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Note

Further improvement of the fluorometric assay for hydroxyproline

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The hydroxyprolines (Hyp) constitute a group of amino acids, uncommon in their structure as well as in their metabolism. Their α nitrogen atom is included in a pyrrolidine cycle, a hydroxyl group being attached to either the carbon 3 of the cycle (3-Hyp) or the carbon 4 (4-Hyp). Both natural position isomers are of the *trans* form (*E*) by reference to the carboxyl group located on carbon 2. These amino acids have a special biosynthetic pathway including a preliminary incorporation of proline residues at specific places in polypeptide chains followed by hydroxylation catalyzed by specific enzyme systems. Free proline cannot be hydroxylated, at least in animals.

This biosynthetic pathway is used during the synthesis of proteins belonging to the group of collagens that comprise some twelve different types, all containing a large amount of 4-Hyp. Two types of collagen contain, in addition, a noticeable amount of 3-Hyp: type IV and type V. Also, 4-Hyp is found in several related proteins such as the C_{1q} component of complement, the enzyme acetylcholinesterase, the lung protein alveolyn and also two proteins found in the connective tissue in the immediate vicinity of collagen: elastin and a structural glycoprotein [1]. In addition, some invertebrate proteins seem to contain 4-Hyp synthesized through a different mechanism, whereas many plant species are rich in either 4-Hyp or 3-Hyp.

This distribution explains why 4-Hyp is a marker for all types of collagen while 3-Hyp should be considered as a marker for type IV (basement membrane) and type V (pericellular) collagens. The evaluation of Hyp is of interest in collagen as well as in plant biochemistry.

In several previous papers we described a new technique for the quantitative evaluation of the various isomers of Hyp and also of proline (Pro) [2—4] using the condensation reaction of these pyrrolidine amino acids with the fluorophor 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD—Cl) followed by

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thin-layer chromatography on silica gel and fluorometric recording. We also published an application of this method for the evaluation of urinary 3-Hyp [5].

The purpose of this new paper is to introduce a modification of the technique that makes it absolutely specific: prior to the formation of the fluorophor by reaction with NBD—Cl, any remaining primary amine is removed by reaction with *o*-phthalaldehyde (OPA).

MATERIAL AND METHODS

Reagents

The usual reagents, all of analytical grade, are purchased from Prolabo, Paris. NBD—Cl is obtained from Aldrich, hydrochloric acid and triethylamine from Merck, *o*-phthalaldehyde from Sigma, Pro, 4-Hyp and 3-methylhistidine from Calbiochem. 3-Hyp is prepared in the laboratory [6]. The standard amino acid mixture is bought from Beckman.

Hydrolysis

Collagen, or protein, or peptide mixture (for instance, originating from urine) is hydrolyzed in 6 *M* hydrochloric acid at 105°C for 18 h and the hydrolyzate evaporated to dryness.

Ion-exchange chromatography

The fluorometric method is inhibited by inorganic ions, so that two steps of ion-exchange chromatography are necessary. They may be conducted as described in a previous paper [5] or as follows in order to accelerate the operations.

The residues of hydrolyzates are dissolved in 1 ml of 0.20 *M* citrate buffer, pH 2.2. A 0.20-ml aliquot is layered at the top of a minicolumn of ion-exchange resin suitable for fast elution by centrifugation. This column is made up in the laboratory by drilling a 1-mm diameter hole through the bottom of a conical plastic eppendorf 1.5-ml microtest tube. The hole is covered with a small patch of glass wool, and 0.8 g of M 72 (Beckman) resin, equilibrated with 0.20 *M* citrate buffer, pH 3.2, is then introduced. The eppendorf microtube is forced into the top of an 8-ml glass centrifuge tube of the same internal diameter and centrifuged at 1000 *g* in a Heraeus Labofuge III centrifuge equipped with horizontal tubes. The resin is washed four times with 1 ml of the same buffer and centrifuged every time using the same conditions. It takes less than 7 min for the pyrrolidine amino acids to get through the resin and to collect in the bottom space. A set of ten minicolumns may be operated by the same technician within a few minutes. In the case of urine analysis, all pigments and the basic amino acids remain on the column which may be regenerated by 1 ml of 0.2 *M* sodium hydroxide followed by 3 ml of 0.20 *M* citrate buffer, pH 3.2.

Then, the effluents of these minicolumns are chromatographed through a 4 × 0.8 cm column of Dowex W-50-X2 in the H^+ cycle. The pyrrolidine amino acids are bound and, after several washes by distilled water, eluted by 15 ml of 2 *M* ammonia solution. This solution is evaporated to dryness

under a stream of nitrogen. The residue is carefully dissolved in a minimal volume of 2 *M* ammonia solution and transferred to an eppendorf conical test tube. A second volume of 2 *M* ammonia solution is used for washing the tube and added to the first one. Then the contents of the eppendorf tube are evaporated to dryness. This method permits the final residue to be dissolved in only 20 μl of distilled water.

Derivatization with o-phthalaldehyde (OPA)

The OPA reagent is obtained by dissolving 8 mg of OPA in 1 ml of a 30% (v/v) triethylamine solution. The addition of 0.02% (v/v) mercaptoethanol, proposed by many authors in the evaluation of primary amines by OPA, is not necessary. To the 20 μl of the above amino acid solution are added 20 μl of OPA reagent. The solution is gently shaken and allowed to stand for 5 min at room temperature.

Derivatization with NBD—Cl

The preparation of the NBD—Cl reagent was described in a previous paper [4]. It contains 6 mg of NBD—Cl per ml of ethanol (30 m*M* solution) and must be stored in the dark. Twenty microliters of this reagent are added to the mixture of amino acids and OPA and allowed to react at 65°C for 30 min in the dark.

Thin-layer chromatography

A 5-μl aliquot of the NBD—amino acids solution is spotted on the silica gel thin-layer plate. The system was fully described in a previous paper [4]. A new solvent system was adopted for 3-Hyp separation: chloroform—triethylamine—methanol (80:10:10, v/v). The migration lasts 60 min. The plate is dried at 65°C for 5 min and the fluorescent spots are scanned in a Farrand spectrofluorometer Mark I equipped with a thin-layer plate recording device.

The excitation wavelength is set at 355 nm with an additional violet filter absorbing light over 500 nm and a slit 10 nm wide. The emitted light is read at 525 nm with an additional yellow filter absorbing radiation under 450 nm. The surface areas of recorded peaks are measured and compared with a set of four standards deposited on the same plate. There is a linear relationship between the surface area of the peak and the amount of Hyp deposited on the plate in the range 1—400 pmol [4].

The above proportions have been given for the evaluation of 3-Hyp. When the evaluation of 4-Hyp is required, one has to dilute the residue of hydrolysis in a 1:10 proportion with distilled water, prior to the condensation with OPA. This is particularly so for urine.

RESULTS

Fig. 1 shows a picture of the separations obtained by thin-layer chromatography of the fluorescent NBD derivatives and Fig. 2 is a scan obtained with a sample of urine after OPA and NBD—Cl reactions. It is evident that

some interfering amino acids remain in the vicinity of 3-Hyp or 4-Hyp when OPA is omitted but that the derivatization with OPA eliminates any interfering material.

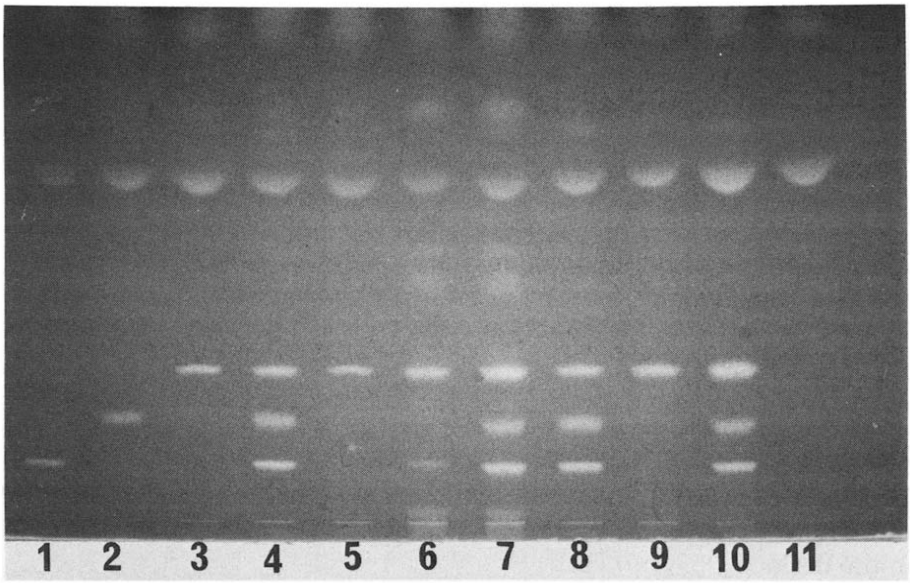

Fig. 1. Thin-layer chromatography of several amino acids after NBD—Cl derivatization. Lanes 1—7: samples directly reacted with NBD—Cl according to the method of ref. 4. Lanes 8—10: samples reacted with *o*-phthalaldehyde (OPA) prior to NBD—Cl (technique described in this paper). All the samples were deposited in 200-pmol amounts. Lane 1: control *trans*-4-Hyp. Lane 2: control *trans*-3-Hyp. Lane 3: control Pro. Lane 4: mixture of 4-Hyp, 3-Hyp and Pro. Lane 5: control *cis*-4-Hyp (kindly provided by Dr. R. Berg, Piscataway, NJ). Lane 6: a mixture of amino acids (Beckman). Lane 7: the same mixture supplemented with 200 pmol each of *trans*-4-Hyp, *trans*-3-Hyp and Pro. Lane 8: the same as lane 4 but previously reacted with OPA. Lane 9: the same as lane 6 but previously reacted with OPA. Lane 10: the same as lane 7 but previously reacted with OPA. Lane 11: reagents.

In order to assess the usefulness of the OPA reaction, we compared the concentrations of 3-Hyp obtained for sixteen samples of urine by three different methods: the ion-exchange technique that we described several years ago [7], the NBD—Cl technique without OPA, and the new improvement. With the fluorometric method performed in the absence of OPA, we found results higher by nearly 30% than with the ion-exchange technique. The results of the OPA + NBD—Cl technique correlated with the colum chromatographic technique (r = 0.98, n = 16). We found that times of contact of OPA with the amino acid solution ranging from 5 to 30 min did not modify the results.

For the evaluation of efficiency, five samples of urine were supplemented with a known amount of 3-Hyp and the results gave 96.3 ± 3.3% of the amount added. The repeatability of the method was studied fourteen times on the same urine, giving the results 6.0 ± 0.4 μmol/l for 3-Hyp and 82.0 ± 5.7 μmol/l for 4-Hyp.

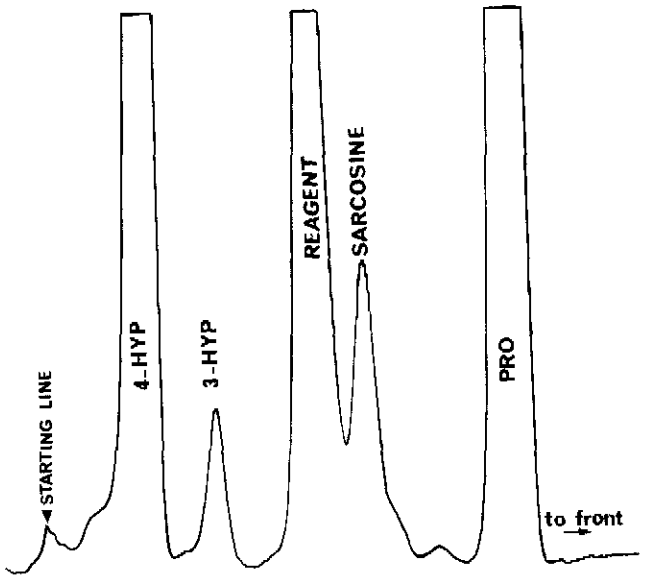

Fig. 2. Scan of thin-layer chromatogram of NBD derivatives of 4-Hyp, 3-Hyp and Pro from a hydrolyzate of urine (100 μl). Silica gel plate. Solvent: chloroform—methanol—triethylamine (80:10:10, v/v). Development: 1 h at 20°C. Scanning: spectrofluorometer Farrand Mark I, sensitivity 0.3, speed 150 mm/min. The surface area of the 3-Hyp peak can easily be measured. Surface areas of 4-Hyp and Pro cannot be measured without prior dilution of the sample.

DISCUSSION

The NBD technique for the evaluation of the hydroxyproline isomers is far more sensitive than any of the previously described ion-exchange techniques. It permits the accurate measurement of picomole amounts of these pyrrolidine amino acids. For full efficiency, the reaction must operate in the absence of mineral ions. The fluorescence of primary amino acids is ten to a hundred times lower. Nevertheless, these amino acids may interfere when they occur in very large amounts in the initial medium or when this medium has to be strongly concentrated for improving the sensitivity. In this paper, we described a fast method for completely desalting the solutions or urines and we largely improve the specificity by the use of the OPA reagent. In these conditions, we may apply the technique to larger volumes of initial solution and then increase the sensitivity.

The data obtained for urinary 3-Hyp confirm the reference values that we had previously established by another technique [7]. The values that we obtain for urinary 4-Hyp are lower than those given by the classical reaction with *p*-dimethylaminobenzaldehyde [8, 9]. We suspect that some non-specifically reacting substances such as pyrrole radicals exist in the urine and increase artefactually the results given by this colorimetric technique.

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