Detection and Estimation of Collagen[a]

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Collagen exists in at least five genetically distinct forms and the relative abundance of these is determined by the type and age of the tissue. Methods are presented for the detection and estimation of these collagens based on appropriate chemical, physical, histochemical and immunological techniques. The specific difficulties in providing accurate quantitative data for collagen in meat and meat products are discussed.

1. Introduction

Collagen is the most abundant protein in the body and is present mainly in bone, skin and tendon. It is found in muscle as 1–9% of the dry, fat-free mass[1] where it exists as networks of fibres. In the living muscle these fibres resist over-extension which may cause damage to the tissue. The force of contraction is transmitted to the tendons through sheets of intramuscular connective tissue which enclose the individual muscle fibres. Three collagenous structures can be distinguished morphologically—endomysium, enclosing each fibre, perimysium, surrounding bundles of these fibres, and epimysium, surrounding the whole muscle (Figure 1). The subunit of the collagen fibre

Figure 1. Connective tissues of muscle. Each muscle fibre is enclosed in a delicate collagenous meshwork, the endomysium. Bundles of these muscle fibres, fasciculi, are surrounded by a thicker sheath of connective tissue, the perimysium. The strong outer layer of connective tissue, the epimysium, is continuous with the peritendineum. The collagen fibres in the tendon appear to connect with both the perimysium and the endomysium.
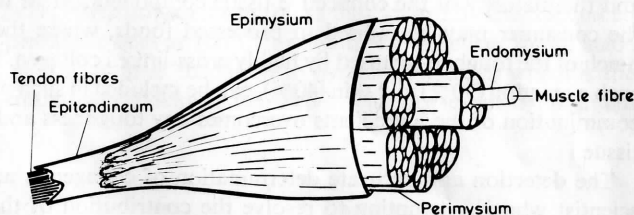
is tropocollagen, or the collagen monomer, which is a long rod-like molecule, 1.5 μm in diameter and 300 μm long. Tropocollagen has a molecular weight of about 300 000 daltons and is comprised of three polypeptide chains wound into a helix. These have a high content of glycine, proline and hydroxyproline located in regular repeating sequences of Gly, Pro, Y and Gly, X, Hyp.[2] In the collagen fibril the tropocollagen molecules are aligned in parallel and held together by covalent intermolecular cross-links. The structural features of collagen are shown in Figure 2.

The development of these cross-links is an age-related process and it is the increase in the number of the heat-stable covalent attachments that makes meat from older animals much tougher on cooking.[3] Therefore, in assessing the quality of meat it is necessary to consider both the quantity

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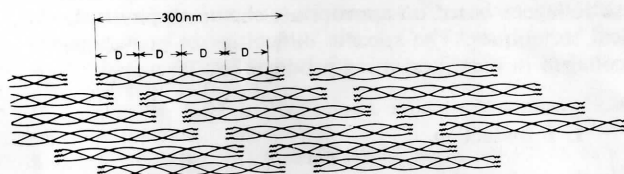
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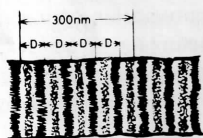
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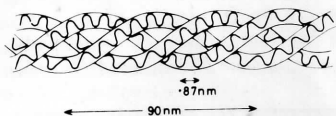
3. COLLAGEN FIBRIL

4. COLLAGEN FIBRE

Figure 2. Structure of collagen. (1) In the primary sequence of the α-chain glycine is found predominantly at every third residue. In these triplets with glycine at the first position, proline is located generally at the second and hydroxyproline at the third position. (2) The α-chain takes up a tight helical conformation and the three α-chains of tropocollagen are wound together in a super triple helix. (3) The collagen fibril is composed of many rod-like tropocollagen molecules aligned in parallel but showing a regular quarter-stagger end overlap with the repeat distance (D) being 64 nm. The fibril is stabilised by covalent cross-links. These cross-links (not shown) are generated at the non-helical ends of the molecule and connect with the helical region of adjacent molecules. (4) It is this precise arrangement of the tropocollagen molecules in the fibre that produces the regular cross-striations typically revealed in the electron microscope.

and the maturity of the connective tissue component. Meat that is less desirable for direct sale to the consumer may be utilised in processed foods, where the high cooking temperature reduces much of the toughness caused by highly cross-linked collagen. Furthermore, tissues rich in collagen, such as tendon (90%) and skin (40%), can be included in such products as sausages where mechanical comminution of the ingredients overcomes any toughness and effectively disperses the collagenous tissue.

The detection and accurate determination of collagen is an essential requirement for the meat scientist who is attempting to resolve the contribution of the connective tissues to the structure and quality of meat. The food technologist also requires reliable analytical techniques to help maintain a uniform product that will also conform to any regulations governing composition. There are several experimental approaches to the detection and estimation of collagen, depending upon the nature of the investigation. These various methods will be considered separately.

2. Chemical methods

Proline is incorporated into collagen to >20% of the total amino acids and nearly half is hydroxylated to 4-hydroxyproline immediately following synthesis of the polypeptide chain. The large amount of hydroxyproline in collagen is a unique feature of this protein and is generally found to be about 14% of the dry weight of collagen. Therefore, chemical methods for the detection of collagen are conveniently based on the determination of protein-bound hydroxyproline in the tissues. It is because of this unusual composition that most interest has been given to the development of accurate methods for measuring this imino acid. In recent years different phenotypes of collagen have been identified and purified and the amino acid composition for each collagen type is now known.[4, 5] The hydroxyproline content is lowest in type I collagen, found mainly in bones

and tendons, and highest in the type III collagen which is abundant in arterial walls and embryonic tissues. The recent data on composition show that hydroxyprolyl residues are present in the major phenotype of collagen, type I, to the extent of approximately 11.3% by weight. The weights of the hydroxyprolyl residue in type II cartilage collagen and the type IV collagen of basement membranes are found to be 12.9 and 14.3%, respectively,[4] and about 15.0% in type III collagen.[4,5] However, all chemical determinations are made on the free imino acid after hydrolysis of the protein. When a correction is made for the addition of water, the hydroxyproline value of type I collagen is raised from 11.3 to 13.1%. The corrected values for the free imino acid of the other collagens are: type II—15.0%, type III—17.4% and type IV—16.6%. Clearly then, the actual hydroxyproline value for a mixed connective tissue will vary according to the ratio of these different types. Overall, the traditionally applied value of 14% is probably a fair approximation for muscle connective tissue.

Hydroxyproline is also found in small amounts in other proteins such as the collagen-like sequences found in acetylcholinesterase and the subcomponent of serum complement, Clq.[6] Elastin (1.5% hydroxyproline) is found mainly in ligaments, arterial walls and, to a lesser extent, in lung and skin.[6] Unless this is physically separated out it can interfere in the collagen hydroxyproline determination. The amount of elastin in whole muscle is generally low but where significant amounts are believed to be present, as in processed meats, the elastin hydroxyproline is best determined separately. Elastin is insoluble in hot alkali[7] and this extractant can be used to remove all of the collagen as soluble gelatins. The residue of insoluble elastin is then analysed for hydroxyproline and the amount deducted from the value determined for the whole tissue.

For the determination of muscle connective tissue some laboratories prefer to extract as much of the myofibrillar protein as possible before analysing for hydroxyproline. Extraction is performed with a strong potassium iodide solution and this may be followed by chloroform–methanol which is used to remove any lipids.[8] In this laboratory the preferred method is to extract initially with Hasselbach–Schneider pyrophosphate solution[9] which dissociates the actomyosin complex and facilitates treatment with KI. The main disadvantage of this extraction procedure is that some poorly cross-linked collagen, particularly in the muscles of young animals, may be lost.

Tissues to be analysed are first acid hydrolysed to release the hydroxyproline from peptide linkage. This is generally achieved using 6M HCl at 110°C for 18–24 h, either in sealed tubes or under reflux with an air condenser. For rapid hydrolysis it was found that 3 h at 130°C is adequate.[10] Other workers have employed 72% perchloric acid at 100°C for 2–4 h.[11,12] The free imino acid is most conveniently determined colorimetrically after oxidation to pyrrole (Figure 3) which is then reacted specifically with p-dimethyl-amino-benzaldehyde (Ehrlich's reagent) to yield an intense red-brown colour.
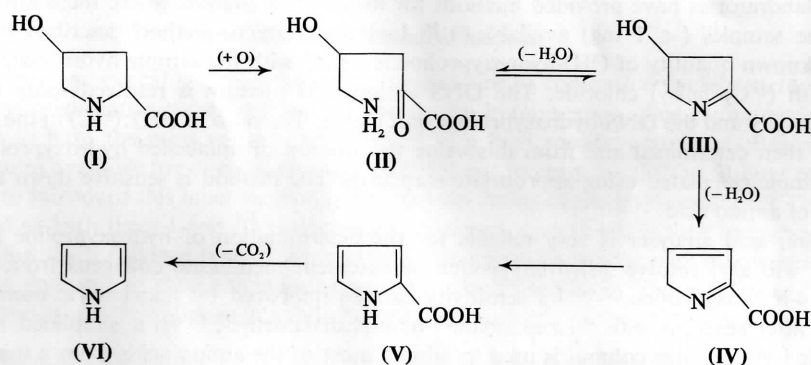

Figure 3. Postulated mechanism for the oxidation of hydroxyproline to pyrrole. Hydroxyproline (I) is oxidised first to the linear α-keto-γ-hydroxy-δ-aminovaleric acid (II), which is in equilibrium with the cyclic structure Δ'-pyrroline-4-hydroxy-2-carboxylic acid (III). Loss of water gives an unstable structure (IV) which spontaneously rearranges to pyrrole-2-carboxylic acid (V). The final decarboxylation step to pyrrole (VI) occurs during heating after the addition of the chromogenic reagent for pyrrole, p-dimethylaminobenzaldehyde.

In 1970 Mitchell and Taylor[13] critically examined the available methods and concluded that no single method could combine accuracy with freedom from errors caused by interferring substances. Consequently, several different experimental approaches can be found in the literature, depending upon the nature of the test material.

Chloramine T is now generally preferred for use as an oxidant in the formation of pyrrole. For collagen-rich materials three methods[10, 14, 15] are in common use and that described by Woessner[10] is probably the most popular. The International Standards Organisation method[16] has been developed from these specifically for the determination of hydroxyproline in meat or meat products. Here, $SnCl_2$ is incorporated into the acid used for hydrolysis and reduces the losses caused by side reactions. The colour yield from hydroxyproline can be depressed by such substances as other amino acids and salts. However, in tissues low in collagen, such as muscle, there is usually an overestimate of the amount of hydroxyproline, and in this laboratory the colour value for muscle hydroxyproline is found to be increased by as much as 15–20%. The reasons for this are unclear but may be caused by the weak chromogenic reaction of certain amino acids, e.g. tyrosine. Woessner[10] claimed that for hydrolysates of tissues in which hydroxyproline is <2% of the total amino acid content the colour value could not be accepted as accurate. Woessner's method II[10] eliminates this excess colour by using benzene as the solvent extractant—this removes any contaminants and leaves only the chromogen derived from hydroxyproline in the aqueous layer. Bergman and Loxley[17] overcame much of the problems arising from contaminants by modifying the conditions for chromogen formation. They reacted the oxidised hydroxyproline with p-dimethylamino-benzaldehyde reagent overnight at room temperature, instead of at 60°C for 20 min as in Woessner's procedure.

Several accurate methods exist to correct for the depression of colour yield by such interferring substances as usually occur in urine.[18–20] In these methods colour development is made in the presence of added alanine and saturated KCl and the hydroxyproline chromogen is separated from other pigments by extraction with toluene. Prockop and Udenfriend[18] also included a charcoal–resin step to adsorb the urinary pigments although in more recent methods[19, 20] this is not necessary. These elaborate methods are less suited for routine assay, but where accuracy is required they do provide a reliable means for the determination of hydroxyproline in whole tissues or processed foods.

Where the need is for routine analysis without correction, then autoanalyser methods offer a convenient means for handling large numbers of samples. The method described by Grant,[21] derived from Woessner's manual method I, is used in this laboratory and is accurate in the range 1–8 μg ml^{-1} hydroxyproline for neutralised hydrolysates of collagenous tissues. The method described by Bannister and Burns[22] is based on Bergman and Loxley's manual method,[17] but the sensitivity is reduced as the reaction times are shorter.

Several laboratories have provided methods for micro-scale analysis where there are only very small tissue samples (< 1 mg) available.[23–25] In the ultramicro-method described by Airhart et al.,[26] a known quantity of (^{3}H)-hydroxyproline is mixed with the sample hydrolysate and then reacted with (^{14}C)-dansyl chloride. The DNS amino acid mixture is resolved using thin layer chromatography and the DNS-hydroxyproline spot eluted. The ratio of (^{3}H):(^{14}C) in the recovered fraction is then determined and from this value the amount of unlabelled hydroxyproline in the original sample calculated using appropriate standards. The method is sensitive down to as little as 2 pmol of amino acid.

The amino acid analyser is very reliable for the determination of hydroxyproline in the μM range and will also resolve 3-hydroxyproline of basement membrane collagens from the more abundant 4-hydroxyproline.[27, 28] Its sensitivity can be improved by fluorimetric monitoring of the eluate after reaction with fluorescamine[29] or o-phthalaldehyde.[30] In a simplified method, a short acidic ion exchange column is used to adsorb most of the amino acids from a tissue hydrolysate and the unadsorbed hydroxyproline then determined using a standard colorimetric method.[31] Several published methods exist for the sensitive determination of both 3-hydroxyproline and 4-hydroxyproline by gas–liquid chromatography[32–35] using volatile derivatives, such as the trifluoracetyl butyl esters[32, 33, 35] or N-heptafluorobutyryl isobutyl esters,[34] which can be prepared quantitatively after acid hydrolysis of the collagen.

3. Physical methods

Recently a nuclear magnetic resonance technique was investigated as a means for determining hydroxyproline in meat and meat products.[36] The method examines the peak heights of the ^{13}C-Fourier transform spectrum in which the peak for the carbon-4 in hydroxyproline is compared with the β-carbon of the internal standard (β-hydroxy-α-methyl phenethyl ammonium chloride). The method is expensive to operate and is not immediately applicable to routine analysis but with further development it could be applied directly to unhydrolysed materials. High levels of carbohydrate were found to obscure the reference signals in the spectra which limited the potential usefulness of this method for certain processed foods.

4. Histochemical methods

The structural organisation of the connective tissues can be examined by histochemical techniques. Van Gieson's stain[37] was introduced during the last century for the specific detection of collagen fibres. Under the microscope collagen present in connective tissues appears bright red. More recently, Sirius supra red F3BA was introduced as an alternative to Van Gieson's stain.[38] A method is now available for the spectrophotometric determination of soluble collagen as a conjugate with Sirius supra red F3BA.[39] Lillie *et al.*[40] have examined the specificity of Victoria Blue R stain. In combination with phosphomolybdic acid or phosphotungstic acid, two histochemically distinct forms of collagen have been revealed. They claim that dense fibrous tissue (dark blue) can be distinguished from the fine fibres of fatty and areolar tissues (light green). Basement membranes assume a deep purple tint when the PAS reaction is subsequently performed on stained sections.

At least five genetically distinct forms of collagen are now known to exist and the locations of the different collagen types have been examined by immunohistochemical techniques. Bailey *et al.* have prepared specific antibodies for the five collagen types from several different species. The indirect immunofluorescent or sandwich method was used to investigate the distribution of the collagen types in muscle. Type I collagen was located in the epimysium and perimysium. The fine lace-like fibres of type III collagen were located only in the perimysium. The reticulin is now believed to be type III collagen. Type V collagen is associated with basement membranes and in muscle this collagen type was found specifically in the endomysium which encloses each muscle.[41] These authors also found that the tougher muscles in the carcass possessed an increased amount of perimysial type III collagen. The relationship, if any, of this increased content of type III collagen to toughness is not known. Immunohistochemical techniques are proving increasingly useful for qualitative assessment of the distribution and organisation of the different collagen phenotypes.

5. Immunochemical methods

The immunohistochemical techniques for the examination of connective tissue structure are now allowing the quantitative determination of collagen by immunoassay. For radioimmunoassay it is essential to have the pure antigen radiolabelled to a high specific activity. Collagen has very few side chains available for direct iodination with ^{125}I but recently the Bolton–Hunter reagent has been used to introduce this label by modifying the side chains of the lysyl residues.[42] The method was applied to both types I and III collagens which were shown to retain their immuno-reactivity after labelling. Each collagen type in solution was determined by mixing with a known amount of ^{125}I-labelled antigen and then precipitating with the specific antibody. The amount of ^{125}I in the immune complex was found to be inversely related to the amount of the unlabelled sample collagen present.

The enzyme-linked immunosorbent assay (ELISA) pioneered by Engvall and Perlamann[43] may, with suitable modification, eventually supersede radioimmunoassay for the determination of antigenic components.[44]

Recently, an ELISA technique was described for the specific assay of various connective tissue components including collagen types I, II, III and IV[45] (Figure 4). The method can be applied only
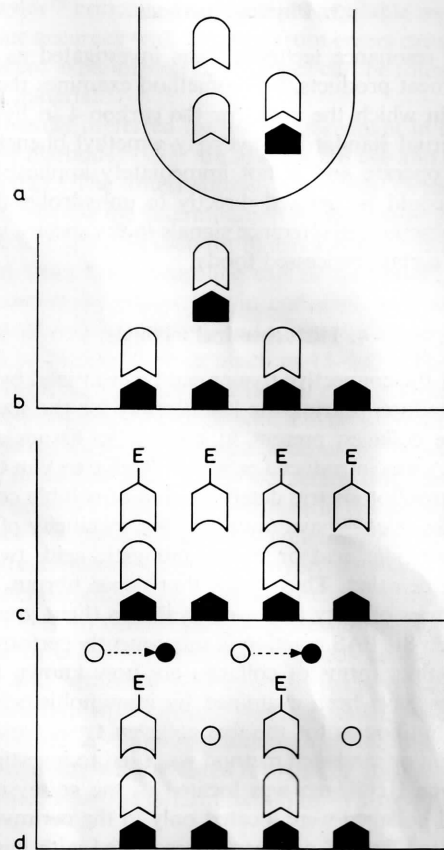
Figure 4. The enzyme-linked immunosorbent assay (ELISA) technique for immunoassay can be modified for the assay of collagen antigens. (a) An unknown amount of type-specific soluble collagen antigen (solid symbol) is mixed with a known and excess amount of the appropriate antibody (open symbol) in a suitable reaction tube. (b) The mixture of antigen–antibody complex and excess antibody is then added to a microtitre well in which a known amount of antigen is adsorbed. Here all of the excess antibody complexes with part of the available antigen. (c) Enzyme linked to a second antibody, raised against the first antibody, is then applied and binds only to the sites where the original antigen is complexed. (d) The amount of immobilised enzyme is then determined by a standard chromogenic assay. The system is calibrated by mixing the antibody in (a) with known amounts of antigen.

to antigens in solution, but the ELISA system has the advantage that any suitable enzyme can be used for the estimation—and with potentially greater sensitivity than radioimmunoassay. Furthermore, ^{125}I has a short half-life whereas the enzyme-labelled antibody should have a longer storage life.

In these assays there was no cross reaction—this is essential for the assay of phenotype. However, where the need is for determination of the total collagen content there is no suitable non-specific immunoassay available. There may be common antigenic sites in the different collagen types which could be selected for such an assay, but this has not yet been determined.

6. Conclusions

In recent years it has become clear that collagen is not a single biochemical entity but exists in several genetically distinct forms which may be closely associated with varying amounts of glycoprotein, lipids, salts and of course, water. The analytical methods for the detection of collagen,

therefore, must be selected according to the type of tissue. Accurate determination of collagen is not always possible using the simpler colorimetric methods because the level of interferring substances may be quite high and for some materials, e.g. processed foods, the nature of these substances is not necessarily known or whether they have any predictable effect on chromogen formation. The use of internal standards is advisable where a high degree of accuracy is required. Alternatively, a chromatographic method may be more suitable. If new and more stringent legislation is introduced, such that the collagen content of meat products must be known with certainty, then this will increase the need for routine methods of analysis which are simple, reliable and sufficiently rapid to avoid undue delays during processing.

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