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IMPROVED METHOD FOR AMINO ACID ANALYSIS OF STAINED COLLAGEN BANDS FROM POLYACRYLAMIDE GELS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

YOKO HASHIMOTO

Department of Biochemistry, School of Dentistry, Aichi-Gakuin University, 1-100 Kusumoto-cho, Chikusa-ku, Nagoya 464 (Japan)

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SUMMARY

A procedure is described for the determination of the amino acid composition of stained collagen bands separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis using high-performance liquid chromatography and post-labelling with *o*-phthalaldehyde. Six α-chain bands from collagens I, III and V were analysed directly after gel electrophoresis, Coomassie blue staining and destaining. It was shown that good accuracy and reproduciblity can be attained using this method, which makes it possible to determine simultaneously nineteen amino acids: Asx, Thr, Ser, Glx, Pro, Cys, Gly, Ala, Val, Met, Ile, Leu, Tyr, Phe, His, Lys and Arg together with 4-Hyp and Hyl, specific amino acids found in collagen.

INTRODUCTION

The procedure described previously[1] for amino acid analysis with *o*-phthalaldehyde (OPA) of stained protein bands, in which the stained gel slices were directly hydrolysed with hydrochloric acid, was suitable for the determinaton of the amino acids aspartic acid, threonine, serine, glutamic acid, proline, cysteine, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine and arginine. However, there was a "buffer-change" peak having the same retention time as histidine, so subtraction of its peak value in the determination of histidine was necessary. Also, one could not determine the amino acid compositions of collagen samples by this method because (1) the hydroxylysine content might be slightly above its true value, (2) the arginine peak was eluted as a shoulder on the ammonia peak and (3) the resolution of 4-hydroxyproline from aspartic acid was not always satisfactory. The method described here served for the analysis of nineteen amino acids, including 4-hydroxyproline and hydroxylysine, by a single-step procedure without any correction, using six α-chain bands derived from collagens I, III and V.

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EXPERIMENTAL

Chemicals

Amino acid standard CH was purchased from Pierce (Rockford, IL, U.S.A.) and acrylamide and N,N′-methylenediacrylamide were from Merck (Darmstadt, F.R.G.). Coomassie Brilliant Blue R (CBB) and sodium dodecyl sulphate (SDS) were obtained from Sigma (St. Louis, MO, U.S.A.), thioglycolic acid, super special grade constant-boiling hydrochloric acid, OPA and sodium citrate buffer solution (pH 2.2) for sample preparation from Wako (Osaka, Japan), 10% sodium hypochlorite solution from Yoneyama Yakuhin Kogyo (Osaka, Japan) and distilled water for injection from Otsuka Pharmaceutical (Tokyo, Japan).

Sample preparation

Human collagens I, III and V were obtained from post-burn granulation tissues by pepsinization and salt fractionation[2]. The surgically obtained tissues were provided by Dr. H. Aoyama of Aichi Medical College. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method reported by Laemmli[3]. About 40 μg of each type of collagen sample were subjected to slab gel (1 mm thick) electrophoresis to separate α-chains. The gel was visualized by staining with CBB. The Coomassie blue-stained gel slices, washed with destaining solution and acetone and then dried by flushing with nitrogen gas were directly hydrolysed under reduced pressure at 110 ± 1°C for 20 h with 5 μl of thioglycolic acid and 0.5 ml of 6 *M* constant-boiling hydrochloric acid. The hydrolysate was evaporated and dissolved in 50 μl of 0.067 *M* sodium citrate buffer (pH 2.2) (stock sample solution); after centrifugation to remove the insoluble material, an aliquot of the stock sample solution was subsequently diluted with the same buffer to give a concentration of 20–40 μg/ml of protein (diluted sample solution). Then, 25 μl of the sample were injected into the column.

Apparatus

The chromatographic equipment was obtained from Shimadzu (Kyoto, Japan). An LC-4A high-performance liquid chromatography (HPLC) apparatus was connected to an RF-540 spectrofluorimeter and a Model C-R3A recording integrator. Detection was accomplished by post-column reaction with OPA at 55°C using a Model PRR-2A minipump post-column reactor.

Chromatography

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, were used for the separation of amino acids. Amino acids were separated using a gradient programme of the mobile phase (Fig. 1) in a Shimadzu Model LC-4A HPLC apparatus using solutions A, B and C, where solution A was 7% (v/v) ethanol in 0.067 *M* sodium citrate (pH 3.15), solution B was 0.2 *M* sodium citrate (pH 10.0) and the solution C was 0.2 *M* sodium hydroxide. The flow-rate was 0.3 ml/min at 55°C. The post-column labeling method was essentially the same as that reported by Ishida *et al.*[4], except that the OPA concentration was raised from 0.08 to 0.4%[1]. Sodium hypochlorite reagent was

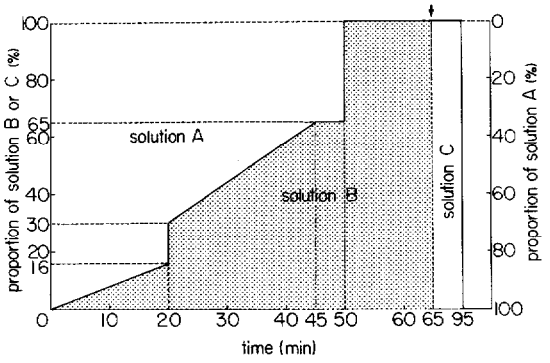

Fig. 1. Gradient profile of the mobile phase. The arrow indicates the switching point from solution B to solution C.

prepared by adding 0.2 ml of 10% sodium hypochlorite to 500 ml of buffer solution (pH 10.0) containing sodium carbonate (0.384 *M*), boric acid (0.216 *M*) and potassium sulphate (0.108 *M*). A fluorescence reagent was prepared by mixing 2.0 g of OPA in 15 ml of ethanol and 1 ml of 2-mercaptoethanol and 2 ml of 10% Brij 35 in 500 ml of the above alkaline buffer. The flow-rates of the sodium hypochlorite and OPA solutions were set at 0.2 ml/min. The fluorescence intensity of the effluent was measured at excitation and emission maxima of 348 and 450 nm, respectively.

RESULTS AND DISCUSSION

Fig. 2 (lanes a, b and c) shows the SDS-PAGE patterns of collagens I, III and V, respectively, obtained by the Laemmli method[3], which uses Tris–glycine buffer as the electrode buffer. These six α-chains were analysed. Typical chromatograms of each α1-chain collagen hydrolysate, which was derived from collages I, III and V, are shown in Fig. 3b, c and d, respectively. One sample analysis took about 80 min.

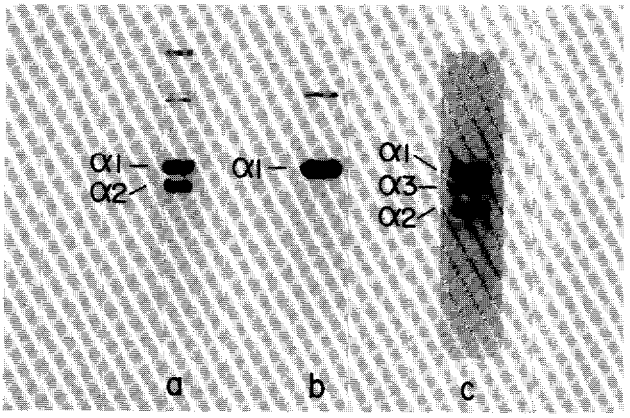

Fig. 2. SDS-PAGE of about 40 μg each of type I (lane a), type III (lane b) and type V (lane c) collagens by the Laemmli method[3]. Acrylamide concentrations were 8% for lanes a and b and 5% for lane c.

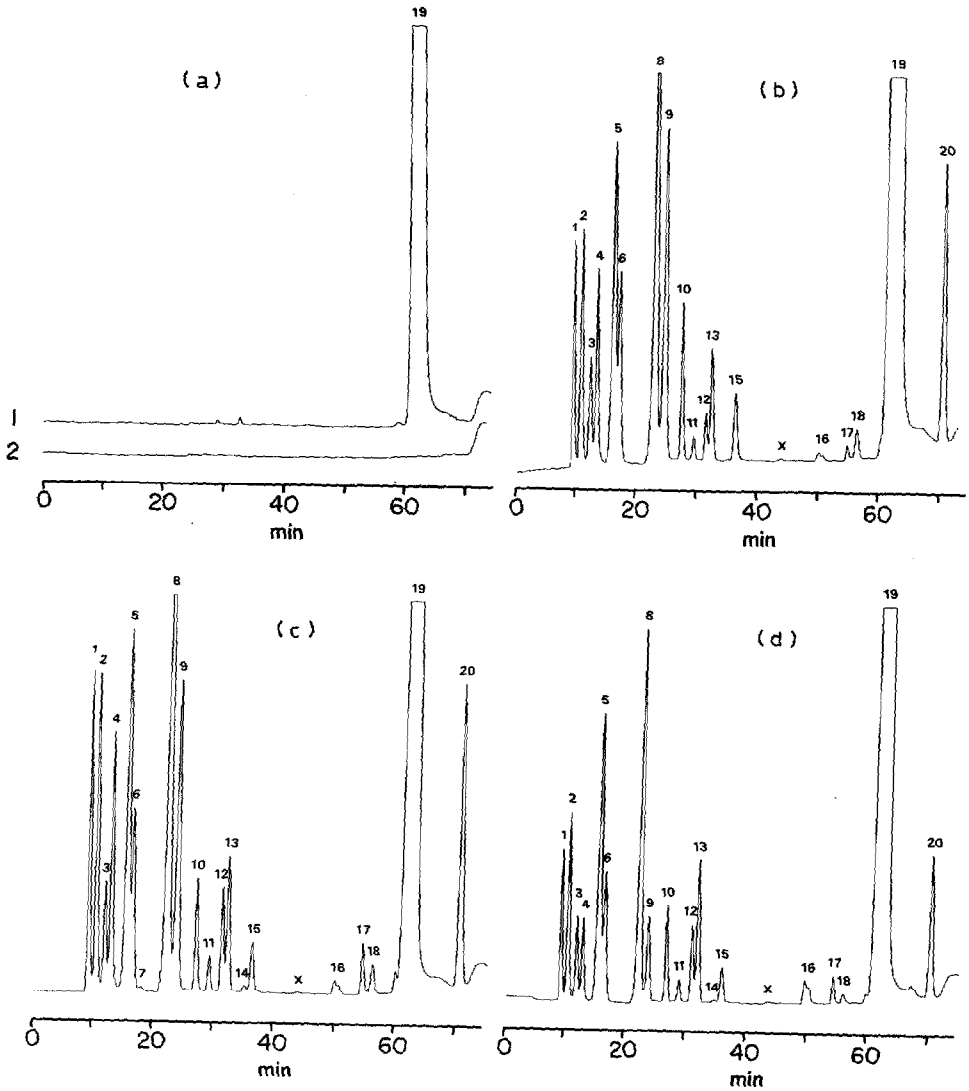

Fig. 3. Elution chromatograms showing relative fluorescence of each amino acid of the empty gel and those of α1-chains derived from collagens I, III and V. a-1, empty gel + CBB (2 μg); a-2, buffer alone; b, α1 (I) chain; c, α1 (III) chain; d, α1 (V) chain. Peaks: (1) 4-Hyp; (2) Asx; (3) Thr; (4) Ser; (5) Glx; (6) Pro; (7) Cys; (8) Gly; (9) Ala; (10) Val; (11) Met; (12) Ile; (13) Leu; (14) Tyr; (15) Phe; (16) Hyl; (17) His; (18) Lys; (19) NH_3; (20) Arg.

One could not determine the amino acid compositions of collagen samples by the method reported previously[1], for the following reasons: (1) the hydroxylysine content might be slightly above its true value, because methionine sometimes decomposes into an unidentified compound which elutes just after histidine and overlaps with the hydroxylysine peak[5]; (2) as the ratio of gel to each α-chain of collagen was high, the arginine peak was eluted as a shoulder on the ammonia peak, so another run was

needed in order to obtain better resolution of arginine by switching the buffer system from the pH gradient type to one at pH 5.28[5]; and (3) the resolution of 4-hydroxyproline from aspartic acid was not always satisfactory. The method described here was able to overcome these disadvantages by changing the gradient programme of mobile phase.

Fig. 1 shows the gradient programme used for the mobile phase. When the concentration of solution B gradually rose from 30% to 65%, the peak of hydroxylysine separated from that of the breakdown product derived from methionine decomposition (16 and X in Fig. 3b, c and d), and the "buffer-change" peak disappeared (Fig. 3a-2). When solution B was maintained at 65% for 5 min (Fig. 1), the arginine peak was separated well from the ammonia peak without eluting as a shoulder on the ammonia peak (19 and 20 in Fig. 3b, c and d). After washing in solution C for 30 min and equilibrating in solution A for another 30 min, analysis was performed in accordance with the gradient. In this way the 4-hydroxyproline and aspartic acid could be separated consistently (1 and 2 in Fig. 3b, cand d). Under these conditions, the hydroxylysine, both in the hydrochloric acid-hydrolysed sample and the L-hydroxylysine standard, showed two neighbouring peaks, for unknown reasons (16 in Fig. 3b, c and d).

The electrode buffer components, Tris and glycine, were reportedly removed by simply washing the gel slices several times with destaining solution and then five times with 3 ml of acetone following the destaining procedure[1]. As shown in Fig. 3a-1, the empty gel and CBB (2 μg) used showed negligible contamination, if any, by amino acids, and they were free from glycine in particular.

The amino acid compositions of the six α-chain samples are summarized in Table I. Fairly satisfactory results were obtained for the amino acid compositions of the six α-chains derived from collagens I, III and V. Every collagen sample has high glycine, proline and hydroxyproline contents, and collagen III has the highest levels of glycine and hydroxyproline and contains cysteine. Table I indicates that the mean values of the glycine, proline and hydroxyproline contents in the six α-chain collagens were about 34%, 11% and 10%, respectively. These concentrations are virtually the same as the reported values, indicating that they are all collagenous. The data are reasonably consistent with the previously reported values. Thioglycolic acid provided effective protection against the decomposition of tyrosine, cysteine and methionine; however, the recovery of methionine was inconsistent[1].

Amino acid analysis with the OPA reagent is fairly sensitive, and one must be very careful to minimize background contaminants through all the steps of the preparative procedure. Contamination by serine and glycine has been traced to fingerprints and micron-size particles of skin[9], and glass tubes sometimes contain glycine and other amino acids (>10 pmol per tube)[10]. Most of the above problems can be solved by protecting the gels from fingerprints by wearing rubber gloves and pyrolysing the glassware, especially the hydrolysing and evaporating tubes, in a flame before use[1].

CONCLUSIONS

A method has been developed for amino acid analysis with a single-stained collagen band directly hydrolysed with a polyacrylamide gel slice using HPLC and

TABLE I

AMINO ACID COMPOSITIONS DETERMINED FROM STAINED GEL BANDS OF THE SIX α-CHAINS OF COLLAGENS I, III AND V

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

Amino acid	*α1 (I)*		*α2 (I)*		*α1 (III)*		*α1 (V)*		*α2 (V)*		*α3 (V)*	
	Mean (n=3)	*Reported*[6]	*Mean (n=3)*	*Reported*[6]	*Mean (n=4)*	*Reported*[7]	*Mean (n=3)*	*Reported*[8]	*Mean (n=3)*	*Reported*[8]	*Mean (n=3)*	*Reported*[8]
3-Hyp	ND*	1	ND	1	ND	0	ND	0.9	ND	1.1	ND	0.9
4-Hyp	97	108	84	93	121	125	107	99	106	107	96	91
Asx	40	42	43	44	47	42	48	50	49	50	47	42
Thr	17	16	19	19	16	13	20	22	30	26	23	19
Ser	31	34	28	30	36	39	20	23	32	34	30	34
Glx	75	73	67	68	72	71	100	100	86	88	104	98
Pro	125	124	106	113	100	107	126	125	105	105	103	99
Cys	0	0	0	0	2	2	0	1.2	0	ND	0	1.3
Gly	343	333	344	338	359	350	336	325	343	325	334	332
Ala	114	115	108	102	89	96	40	41	54	57	45	49
Val	21	21	37	35	13	14	20	21	30	31	27	29
Met	3	7	4	5	4	8	6	6.8	11	10	7	8.1
Ile	7	6	17	14	13	13	18	20	14	18	17	20
Leu	21	19	34	30	23	22	42	44	36	39	53	56
Tyr	Trace	1	2	4	2	3	2	2.5	Trace	2.1	2	2.4
Phe	13	12	11	12	8	8	11	12	11	11	11	9.2
Hyl	8	9	12	12	12	5	41	46	19	23	35	43
His	3	3	12	12	7	6	7	6.3	10	9.9	14	14
Lys	28	26	18	18	23	30	14	15	12	13	13	15
Arg	55	50	56	50	53	46	43	42	55	52	42	42

* ND = Not detected.

a post-labelling method with OPA. By this method, it is possible to determine nineteen amino acids, including 4-hydroxyproline and hydroxylysine, by a single-step procedure. This method will be very useful for the amino acid analysis of collagens composed of heterogeneous α-chains, especially those which can be resolved only by PAGE.

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