THE NISHIHARA TECHNIQUE FOR THE SOLUBILIZATION OF COLLAGEN

APPLICATION TO THE PREPARATION OF SOLUBLE COLLAGENS FROM NORMAL AND RHEUMATOID CONNECTIVE TISSUE

BY

F. S. STEVEN

*Department of Physiology and Biochemistry, St. Salvator's College,
University of St. Andrews, Scotland*

Aged tendon and connective tissue contain very little tropocollagen which may be extracted by dilute solutions of neutral salts or acetic acid (Jackson and Bentley, 1960). Collagen from these tissues is usually prepared by mechanical removal of non-collagenous materials (Neuman, 1949), or by the thermal conversion of the insoluble collagen to its derived soluble gelatin (Jackson, 1957).

A recent Japanese patent (No. 9295/63, Nishihara, 1963) describes the isolation of soluble collagen from ox-hide after pre-treatment of the minced tissue with α -amylase and extraction of the collagen in dilute hydrochloric acid. This technique seems to offer great advantages over the previous methods and it has been applied successfully to the large scale isolation of soluble collagen from connective tissue obtained from normal and rheumatoid arthritic patients. Hip joint tissue used in this work was kindly provided by Mr. J. Charnley of the Wrightington Hospital, Wigan. Knee joint tissue was provided by Mr. Jack Stevens, Western Infirmary, Glasgow.

Method

The technique consists of homogenizing the tissue in 5 per cent. w/v NaCl and repeatedly extracting in this solution until the supernatant fraction is protein-free. The tissue is then washed exhaustively with water and then suspended in 0.22 M NaH_2PO_4 with the pH adjusted to 5.4 (pH meter). To the suspension is added a solution containing 0.3 per cent. w/w crude α -amylase calculated on the basis of the weights of enzyme and the original tissue. The reaction mixture is stirred at room temperature (ca. 18° C.) for 90 hrs. The suspension is then washed repeatedly with 5 per cent. NaCl followed by water and finally homogenized in a large volume of 0.2 M acetic acid. The pH of the suspension is adjusted to pH 3.1–3.3 with *N* acetic acid and the mixture stirred continuously for 24 hrs. The supernatant solution containing dissolved collagen is removed by centrifugation and the residue re-extracted with acetic acid. The

combined supernatant fractions are brought to pH 7–8 with *N* NaOH and the collagen is collected on the end of a vigorously rotated spatula. The tissue residue after two acid extractions is virtually collagen-free, and can be discarded.

Results

The collagen isolated by the method appears to differ from the normal acetic acid-soluble tropocollagen prepared from the skins of young animals in its reduced solubility in dilute acetic acid and is about five to ten times less soluble than soluble calf-skin collagen. Dilute solutions of calf-skin collagen may be readily filtered through cotton wool without precipitation of protein on the filter pad; however, the soluble collagens prepared from aged human tissues rapidly precipitate on cotton-wool, muslin, and other surfaces with the formation of a mass of fibrous collagen, and only a small amount of soluble collagen passes through the filter. This filtrate showed a tendency to precipitate spontaneously in long fibrils on standing at room temperature. Collagen prepared as described above, when dissolved in 0.2 M lactic acid, exhibits intense streaming birefringence and forms a fine network of fibres when stirred.

It is probable that collagen isolated by the Nishihara technique from aged human tissue is largely composed of highly polymerized collagen molecules which readily form nuclei for fibril formation when correctly orientated at neutral pH. This would be expected from the mass of evidence for the increase of chemical cross-linking in collagenous tissues during the ageing process (Jackson and Bentley, 1960).

Discussion

Proteolytic enzymes have previously been employed to pre-treat ox-hide before extraction of collagen (Nishihara, 1962). The resultant collagen

preparations have lower intrinsic viscosities than tropocollagen obtained from young animals. The same reduction in viscosity was observed when tropocollagen was digested with pepsin (Nishihara, 1962; Nishihara and Miyata, 1962; Steven, 1963) and this was attributed to the limited attack of the enzyme on the teleopeptides appended to tropocollagen (Hodge, Highberger, Deffner, and Schmitt, 1960; Rubin, Pfahl, Speakman, Davison and Schmitt, 1963).

In the technique described above, the α -amylase was demonstrated to possess no proteolytic activity when assayed at pH 5.4 in the pH-stat against gelatin. This suggests that the action of the crude amylase preparation used in this study acted on some type of bond other than the peptide bond, with the result that the previously insoluble collagen of the connective tissue became capable of solution in acetic acid. This suggestion infers that the mature collagen of aged connective tissue is stabilized either by covalent linkages which are absent from tropocollagen or by a protective matrix (possibly carbohydrate in nature) which prevents the solution of collagen by acetic acid. The latter explanation seems unlikely in view of the fact that the tendon collagen swells in acetic acid but does not dissolve. Further evidence on the depolymerization of Nishihara solubilized collagens is to be published elsewhere.

The present technique allows the isolation of highly polymerized collagen from small samples of connective tissue. The chemical structures of intact collagens obtained from normal and rheumatoid joints may now be compared by the technique of fingerprinting (Ingram, 1956) their respective collagenase digests. Previously it has been possible to compare only the structures of the derived gelatins; Steven (1964) used proteolytic enzymes to purify the collagens from which the gelatins were obtained, and as a consequence the gelatins may well have been deficient in their teleopeptides which are known to be degraded by proteolysis under these conditions. End-to-end polymerization of tropocollagen to form native type fibrils is possible only if the teleopeptides are intact (Hodge and others, 1960) and it is conceivable that alterations in the chemical structure of these teleopeptides may define whether normal or pathological types of collagen are laid down in connective tissue. It should now be possible to prepare teleopeptides from collagens isolated from normal and rheumatoid connective tissue.

Summary

A method has been described for the extraction of native soluble collagen from aged tendon. Pre-

treatment of the tissue with α -amylase resulted in the destruction of covalent linkages, which stabilized the connective tissue collagen, with the subsequent extraction of collagen in dilute acetic acid. This collagen appeared to be a highly polymerized form of tropocollagen and has its teleopeptides intact.

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Le procédé de Nishihara pour la solubilisation du collagène.—Son application à la préparation de collagènes solubles à partir des tissus conjonctifs normal et rhumatoïde

RÉSUMÉ

On décrit une méthode pour extraire le collagène soluble natif du vieux tendon. Le traitement préalable du tissu par l' α -amylase produit la destruction des liaisons covalentes, ce qui stabilise le collagène du tissu conjonctif, après quoi on peut extraire le collagène dans l'acide acétique dilué. Ce collagène semble être une forme très polymérisée du tropocollagène et ses teleopeptides sont intacts.

El procedimiento de Nishihara para solubilizar el colágeno.—Su aplicación a la preparación de colágenos solubles de tejidos conectivos normal y reumatoide

SUMARIÛ

Se describe un método para extraer el colágeno soluble nativo del viejo tendón. Un tratamiento previo del tejido por α -amilase produce la destrucción de los nexos covalentes, lo que estabiliza el colágeno del tejido conectivo con su extracción subsiguiente en el ácido acético diluido. Este colágeno parece ser una forma altamente polimerizada del tropocollágeno y tiene sus teleopeptidos intactos.