Journal of Chromatography, 183 (1980) 221—225
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 596

Note

Determination of specific urinary hydroxyproline-containing peptides in patients with lung cancer

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(Received February 12th, 1980)

Urinary hydroxyproline is usually taken as a marker of collagen metabolism which has been shown to be altered in a number of diseases (for review see ref. 1). In urine both free and peptide-bound hydroxyproline can be found; this is because some of the hydroxyproline-containing sequences occurring in collagen are not susceptible to cleavage by liver proteases. Early papers [1] were restricted to the estimation of total urinary hydroxyproline. Considerable progress was achieved when, as well as free hydroxyproline, the hydroxyproline-containing urinary peptides were separated by chromatographic methods. Since a very complex mixture is being dealt with, one step separations like gas chromatography failed [2], and several subsequent liquid column chromatographic separations have been used instead [3]. According to the present point of view, however, urinary hydroxyproline does not strictly reflect collagen metabolism since it has been shown that this amino acid is present in other proteins as well; for example, in the first complement subcomponent C1q. These findings resulted in the effort to determine the sequences of urinary hydroxyproline-containing peptides so that they could be attributed to some part of the collagen molecule. At the beginning of these studies it was anticipated that the hydroxyproline-containing sequence most likely to occur in urine would be Gly-Pro-Hyp.

Later, Dubovský and Meyer [4], using molecular sieving on a Bio-Gel P-2 column, demonstrated that urinary hydroxyproline-containing peptides can be categorized into two groups differing in their molecular weight. In a very detailed study by Szymanowicz et al. [3] it was shown that the fraction of low molecular weight peptides represents about 85% of all hydroxyproline-containing peptides while the remaining 15% are bound into much larger

0378-4347/80/0000—0000/$02.25

sequences. In the same paper the sequences of 96% of the low molecular weight peptides were established. However, none of the efforts to separate and purify the high molecular weight peptides were successful Also it has been shown that the difference in the profile of hydroxyproline-containing peptides in healthy individuals and in patients with Paget's disease is limited to a change in the ratio of the low and high molecular weight fractions. Concomitantly it was shown that the low molecular weight fraction is devoid of the peptide Gly-Pro-Hyp.

In the present paper we have compared the fraction of urinary low molecular weight hydroxyproline-containing peptides in patients with lung cancer with that present in the urine of healthy individuals.

MATERIALS AND METHODS

Urine was collected from patients over a period of 24 h; control urine samples (24-h collections) were obtained from the laboratory staff. The filtered urine was stored frozen at −20°C until used for chromatography. All urine samples were concentrated ten times on a rotary evaporator at 50°C. The concentrate was acidified by adding acetic acid to a final concentration of 2.5 *M*.

Bio-Gel P-2 chromatography

A 2.5-ml portion of the concentrated sample was loaded on a Bio-Gel P-2 (Bio-Rad, Richmond, CA, U.S.A.) column (50—100 mesh, 40 cm × 2.5 cm; 0.2 *M* acetic acid was used as mobile phase and 2.5-ml fractions were collected. A flow-rate of 1 ml/min was ensured by a low-pressure piston pump (Mikrotechna, Prague, Czechoslovakia). From every fraction 0.5 ml was taken for the hydroxyproline assay using the Hypronosticon test (Organon, Oss, The Netherlands) [5].

QAE-Sephadex A-25 chromatography

Pooled fractions 25—29 from seven subsequent Bio-Gel runs containing small molecular weight peptides were subjected to further fractionation according to the procedure of Szymanowicz et al. [6]. The sample was taken almost to dryness and redissolved in 5 ml of γ-picoline—morpholine—pyridine—water (80:60:40:3820, v/v) mixture to which concentrated acetic acid was added to reach the final pH 9.4. A 1-ml aliquot of this solution was layered on top of a 40 cm × 2 cm QAE-Sephadex A-25 column (Pharmacia, Uppsala, Sweden) that had previously been washed with the above solvent mixture. A complex Varigrad system was used for elution. Chambers 1—3 were filled with 140 ml of the γ-picoline buffer the pH of which was adjusted with concentrated acetic acid to 9.5, 8.5 and 6.5, respectively. Chambers 4 and 5 contained the same volume of 0.5 *M* and 2.0 *M* acetic acid. Fractions of 5 ml were collected. The fractions were evaluated for hydroxyproline content and total ninhydrin value [6]. The flow-rate of 1 ml/min was ensured by a low-pressure piston pump (Mikrotechna).

Chromatography on Dowex 50 M 82

Peptides emerging from the QAE-Sephadex A-25 column between 210 and

300 ml of the eluate were accumulated from repeated runs of the lung cancer urine and separated further on the Dowex 50 M 82 column (Beckman, Munich, G.F.R.).

Amino acid analyses were done on an automated amino acid analyzer (Mikrotechna), using a 50 cm × 1 cm column. Elution was done with a stepwise gradient as follows: 0.2 *M* citrate buffer pH 3.23 for 120 min, 0.2 *M* citrate buffer pH 4.1 for 65 min, and 0.2 *M* citrate buffer 1 *M* with respect to NaCl pH 5.0 for an additional 110 min. The column was operated at 40° C for the first 40 min, then the temperature was raised to 55° C. The flow-rate was kept at 70 ml/h.

Sequence analysis

This was done using the original procedure of Gray and Hartley [7] with the identification of the N-terminal amino acid in the form of the dansyl derivative [8] on silica gel thin layers (Eastman Chromatogram sheets K301R). Standard solutions of dansyl amino acids were prepared by adding 1 ml of the dansyl reagent (6 mg of dansyl chloride in 1 ml of acetone) to an equal volume of amino acid (or peptide) solution. The concentration of the amino acids was 6.5 μmol/ml and the sample was dissolved in 1 ml of 0.1 *M* $NaHCO_3$ to ensure the alkaline reaction of the mixture. The mixture was left overnight; then 8 ml of acetone were added and the diluted sample was centrifuged. The supernatant was spotted directly onto the chromatogram. Dansyl derivatives of peptides were prepared in a similar way. After the reaction was completed, peptides were hydrolyzed in 6 *N* hydrochloric acid for 12 h and the resulting hydrolysate was chromatographed. For two-dimensional development the following solvents were used [8]: first run: benzene—pyridine—acetic acid (16:4:1); second run: chloroform—benzyl alcohol—acetic acid (70:30:3).

The nature of the C-terminal amino acid in tripeptides and hexapeptides was determined enzymatically [9].

RESULTS AND DISCUSSION

As expected the peptide mixture was separated by chromatography on Bio-Gel P-2 into two complex fractions. The low molecular weight fraction showed a clear tendency to separate further into two subfractions (Fig. 1). Of these two overlapping peaks, material occurring in the faster fraction was accumulated and subjected to further investigation.

The peptidic material obtained in this way was transferred to a QAE-Sephadex A-25 column. The result of the separation is shown in Fig. 2. The profile of hydroxyproline-containing peptides obtained from the urine of patients with lung cancer differed from that of controls in the presence of two unusual peaks emerging with the retention volume around 250 ml. This material was accumulated again (as shown by the solid bar in Fig. 2) and subjected to chromatography on a Dowex 50 M 82 column (Fig. 3). The separation of the fraction with the lower retention time is shown in the upper part of the figure, while beneath the profile of the more delayed material is shown.

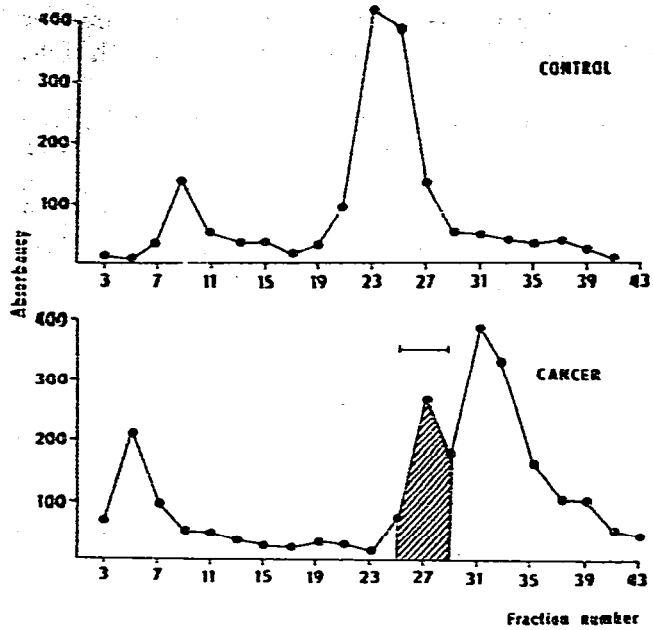

Fig. 1. Chromatographic separation of hydroxyproline-containing peptides on Bio-Gel P-2. Evaluation according to the hydroxyproline content in individual fractions by the hypronosticon test.

Material of all peaks that appeared in this last separation step was accumulated and subjected to routine amino acid analysis and to manual Edman's sequencing procedure. Appearance of a single N-terminal amino acid during this procedure as well as simple ratios obtained during amino acid analysis was considered sufficient for proving the homogeneity of the analysed peptide.

From the data presented the two urinary tripeptides occurring in patients with lung cancer were assigned the sequence Pro-Hyp-Gly and Ala-Hyp-Gly. The sequence of the hexapeptide (Fig. 3, upper part) is assumed on the basis of the obtained results and the data published by Szymanowicz et al. [3] (Ala-Hyp-Gly-Ala-Hyp-Gly).

It can be concluded that among hydroxyproline-containing peptides two additional tripeptides are formed in the urine of patients with lung cancer, namely Ala-Hyp-Gly- and Pro-Hyp-Gly. The presence of these two previously undetected urinary hydroxyproline-containing peptides was assayed in five controls and ten patients with bronchogenic carcinoma. While in healthy individuals all assays were negative, in the set of patients followed 0.8–1.4% of peptide-bound hydroxyproline was recovered in these peptides. These figures are, however, rather semiquantitative due to the complex separation procedure. It has to be stressed that both these tripeptides belong to the category of sequences frequently occurring in all collagen types that have so far been described in vertebrates. This finding is not in contradiction with the previously expressed hypothesis that normal collagen types are synthesized in neoplastic tissues, albeit, in different ratios than unaffected tissues. On the

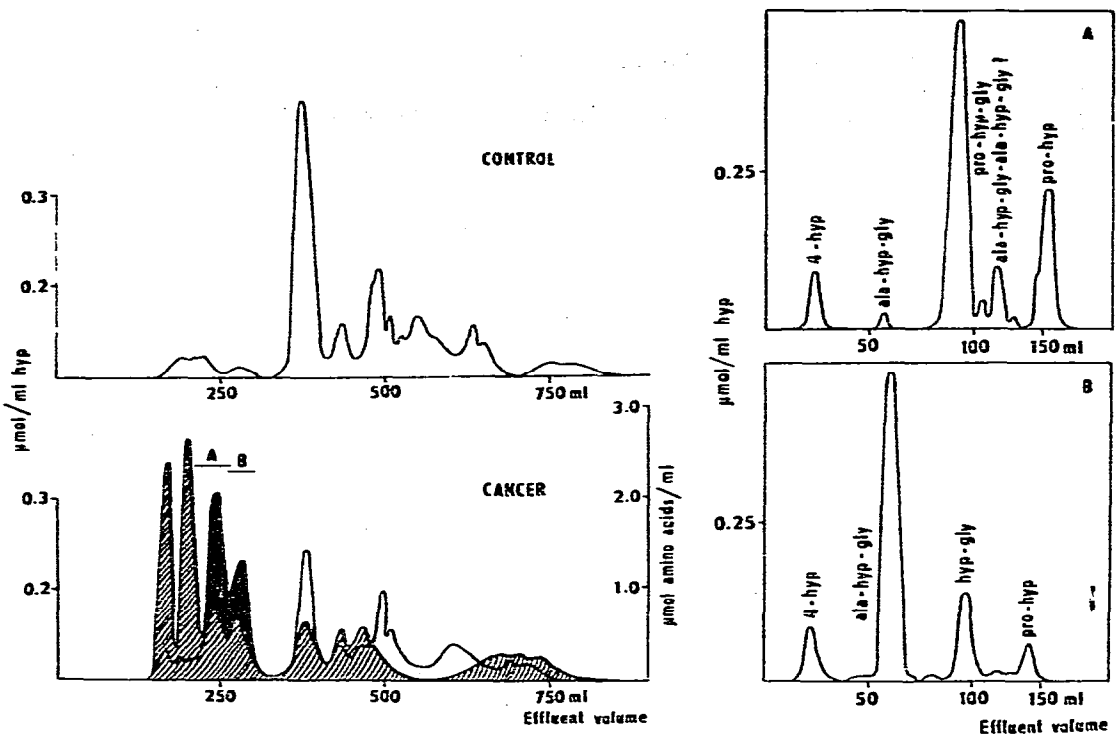

Fig. 2. Chromatography on QAE-Sephadex A-25 of the pooled fractions 25—29 from the Bio-Gel P-2 separation. The shaded area represents ninhydrin-reacting substance concentration in µmol/ml isoleucine; non-shaded urea represents hydroxyproline concentration in µmol/ml. Solid bars indicate pools used for further fractionation.

Fig. 3. Dowex 50 M 82 chromatographic profile of peptides present in fractions A and B from the QAE-Sephadex A-25 separation (Fig. 2) indicating the presence of Ala-Hyp-Gly and Pro-Hyp-Gly.

other hand, it can be concluded that in lung cancer considerable changes occur in collagen catabolism, which are reflected in alterations of hydroxyproline-containing urinary peptides.

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