Journal of Chromatography, 311 (1984) 375—379
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2280

Note

Rapid and sensitive amino acid analysis of human collagens using high-performance liquid chromatography

HIROSHI KONOMI

Division of Mental Retardation and Birth Defect Research, National Center for Nervous, Mental and Muscular Disorders, 2620, Ogawahigashi-Machi, Kodaira, Tokyo 187 (Japan) and Department of Biochemistry, UMDNJ-Rutgers Medical School, University Heights, Piscataway, NJ 08854 (U.S.A.)

TOSHIHIKO HAYASHI*

Department of Tissue Physiology, Medical Research Institute, Tokyo Medical and Dental University, Kanda-surugadai, Chiyoda-ku, Tokyo 101 (Japan)

and

YOSHIHIRO TAKEHANA and MASATAKA ARIMA

Division of Mental Retardation and Birth Defect Research, National Center For Nervous, Mental and Muscular Disorders, 2620, Ogawahigashi-Machi, Kodaira, Tokyo 187 (Japan)

(First received April 25th, 1984; revised manuscript received June 6th, 1984)

A fluorescence detection system has been used for the highly sensitive analysis of amino acids after reaction with o-phthalaldehyde (OPA) [1, 2]. By using the post-column fluorescence-labelling method with OPA after converting imino groups into amino groups in alkaline sodium hypochlorite [3, 4], the determination of proline and hydroxyproline is possible by high-performance liquid chromatography (HPLC). This report describes a rapid and highly sensitive amino acid analysis by HPLC, using post-column fluorescence labelling with OPA and sodium hypochlorite, of human collagens types I, II, III and V, which contain distinct amino acid compositions as well as large amounts of imino acids compared to other proteins.

0378-4347/84/$03.00

EXPERIMENTAL

Chemicals

L-Hydroxyproline and L-hydroxylysine of special reagent grade for amino acid analysis were obtained from Azinomoto (Tokyo, Japan). Commercial 10% sodium hypochlorite was purchased from Yoneyama Yakuhin Kogyo (Osaka, Japan). The solution of standard amino acids and sodium citrate for the amino acid autoanalyser, *o*-phthalaldehyde (OPA) and all the other reagents of analytical grade were purchased from Wako (Osaka, Japan).

Equipment

The apparatus, a Model LC-3A HPLC system manufactured by Shimadzu (Kyoto, Japan) for amino acid analysis, was used with a step gradient programmer Model SCR-1A. A sulphonated polystyrene cation-exchange resin column, 150 × 4 mm I.D. (Shimadzu gel ISC-07/S1504, particle size 7 μm), together with a Dowex 50W-16 resin column 250 × 4 mm I.D. (Shimadzu gel ISC-50) as the precolumn [5], were used for the separation of amino acids.

Procedure

The elution buffer system and the time programme are shown in Table I. After elution with the fifth buffer, the column was recycled by use of the sixth buffer for 15 min and then the first buffer for 60 min or more. The flow-rate was 0.4 ml/min and the pressure was 10.8—11.8 MPa (60—70 kg/cm^2) at 55°C. OPA solution was prepared by mixing 400 mg of OPA in 7 ml of ethanol with 500 ml of 0.5 *M* borate—sodium carbonate buffer (pH 10.2), 1 ml of 2-mercaptoethanol and 2 ml of 10% Brij 35. Sodium hypochlorite solution was prepared by adding 0.5 ml of the 10% sodium hypochlorite to 500 ml of 0.5 *M* borate—sodium carbonate buffer (pH 10.2). The flow-rates of the hypochlorite solution and the OPA solution were set at 0.2 ml/min with a minipump Model PRP-2A. The length of the oxidative reaction coil and that of the fluorogenic reaction coil was 1 m and 2 m, respectively. The reaction temperature was set at 55°C. The results were displayed on a Model C-R1A recording integrator.

TABLE I

BUFFER SYSTEMS AND TIME PROGRAMMES FOR AMINO ACID ANALYSIS

Step	pH	Buffer*	Time (min)
1	2.70	0.067 *M* Sodium citrate—perchloric acid in 7% ethanol	20
2	3.25	0.067 *M* Sodium citrate—perchloric acid	10
3	4.25	0.067 *M* Sodium citrate—perchloric acid	32
4	6.50	0.27 *M* Sodium citrate—perchloric acid	30
5	9.00	0.27 *M* Sodium citrate—sodium hydroxide and 0.016 *M* boric acid	20
6		0.2 *M* Sodium hydroxide	15
Recycle		Buffer 1	60

*Buffers 1—5 contain 0.01% *n*-caprylic acid.

Sample preparation

Human collagens, type I, type III and type V were obtained from placenta by pepsinization and salt fractionation. Human type II collagen was extracted from cartilage by pepsin treatment following 4 *M* guanidine—hydrochloric acid treatment [6]. About 100 μg of each type collagen were hydrolysed in 1 ml of 6 *M* hydrochloric acid under vacuum at 110°C for 24 h. Dried samples were dissolved in 1 ml of buffer 1. The sample solution was passed through a Millipore filter, then 10 μl of the sample were injected.

RESULTS AND DISCUSSION

Typical chromatograms of the mixture of standard amino acids and human type I collagen hydrolysate are shown in Fig. 1. It took about 120 min for the analysis of one sample. Table II shows the amino acid composition of the human collagens (type I, II, III and V) obtained using the post-column labelling method with sodium hypochlorite and OPA, together with the reported data obtained by a conventional automated amino acid analyser with ninhydrin reaction. The values for human type I collagen were calculated from the data

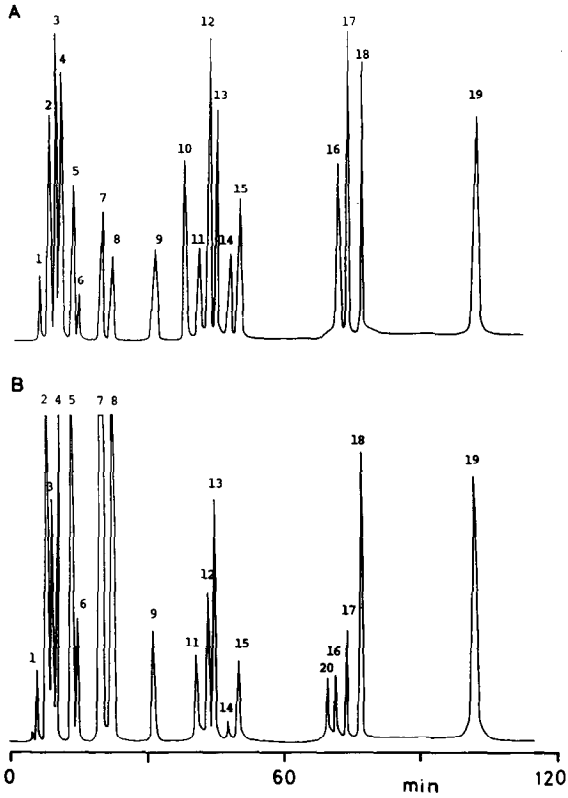

Fig. 1. Chromatograms of (A) a mixture of standard amino acids and (B) human type I collagen hydrolysate. Amino acid analysis was performed as described in the text. The mixture of standard amino acids (each at a concentration of 0.25 nmol) contained: 4-Hyp (1), Asp (2), Thr (3), Ser (4), Glu (5), Pro (6), Gly (7), Ala (8), Val (9), Cys (10), Met (11), Ile (12), Leu (13), Tyr (14), Phe (15), His (16), Hyl (17), Lys (18), Arg (19), and NH_3 (20). For abbreviations see Table II.

TABLE II

AMINO ACID COMPOSITION OF HUMAN COLLAGEN

Results are expressed as the number of residues per 1000 total residues.

Amino acid*	Type I		Type II		Type III		Type V	
	OPA method§	Ninhydrin method [7]	OPA method§	Ninhydrin method [8]	OPA method§	Ninhydrin method [9]	OPA method§	Ninhydrin method
3-Hyp	Trace	1	ND**	2	Trace	NR***	Trace	1
4-Hyp	85 (106)	109	81 (101)	99	111 (125)	125	86 (107)	101
Asp	42	45	41	42	45	42	46	50
Thr	12	18	15	20	9	13	17	23
Ser	35	33	31	27	39	39	26	26
Glu	70	74	91	89	70	71	90	96
Pro	114	115	109	121	109	107	113	118
Gly	367 (337)	325	368 (332)	333	377 (331)	350	383 (345)	325
Ala	113	113	101	100	89	96	37	46
Val	22	24	14	18	12	14	21	24
Cys	ND	ND	ND	NR	Trace	2	1	1
Met	8	5	19	9	7	8	11	8
Ile	9	11	9	9	13	13	17	19
Leu	25	24	27	26	23	22	40	42
Tyr	2	2	1	1	1	3	2	2
Phe	13	12	12	13	9	8	12	11
Hyl	5	11	14	14	5	5	31	38
Lys	26	24	18	22	30	30	8	7
His	5	6	2	2	7	6	15	14
Arg	48	49	46	51	46	46	44	52

*3-Hyp = 3-hydroxyproline, 4-Hyp = 4-hydroxyproline, Asp = asparate, Thr = threonine, Ser = serine, Glu = glutamate, Pro = proline, Gly = glycine, Ala = alanine, Val = valine, Cys = cysteine, Met = methionine, Ile = isoleucine, Leu = leucine, Tyr = tyrosine, Phe = phenylalanine, Hyl = hydroxylysine, Lys = lysine, His = histidine, Arg = arginine.

**ND = not detected.

***NR = not reported.

§Numbers in parentheses are the values after correction for glycine and hydroxyproline using correction factors obtained experimentally for type I collagen by ninhydrin and OPA methods (see text).

of Burgeson et al. [6] on α1 (I) and α2 (I) chains (two-thirds α1 + one-third α2).

The contents of glycine and hydroxyproline residues were about 10% higher and 25% lower, respectively, than those previously reported (Table II). The values for the other amino acids were comparable within the error by the present analysis. Even though relatively high errors in glycine and hydroxyproline occurred, the overall amino acid composition determined by the hypochlorite—OPA method clearly indicates that the purified protein is collagenous. It also demonstrates a distinct difference in amino acid compositions between interstitial collagens (type I—III) and type V collagen. That is, the latter collagen contains fewer than 50 alanine residues, about 100 hydrophobic amino acid residues (Val, Met, Ile, Leu, Tyr and Phe) and 30—40 hydroxylysine residues, while collagens I—III contain about 100 alanine residues, about 80 or less hydrophobic amino acid residues and fewer than 15 hydroxylysine residues. In this respect, the highly sensitive amino acid analysis by OPA reaction after sodium hypochlorite treatment was useful. Improvement of the method for the determination of glycine and hydroxyproline by increasing the oxidation reaction by sodium hypochlorite is now under investigation.

For practical usage in determining the amino acid composition of a collagenous protein which contains about 10% hydroxyproline and 33% glycine, correction factors which are experimentally determined for the same sample of type I collagen by both the conventional ninhydrin method and the present hypochlorite—OPA method should be used. The correction factors for glycine and hydroxyproline under the present condition are 0.9 and 1.25, respectively. The corrected values for the contents of type I, II, III and V collagens are listed in parentheses in Table II. The data are reasonably consistent with the previously reported values.

The reason that the quantification by the OPA method after sodium hypochlorite reaction gives rise to larger errors in the glycine and hydroxyproline content may be as follows. The overestimation of glycine may be due to a relatively smaller number of amines being degraded during the oxidation by sodium hypochlorite under the present conditions. The underestimation of hydroxyproline, on the other hand, may be due to relatively less conversion into amines during hypochlorite oxidation. A high hydroxyproline content which becomes reactive with OPA only after hypochlorite oxidation may have not been completely converted into primary amines.

Other than the high sensitivity for amino acid analysis of collagenous proteins which contain abundant imino acids, the HPLC method has the further advantage of shortening the analysis time (120 min for one sample).

REFERENCES

1 P.E. Hare, Methods Enzymol., 47 (1977) 3.
2 J.R. Benson, Methods Enzymol., 47 (1977) 19.
3 P. Bohlen and M. Mellet, Anal. Biochem., 94 (1979) 313.
4 M. Sato and K. Yagi, J. Chromatogr., 242 (1982) 185.
5 E.J. Miller and R.K. Rhodes, Methods Enzymol., 82 (1982) 33.
6 R.E. Burgeson, F.A. Eladli, I.I. Kaitila and D.W. Hollister, Proc. Nat. Acad. Sci. U.S., 73 (1976) 2579.
7 E.J. Miller and L.G. Lunde, Biochemistry, 12 (1973) 3153.
8 E. Chung and E.J. Miller, Science, 183 (1974) 1200.
9 K. Nakazawa, H. Tanaka and M. Arima, J. Chromatogr., 233 (1982) 313.