SEPARATION OF PROLINES AND HYDROXYPROLINES BY GAS CHROMATOGRAPHY

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INTRODUCTION

A recent report describes the presence of new isomers of hydroxyproline in collagen[1]. This finding poses several problems about the usefulness and the specificity of the methods commonly used for the determination of proline and hydroxyproline in normal or pathological tissues. The methods available are usually based upon colorimetric determinations[2–5].

It was therefore interesting, in relation to previous work[6], to develop a more specific procedure by means of gas chromatography for the separation of proline and hydroxyproline congeners.

EXPERIMENTAL

The present method was used for the resolution of the following amino acids: proline; 3,4-dehydroproline; 3-*trans*-hydroxyproline; 3-*cis*-hydroxyproline; 4-hydroxyproline and 4-*allo*-hydroxyproline.

The hydrochlorides of the amino acids (0.5 mg each) were esterified with 10 ml of *n*-butanol in the presence of a strong cationic resin (Amberlyst 15, kindly supplied by Rohm and Haas Co.). The mixture was gently rotated at 120° for one hour. The ratio between amino acids and resin was 1:20 (w/w).

After esterification, the *n*-butanol was decanted and the resin was filtered on glass under slight vacuum. The dry resin was suspended for 15 min at room temperature in 5 ml of benzene to which 1 ml of acetyl chloride had been added. The resin was washed with 5 ml aliquots of benzene until the liquid was neutral.

The dry resin was then mixed with 10 ml of benzene containing 0.2 ml of *n*-butylamine and the mixture was refluxed at 70–80° for 15 min. The resin was discarded, and the benzene solution containing the esters of the imino acids was concentrated under vacuum (22 mm Hg) in an ice bath.

The residue was then trifluoroacetylated at room temperature with 0.2 ml trifluoroacetyl anhydride in 5 ml of methylene chloride with occasional stirring. After 30 min the excess reagent was removed under vacuum (22 mm Hg) in an ice bath.

The final residue was dissolved in 0.25 ml of methylene chloride and 2 μl injected into the chromatographic column with a Hamilton syringe. The gas chromatography apparatus used was a Fractovap C (Carlo Erba, Milano) with a hydrogen-flame ionization detector.

RESULTS

Figs. 1 and 2 illustrate the gas-chromatographic separation of imino acid derivatives using two different stationary phases.

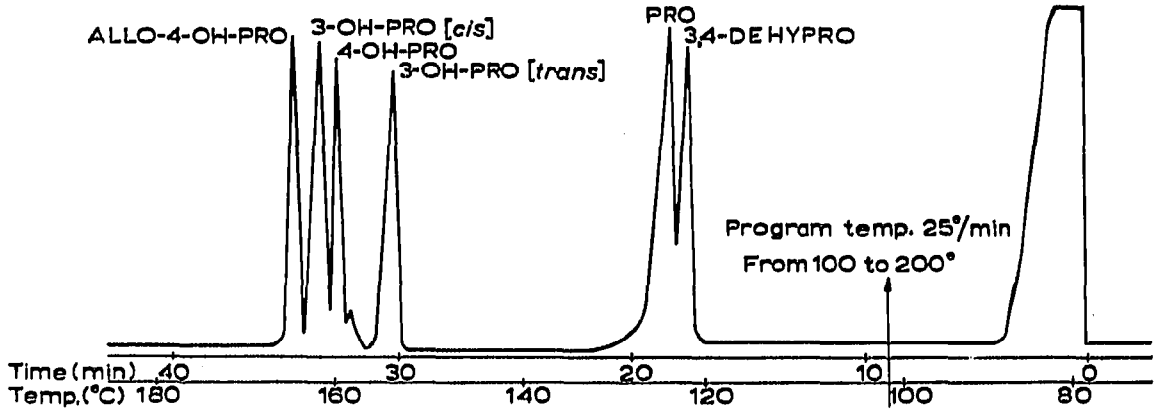

Fig. 1. Gas chromatogram of a mixture of imino acid esters. Column (2 mm internal diam. × 2 m) packed with Carbowax 20 M (1 %) on Chromosorb 80–100 mesh; starting temperature 100° with a programmed temperature increase of 2.5°/min up to 200°. Carrier gas: H_2 at a flow rate of 15 ml/min.

It was only possible to achieve a satisfactory separation with 1 % Carbowax 20 M. When working with 1 % QF 1, there was no resolution between 3-*cis*-hydroxyproline and 4-hydroxyproline. The retention times of the various proline derivatives on the two stationary phases employed are listed in Table I.

The method described has been applied to the determination of proline derivatives in proteins.

Both bovine serum albumin and gelatin contain proline but they differ in the 4-hydroxyproline content.

Gelatin or serum albumin (100 mg) were hydrolysed in HCl (6 *N*) for 24 h at

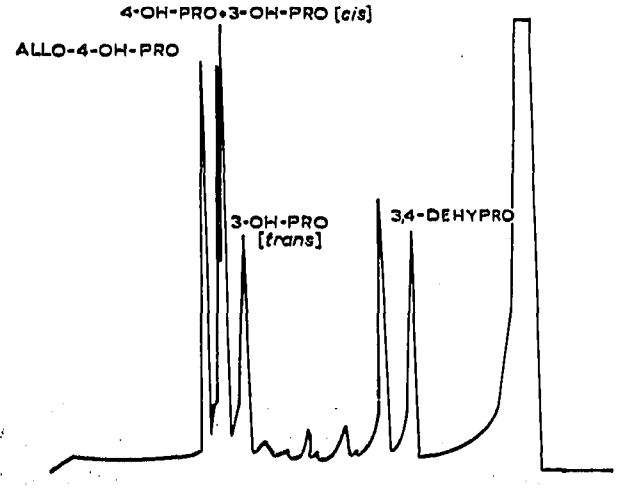

Fig. 2. Gas chromatogram of a mixture of imino acid esters. Column (2 mm internal diam. × 1.5 m) packed with QF 1 (1 %) on Chromosorb 100–120 mesh; starting temperature 125° with a programmed temperature increase of 5°/min to 200°. Carrier gas: H_2 at a flow rate of 15 ml/min.

TABLE I

RETENTION TIMES OF VARIOUS PROLINE DERIVATIVES

Imino acid ester	*Retention time on Carbowax 20 M (1%) (min)*	*Retention time on Q.F. 1 (1%) (min)*
3,4-Dehydroproline	17	4
Proline	18	5
3-*trans*-Hydroxyproline	30	10
4-Hydroxyproline	32	11
3-*cis*-Hydroxyproline	33	11
4-*allo*-Hydroxyproline	34	12

100°. Hydrolysates were then evaporated to dryness under vacuum. Nitrous acid (1 ml/mg of amino acid) was added to the hydrolysate residue to destroy the amino acids but not the imino acids present. In order to avoid the destruction of 3,4-dehydroproline, the nitrous acid must be prepared with sodium nitrite and acetic acid, instead of the usual sodium nitrite and hydrochloric acid. Imino acid N-nitroso derivatives were hydrolysed with hydrochloric acid and then esterified and acetylated according to the procedure previously described.

Figs. 3 and 4 illustrate the gas chromatographic separation of imino acid esters prepared from gelatin and bovine serum albumin. As expected albumin contained

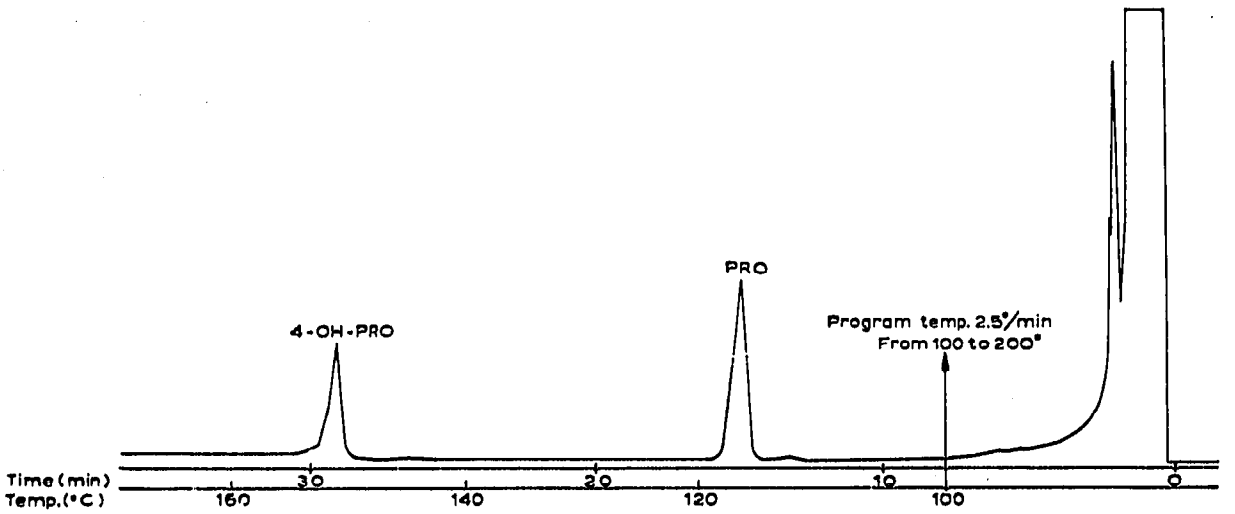

Fig. 3. Gas chromatogram of proline esters obtained on hydrolysis of gelatin. Experimental conditions as in Fig. 1.

only proline, while gelatin contained both proline and 4-hydroxyproline. There was no evidence of the presence of other peaks, at least for the amounts of protein used.

The method described can be used for the identification and the determination of prolines and hydroxyprolines in different tissues in relation to the studies on collagen metabolism.

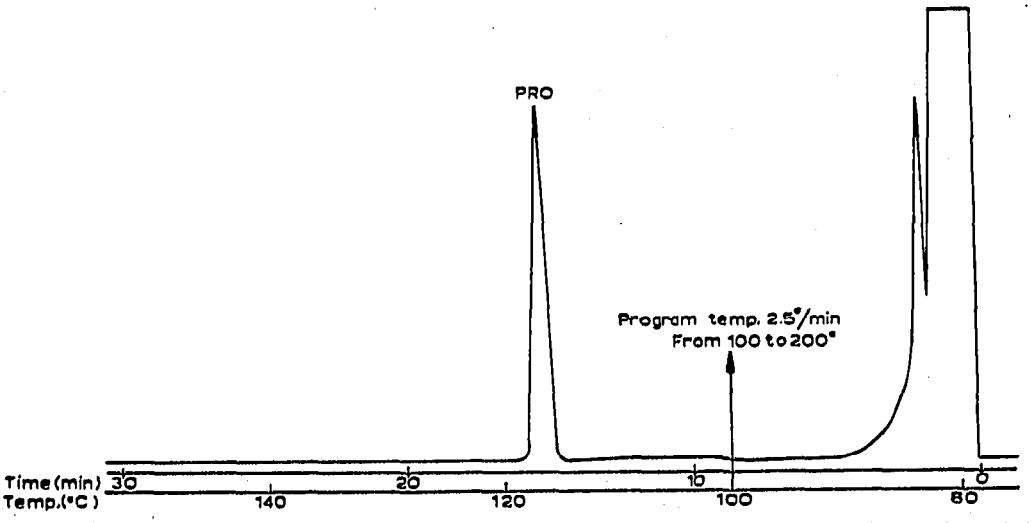

Fig. 4. Gas chromatogram of proline esters obtained on hydrolysis of bovine serum albumin. Experimental conditions as in Fig. 1.

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SUMMARY

A gas-chromatographic method for the separation of proline, 3,4-dehydroproline, 3-*trans*-hydroxyproline, 3-*cis*-hydroxyproline, 4-hydroxyproline and 4-*allo*-hydroxyproline is described. The procedure has been applied to the detection of imino acids in gelatin and bovine albumin.

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