Molecular location of Ehrlich chromogen and pyridinoline cross-links in bovine perimysial collagen from two muscles.

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Abstract

Cross-linked collagenous peptides were isolated from tryptic digests of intramuscular collagen (IMC) prepared from *Longissimus dorsi* (LD) and *Semitendinosus* (ST) muscles. Peptides containing Ehrlich chromogen (EC) were selectively coupled from the cross-linked peptides using an affinity support. Peptides containing pyridinoline (Pyr) were isolated from the uncoupled material. Isolated EC and Pyr cross-linked peptides were purified by chromatographic procedures and subsequently characterised by amino acid analyses, amino acid sequencing and mass spectrometry. The predominant locus of EC in IMC was identical in both muscles, with the prosthetic groups of α2(I)-chain Hyl-933, α1(I)-chain Lys (Hyl)-9^N, and α2(I)-chain Lys (Hyl)-5^N forming the cross-link. Pyr occurred predominantly at one locus in both muscles, forming from an α1(I)-chain Hyl-87 and two α1(I)-chain Hyl-16^C residues. These results provide evidence, at the molecular level, that the IMC cross-linking is identical in these two muscles, supporting the hypothesis of Horgan *et al.* [Meat Science **29** (1991) 251-262] that the major determinant of collagen toughness of different muscles is the amount of collagen rather than its quality.

Introduction

The thermal stability of tendon collagen between 60 and 90 °C, as measured by isometric tension, has been correlated with its content of pyridinoline (Pyr) and Ehrlich-reactive pyrrole cross-links (EC) (Horgan *et al.* 1990). These cross-links occur in high concentrations in intramuscular collagen (IMC) (Horgan *et al.* 1991) so can affect meat texture by influencing the shrinkage and tension generated by the connective tissue matrix during the cooking of meat. Horgan *et al.* (1991) showed that muscles that varied greatly in collagen content had similar concentrations of Pyr and EC on a mole of cross-link per mole of collagen basis, and proposed that the major determinant of collagen toughness in cooked meat was the amount of collagen present. Despite the IMC concentrations of these cross-links being similar in different muscle types, their molecular location may differ which may affect collagen strength; therefore, we compared the molecular location of Pyr and EC in IMC from the *Longissimus dorsi* (LD), a low collagen muscle, and the *Semitendinosus* (ST), a high collagen muscle.

Materials and methods

LD and ST muscles were excised from the right sides of carcasses from twelve steers (22 to 24 months old) of three different breeds (Angus, n=7; Murray Grey, n=4; Shorthorn, n=1) following 24 h ageing at 5°C and were stored frozen at -20°C for 14 days. Thawed muscle was trimmed of overlying connective tissue and fat and diced to about 2 cm^3. Muscles were pooled within each muscle type, and an homogenate of each muscle type prepared by blending 900 g portions of the diced muscle in 4 L of demineralised water for 15 seconds at low then 15 seconds on high in a 5 L stainless steel Waring blender. The homogenate was filtered through 1.5 mm^2 plastic mesh. Material not passing through the mesh was rehomogenised in 800 g portions a further three times and the retained material defined as IMC. The IMC was extracted overnight in 15 L of 8 M urea at 25°C with stirring, then washed exhaustively with water and defatted by two overnight extractions in 12 L of chloroform/methanol (2:1 vol/vol). It was then allowed to dry at 25°C before storage at -70°C.

Dry IMC (200 g) was suspended in 10 L of 0.2 M ammonium bicarbonate containing 1 mM calcium chloride and denatured by heating at 65°C for 20 min. The suspension was cooled to 37°C and then incubated with 2 g of trypsin (TPCK-treated; Sigma Chemical Co.) for 5 h with continuous stirring, after which the suspension was heated to 65°C for 30 min. The digest was adjusted to pH 4.0 with acetic acid, then centrifuged at 9 000 x *g* for 1 h and the supernatant collected. The supernatant was pumped through an ultrafiltration module (Microza AIP-1010; Pall Australia) with a 6 000 molecular weight cut-off and the retentate collected.

EC peptides were isolated from the retentate by affinity coupling to a diazotised support prepared from polyacrylamide beads (Bio-Gel P10; 50-100 mesh; Bio-Rad Laboratories)(Kemp and Scott 1988). EC peptides were purified by molecular-sieve chromatography (Sephadex G50, superfine; 2.6 cm x 95 cm column) then applied to phosphocellulose ion-exchange columns (Whatman P11, 1.6 cm x 15 cm)(Kuypers *et al.* 1994). The uncoupled material was applied to a similar phosphocellulose ion-exchange column, with the fluorescent Pyr fraction excited at 295 nm and detected at 395 nm. Pyr peptides were further purified by DEAE ion-exchange chromatography (Whatman DEAE-cellulose, 1.6 cm x 17 cm) (Yamauchi *et al.* 1982). EC and Pyr peptides were finally purified by reversed-phase high performance liquid chromatography (HPLC) on C18 columns (Waters Delta Pak; 7.8 mm x 30 cm)(Kuypers *et al.* 1994). Purified EC and Pyr peptides were then characterised by amino acid analysis, amino acid sequencing and electrospray mass spectrometry.

Results and discussion

EC peptides isolated from LD and ST IMC produced very similar traces during all chromatographic procedures (all results not shown). Figure 1 shows the similar molecular weight profiles of the LD and ST EC peptides (panels A and B , respectively, Figure 1) which were virtually identical to that reported by Kuypers *et al.* (1994) for EC peptides from LD. Amino acid analyses, amino acid sequencing, and mass spectrometry of the HPLC-purified LD and ST EC peptides revealed that they all involved the α1(I)- and α2(I)-chain N-telopeptides cross-linked to the α2(I)-933 helical site at the C-terminus of an adjacent molecule, and that the prosthetic groups of α1-chain Hyl-or Lys-9^N, α2-chain Hyl- or Lys-5^N, and α2-chain Hyl-933 from type I collagen formed the Ehrlich-reactive pyrrole cross-link (results not shown). This locus has also been shown to be the preferred site of EC formation in tendon (Kuypers *et al.* 1992) and bone (Hanson and Eyre 1996); therefore, pyrrole cross-link formation appears to be ubiquitous at this site in type I collagen.

Pyr peptides isolated from LD and ST IMC produced very similar traces during all chromatographic procedures (all results not shown). The final purification reversed-phase HPLC chromatograms of the Pyr peptides from LD (panels A and C, Figure 2) and ST (panels B and D, Figure 2) show no differences due to muscle type. Amino acid and sequence analyses of these peptides revealed that those of panels A and B (Figure 2) were composed of residues α1(I) 993-22^C X α1(I) 993-22^C X α1(I) 76-90, while the major peaks of panels C and D (Figure 2) were α1(I) 993-22^C X α1(I) 8^C-22^C X α1(I) 76-90. In all these peptides, two prosthetic groups of α1-chain Hyl-16^C and one of α1-chain Hyl-87 formed the pyridinium cross-link (results not shown); therefore, all the Pyr in LD and ST IMC cross-links type I collagen and involves two α1-chain C-telopeptides and the α1-chain 87 N-terminus helical cross-linking site.

The results of this study have shown EC and Pyr both link three collagen α-chains of type I collagen, two telopeptides and one helical domain, but that EC cross-links N-telopeptide to helix while the Pyr cross-links C-telopeptide to helix. Based on these observations, the cross-linking of IMC appears to be the same in LD and ST muscles so the "quality" of the collagen is the same for both muscles. In light of the data presented by Dransfield (1977) which showed that the collagen content of ST muscle is twice that of LD muscle, the hypothesis that the amount of collagen is the major determinant of collagen toughness within and between muscle types (Horgan *et al.* 1991) is supported, on the molecular level, by the findings reported here.

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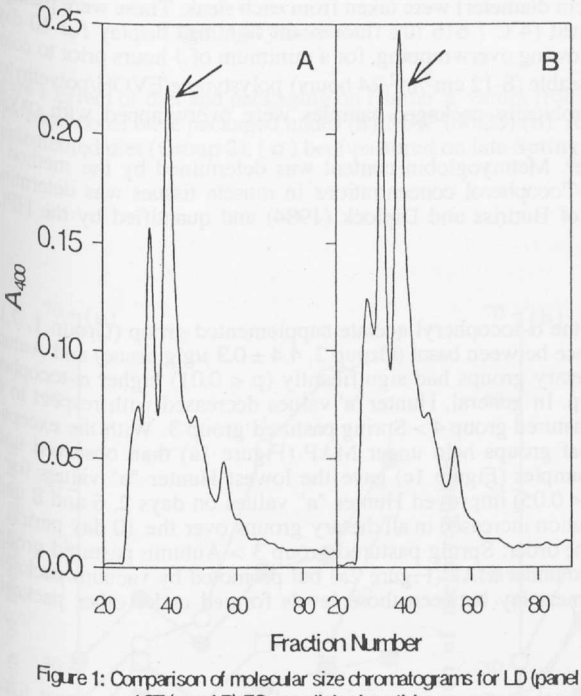

Figure 1: Comparison of molecular size chromatograms for LD (panel A) and ST (panel B) EC crosslinked peptides.

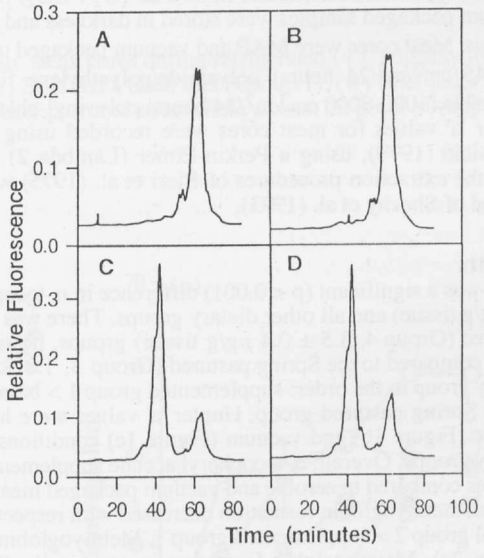

Figure 2: Comparison of HPLC traces of Pyr peptides from LD (panels A and C) and ST (panels B and D) IMC.