Journal of Chromatography, 190 (1980) 457–461

CHROM. 12,564

Note

Improved method for the preparation of 3-hydroxyproline, and some of its chromatographic properties

A. G. SZYMANOWICZ, G. POULIN, N. FONTAINE, J. P. WERQUIN and J. P. BOREL

Laboratory of Biochemistry, Faculty of Medicine, University of Reims, 51 Rue Cognacq Jay, 51095 Reims Cédex (France)

(Received October 29th, 1979)

3-Hydroxyproline (3-Hyp) is a cyclic amino acid, a position isomer of 4-hydroxyproline (4-Hyp), the well known marker of collagen. 3-Hyp is also present in collagen in trace amounts. It is of interest because it is a characteristic marker for some types of basement membrane collagen, that contain more significant amounts of 3-Hyp than other collagens. In addition, 3-Hyp is not uncommon in plants.

The aim of this paper is to demonstrate that the chromatographic analysis of 3-Hyp requires some special refinements. For instance, the ninhydrin colour yields furnished by the two isomers of hydroxyproline are largely different. In every laboratory, there is a need to measure this yield with an appropriate standard solution under the prevailing conditions. For that reason, an improved method for preparing standard 3-Hyp is described.

MATERIALS AND METHODS

The reagents were all purchased from Merck (Darmstadt, G.F.R.) except tetrabutylammonium iodide and potassium tetraborate (BDH, Poole, Great Britain), Bio-Gel P-2 and Dowex 50-X8 resin (200–400 mesh) (Bio-Rad, Richmond, Calif., U.S.A.), Sephadex G-10 (fine) (Pharmacia, Uppsala, Sweden) and the seeds of *Delonic regia* (Setropa, Bussum, The Netherlands).

Preparation of 3-hydroxyproline

The method of preparation of 3-Hyp that we recently described[1] is improved as follows.

The 10% alcoholic extraction mixture obtained from 1 kg of seeds (10 l reduced to 0.1 l) is filtered on Prolabo filter paper (01 761 825). The filtrate is chromatographed in fractions of 10 ml on a Sephadex G-10 (fine) column (90 × 2.5 cm I.D.) equilibrated with distilled water. The 3-Hyp-containing fractions (eluting between 200 and 300 ml) are pooled and concentrated to dryness under a stream of nitrogen. The residue, dissolved in 0.1 *N* HCl is chromatographed on a 45 × 2.5 cm I.D. column of Dowex 50-X8 (H^+) resin. The column is thoroughly washed with 1 l of distilled water and then 3-Hyp is eluted by 1 *N* HCl, under control of the ninhydrin reaction: the elution volume is between 350 and 450 ml.

This fraction is evaporated to dryness and the residue dissolved in 10 ml of 0.1 *N* acetic acid solution. Aliquot fractions of 1 ml are layered on a 54 × 0.9 cm I.D. column of Beckman amino acid analyser filled in with a M-72 resin, equilibrated with a 0.1 *M* acetic acid solution. The column is eluted by a 0.1 *M* solution of acetic acid (pH 2.85) at a rate of 1 ml/min. 50 fractions of 4 ml are collected in a Gilson TD fraction collector. The fractions containing 3-Hyp are localized by the ninhydrin reaction performed on an aliquot.

Finally, 3-Hyp is crystallized from 95% ethanol. The yield is *ca.* 300 mg from 1 kg of seeds.

Kinetics of the ninhydrin reaction with 3-hydroxyproline

Solutions of 3-Hyp, 4-Hyp and proline (Pro) (400 m*M* in a 0.2 *M* sodium citrate buffer, pH 3.23) are prepared. The ninhydrin solution is made up according to the Beckman instruction booklet. A 1-ml volume of ninhydrin solution is mixed manually in a test tube with 2 ml of amino acid solution and heated in a water bath at 100°. The influence of increasing the heating period from 1 to 60 min on the development of the colour is recorded for the three amino acids. The test tube is cooled under tap water for 1 min and the optical density measured in a Beckman Model 25 spectrophotometer, in 10-mm optical pathway cells. The 440 nm wavelength is chosen as the optimum for each of the amino acids. Every measurement is done in quadruplicate.

Column chromatography separation of 3-hydroxyproline

The optimal conditions for separating 3-Hyp from other amino acids of similar elution time, have been determined for a column of 54 × 0.9 cm I.D. M-72 resin in a Multichrom B Beckman analyser.

The elution rate is 65 ml/h, with three buffers: 0.2 *N* sodium citrate (pH 3.22) pumped for 120 min, then 0.2 *N* sodium citrate (pH 4.1) pumped for 90 min and finally 0.2 *N* sodium citrate (pH 5.00), containing 1 *M* NaCl, pumped for 95 min. The initial temperature of the column is set at 39° and is increased to 67° after 40 min. The column is regenerated by 0.2 *N* NaOH for 15 min, then equilibrated with 0.2 *N* sodium citrate (pH 3.22) for 30 min. The whole cycle takes 6 h.

The ninhydrin reagent, prepared according to the Beckman technology, is pumped at a rate of 32.5 ml/h. The length of the reaction coil is 30 m, providing a reaction time of 9 min at 100°. The optical density is measured at 440 and 570 nm.

Thin-layer chromatography of 3-hydroxyproline

In addition to the technique of separation of 3-Hyp on silica gel thin-layer plates after reaction with NBD chloride[2], it is possible to separate and characterize specifically Pro and the two Hyp isomers by thin-layer chromatography (TLC) on cellulose plates. The amino acid samples are spotted on a Merck cellulose plate (Art 5552 DC Alufoliencellulose) and developed successively in the first dimension with the solvent isopropanol–34% ammonia (70:30, v/v) and in the second direction with *n*-butanol–acetone–acetic acid–water (28:28:8:16). The plates are dried for 5 min in an oven at 90°, then sprayed with the NBD reagent (NBD chloride 50 mg, tetrabutylammonium iodide 100 mg, 0.5 *N* HCl 5 ml, ethanol 65 ml, water up to 100 ml) and heated for 3 min at 90°. They are viewed under a Wood lamp and the

yellow fluorescent spots of Pro, 3-Hyp and 4-Hyp are marked with a pencil. The sensitivity of 3-Hyp to this reaction is the same as that of Pro.

RESULTS AND DISCUSSION

This new method of preparation increases the yield of pure 3-Hyp by a factor of 1.5 and reduces the necessary time in comparison with previous[1,3]. The purity of 3-Hyp is demonstrated by the following data. The nuclear magnetic resonance spectrum is identical with that described by Wolff[4] for pure 3 Hyp. Gas–liquid chromatography, performed by Frey[5], shows only one peak. Column chromatography in the Beckman analyser, shows one single peak even for large amounts of purified product layered on the column. TLC, either on silica gel plates as described in an earlier paper[2] or on cellulose plates as described in this paper, shows only one spot, clearly separated from the spot of 4-Hyp.

The ninhydrin reaction with 3-Hyp was reinvestigated because we found that the colour yield was different from that of Pro and 4-Hyp under prevailing conditions. The kinetics is shown on Fig. 1. The colour yield is in practice lower than that of 4-Hyp for incubation periods shorter than 3 min. For longer periods, the yield increases progressively up to a plateau, reached after 30 min heating. The colour yield is double that of 4-Hyp and almost the same as that of Pro when the heating period is no more than 25 min. Over this period, the colour given by 3-Hyp fades quickly whereas the colour given by Pro remains stable for more than 60 min. These differences in colour yield may lead to an overestimation of the amount of 3-Hyp when the colour is referred to a standard of Pro, especially if the heating period is short. On the other hand, a standard of 4-Hyp will give a reliable comparison only with short incubation. In any case, it appears better to measure the colour yield in the conditions used in the laboratory with a standard of 3-Hyp itself.

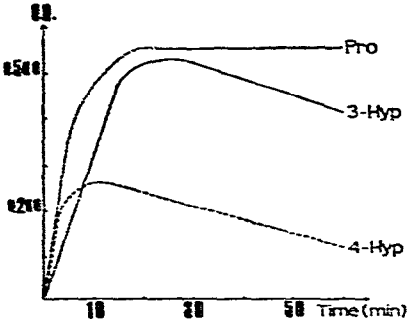

Fig. 1. Kinetics of the reaction of ninhydrin with 3-Hyp, 4-Hyp and Pro.

The optimal conditions for the separation and detection of 3-Hyp on a column of M-72 resin in the multichrom Beckman analyser were reinvestigated. The proposed system of elution permits a complete separation of 3-Hyp and 4-Hyp, all the usual amino acids and, in addition, glucosamine and galactosamine. Table I shows the chromatographic data obtained in this system.

Spraying the TLC plates with NBD reagent permits a very sensitive and specific

TABLE I

DATA OBTAINED FROM FIVE CHROMATOGRAPHIC SEPARATIONS OF STANDARD AMINO ACIDS AND OSAMINES ON M-72 RESIN

S.D. = Standard deviation.

Detected peaks	*Retention time (min)*	*S.D.*	*Ninhydrin colour index*	*S.D.*	*S.D./mean (%)*
Cys-SO_3H	21.5	0.1	2390	145	6.1
3-Hyp	48.1	0.1	1185	30	2.5
4-Hyp	52.4	0.1	624	65	10.4
Asp	54.9	0.1	4898	222	4.5
Thr	62.2	0.1	4947	318	6.4
Ser	65.6	0.1	5023	98	1.9
Glu	77.8	0.3	5728	198	3.5
Pro	83.0	0.3	1347	32	2.4
Gly	97.8	0.1	5139	189	3.7
Ala	104.0	0.2	5244	184	3.5
Cys	113.0	0.8	3414	223	6.5
Val	131.0	1.1	5015	116	2.3
Met	146.5	0.4	2868	348	12.1
Ile	155.4	0.1	5416	60	1.1
Leu	159.2	0.1	5405	9	0.2
Nle	164.1	0.1	5485	12	0.2
Tyr	175.6	0.1	5399	34	0.6
Phe	185.0	0.1	5307	70	1.3
$GlcNH_2$	210.5	0.2	5418	59	1.1
$Gal\,NH_2$	227.5	0.6	3926	351	8.9
Hyl	250.5	0.3	6513	217	3.3
Lys	260.0	0.2	6890	16	0.2
His	272.6	0.3	5436	41	0.7
Arg	312.0	0.2	5378	63	1.2

fluorimetric detection. Amounts as low as 0.1 nmole of 3-Hyp are easily detected. After localizing Pro and the Hyp isomers by this fluorescent reaction, it is still possible to detect the other amino acids by spraying a ninhydrin reagent in the usual conditions[6]. Pro and the Hyp isomers, the N groups of which are blocked by NBD, no longer react with ninhydrin.

CONCLUSION

Several techniques for the evaluation of 3-Hyp have been described recently[7,8]. None of them is based on the reaction capable of detecting 4-Hyp (oxidizing reagent followed by condensation with *p*-dimethylaminobenzaldehyde) because 3-Hyp does not react under these conditions. For that reason, the evaluation of 3-Hyp is based on ion exchange chromatography and the ninhydrin reaction. This demonstrates the need for an accurate calibration, with a standard of 3-Hyp, since the colour yield of 4-Hyp with ninhydrin is different. The new method of preparation will be of benefit for all those studying the structure and metabolism of the collagen proteins, as well as those involved in the field of plant physiology.

ACKNOWLEDGEMENT

This work was supported by grants from the University of Reims, CNRS No. 32670 and INSERM No. 79 1140 3.

REFERENCES

1 A. Szymanovicz, O. Mercier, A. Randoux and J. P. Borel, *Biochemistry*, 60 (1978) 499–503.
2 A. Szymanovicz, C. Chéron and J. P. Borel, *Biochemistry*, 61 (1979) 425–427.
3 M. L. Sung and L. Fowden, *Phytochemistry*, 7 (1968) 2061–2063.
4 J. S. Wolff, J. D. Oggle and M. A. Logan, *J. Biol. Chem.*, 241 (1966) 1300–1307.
5 J. Farjanel and J. Frey, personal communication.
6 R. S. Erssner and I. Smith, in I. Smith and J. W. T. Sekins (Editors), *Chromatographic and Electrophoretic Techniques*, Vol. 1, Heinemann, London, 4th Ed., 1976, Ch. 5, p. 75.
7 E. Adams, S. Ramaswamy and M. Lamon, *J. Clin. Invest.*, 21 (1978) 1482–1487.
8 A. Szymanovicz, G. Poulin, A. Randoux and J. P. Borel, *Clin. Chim. Acta*, 91 (1979) 141–146.