A. Ledward, Biochim. Biophys. Acta 295 (1973) 296–300.
[11] P.E. McClain, E.R. Wiley, J. Biol. Chem. 247 (1970) 692–697.
[12] M.D. Judge, E.D. Aberle, J. Anim. Sci. 54 (1982) 68–71.
[13] G.C. Arganosa, N.G. Marriott, J. Food Sci. 54 (1989) 1173–1176.
[14] SAS Users Guide: Statistics, SAS Institute, Cary, NC, 1996.
[15] D.A. Ledward, R. Chizzolini, R.A. Lawrie, J. Food Technol. 10 (1975) 349–357.
[16] D.J. Horgan, L.B. Kurth, R. Kuypers, J. Food Sci. 56 (1991) 1203–1208.
[17] S.-T. Li, E.P. Katz, Biopolymers 15 (1976) 1439–1460.
[18] E.H. Cordes, W.P. Jencks, J. Am. Chem. Soc. 85 (1963) 2843–2848.
[19] J. Hine, J.C. Craig, J.J.G. Underwook, F.A. Via, J. Am. Chem. Soc. 92 (1970) 5194–5199.
[20] F.A. Carey, R.J. Sundberg, Advanced Organic Chemistry: Structure and Mechanisms, Part A, 3rd ed., Plenum Press, New York, NY, 1993.
[21] A.J. Bailey, The chemistry of intramuscular collagen, Recent Advances in the Chemistry of Meat, vol. 39, The Royal Society of Chemistry, Burlington House, 1984.
[22] E.D. Aberle, E.W. Mills, Recip. Meat Conf. Proc. 36 (1983) 125–130.
[23] J. Kijowski, Thermal transition temperature of connective tissues from marinated spent hen drumsticks, Int. J. Food Sci. Technol. 28 (1993) 587–594.
[24] P.L. Privalov, E.I. Tiktopulo, Biopolymers 9 (1970) 127–139.
[25] D.G. Wallace, Biopolymers 29 (1990) 1015–1026.
[26] N. Aktaş, M. Kaya, Eur. Food Res. Technol. 213 (2001) 88–94.
[27] A.E. Russell, J. Biochem. 131 (1973) 335–342.
[28] H.R. Horton, L.A. Moran, R.S. Ochs, J.D. Rawn, K.G. Scrimgeour, Principles of Biochemistry, Prentice-Hall, Englewood Cliffs, NJ, 1992.
[29] N. Aktaş, M. Kaya, Meat Sci. 58 (2001) 413–419.