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Multiple peaks induced by domain-specific binding of fibrinogen in anion-exchange high-performance liquid chromatography

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Abstract

A domain binding model was developed for explaining the multiple peak chromatograms obtained in the high-performance liquid chromatography of pure fibrinogen on a DEAE polymethacrylate column using different gradients of ammonium chloride. The different peaks for fibrinogen result from the binding of either the D or E domain of fibrinogen to the packing material. This was confirmed by comparing the retention times of the chromatograms for fibrinogen, fragment D_1 and fragment E. Native and denatured forms of fibrinogen are proposed to be important to fibrinogen's interaction with the column, hiding or exposing the E domain, respectively. Different gradient speeds resolve a different number of peaks for fibrinogen, with slow gradients yielding essentially one peak and fast gradients 10 or more peaks. Temperature studies were done to confirm the model. Different commercial sources of fibrinogen showed different proportions of native and denatured/degraded forms.

Keywords: Protein–stationary phase binding; Retention model; Fibrinogen; Proteins

1. Introduction

Increasingly, high-performance liquid chromatography is being utilized in the determination of macromolecular species. In comparison to the chromatography of small analytes, chromatographic behavior of macromolecular species is far more complex and more difficult to characterize. The effect of protein structure on chromatographic behavior has been reviewed [1]. One aspect that has not been extensively studied is the effect of orientation of the macromolecule as it interacts with the stationary phase. This aspect may be contributory to the mechanism of retention of a macromolecule, which has many potential sites of interaction, as opposed to a small molecule, which would interact with the stationary phase through one site on the molecule (e.g., interaction of a charged site on an analyte with the ion-exchange ligand on an ion-exchange packing material) or through the entire structure of the molecule (e.g., a partitioning mechanism in reversed phase).

The present work provides evidence for different domains on a protein molecule binding to the stationary phase, which is used to explain the observed chromatographic pattern. Fibrinogen was chosen as a model protein because its structural features lend itself to studying a domain-dependent hypothesis of protein retention. These features are distinct domains in the protein and a well-defined

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proteolysis pattern, yielding fragments that consist predominantly of intact domain structures (such that chromatography of the protein can be compared with that of the domain structure).

The properties of human fibrinogen have been extensively studied including physiological function and structural characteristics [2–5]. A review has been written covering various aspects of fibrinogen and proteolysis of fibrinogen [6]. Fibrinogen is a dimer, with identical monomer units consisting of three polypeptide chains (Aα, Bβ, and γ) interconnected via a network of disulfide bonds. Each monomer also contains a pair of M_r $2.5 \cdot 10^3$ N-linked biantennary oligosaccharides. The structure of fibrinogen is given in Fig. 1. It contains three globular domains, consisting of a central E domain connected by coiled coils to two identical terminal D domains. The Aα chain extends from the D domain and is hypothesized to interact with the E domain [4,7,8].

An important characteristic of fibrinogen for the present study is its pattern of proteolytic cleavage by plasmin to form fibrinogen degradation products (FDPs), which is also shown in Fig. 1. The sequence of proteolytic steps by plasmin has been intensively studied [9–11]. Under action of plasmin, the transient degradation products fragment X and fragment Y are formed first. At the end stage, the major digestion products formed are fragments D and E. Throughout the digestion, the D and E domains are kept essentially intact, although peptides extending from the domains are extensively cut. Fragment D consists of the D domain and a portion of the coiled

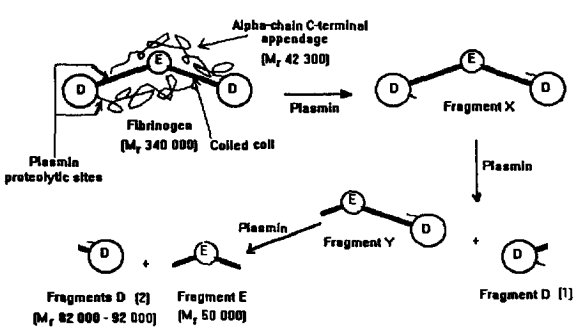

Fig. 1. Schematic representation of the degradation of fibrinogen by plasmin, producing intermediate fragments X and Y, which are further degraded to the final products, fragments D and E. Fragments D and E consist predominantly of the D and E domains, respectively.

coil, while fragment E consists of the E domain with two residues of the coiled coil. Fragment E has a molecular mass of about 50 000. Spectroscopic studies have been done which reveal secondary structural aspects of fragments D and E [12].

Fragment D can be further differentiated into 3 forms: D_1, D_2, and D_3; which have molecular masses of 92 000, 86 000 and 82 000, respectively [13]. The conversion of D_1 to D_2 to D_3 occurs at the later stage of plasmin digestion, through the successive cleaving of segments from the C-terminal portions of the γ-chains. In the presence of calcium, however, the conversion of $D_1 \rightarrow D_3$ is hindered [14,15].

Chromatographic techniques for fibrinogen have largely been limited to low-performance methods. Finlayson and Mosesson [16] observed three overlapping peaks for purified human fibrinogen (Blombäck fraction I-4) chromatographed on DEAE–cellulose. They confirmed the heterogeneity of fibrinogen by rechromatography of the first and second fractions. Three resolved peaks were also observed by Francis et al. [17] who applied commercial fibrinogen to DEAE–Sephacel. The separation of fragment D and fragment E has been achieved by a DEAE–cellulose column [18] and a chromatofocusing technique [19]. However, the separation of fibrinogen from fragments D and E using ion-exchange chromatography has not been reported.

The drawback of low-performance chromatography is low resolution and long analysis times. Recently, this laboratory has extended fibrinogen methodology to HPLC, reporting the determination of fibrinogen in plasma by high-performance immunoaffinity chromatography [20]. This work successfully addressed the major problem encountered in the HPLC of fibrinogen, namely irreversible retention of fibrinogen. This was solved through use of a polymethacrylate HPLC support. Use of polymethacrylate supports for ion-exchange HPLC can now be done, extending the capabilities of HPLC in fibrinogen analysis.

In the present work, the chromatographic behavior of fibrinogen in anion-exchange HPLC has been examined. Fibrinogen was found to give multiple peaks, with the chromatographic pattern being strongly dependent on the eluting conditions. A domain-dependent retention model is proposed to

explain both the multiple peaks observed and the shift among these peaks when the gradient speed was varied. The model was established by comparing the chromatographic behavior of fibrinogen with that of its proteolytic degradation products.

2. Experimental

2.1. Materials and preparation of samples

Fibrinogen from Calbiochem (human plasma, plasminogen-free, clottable proteins >95%, Cat. No. 341578, Lot. 229491, La Jolla, CA, USA) and from ICN (human plasma, 90%, Cat. No. 151123, Lot. 54304, Cleveland, OH, USA) was used.

To generate the plasmin-induced FDPs fragment D_1 and fragment E, fibrinogen and plasminogen were co-purified as described [21] with ϵ-amino-caproic acid omitted from the procedure, then the fibrinogen (4 mg/ml) was coagulated with human thrombin (4 U/ml) from Ortho Diagnostics (Raritan, NJ, USA) in the presence of streptokinase (4000 U/ml) from Behringwerke (Marburg, Germany) to activate the plasminogen; with (1) 2.5 m*M* calcium added to prevent degradation of fragment D_1 [14,15] and (2) 50 μM methylmercuric bromide from AlfaAesar (Ward Hill, MA, USA) added as an inhibitor of factor XIII to block formation of D_1 dimers. After overnight incubation at 37°C (approximately two-times the lysis time), plasmin was inactivated using both phenylmethylsulfonylfluoride (0.1 m*M*) and aprotinin (20 U/ml), both from Sigma (St. Louis, MO, USA), and the degradation products were dialyzed into starting buffer and purified by chromatography on DEAE–Sepharose according to Yoshida et. al [22]. As assessed by SDS–PAGE electrophoresis, fragment D_1 exhibited a single peak and fragment E had only a 10% contamination of D_1.

In addition to the purified fragments D_1 and E, one experiment used FDPs without separation of individual fragments (Fig. 6b). A procedure similar to that previously reported was employed [23,24]. Plasminogen (Cat. No. 191342), aprotinin (Cat. No. 190382), and streptokinase (Cat. No. 101114) were obtained from ICN (Cleveland, OH, USA). Fresh fibrinogen (Calbiochem) solution (10 mg/ml) was prepared with buffer P for fibrinolysis. Digestion was activated by addition of streptokinase (40 U/ml, final concentration) to the fibrinogen solution, which was mixed with plasminogen (0.5 U/ml, final concentration). The process was performed at 37°C and was stopped with aprotinin (510 kIU per 500 μl digest solution). The digest was stored at −20°C for chromatography later on.

All other chemicals used for this work were ACS certified reagents including tris[hydroxymethyl] amino-methane and urea from Sigma, ammonium chloride from Aldrich (Milwaukee, WI, USA) and sodium chloride from Fisher (Fair Lawn, NJ, USA).

Bulk anion-exchange packing material was purchased from Millipore Corporation (Cat. No. 35688, Milford, MA, USA). The packing material was diethylaminoethyl-functionalized polymethacrylate, with a pore size of 1000 Å and an average particle diameter of 15 μm.

2.2. Composition of injected samples

A buffer solution (buffer P), consisting of 25 m*M* Tris–HCl, 0.15 *M* NaCl, pH 7.40, was used for the preparation of all protein solutions. To remove possible low M_r components, fibrinogen samples were prepared with buffer P (1.0 ml of 10 mg/ml) and then dialyzed at 4°C against 500 ml buffer P for 6 h, using a dialysis membrane (Spectra/Por 7, M_r cut-off 25 000) from Spectrum (Houston, TX, USA). Fibrinogen solutions (10 mg/ml) were stored at −20°C until use. Buffer P was different than the starting mobile phase (buffer A1 or A2, described below). However, there was no difference in the chromatographic pattern for fibrinogen prepared in buffer P compared to fibrinogen prepared in starting buffer (A1 or A2). Buffer P was used because it stabilized fibrinogen in solution.

Fragment D_1 (1.1 mg/ml) and fragment E (0.42 mg/ml) were stored at −70°C until use. Prior to chromatography, fragments D_1 and E were ultrafiltered twice to change the original buffer to buffer P (replacing filtered solution with an equal amount of buffer P after each ultrafiltration). The ultrafiltration was performed at 4°C using Centricon-30 (M_r cut-off 30 000) from Amicon (Beverly, MA, USA).

For the rechromatography experiments, each peak fraction was concentrated to approximately 150 μl, subsequently diluted to 750 μl with starting buffer

A1, and reconcentrated by ultrafiltration (as described above). This process was repeated once more, with the final concentrated solution being chromatographed.

2.3. Chromatography

Chromatography was performed on a system consisting of two Model 112 solvent delivery module pumps equipped with a Model 421 system controller from Beckman (Berkeley, CA, USA), a variable wavelength detector from Dionex (Sunnyvale, CA, USA) connected to a WINner system consisting of an SP 4270 integrator from Thermo Separation Products (San Jose, CA, USA) and a 386SX computer. A Rheodyne Model 7125 injection valve from Rainin (Emeryville, CA, USA) was used. The injection volume of fibrinogen solution was 20 μl except for the fractions that were rechromatographed, in which case approximately 100 μl was used. A Model EX200 constant temperature water bath from Neslab Instruments (Portsmouth, NH, USA) was used for the column temperature studies. DEAE anion-exchange columns (50×4.1 mm I.D.) were packed at a pressure of 500 p.s.i. (1 p.s.i.= 6894.76 Pa) with a buffer of 0.5 *M* NH_4Cl, 25 m*M* Tris–HCl, pH 7.4, using a HPLC slurry packer from Alltech (Deerfield, IL, USA).

Chromatographic mobile phases were: buffer A1 (starting buffer 1), 25 m*M* Tris–HCl, pH 7.40; buffer A2 (starting buffer 2), 25 m*M* Tris–HCl, 3.0 *M* urea, pH 7.40; and buffer B (eluting buffer), 25 m*M* Tris–HCl, 2.0 *M* NH_4Cl, pH 7.2. Use of mobile phase urea above 3 *M* was avoided in the chromatography of fibrinogen due to urea's strong denaturing properties. For example, chromatographed fibrinogen samples containing 8 *M* urea (in place of buffer P) did not elute fibrinogen from the column to any appreciable extent. All buffer solutions were stored at 4°C.

Unless stated otherwise, gradient programs included a preliminary wash with buffer A1 or buffer A2 for 15 min at 0.8 ml/min to pre-equilibrate the column, and then upon injection of sample, linear gradients in buffer B were run, including: 0 to 20% over 40 min at a flow-rate of 0.6 ml/min (fast gradient); 0 to 20% over 80 min at a flow-rate of 0.3 ml/min (intermediate gradient); and 0 to 24% over 144 min at a flow-rate of 0.15 ml/min (slow gradient). The column was usually cleaned by further applying an eluent containing 40% buffer B at 0.6 ml/min for 10 min prior to subsequent chromatography.

To study the effects of the column temperature, the column plus 15 cm of tubing prior to the column were inserted into the temperature-controlled water bath. The column was at ambient temperature for all other experiments.

3. Results

3.1. Chromatography of fibrinogen

3.1.1. Multiple peaks

Fibrinogen applied to a high-performance anion-exchange column yielded multiple peaks with the intermediate gradient, as shown in Fig. 2a and Fig. 3a. Each peak was collected and rechromatographed. The results in Fig. 2(b–d) reveal that each fraction could behave as the other fractions in properties of retention, although the distribution was skewed to the peak fraction originally collected.

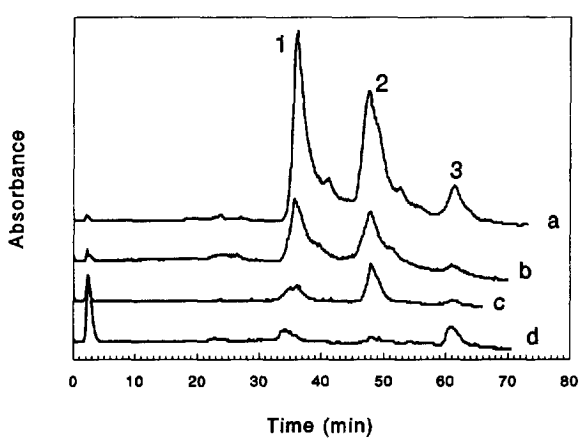

Fig. 2. Comparison of the chromatogram of fibrinogen (a) with chromatograms of rechromatographed peak 1 (b), peak 2 (c) and peak 3 (d) from the fibrinogen chromatography. Starting mobile phase was buffer A1. The intermediate gradient method was used. Injection amount for (a) was 200 μg of fibrinogen (ICN). AUFS is 0.0500 AU.

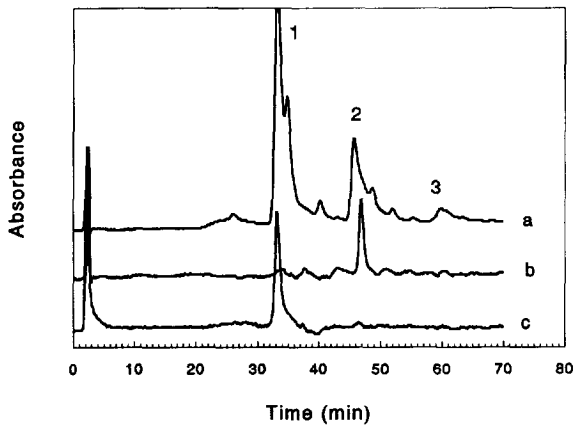

Fig. 3. Comparison of the chromatograms of fibrinogen (a), fragment E (b) and fragment D_1 (c). Starting mobile phase was buffer A2. The intermediate gradient method was used. Injection amounts were 75 μg of fibrinogen (Calbiochem), approximately 25 μg of fragment D_1 and approximately 25 μg of fragment E. AUFS is 0.0500 AU.

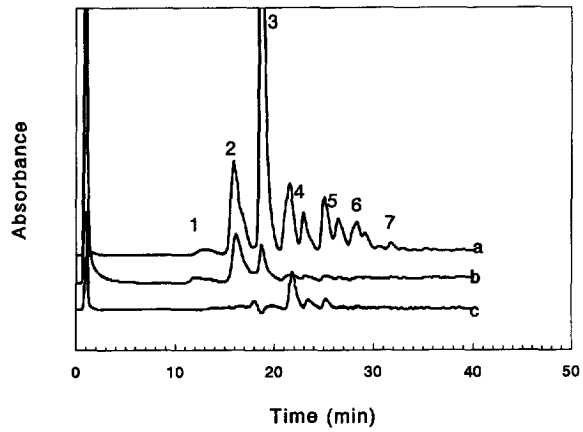

Fig. 5. Comparison of the chromatograms of fibrinogen (a), fragment D_1 (b) and fragment E (c). Starting mobile phase was buffer A2. The fast gradient was used. Injection amounts were 200 μg of fibrinogen (Calbiochem), approximately 35 μg of fragment D_1 and approximately 30 μg of fragment E. AUFS is 0.0625 AU.

3.1.2. *Effect of gradient speed*

Different gradient speeds produced different chromatographic patterns. More peaks were resolved when fibrinogen was chromatographed with a fast gradient, as shown in Fig. 4a and Fig. 5a. Conversely, use of a slow gradient produced predominantly one peak as shown in Fig. 6a. For the slow gradient, the increase in the area of the late eluting peak band appears to be at the expense of the early eluting peak bands. Fig. 6b shows the chromatogram of an end-stage plasmin digest of fibrinogen with the slow gradient, showing that the shift from the early to late eluting peak(s) did not occur for FDPs as it did for

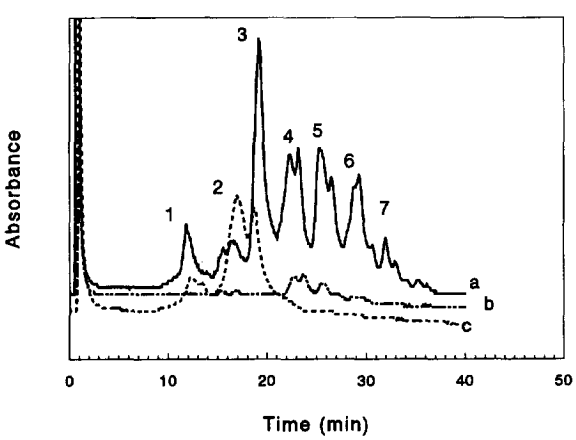

Fig. 4. Comparison of the chromatograms of fibrinogen (a), fragment E (b) and fragment D_1 (c). Starting mobile phase was buffer A1. The fast gradient was used. Injection amounts were 200 μg of fibrinogen (ICN), approximately 40 μg of fragment D_1 and approximately 30 μg of fragment E. AUFS is 0.0250 AU.

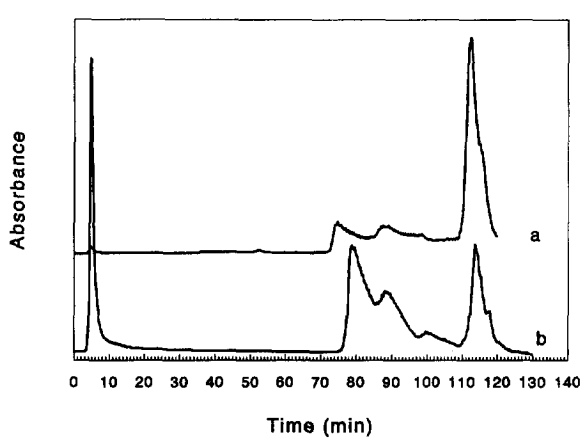

Fig. 6. Comparison of the chromatograms of fibrinogen (a) and a 300 min plasmin digest of fibrinogen (b). Starting mobile phase was A1. The slow gradient was used. Injection amounts were 140 μg of fibrinogen (Calbiochem) and 100 μg of total protein in the digest. AUFS is 0.0875 AU.

fibrinogen (Fig. 6a). As seen in Fig. 6b, chromatography of FDPs leads to a substantial void peak, which may be due to small peptide fragments generated by plasmin. The shift from the early eluting peak band(s) to the late eluting peak band was also observed when a shallow pH gradient was employed (data not shown). The chromatographic pattern is reproducible on a day-to-day basis for a given gradient speed.

3.1.3. Urea and fibrinogen source effects

Comparison of Figs. 4 and 5 shows increased resolution with urea-containing mobile phases, as minimally resolved doublets of peaks 4 and 5 in Fig. 4 are well resolved in Fig. 5. The difference in peak proportions in comparing Fig. 4 and Fig. 5 is attributed to the different commercial sources of fibrinogen and not to the difference in the urea content of the mobile phase. Peak proportions were similar in runs using mobile phases with and without urea when fibrinogen from the same source was used. This difference in peak proportions for fibrinogen from different sources is also noted in comparing Fig. 2 and Fig. 3.

3.1.4. Self-association studies

Fibrinogen molecules have strong adsorptive properties. Therefore, it was necessary to check whether fibrinogen self-association was occurring, which has been reported to cause multiple peaks in chromatography [25]. Chromatographic profiles of fibrinogen did not change significantly when different concentrations of fibrinogen (20 μl, 20–0.75 mg/ml) were injected, implying that the multiple peaks were not induced by aggregation. Also, self-association would not occur in urea-containing solutions, as urea has been shown to dissociate fibrin complexes [26]. Thus, the multiple peak pattern would not be expected in chromatography using mobile phases with urea. However, Fig. 3a shows multiple peaks for fibrinogen chromatographed in urea-containing mobile phases.

3.1.5. Dwell time

The effect of the column dwell time (time in which analyte is adsorbed to the packing material) on chromatographic behavior of fibrinogen was also examined. A fibrinogen sample was injected into the starting buffer (flow-rate of 0.15 ml/min), which was subsequently pumped for 50 min. The intermediate gradient was then employed, which yielded the same results as given in Fig. 2a, except for a slight decrease in recovery. This result indicates that the shift of peak area noted for slow gradients from the early to late eluting peaks (Fig. 6a), was not caused by an extended adsorption effect but rather by an extended elution effect.

3.1.6. Temperature effects

The effect of changing column temperature on chromatographic behavior of fibrinogen is shown in Fig. 7. Increasing the temperature of the column greatly reduced the area of the early eluting peak (peak 1), while the area of the late eluting peaks (peaks 2 and 3) were either maintained or increased.

3.1.7. Chromatography of fragments D_1 and E

Chromatography of purified samples of fragments D_1 and E were separately done using the different gradient speeds. Assignment of many of the peaks in the fibrinogen chromatogram to a particular domain binding could thus be made from these experiments. Fig. 3 shows the results for the intermediate gradient, where fragment D_1 corresponded to peak 1 of fibrinogen and fragment E corresponded to peak 2 of fibrinogen. Peak 3 of the fibrinogen chromatogram

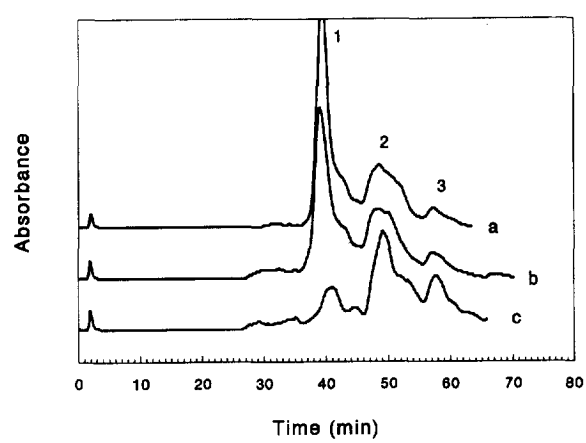

Fig. 7. Comparison of the chromatograms of fibrinogen obtained at column temperatures 12°C (a), 25°C (b) and 40°C (c). Starting mobile phase was buffer A1. The intermediate gradient method was used. Injection amount was 100 μg of fibrinogen (Calbiochem) for each run. AUFS is 0.0500 AU.

does not appear in the chromatograms of fragment D_1 or fragment E.

Increasing the gradient speed resulted in an increased number of peaks for fragment D_1 and fragment E, which is similar to the results obtained for fibrinogen. Fig. 4 and Fig. 5 show the chromatography of fragments D_1 and E compared to the chromatography of fibrinogen using a fast gradient. Fig. 5 shows that the fragment D_1 peaks corresponded to peaks 1, 2 and 3 of fibrinogen, while the fragment E peaks corresponded to the fibrinogen peak 4 doublet (both peaks) and the first peak of the peak 5 doublet. Peaks 5 (second peak in the doublet), 6 and 7 of fibrinogen do not appear in the chromatograms of fragment D_1 or E.

4. Discussion

4.1. Possible explanations for multiple peaks

Heterogenous forms of fibrinogen have been reported extensively in the literature [6,27]. Variations have been reported in molecular mass [28,29], charge [30,31], sialic acid content [31], γ-chain composition [17,30,32–35], degree of proteolysis of the Aα chain [17,36,37] and other properties such as solubility and coagulability [38–40]. Heterogeneous forms have been separated on low-performance DEAE columns [16,17]. However, the above-mentioned heterogeneity is not the over-riding explanation for the multiple peaks observed in this study, since a collected peak yields a similar multiple peak pattern upon rechromatography (Fig. 2). Also, reducing gradient speed shifts the peak distribution to essentially one late eluting peak (Fig. 6), which would not occur if fixed heterogeneous forms were present.

Another explanation given for multiple peaks is the presence of different conformational forms of the protein [25,41–48], which is consistent with the observations of the present work. Normally, if a protein can bind in a variety of conformations, a broad peak would be observed, indicating a continuum of such forms [49]. The results here are unusual in that discrete peaks are obtained instead of a continuum. In general, early eluting peaks seem to be associated with binding through the D-domain of fibrinogen and late eluting peaks with E-domain binding. The latest eluting peaks, however, do not correspond to binding of any one domain.

4.2. Models in the literature

The feasibility of differential protein retention due to site-specific interaction with the column is reasonable, according to mechanisms proposed by others for protein retention in ion-exchange chromatography, as discussed below. The binding of a protein in ion-exchange chromatography usually involves electrostatic interaction between the stationary phase and multiple charged sites on the protein. At low ionic strength, there is little partitioning of the protein between the stationary and mobile phases due to the low probability of simultaneous dissociation of the multiple binding sites. Regnier and co-workers [50,51] derived an expression relating protein retention to salt concentration of the mobile phase. The capacity factor for the protein is inversely proportional to salt concentration raised to the Z power, with Z being the number of charged sites on the protein that bind to the stationary phase. For Z values>1, the protein is able to partition between the mobile and stationary phases within only a narrow gradient window. This window becomes even more narrow as the Z value increases. In the other parts of the gradient, the protein is strongly adsorbed (low salt) or completed released (high salt). This is the 'on–off' mechanism of protein chromatography.

Binding of a protein to the ion-exchange stationary phase can only occur through a particular 'face', not through every charged site present on the protein's three-dimensional structure [1]. Thus, orientation of the protein is important, since a particular protein can be adsorbed to a surface through different regions on the protein [52]. However, it has been suggested that an ion-exchanger can 'steer' the orientation of a protein so that binding occurs through domains having a high charge density [50]. The effect of protein domain in dictating retention of the entire protein has been reported for hydrophobic interaction chromatography of different isoforms of estrogen receptors [53].

The appearance of multiple peaks in the present work can be explained by the two mechanistic aspects of protein binding described above (binding

through specific regions and the 'on–off' retention). Different adsorbed orientations would elute as separate peaks, given the non-partitioning mechanism of the 'on–off' mechanism and assuming the protein does not interact with the column further.

4.3. Kinetic versus thermodynamic effects on binding orientation of fibrinogen

A model depicting the chromatography of fibrinogen is shown in Fig. 8. Binding via the E domain is thermodynamically favored, since fragment E has a longer retention time than that of fragment D_1. The E domain does, in fact, have a greater negative charge density than the D domain [3]. Conversely, binding through the D domain is kinetically favored, since the D domains are more numerous and are sterically accessible. It is hypothesized here that activation energy is required to change the conformation of native fibrinogen to a form having an exposed E domain. This is supported by the work of several investigators who propose a structure for native fibrinogen which has the Aα chain folding over the E domain [4,7,8], making the E domain sterically unavailable for binding. Francis et al. [17] noted a shift to late eluting peaks in anion-exchange chromatography when the Aα chain was significantly degraded. In the present work, a steady increase in the E-binding peaks is seen with increased time of storage of fibrinogen solutions (data not shown). This suggests that the process of denaturation is occurring, which exposes the E domain. In addition, rechromatography studies given in Fig. 2 suggest that there is interconvertibility between the peaks, indicating the presence of different conformational forms.

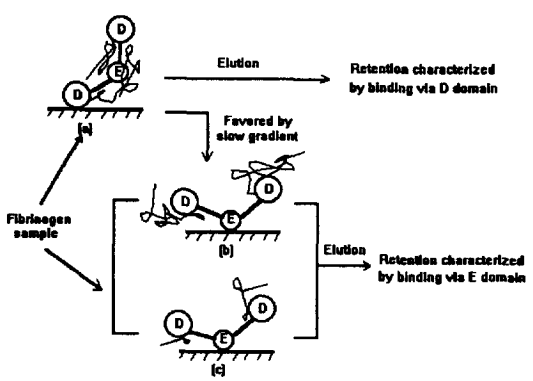

Fig. 8. Schematic representation of the binding model for fibrinogen in anion-exchange chromatography. Three forms of fibrinogen are proposed: (a) native fibrinogen which binds via the D domain, (b) denatured fibrinogen and (c) degraded fibrinogen which both bind via the E domain.

It is thus hypothesized here that there are at least two binding forms of fibrinogen; a native form, which shows D-binding retention characteristics and an E-domain-exposed form (Aα-denatured and Aα-degraded fibrinogen forms), which shows E-binding retention characteristics. The presence of these forms is demonstrated by comparing chromatograms of fibrinogen from two different commercial sources. Fig. 2 and Fig. 3 show that the binding pattern for fibrinogen differs according to commercial source. Fibrinogen from ICN (Fig. 2) has an increased proportion of peak 2 compared to fibrinogen from Calbiochem (Fig. 3), thus indicating an increased proportion of denatured and/or degraded forms in the fibrinogen from ICN. This difference in peak proportions for fibrinogen from different sources is also seen for the fast gradient runs in Fig. 4 and Fig. 5.

It is possible to find a salt concentration which is sufficiently high to cause desorption of fibrinogen bound through the D-domain, but not high enough to cause desorption of fibrinogen bound through the E-domain. This explains the multiple peaks observed with the intermediate and fast gradients (Figs. 2–5). Fibrinogen adsorbed via the D-domain is desorbed earlier in the salt gradient than E-bound fibrinogen. For an intermediate/fast gradient there is insufficient time for desorbed D-bound fibrinogen to reorient to a conformation favoring re-binding through the E-domain. Thus, early eluting peaks are D-bound fibrinogen and late eluting peaks are E-bound fibrinogen. By contrast, if the gradient speed is sufficiently low, then the desorbed D-bound fibrinogen has enough time to undergo a conformational change, permitting readsorption through the E-domain. This accounts for the shift of essentially all fibrinogen to one E-binding peak with the slow gradient (Fig. 6a). In contrast, there is no similar shift of the early eluting peaks in the chromatography of FDPs (Fig. 6b). This is because the early eluting peaks in the FDPs chromatogram presumably result from fragment D,

which does not have a stronger binding site to further interact with the column after desorption occurs.

Not considered in this model is the possibility of DE or DD binding. Such orientations could conceivably account for the unidentified late-eluting peaks for the fibrinogen profiles in the present work. Other workers have reported that the D-dimer fragment of fibrinogen elutes in two different peaks in hydrophobic interaction chromatography [54]. This could reflect different binding orientations of the D-dimer, possibly single (D domain) and double (D–D domain) binding, supporting the proposed hypothesis for the latest eluting peak(s).

4.4. Temperature effects

The D domain is different than the E domain in thermal stability. This property of fibrinogen offers an additional opportunity to test the binding model. Scanning microcalorimetric studies of fibrinogen and its proteolytic degradation products reveal that the D domain is denatured at approximately 55°C or lower, while the coiled coil portion and E domain are stable to approximately 70°C or higher [5]. The results of the chromatography of fibrinogen at different temperatures are shown in Fig. 7. The peak corresponding to D-domain binding (peak 1) decreases substantially with increased temperature. This is an expected result, given the thermal lability of the D domain. On the other hand, the relative proportion (compared to D-bound peak) of the late eluting E-bound peak (peak 2) is seen to increase with increasing temperature. The increase in temperature provides fibrinogen with energy, which could promote the change in conformation needed to expose the binding regions of the E domain.

4.5. Implications for protein chromatography

In cases where there is evidence of alternative protein conformations, it is important to determine if the conformations exist naturally in solution or if they are artifacts resulting from contact with the stationary phase. For example, tumor necrosis factor seems to dissociate from a trimer to monomers upon binding in hydrophobic interaction chromatography, which reassociate in solution to the trimer [55]. In the case of anion-exchange of fibrinogen, when a peak is collected and rerun, the same 3-peak pattern is obtained (Fig. 2). However, the resulting distribution is skewed toward the peak originally collected. The conformational shift is thus slow relative to the time scale of the chromatography. This implies that fibrinogen assumes alternative conformations that really exist in solution and are not artifacts of contact with the stationary phase. The difference in chromatographic profiles for fibrinogen from different commercial sources may also be evidence for the actual presence of different conformations of fibrinogen in solution, although different proportions of degraded fibrinogen could also explain the different profiles.

In contrast to the 'steering effect' model, in which the macromolecule orients on the stationary phase via the strongest binding site [50], our data suggest that there are circumstances where macromolecules can bind in metastable orientations. Under favorable conditions, the macromolecules can subsequently change conformation, leading to a more stable binding. These factors are important since they can lead to elution in broad bands [49] or even discrete peaks (as seen in the present work). Fibrinogen is a special case; having well-defined, isolated domains. However, these factors are worth examining for other proteins as well.

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