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A MICRO-METHOD FOR THE DETERMINATION OF HYDROXYLYSINE AND ITS GLYCOSYLATED DERIVATIVES

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SUMMARY

A simple method is described, using high voltage electrophoresis for the estimation of hydroxylysine, hydroxylysine galactoside and hydroxylysine glucosidogalactoside in alkaline hydrolysates of proteins.

Hydroxylysine and their glycosylated derivatives have characteristic migration properties; high amounts of salts (even 10–15 times the weight of protein) do not interfere with their separation and quantitative estimation.

INTRODUCTION

Collagen contains small quantities of carbohydrates[1] in the form of galactose and 2-O-α-D-glucopyranosyl-O-β-D-galactopyranose, linked by an O-glycosidic bond to the hydroxylysine[2] of the polypeptide chain. The ratio of the mono- and disaccharide units may be involved in the tissular organisation, as can be concluded from the variations of this ratio in collagens from different organs or species representing different levels of evolution[3–9]. It may also be characteristic for the pathological alterations of collagen[10,11].

The methods described for the detection and determination of hydroxylysine galactoside and hydroxylysine glucosidogalactoside involve the alkaline hydrolysis of the tissue[2,7] or its enzymatic digestion[4,12].

The O-glycosidic linkage is quite stable during the alkaline hydrolysis. After 24 h at 100° in 2 *N* NaOH about 90% of the hexoses can be recovered[13]. Consequently, a quantitative estimation of the hydroxylysine glycosides is possible in these hydrolysates. The methods reported imply the use of automated ion-exchange techniques, but in the case of alkaline hydrolysates the Hyl-Gal peak overlaps with other amino acids[7]. Therefore, for the determination of the Hyl-Gal-Glc/Hyl-Gal ratio it is necessary to perform a second analytical run on an acid hydrolysate[7].

A semi-quantitative estimation of the Hyl-Gal-Glc/Hyl-Gal ratio has been obtained by high voltage electrophoresis of the hydrolysate followed by photodensitometry of the ninhydrin positive spots or by determination of the weight of the

eluted substances[4,5]. All the methods described require previous desalting of the hydrolysate.

In the present study a quantitative paper electrophoretic method is described for the separation and determination of the ratio of lysine, hydroxylysine, hydroxylysine-galactoside and hydroxylysine-glucosido-galactoside in a crude alkaline hydrolysate of the proteins.

Electrophoretic techniques, although less accurate than the ion-exchange methods for the determination of amino acid derivatives, have the advantage of rapidity and permit several simultaneous determinations to be carried out.

MATERIALS AND METHODS

Material

Hydroxylysine-galactoside and hydroxylysine-glucosido-galactoside were prepared from the low molecular weight glycopeptide fractions of the calf corneal stroma[1] and pig aorta[14] by a preparative electrophoretic separation of the desalted alkaline hydrolysates. The substances were analysed for sugars and amino acids. One of the substances was composed of galactose, glucose and hydroxylysine, the other one of galactose and hydroxylysine, in the ratios 1:1:1 and 1:1, respectively. The electrophoretic and chromatographic mobilities of these compounds were identical to those of an authentic sample[2,4].

Hydrolysis of the samples

Samples of 1–2 mg of protein or tissue containing about 40–60% collagen were hydrolysed in 50–100 μl 2 *N* NaOH at 105° for 24 h in sealed pyrex or capped polypropylene tubes. The hydrolysate was acidified with 2.2 volumes of 1 *N* acetic acid and centrifuged. The supernatant was evaporated to dryness *in vacuo* over KOH pellets and dissolved in 15 μl water/1 mg protein. If the hydrolysis was carried out in glass tubes, the precipitated hydrated silica renders the quantitative recovery of the hydrolysis products difficult, but the determination of the Hyl-Gal/Hyl-Gal-Glc ratio is still possible.

Electrophoretic separation

Whatman 3 MM paper strips, 120 cm long and 5 cm wide, were used in a Gilson Electrophoretic Model D. 15–20 μl of the solutions prepared as described above were deposited as an 8–10 mm starting line, about 40 cm from the anodic end of the strips. Two samples were applied on each strip. Up to seven strips were used in a single run. The paper was sprayed with the buffer pyridine–acetic acid–water (1:10:89) pH 3.8, and 4000–5000 V were applied for 1.5–2 h.

Staining of the spots and quantitative evaluation

After electrophoresis, the air-dried paper strips were dipped in the ninhydrin–cadmium reagent[15] (cadmium acetate 0.1 g, ninhydrin 1 g, water 10 ml, acetic acid 5 ml, acetone 50 ml; diluted 1:5 with acetone before use). The colour was developed by heating the strips at 50° for 30 min. The intensities of the developed spots were either evaluated by photodensitometry or by elution of the spots with 1.5 ml of methanol and determination of their optical density at 500 mμ.

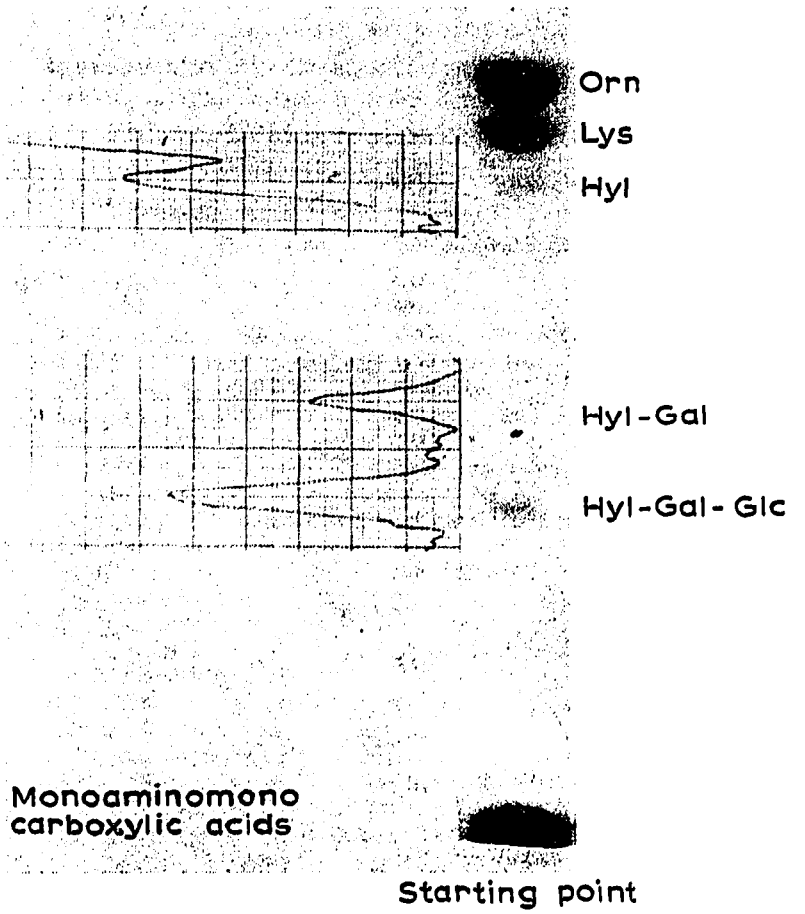

Fig. 1. Electropherogram and photodensitometric recording of hydroxylysine, galactosylhydroxylysine and glucosyl-galactosylhydroxylysine visualised by the ninhydrin–cadmium reagent[15] from an alkaline hydrolysate of 150 μg of calf corneal stroma.

RESULTS AND DISCUSSION

Photodensitometry may be less accurate but more sensitive than the elution of the coloured spots. Samples corresponding to 0.1–0.2 mg of collagen equivalent to 0.5–1 μg (4–6 μM) of the Hyl-glycoside gave readily reproducible densitometric recordings. For the colorimetric determination of the methanolic eluates, samples corresponding to about 0.3–0.6 mg should be used.

However the ratio of hydroxylysine to lysine could not be evaluated by photodensitometry, because of the big difference in the colour intensity of the spots. To obtain comparable optical densities the elution method had to be used, the lysine spot being eluted with 3 times more methanol than the volume used for the elution of the hydroxylysine derivatives.

An electropherogram and the corresponding densitometric recording of the hydrolysate of the corneal stroma of the calf embryo[8] are illustrated in Fig. 1.

The presence of the sodium salts under the conditions used does not interfere with the separation; however higher NaOH–protein ratios should be avoided.

Arginine has the same electrophoretic mobility as hydroxylysine in the buffer used. Arginine is, however, quantitatively hydrolysed during the alkaline treatment

TABLE I

DETERMINATION OF THE RATIO OF THE HYDROXYLYSINE AND ITS GLYCOSYLATED DERIVATIVES IN THE ALKALINE HYDROLYSATES OF THE HUMAN CORNEAL STROMA

Experimental details, see text.

	Run	*Hyl-Gal-Glc*	*Hyl-Gal*	*Hyl*
(a) Photodensitometric recording. Peak areas are related to Hyl-Gal-Glc = 100				
Hydrolysate No. 1	1	100	56	108
(1 mg dry substance)	2	100	48	109
	3	100	55	104
$\bar{m} \pm \sigma$			53 ± 2.5	107 ± 1.6
Hydrolysate No. 2	1	100	50	100
(1.2 mg dry substance)	2	100	56	105
	3	100	58	103
$\bar{m} \pm \sigma$			54.7 ± 2.4	102.7 ± 1.3
(b) Spectrophotometric readings of the eluted spots (1.5 ml methanol) at 500 mμ				
Hydrolysate No. 3 (0.8 mg dry substance) optical density		0.18	0.085	0.175
Ratio related to Hyl-Gal-Glc = 100		100	47	97
Hydrolysate No. 4 (1.2 mg dry substance) optical density		0.29	0.14	0.27
Ratio related to Hyl-Gal-Glc = 100		100	48	93

giving ornithine and urea[16]. The complete decomposition of the arginine during the alkaline hydrolysis of several samples of collagen-containing tissues was checked by eluting the hydroxylysine spot from the electropherograms and submitting this eluate to thin-layer chromatography (microcrystalline cellulose, developed by: (1) pyridine–ethyl acetate–acetic acid–water (5:5:1:3) and (2) *n*-butanol–acetic acid–water (12:3:5)). By this method no amino acid other than hydroxylysine could be detected.

Those spots migrating with average electrophoretic mobilities of 0.65 and 0.52, relative to the lysine spot, are the hydroxylysine glycosides: the Hyl-Gal and Hyl-Gal-Glc, respectively, present in the alkaline hydrolysates. The colour developed with the ninhydrin–cadmium reagent at 50° for 30 min[14,15,17] gave the same intensity as the usual method (24 h at room temperature)[15,17]. The two hydroxylysine glycosides behave like other amino acids in this colour reaction[7,13,15,17–19].

Table I presents the results of the photodensitometric and colorimetric evaluation of the electropherograms of an alkaline hydrolysate of human corneal stroma. The peak areas were calculated by multiplying the height of the peak by its width at midheight. Densitometric evaluation always gives somewhat higher hydroxylysine galactoside values related to the hydroxylysine disaccharide and hydroxylysine than spectrophotometric estimation of the intensity of the colour eluted. This may be attributed to the slight irregularity of the densitometric tracings, due to irregular paper structure.

The standard error of the means from 3 determinations is usually of the order of 1.5–3% for the glycosylated hydroxylysine derivatives, and 2–7% for the free hydroxylysine due to the proximity of the strong lysine peak.

It is interesting to compare the value of 70:30 for the Hyl-Gal-Glc/Hyl-Gal ratio obtained by us from the calf corneal stroma to that of 65:35 reported by SPIRO[7] for the citrate soluble collagen of calf cornea.

This method is recommended for the determination of the ratio of hydroxylysine and its glycolylated derivatives in collagen and connective tissue constituents. It has the advantage of a direct estimation, of rapidity, and requires little starting material.

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