PREPARATION AND ISOLATION OF THE *S*-CARBOXYMETHYL DERIVATIVE CHAINS OF HUMAN FIBRINOGEN

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1. Introduction

Fibrinogen is composed of three pairs of polypeptide chains: α(A), β(B), and γ [1, 2]. Fibrinopeptides A and B are cleaved off the α(A) and β(B) chains respectively by thrombin. The resulting product is fibrin [3, 4]. Sulfitolysis of fibrinogen results in the cleavage of the disulfide bridges [5], and the resulting S-sulfo chain derivatives have been separated by paper [5], starch gel electrophoresis [6] and column chromatography [5, 7]. More recently, the S-sulfo chain derivatives from bovine [8] and human [9] fibrinogen have been isolated on carboxymethyl cellulose using a stepwise [8] and gradient [9] sodium acetate buffer system.

In this study, the *S*-carboxymethyl derivatives of the three human fibrinogen chains were separated on carboxymethyl cellulose, with an exponential sodium acetate buffer gradient, and a concomitant pH gradient, in the presence of 8 M urea. Each chain was characterized and identified by *N*-terminal amino acid analysis, polyacrylamide gel electrophoresis, fingerprinting, and amino acid analysis.

2. Materials and methods

Fibrinogen: A 3.67% solution of human fibrinogen, 98% clottable [10], in 0.3 M NaCl was lyophylized.

Dithiothreitol (DTT) [11, 12] was obtained from P-L Biochemicals Inc. Milwaukee, Wis. Lot No. 72–17.

Iodoacetic acid (Kebo) was recrystallized 5X from CCl_4, and stored in the dark. This product was called: Carrier.

2-^{3}H-Iodoacetic acid (Radiochemical Centre, Amersham, England) Lot No. B 10, specific activity: 50–150 mCi/mmole, was diluted appropriately with carrier iodoacetic acid before use.

^{3}H-Iodoacetic acid-carrier mixture was prepared fresh by mixing 4 ml of 1 M iodoacetic acid carrier (186 mg/ml of 0.1 N NaOH) with 6 ml of 2-^{3}H-iodoacetic acid (1 mCi/ml of 0.1 N NaOH).

Carboxymethyl cellulose (CM-52) was precycled as recommended by the manufacturer (H. Reeve Angel and Co., London, England).

Radioactivity measurements were performed with a Beckman liquid scintillation system (CPM 200).

N-Terminal sequence amino acid analysis: The three stage phenylisothiocyanate method of Edman was used [13–16] using phenylisothiocyanate-^{35}S as the coupling agent [17]. This procedure permitted the analysis to be performed on 2–5 mg quantities of each chain.

Amino acid analysis [18] was performed on a Technicon autoanalyzer. Caution was exercised in evacuating the samples thoroughly in preparation for 22 hr acid hydrolysis in an atmosphere of N_2. This insured minimal destruction of *S*-carboxymethyl cysteine.

Polyacrylamide gel electrophoresis was carried out at acid pH in a disc electrophoresis analytical apparatus (Shandon) as described [19], with slight modifications. A 7.5% gel solution was used, and the samples were electrophoresed for 2.5 hr.

Trypsinolysis and fingerprinting were performed on thin-layer plates (Whatman CC-41) as described [20], using Trypsin-TPCK (Worthington) with a 12 hr digestion period.

Antibody production and purification: Antibodies

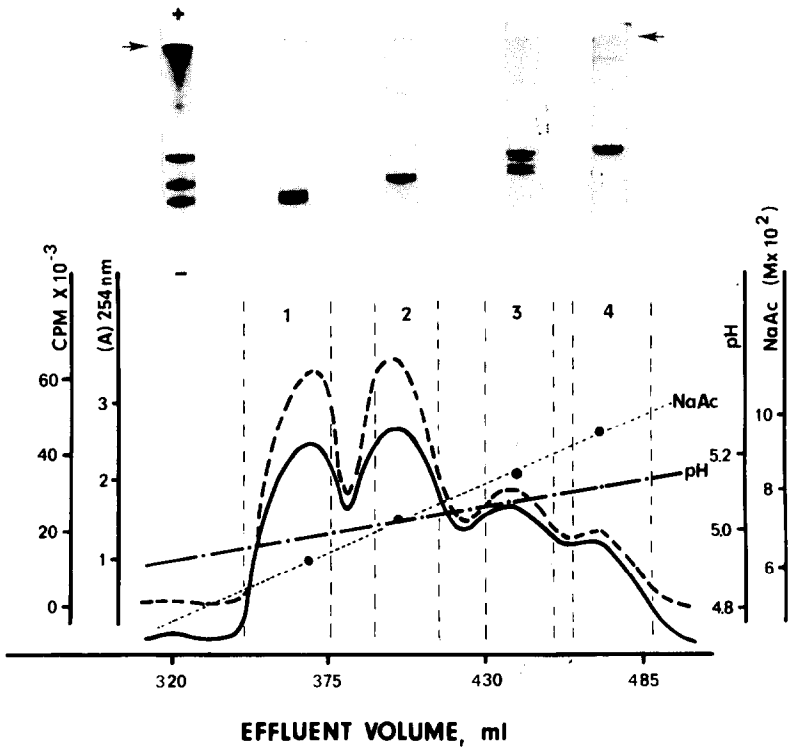

Fig. 1. Chromatographic profile of human *S*-carboxymethyl fibrinogen on Whatman cellulose (CM-52), using a sodium acetate and pH gradient, in the presence of 8 M urea. (———) (A) 254 mm. (– – –) cpm $\times 10^{-3}$. Polyacrylamide gel electropherograms, performed in 5% acetic acid, appear directly above each peak. The arrows indicate the origin. The electropherogram to the far left, containing three major components, is typical for *S*-carboxymethyl fibrinogen prior to chromatography.

to the individual chains were elicited in rabbits by routine foot-pad immunization and purified as described [21] using BrCN activated Sephadex [22] for the preparation of the immunoadsorbents.

All other chemicals were reagent grade.

2.1. *Reduction and S-carboxymethylation*

The lyophylized, salt containing fibrinogen product (1.168 g, containing 760 mg pure fibrinogen) was dissolved in 45 ml of deareated 8.4 M guanidine chloride (Kebo) pH 8.5. Once the product was in solution, the pH was readjusted to 8.5 with 1 N NaOH. The mixture was incubated at 40° for1 hr. DTT (97 mg) in 1 ml of deaerated distilled water was added (10 moles/mole disulfide). The pH was adjusted to 8.8 ± 0.1 with 1 N NaOH. The vessel was flushed with nitrogen, capped, and incubated at ambient temperature for 1.5 hr. The vessel was wrapped in aluminium foil and carboxymethylation commenced by the addition of 7.8 ml of 2-^{3}H-iodoacetic acid-carrier mixture (5 moles/mole DTT). The pH usually dropped below 6.0, and was immediately adjusted to 8.3 ± 0.1 with 2 N NaOH. The reaction was allowed to proceed for precisely 15 min. Glacial acetic acid was added to a final concentration of 50%, and the sample applied directly to a Sephadex G-25 (Pharmacia) column 5 × 70 cm, previously equilibrated with 50% (v/v) acetic acid. The column was wrapped in aluminium foil, and developed at a flow rate of 150 ml/hr. The carboxymethylation reaction and subsequent chromatography were carried out in the dark to avoid oxidation of iodide to iodine, thereby minimizing modification of tyrosine, histidine and tryptophan residues. Complete separation of salts and excess reagents from the protein was attained. The sample was lyophylized. The yield was quantitative. This product was called: CM-fibrinogen.

2.2. *Chromatography on CM-cellulose*

The buffers utilized in this system were always prepared fresh. A column of cellulose 2.5 × 15 cm was

Table 1
Amino acid composition of *S*-carboxymethyl fibrinogen (human) and its *S*-carboxymethyl chain derivatives (μmoles/mg)*.

Residue	CM-fibrinogen	Peak 1 γ chain	Peak 2 β (B) chain	Peak 3 α (A) +? chain	Peak 4 α (A) chain
CM-Cys	0.127	0.142	0.139	0.102	0.071
Asp	1.011	1.031	1.035	0.975	0.801
Thr	0.466	0.440	0.339	0.493	0.517
Ser	0.615	0.412	0.458	0.836	0.875
Glu	0.989	0.967	1.033	0.936	0.814
Pro	0.397	0.266	0.377	0.507	0.455
Gly	0.761	0.617	0.699	0.865	0.819
Ala	0.412	0.476	0.412	0.304	0.279
Val	0.387	0.307	0.426	0.419	0.342
Cys/2	0	0	0	0	0
Met	0.176	0.159	0.257	0.152	0.117
Ile	0.332	0.432	0.285	0.277	0.209
Leu	0.479	0.484	0.479	0.470	0.363
Tyr	0.269	0.335	0.316	0.129	0.106
Phe	0.251	0.296	0.172	0.221	0.224
Try	–	–	–	–	–
Lys	0.608	0.580	0.522	0.525	0.474
His	0.169	0.152	0.107	0.142	0.165
Arg	0.420	0.198	0.408	0.538	0.467

* Represents an average of 4 analyses on two separate products.

equilibrated with starting buffer (0.025 M sodium acetate–8 M urea, pH 4.8) at a flow rate of 12–14 ml/hr at ambient temperature. CM-fibrinogen (320 mg) was dissolved in approximately 6 ml of starting buffer, and applied on top of the cellulose bed with several 2 ml washes. The column was developed with a gradient formed by a Technicon 'autograd'. Chambers 1–3 contained 400 ml of starting buffer, chambers 4–6 contained 400 ml of: 0.175 M sodium acetate–8 M urea, pH 5.2 buffer. The effluent was collected in 5.5 ml fractions and the protein zone monitored by automatic absorbance measurements at 254 nm. (LKB Uvicord II, and LKB Recorder). The peaks were pooled as indicated in the chromatographic profile (fig. 1). Salts were removed by extensive dialysis against distilled, deionized water (alkaline with NH_3 to minimize precipitation). The protein solution was lyophilized. For analytical purposes, each peak fraction from several runs (100–150 mg) was rechromatographed on a fresh column under the same conditions and pooled sharply.

3. Results and discussion

The yield (total protein) from a typical chromatography was about 85%. The *S*-carboxymethyl products were insoluble in water, but soluble in 10% acetic acid or 8 M urea solutions. The amino acid analysis (table 1) revealed that all disulfide bridges were reduced and carboxymethylated. No side reactions, such as the carboxymethylation of the ε-amino group of lysine or the oxidation of methionine to sulfoxides were observed. Fig. 1. is a typical chromatographic profile. Radioactivity measurements were concurrent with the carboxymethyl cysteine content calculated from the amino acid analyses (table 1). Polyacrylamide gel electrophoresis (fig. 1) showed peaks 1, 2 and 4 to be virtually homogeneous. The width of the electrophoretic band of peak 1 indicated a minor heterogeneity, probably of the type recently described for the γ chain [8]. Peak 3 contained 4 molecular species, one of which corresponded to peak 4.

N-Terminal amino acid analysis on CM-fibrinogen compared well with that of native fibrinogen. The Ala + Asp/Tyr ratio was 0.85 ∓ 0.1/1.0. Both products contained traces (2–5%) of glutamic acid and glycine.

Table 2
N-Terminal amino acids.

Peak No.	Chain identification	*N*-Terminal residues	
		Step 1	Step 2
1	γ	Tyr (Gly, Glu, Asp, Ala)*	Val
2	β (B)	None	None
3	α (A) +?	Ala 21% 15% 9% ** (Asp, Glu, Gly)	Asp (Ser)*
4	α (A)	Ala 22% 14% 14% ** (Asp, Glu, Gly)	Asp (Ser)*

* Traces (less than 5%).
** Percent of Ala.

The *N*-terminal sequence analysis (table 2) confirmed the homogeneity of peak 1, 2 and 4, and identified them as: γ, β (B) and α (A) chains respectively [23]. The pyroglutamyl *N*-terminus on the β (B) chain does not permit coupling with phenylisothiocyanate. Peak 3, although heterogeneous in polyacrylamide gel electrophoresis, displayed the same *N*-terminal amino acid profile as peak 4.

From the *N*-terminal sequence analysis (table 2) and from the amino acid analysis data, it was postulated that peak 3 might in effect contain structural variants of the α (A) chain (peak 4). This being the case, in the fingerprint profiles, peak 3 should exhibit substantial similarities to peak 4. This is precisely what was observed (fig. 2, A and D). Furthermore, immunologic studies performed with the double diffusion technique of Ouchterlony showed that antibodies against peak 4 (α (A)) reacted with immunologic identity between peak 3 (α (A) +?) and peak 4 (α (A)).

Extensive physico-chemical and immunologic studies characterizing the three chains, including the 'variants' in peak 3, are in progress.

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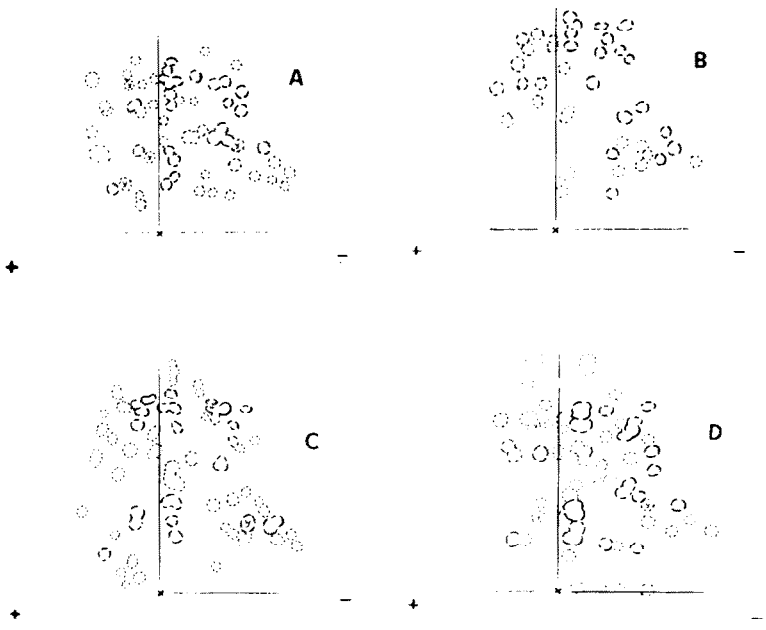

Fig. 2. Fingerprint profiles of the tryptic digests of the four peak components shown in fig. 1. Electrophoresis (⟷). chromatography (↕). A = peak 4 (α(A) chain); B = peak 2 (β(B) chain); C = peak 1 (γ chain); D = peak 3 (α(A) +? chain(s)). The plates were developed with ninhydrin. Each spot is marked according to intensity. The spots marked *y* appear yellow, and are usually indicative of proline and/or glycine. They serve as excellent landmarks for purposes of comparison. *x* = origin.

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