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# ISOLATION AND COMPARATIVE PEPTIDE MAPPING OF FIBRINOGEN SUBUNITS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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## SUMMARY

Comparative peptide mapping represents one approach to identification of structural defects in variant human fibrinogens. In view of the large size of this protein, we chose to generate peptide maps of fibrinogen subunits. A reversed-phase high-performance liquid chromatographic method was developed to isolate the subunits: fibrinogen was reduced with dithioerythritol and alkylated with iodoacetamide. Subunits were isolated on a Vydac TP, $C_4$ column (25 × 1.0 cm). Eluent A was 0.1% aqueous trifluoroacetic acid (TFA); eluent B was 0.1% TFA in acetonitrile. Initial conditions were 65% A, 35% B, at 2 ml/min. The reduced-alkylated subunits were lyophilized, redissolved in 0.1% TFA plus 4–8 *M* guanidine–HCl, and chromatographed using a linear gradient (1%/min) to 50% B. This procedure provides homogeneous subunits in yields exceeding 90%, and is therefore superior to conventional cation-exchange chromatography. For comparative peptide mapping, the same stationary and mobile phases were used, except that the initial conditions were 90% A/10% B, and a linear gradient to 60% B (1%/min) was used. Alternatively, peptide maps were generated with a 10 × 0.46 cm Spherisorb ODS-2 column and very shallow gradients. The mapping procedure resolves 45–60 peptides with excellent reproducibility, and has been applied to the identification of an apparent polymorphism in fibrinogen Baltimore II, and the structural defect in fibrinogen Baltimore IV.

## INTRODUCTION

Fibrinogen is a coagulable, 340-kDa plasma glycoprotein containing three pairs of non-identical subunits, $(A\alpha, B\beta, \gamma)_2$, connected by disulfide bonds[1,2]. Congenital dysfibrinogenemia is a disease in which a structurally and functionally altered form of

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fibrinogen is responsible for coagulation abnormalities such as excessive bleeding or thrombosis[3]. The molecular defects responsible for the abnormal coagulability of these proteins have been described for only about 10% of the 150–200 cases reported worldwide.

One approach to the identification of amino acid substitutions in dysfibrinogens involves comparative peptide mapping by reversed-phase high-performance liquid chromatography (HPLC). This is not feasible for a protein as large as fibrinogen, because the number of peptides generated in a typical endoproteolytic digest (> 100) exceeds the selectivity limits of presently-available stationary phases. Consequently, it is advisable to reduce fibrinogen to its component subunits and —to improve solubility and prevent re-formation of disulfides— to alkylate the resultant cysteines with iodoacetic acid or iodoacetamide.

Conventional cation-exchange chromatography has been used to isolate fibrinogen subunits[4–6]. While this technique results in homogeneous subunit preparations, yields are low due to substantial overlap among the subunit-containing peaks, and the product is obtained in a solution containing 6–8 *M* urea. These limitations are unacceptable when, as is often the case in dysfibrinogenemia, only 10–20 mg of fibrinogen are available for analysis.

A method for isolating fibrinogen subunits by reversed-phase HPLC has been published[7]. However, this method failed to resolve all three subunits to baseline, resulted in slight contamination of the B$\beta$-subunit with $\gamma$-subunit, and was applied only to the isolation of microgram quantities of material.

In the present report we describe a semi-preparative method for isolating reduced or reduced-alkylated fibrin(ogen) subunits in high yield, and two systems which have been successfully applied to comparative peptide mapping of variant human fibrinogens.

## EXPERIMENTAL

### *Materials*

A pooled preparation of human fibrinogen was obtained from Kabi Vitrum, Stockholm, Sweden. Glycine, tosylphenylchloroketone (TPCK)-trypsin, porcine pepsin (Cat. No. P-7012), lysine-Sepharose, dithioerythritol, dithiothreitol, and iodoacetamide were purchased from Sigma, St. Louis, MO, U.S.A. Sephadex G-50SF was obtained from Pharmacia, Piscataway, NJ, U.S.A. HPLC-grade acetonitrile was from Baker, Phillipsburg, NJ, U.S.A. and trifluoroacetic acid (TFA) was from Eastman Kodak, Rochester, NY, U.S.A. Guanidine–HCl was from Pierce, Rockford, IL, U.S.A. A Vydac 214-TP510 butylsilane column (25 × 1.0 cm) was obtained from the Separations Group, Hesperia, CA, U.S.A. and a Spherisorb ODS-2 column (10 × 0.46 cm) was from Alltech Assoc., Deerfield, IL, U.S.A.

### *Isolation of fibrinogen subunits*

A commercial preparation of fibrinogen (Kabi, Grade L) was preincubated with lysine-Sepharose[8] to remove plasmin(ogen), and further purified by a single glycine precipitation step[9]. This material served as "control" fibrinogen. Abnormal human fibrinogens were purified from patient plasma by repetitive glycine precipitation as described previously[10,11]. All fibrinogen preparations were found to be >98% pure

on the basis of sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)[12] and a functional test for coagulability[13].

In accord with the method of Murano *et al.*[4], fibrinogen (5–10 mg/ml) was dissolved in 0.5 *M* Tris–HCl (pH 8.5) containing 6 *M* guanidine–HCl (buffer A), reduced with dithioerythritol or dithiothreitol (in ten-fold molar excess over the 29 disulfides per fibrinogen molecule) for 90 min at 37°C, and alkylated in the dark for 20 min with a five-fold molar excess (over reducing agent) of iodoacetamide or iodoacetic acid. After removal of reagents by Sephadex G-50SF gel chromatography in 10% aq. acetic acid, the reduced-alkylated subunits were lyophilized and stored at −20°C. We have found fibrin(ogen) to be resistant to complete reduction by β-mercapthoethanol (2%, v/v) under these conditions, unless incubation is carried out for at least 6 h at 37°C. Thus, reduction by dithioerythritol or dithiothreitol is preferred. However, once reduction has been accomplished, we have found no evidence of subunit reassociation during HPLC.

To prepare fibrin subunits, fibrinogen (5 mg/ml) was incubated with human thrombin (1 U/ml) for 60 min at 30°C. The resulting clot was removed by centrifugation, washed with 0.15 *M* sodium chloride and distilled water, redissolved in 2–5 ml of buffer A, and reduced and alkylated as above.

The lyophilizate [containing reduced or reduced-alkylated fibrin(ogen)] was dissolved in a minimal volume of 4–8 *M* guanidine–HCl in 0.1% aq. TFA. Aliquots of up to 0.5 ml (*ca.* 5 mg of protein) were injected onto a Vydac butylsilane column protected by a 0.5-μm in-line filter (Rheodyne No. 7350; Thomson Instrument, Newark, DE, U.S.A.) and equilibrated with 65% eluent A (0.1% aqueous TFA) and 35% eluent B (acetonitrile). The subunits were resolved by using the gradient listed in Table I. Protein-containing peaks were pooled and concentrated using a Savant Speed-Vac concentrator (Savant Instruments, Farmingdale, NY, U.S.A.).

*Comparative peptide mapping of the β-subunit*

To minimize heterogeneity, it is important to use conditions which yield a complete digest of the protein or subunit in question. For trypsin hydrolysis, the reduced-alkylated subunit (2–5 mg) was dissolved in 1 ml of a solution containing 0.1 *M* sodium phosphate buffer (pH 6.5) and 2 *M* urea. Proteolysis was carried out for 24 h

TABLE I

GRADIENT PROGRAM FOR ISOLATION OF FIBRINOGEN SUBUNITS

| *Time (min)* | *Eluent A (%)* | *Eluent B (%)* | *Flow (ml/min)* |
|---|---|---|---|
| 0 | 65 | 35 | 2.0 |
| 5 | 65 | 35 | 2.0 |
| 20 | 50 | 50 | 2.0 |
| 22 | 50 | 50 | 2.0 |
| 24 | 40 | 60 | 4.0 |
| 27 | 40 | 60 | 4.0 |
| 31.5 | 65 | 35 | 4.0 |
| 34.5 | 65 | 35 | 4.0 |
| 35 | 65 | 35 | 2.0 |

at 30°C in the presence of TPCK-trypsin (1%, w/w). For pepsin hydrolysis, the reduced-alkylated subunit (2–5 mg) was dissolved in 100 μl of 50% aldehyde-free acetic acid. This solution was diluted ten-fold with hydrochloric acid (1 m*M*). Proteolysis was carried out for 60 h at 30°C in the presence of pepsin [1%, w/w) in 1 m*M* hydrochloric acid]. The digests were evaported to dryness and dissolved in a solution of 95% eluent A, 5% eluent B. Peptide mapping was carried out on a Varian Model 5500 liquid chromatograph (Varian, Walnut Creek, CA, U.S.A.) and the data were analyzed by a Varian CDS-402 chromatography data system.

*Other methods*

The concentration of fibrinogen was determined by the method of Lowry *et al.*[14] or was measured spectrophotometrically ($A^{1\%}_{280\,nm,\,1\,cm} = 15.1$)[15]. SDS-PAGE was performed on a vertical slab gel electrophoresis apparatus using the Laemmli system[12] and a 10% resolving gel. Gas phase sequencing (Applied Biosystems, Foster City, CA, U.S.A.) was performed according to the manufacturer's specifications on a sequenator equipped with an on-line amino acid analyzer.

## RESULTS

The three fibrinogen subunits were completely resolved (Fig. 1) with the Vydac butylsilane column using the conditions described in Experimental and Table I. Protein yields (based on the Lowry *et al.* method[14]) exceeded 90%. The heterogeneity

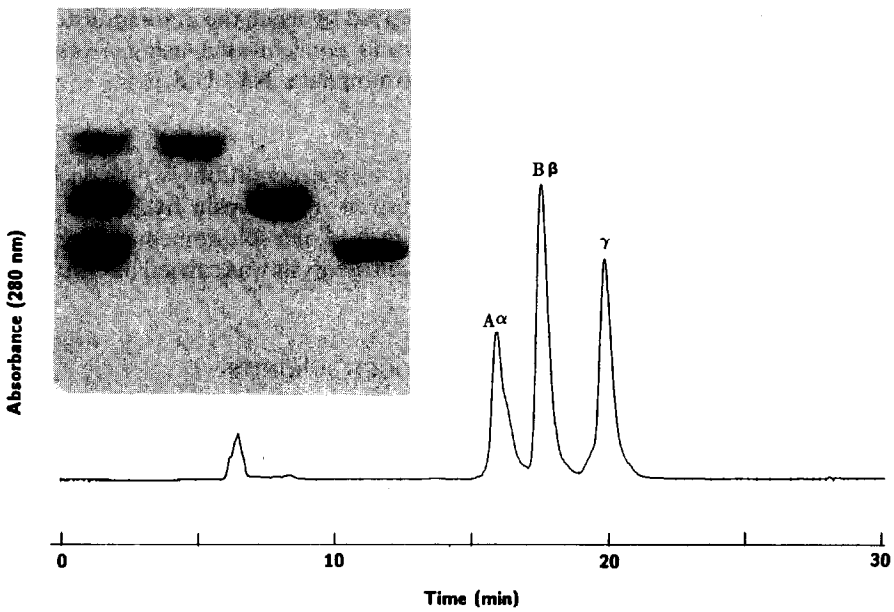


Fig. 1. Isolation of fibrinogen subunits. Approx. 2 mg of reduced-carboxyamidated fibrinogen in 0.25 ml of a solution containing 4 *M* guanidine–HCl plus 0.1% TFA was applied to a VYDAC TP-butylsilane column (25 × 1.0 cm). The column was developed with the gradient described in Table I (eluent A, 0.1% TFA in water; eluent B, acetonitrile). The subunits eluted over the range 38–45% eluent B. Inset: SDS-PAGE of fibrinogen. From left to right: reduced-carboxyamidated fibrinogen, HPLC-purified Aα, Bβ and γ subunits.

evident from the shoulder on the Aα-subunit peak is apparently due to partial proteolysis which is known to occur during purification of fibrinogen from plasma. This heterogeneity was not observed when we prepared fibrinogen from normal and patient plasma[10,11]. The subunits were judged to be homogeneous on the basis of SDS-PAGE (Fig. 1, inset). Similar results were obtained when the subunits were reduced but not alkylated and when fibrin subunits were generated by pre-incubation of fibrinogen with thrombin prior to reduction and alkylation.

Gas phase sequencing of the Aα- and γ-subunits disclosed a single amino acid sequence corresponding to that known for each protein. The Bβ-subunit is blocked at the N-terminus with pyroglutamic acid; however, when a new N-terminus was exposed after incubation of fibrinogen with thrombin, a single sequence corresponding to that of the β-subunit was obtained. Thus, the sequencing data support the conclusion that each subunit has been purified to homogeneity.

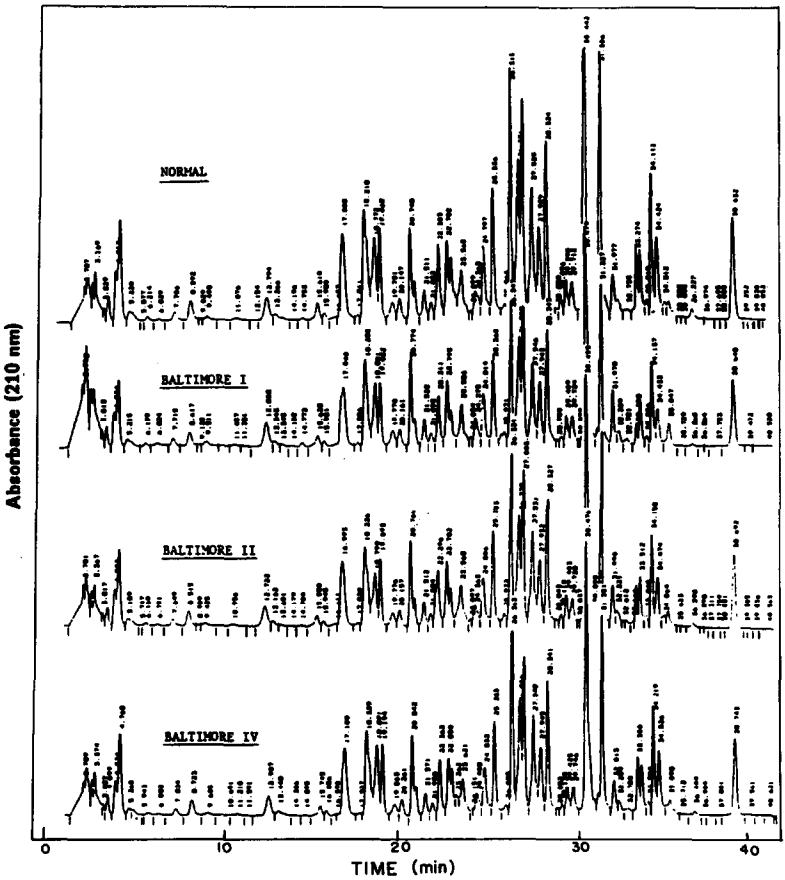


Fig. 2. Comparative peptide mapping of β-subunits on a Vydac butylsilane column. Fibrinogen was proteolyzed with thrombin, reduced, alkylated with iodoacetamide, and the subunits were isolated as described in Experimental. The β-subunits from four different fibrinogen preparations were digested with TPCK-trypsin. HPLC was performed with the stationary and mobile phases described in Fig. 1. The column was developed with the gradient described in Table II. At 90 min intervals, 30–50 μg of protein were injected.

TABLE II

GRADIENT PROGRAM FOR COMPARATIVE PEPTIDE MAPPING

| *Time (min)* | *Eluent A (%)* | *Eluent B (%)* | *Flow (ml/min)* |
|---|---|---|---|
| 0 | 90 | 10 | 2.0 |
| 3 | 90 | 10 | 2.0 |
| 53 | 40 | 60 | 2.0 |
| 62 | 40 | 60 | 2.0 |
| 65 | 30 | 70 | 4.0 |
| 75 | 30 | 70 | 4.0 |
| 78 | 90 | 10 | 3.0 |
| 88 | 90 | 10 | 3.0 |
| 89 | 90 | 10 | 2.0 |

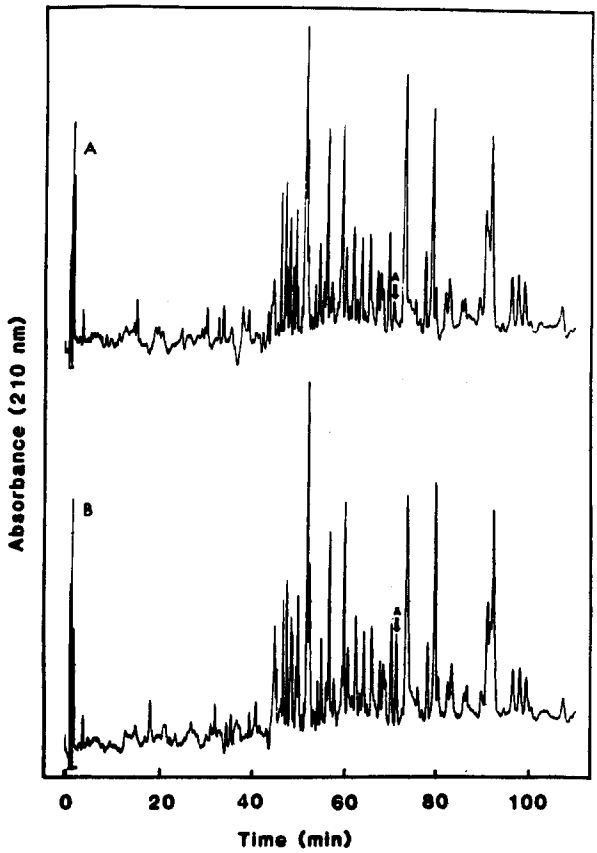


Fig. 3. Comparative peptide mapping of B$\beta$-subunits on a Spherisorb ODS-2 column. Fibrinogen was reduced, alkylated with iodoacetamide, and the subunits were isolated as described in Experimental. The B$\beta$-subunits from normal fibrinogen (profile A) and fibrinogen Baltimore II (profile B) were digested with pepsin and chromatographed under the following conditions: eluent A, 0.1% aq. TFA; eluent B, 0.1% TFA in acetonitrile; initial conditions, 95% A, 5% B; flow, 1 ml/min. After 5 min, a linear gradient to 30% B (0.22%/min) was started. The column was washed with 70% B before returning to the initial conditions.

The same mobile and stationary phases utilized to isolate the fibrinogen subunits were used to generate comparative peptide maps of trypsin-generated fragments of the β-subunit (Fig. 2). Approximately 51 peaks were obtained, and no abnormal peptides were detected. Between-run variations in peak retention times were generally less than 0.1 min, and when the profiles were compared by overlaying the chromatograms on a lightbox, most peak patterns were superimposable. With minor modifications, this peptide mapping procedure has been used to determine the structural defect in fibrinogen Baltimore IV (manuscript in preparation).

Incompletely resolved peptides could be separated by rechromatography on a Spherisorb ODS-2 column (data not shown) using the following conditions: Eluent A, 5 m*M* potassium phosphate (pH 6.0); eluent B, 50% acetonitrile in eluent A; flow 1 ml/min; initial conditions, 95% A, 5% B. Most peptides are resolved by a linear gradient (2%/min) to 100% eluent B.

An "abnormal" peptide was detected in a comparative peptide map generated from a pepsin digest of the Bβ-subunit derived from fibrinogen Baltimore II (Fig. 3). This peptide, which elutes at 72 min, was absent from other individual fibrinogen preparations, and present in very small quantities (or not detected) in commercial preparations of pooled fibrinogen. The peptide contains the sequence, Tyr-Ser-Met-Lys-Lys-Met-Ser-Met, which corresponds to residues 445–452 of the Bβ-subunit, except that Arg is replaced by Lys in position 448. Additional details of this apparent polymorphism will be published elsewhere[16].

## DISCUSSION

A major premise of the present study is that reversed-phase HPLC can be used to discriminate between a peptide containing a molecular defect and normal or wild-type peptides. We think the probability that this premise will be valid increases when: (i) the amino acid substitution is from one class to another (*e.g.*, polar to non-polar); (ii) chromatographic conditions are highly reproducible; (iii) the peptide containing the defect is fewer than 25 amino acids in length; and (iv) peptide heterogeneity due to post-translational or chemical modifications is minimal. It is important to note that individuals with dysfibrinogenemia are usually heterozygous for the trait; thus, the expected result is half the normal amount of a given peptide, plus a new (variant) peptide. These considerations have guided the development of our approach.

The method described for isolating fibrinogen subunits by reversed-phase HPLC resolves all three subunits rapidly, and in high yield. It is most appropriate when the supply of fibrinogen is limited to 10–20 mg, and provides subunits in less than 2 ml of a volatile solvent which is easily removed by vacuum drying or lyophilization.

Some important features of our methods for comparative peptide mapping are illustrated in Fig. 2. (i) Peptide heterogeneity is expected due to prosthetic groups (*e.g.*, phosphate, carbohydrate). Such heterogeneity might lead to the selection of false-positives for further study. However, by evaluating several dysfibrinogens simultaneously, we were able to distinguish between normal heterogeneity (such as that which occurred in peaks at 23 and 33 min) and true positives. If several different dysfibrinogens are not available, the same effect can be achieved by concurrent mapping of fibrinogen prepared from individual donors. (ii) Peak elution times and areas must be extremely reproducible. Among the four chromatograms shown in Fig.

2, elution times for a given peak rarely varied by more than 4 s. This is accomplished by autosampling, which standardizes the time between sample injections. (iii) By using a semi-preparative column and injecting only about 5% of the digest, scaling up to isolate peaks of interest simply involves the injection of more material onto the same column.

For the comparative peptide map illustrated in Fig. 3, two major modifications were introduced: The stationary phase was changed from butylsilane (5-$\mu$m silica, 300-Å pores), to octadecylsilane (3-$\mu$m silica, 80-Å pores), and an extremely shallow acetonitrile gradient (0.22%/min) was used. This very efficient chromatographic system provided excellent resolution of about 45 peaks, and made possible the identification of an apparent polymorphism in fibrinogen. It has also been used in isocratic mode to resolve peptides generated from fibrinogen by thrombin[17].

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