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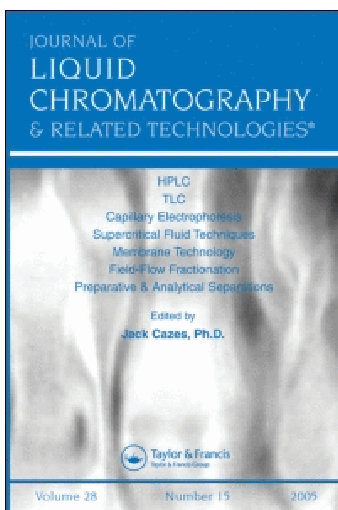


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JOURNAL OF LIQUID CHROMATOGRAPHY, 6(2), 229-248 (1983)

# CHARACTERIZATION OF PROTEIN-PROTEIN AND PROTEIN-LIGAND INTERACTIONS BY HIGH PERFORMANCE SIZE EXCLUSION CHROMATOGRAPHY

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## ABSTRACT

HPLC has been used in our laboratory to characterize a wide range of protein-protein and protein-ligand interactions. In a study of the dissociation and recombination of human chorionic gonadotropin subunits, HPLC provided a fast and sensitive method for directly observing the state of association of samples equilibrated under various conditions. The $\alpha$ subunit (15 Kd) was easily resolved from the $\beta$ subunit (23 Kd) using a Toyo Soda type SW 3000 column (0.8 x 60 cm) eluting at 1 ml/min. The subunit was poorly resolved from the intact hormone (38 Kd) in agreement with results obtained using conventional exclusion media. In another study, the same column was used to assess the degree of aggregation of various protease inhibitors (antithrombin III (AT III), C1-inactivator and $\alpha_1$-proteinase inhibitor) after heating, as part of an effort to determine conditions under which these potentially therapeutic proteins might withstand pasteurization to reduce the risk of transfusion hepatitis. The ability of AT III (65 Kd) to bind heparin (5-20 Kd) and thrombin (37 Kd) was also readily ascertained by HPLC. When native inhibitor was premixed with excess heparin, its elution shifted toward the void and became broader due to the polydispersity of the mucopolysaccharide. By contrast, formation of a complex with thrombin only slightly increased the rate of elution of AT III. Nevertheless, the extent of complex formation could be determined from the depletion of the much slower moving thrombin peak. The latter approach proved useful for characterizing thrombin after covalent attachment of fluorescent probes.



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## INTRODUCTION

Our interest in HPLC was triggered by the advent of an exclusion column through which at least some of the proteins being studied in our laboratory would pass freely. The ability to ask simple short term questions regarding the state of association of a macromolecule and realizing the answer in only 20-30 min has had a definite impact on our program. Most of our experience is with systems involving strong complexes which are stable on the column. These include (1) protease inhibitors, which form covalent complexes with their target enzymes, and some of which interact with heparin, a mucopolysaccharide which binds strongly but reversibly, shifting the elution of the inhibitors to shorter times, (2) human chorionic gonadotropin (hCG) subunits which interact reversibly to form an active hormone with an association constant $> 10^8 M^{-1}$ at room temperature; and (3) heated proteins whose denaturation is sometimes detected by the formation of aggregates, or by loss of ability to interact with other macromolecules, either event being amenable to detection and characterization by HPLC . The latter application is part of a program to determine conditions which prevent denaturation so that potentially therapeutic human plasma proteins can be pasteurized to reduce the risk of transfusion hepatitis. The purpose of this report is to share our experience with high-performance size-exclusion chromatography so that others might appreciate the power of this tool. The results presented here

have been published in preliminary form as part of the abstracts of the International Symposium on HPLC of Proteins and Peptides, November, 1981, Washington D.C.

## MATERIALS AND METHODS

Highly purified hCG and its subunits were obtained from Dr. Robert Canfield of Columbia University via the Center for Population Research, National Institute Child Health and Human Development, NIH. Antithrombin III and C1-Ina were gifts from Dr. Milan Wickerhauser of this institution. The purification and characterization of AT III has been described (1). C1-Ina was partially purified from plasma by ion exchange chromatography and further purified as described herein. Alpha$_1$-proteinase inhibitor was obtained from Dr. Charles Glazer of the Institutes of Medical Sciences, San Francisco. C1s was partially purified by a modification of the procedure of Bing et al., (2) and Taylor et al., (3). Thrombin was a gift of Dr. John Fenton, of the New York State Department of Health, Albany. Bovine Pancreatic Trypsin was purchased from Cal Biochem and used without further purification.

All chromatography experiments were conducted at room temperature using 0.8 x 60 cm exclusion columns donated by or purchased from Toyo Soda Manufacturing (TSK G3000SW). Unless otherwise stated, the eluting buffer was 0.02 M potassium phosphate pH 7.35, plus 0.15 M NaCl. Elution was controlled at 1

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ml/min with a Waters M600A pump. Samples were injected with a Waters U6K injector and protein elution was monitored at 280 nm with a Waters Model 450 Variable Wavelength detector. Other details of the experimental conditions can be found in the figure legends and in the appropriate references given in the text.

## RESULTS

### Human Chorionic Gonadotropin

Human chorionic gonadotropin (hCG) is a glycoprotein comprised of two nonidentical subunits, α and β, held together by noncovalent bonds (4,5). The dissociated subunits are biologically inactive but activity can be recovered by combining the isolated subunits under physiological conditions (6). Studies of the kinetics of dissociation and recombination would be facilitated by the availability of a fast and sensitive method to assess the state of association of a given sample. The TSK G3000SW column has proven useful for this purpose and we have utilized HPLC along with that of fluorescence polarization, to study the reversible dissociation of hCG subunits at elevated temperatures and neutral pH (7).

Figure 1A illustrates the elution of several samples of hCG which were incubated in 0.01 M potassium phosphate pH 7, at various temperatures until equilibrium was established. Profile A refers to the intact native hormone which elutes as a single symmetrical peak, while profile B refers to a sample incubated

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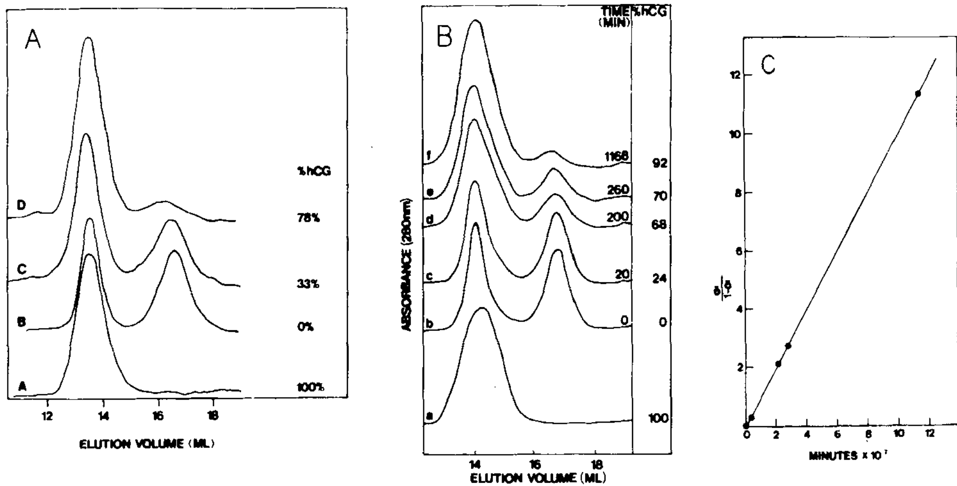


Figure 1 - Analysis of the state of association of human chorionic gonadotropin subunits using Toyo Soda exclusion column TSK G3000SW.

A) Determination of the extent of dissociation of hCG (54 M) following prolonged incubation at neutral pH at various temperatures a) 25°C (100% associated), b) 80°C (100% dissociated), c) 65°C (33% associated), d) dissociated subunits incubated at 37°C for 18 hrs (78% asssociated).

B) Analysis of the time couse for recombination of acid-dissociated hCG subunits (22 μM) a) intact native hormone, b) dissociated subunits obtained by incubation at pH 2.5 and 37°C for 1 hr, c-f) samples withdrawn at the indicated times after adjustment of pH to 7.

C) Second order kinetic plot of the recombination data shown in panel b. k = $385 M^{-1} min^{-1}$.

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at 80° C resulting in complete dissociation. The α subunit elutes several minutes later than either the intact hormone or the β subunit, the latter two being unresolved on this column. A similar lack of resolution between hCG and hCG-α is obtained by conventional chromatography using Sephadex G-100 (8). The rate of subunit recombination at room temperature under the conditions of elution in Fig. 1A is too slow to cause a significant change in the state of association during the time required for the analysis. The relative areas under the two peaks in profile B is consistent with the known extinction coefficients of the subunits. Profile C refers to a sample equilibrated at 65° C. Analysis of the areas indicates that 33% of the hormone is in the associated state, corresponding to a dissociation constant of $7.3 \times 10^{-5}$M. Using this approach it was possible to determine the dependence of the subunit dissociation constant on temperature (7). Profile D illustrates the reversibility of the reaction. This profile refers to a sample which was first equilibrated at 80° C causing complete dissociation and then held overnight at 37° resulting in 78% recombination; longer times are required for complete recombination under these conditions.

Figure 1B illustrates the time course for recombination of acid-dissociated hCG subunits in 0.01 M potassium phosphate pH 7 at 37° C. The intact hormone (profile A) was dissociated by incubating at pH 2.5 and 37° (profile B) and then neutralized. A sample withdrawn immediately after neutralization had the same

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profile. Samples withdrawn at various times after neutralization showed increasing amounts of recombination (profiles C-F). A second order plot of the kinetic data obtained in this fashion is shown in Figure 1C. The rate constant obtained from the slope (k = $385M^{-1}min^{-1}$) is in good agreement with that obtained previously by spectral methods (9).

The lack of resolution between hCG and hCG-β on this column as well as on conventional exclusion columns could be due to an unfolding of the isolated β subunit resulting in a larger exclusion radius. However, an unfolded conformation seems unlikely in view of the high polarization of tyrosyl fluorescence (8). Studies with the homologous hormone, ovine lutropin, suggested that most of the unfolding which accompanies subunit dissociation occurs in the α subunit (10). An alternative explanation for the peculiar elution behavior would be the existence of a highly asymmetric but perhaps rigid conformation for hCG-β which combines with a flexible α subunit to form a more spherical complex whose exclusion radius is similar to that of isolated β. It is of interest that the elution peak for hCG-β is narrower than that of the intact hormone suggesting a lower diffusion coefficient for the former, consistent with an asymmetric conformation.

## Antithrombin III

Antithrombin III (AT III) is a circulating protease inhibitor which inactivates thrombin and most serine proteases of

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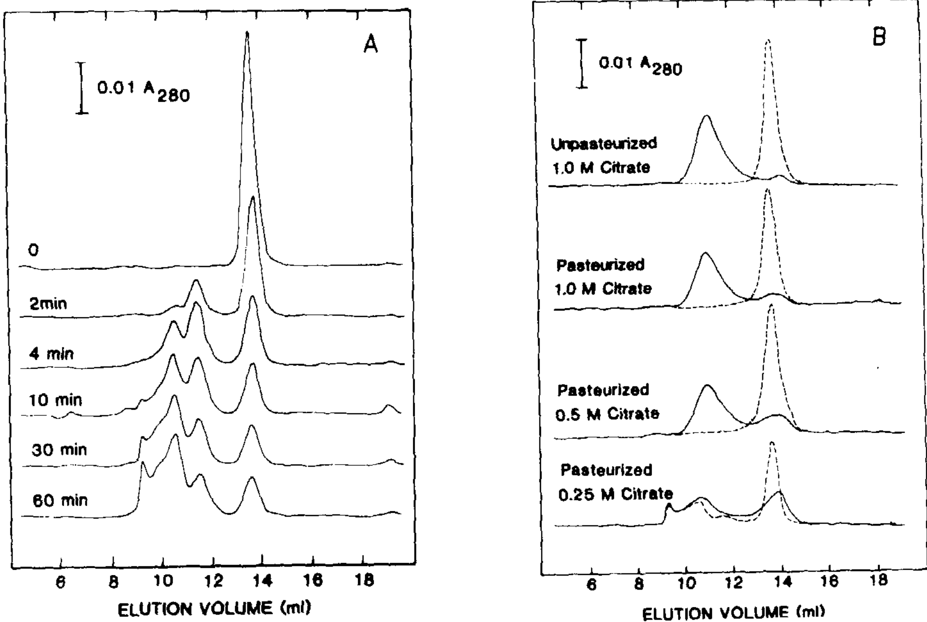


Figure 2 - A: Formation of high molecular weight aggregates of antithrombin III as a function of time at 60°C. AT III samples (2 mg/ml) were heated in 0.02 M potassium phosphate buffer, pH 7.35 containing 0.15 M NaCl, removed at indicated times, stored at 2°C and subsequently applied to the column. B: Measurements of heparin binding to samples of AT III in the presence of citrate. AT III samples (2 mg/ml) containing the indicated concentrations of sodium citrate were heated at 60°C in 0.02 M potassium phosphate buffer, pH 7.35, containing 0.15 M NaCl, removed after 10 hr, cooled to 2°C and subsequently applied to the HPLC. The heated and control samples were dialyzed against 0.02 M potassium phosphate buffer, pH 7.35, plus 0.15 M NaCl, mixed with heparin for a final concentration of 0.7 mg/ml AT III, 12 mg/ml heparin and applied to the column. (----), no heparin; (———), heparin added. Adapted from Busby et al., (18).

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the blood coagulation system (11,12). It is of potential therapeutic interest for replacement therapy in patients with inherited or acquired deficiencies. A major risk associated with such therapy is transfusion hepatitis and one approach to reduce this risk is to determine conditions under which the protein can be pasteurized (for 10h at 60°) to inactivate the virus. In the case of AT III, the presence of 0.5 M citrate is sufficient to preserve most of the activity (13). In the absence of stabilizers, the inhibitor undergoes extensive aggregation and loses its ability to bind the mucopolysaccharide heparin and inhibit thrombin. All three of these events can be readily monitored by HPLC.

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Figure 2A illustrates the time course of aggregation in a sample of AT III which was heated at 60° C. Higher molecular weight forms are detectable after 2 min and by 60 min very little monomer remains. The multiple peaks presumably correspond to different oligomers which are formed and can be resolved by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (data not shown). This aggregation can be entirely prevented, even after 10h at 60° C, by addition of sodium citrate at concentrations $\geq$0.5 M. This is illustrated by the dashed profiles in Figure 2B where it can be seen that the samples pasteurized in 0.5 or 1.0 M citrate are indistinguishable from the unpasteurized control, whereas that pasteurized in 0.25 M citrate showed substantial aggregation.

The solid profiles in Figure 2B illustrate the manner in which HPLC can be used to assess the ability of AT III to bind heparin. Premixing with an excess of the mucopolysaccharide, which is essentially transparent at 280 nm, shifts the absorbance profile of unpasteurized AT III to an earlier elution time with a concomitant broadening of the peak. This broadening is presumably due to the polydisperse nature of the crude heparin used in these experiments ($M_r$ = 5000-20000) Similar results are seen with the sample pasteurized in 1.0 M citrate whereas the sample pasteurized in 0.5 M citrate appeared to contain slightly more monomer which did not shift. The 0.25 M citrate sample contained appreciable amounts of higher molecular weight species even in the absence of heparin and addition of heparin caused only a partial depletion of the monomer peak. This approach obviously has considerable potential for characterizing the interaction of AT III with various heparin preparations and for rapid assessment of the ability of various modified antithrombins to interact with heparin.

HPLC has also been used to monitor the formation of complexes between AT III and one of its target enzymes, thrombin. This is part of an effort in our laboratory to prepare fluorescent-labeled derivatives of AT III and thrombin which retain their ability to form enzyme-inhibitor cmplexes in a time dependent reaction whose rate could be directly monitored by observing changes in fluorescence polarization. The elution

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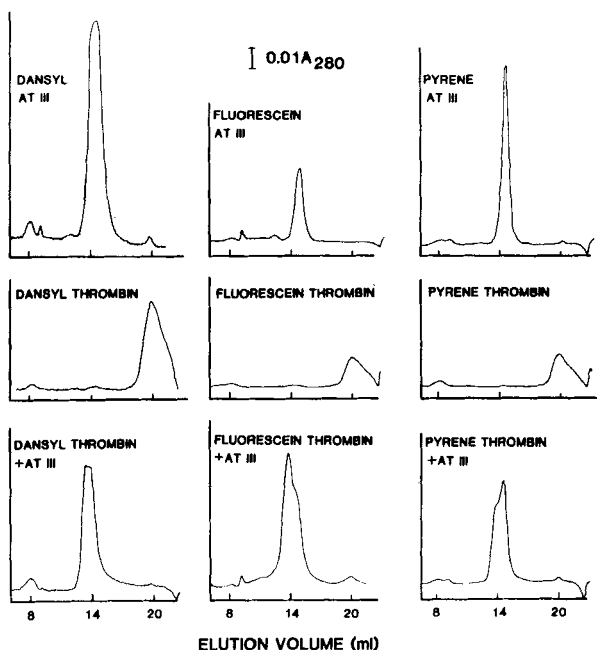


Figure 3 - High performance size exclusion chromatography of various fluorescent-labeled derivatives of thrombin, antithrombin, and their mixtures. Samples (25 µl) of thrombin (1 mg/ml) or AT III (1-2 mg/ml) were injected into the HPLC and eluted with 0.01 M K phosphate pH 7.35, 0.1 M NaCl. Complex formation was observed in mixtures containing labeled thrombin (0.5 mg/ml) and an excess of unlabeled AT III (1.0 mg/ml). These mixtures were incubated 15 minutes at 37°C prior to injection (50 µl).

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profiles of several labeled derivatives of AT III and thrombin are shown in the upper and middle portion of Figure 3. Thrombin, which has a molecular weight of about 37,000, elutes close to the salt volume ( 22 ml) suggesting a tendency to interact with the column. When mixed with excess unlabeled AT III, the thrombin peak disappeared and was replaced by a new peak emerging slightly

ahead of AT III, as shown in the lower three panels of Figure 3. Thus, the presence of the labels did not interfere with the ability to form complexes.

The small difference between the elution of AT III and that of the AT III-thrombin complex could be due to retardation of the latter due to interaction of the thrombin moiety with the column or to conformational changes accompanying complex formation which result in a more compact structure. This behavior is reminiscent of that seen with the combination of gonadotropin subunits, as discussed above and is in contrast to the behavior seen with heparin which caused a substantial shift in the elution of AT III. The latter effect can be attributed to the formation of a heparin:AT III complex from which substantial portions of the polysaccharide chain protrude, producing a larger effective radius.

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## $Alpha_1$-Proteinase Inhibitor

$Alpha_1$-proteinase inhibitor ($\alpha_1$-PI, also termed $\alpha_1$-antitrypsin) is the most abundant protease inhibitor in plasma (14). Individuals with inherited deficiencies of this protein are predisposed to pulmonary emphysema and might benefit from replacement therapy with the purified protein (15). As with AT III, pasteurization to inactivate hepatitis virus results in loss of activity and extensive aggregation. Treatment for 10h at 60$^{o}$C in the absence of stabilizers shifted all of the protein into

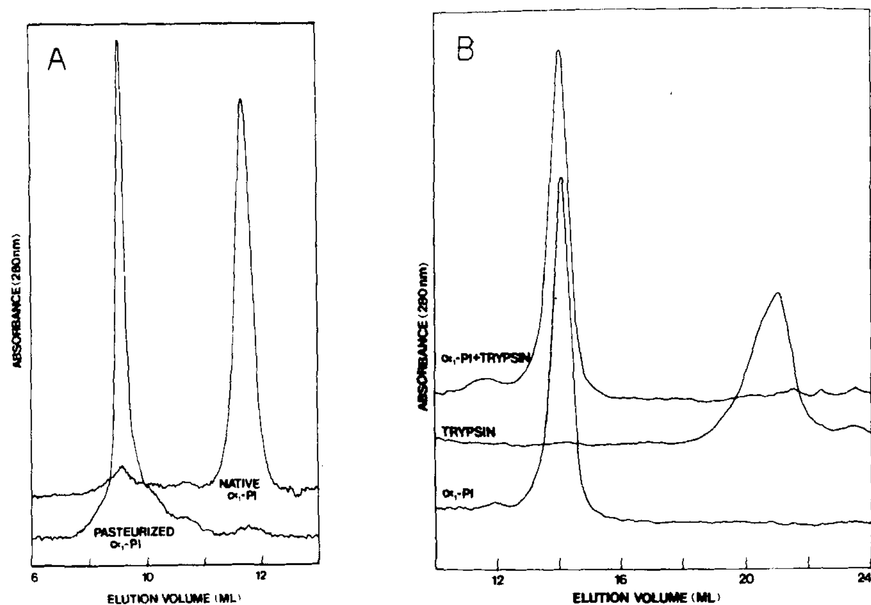


Figure 4 - A: Heat induced aggregation of $\alpha_1$-proteinase inhibitor ($\alpha_1$-PI). The protein (0.50 mg/ml) was "pasteurized" at 60°C for 10 hr in 0.02 M potassium phosphate buffer, pH 7.35 containing 0.15 M NaCl. B: complex formation between $\alpha_1$-PI (0.56 mg/ml) and trypsin (1.0 mg/ml) incubated together for 10 min at 25°C. Note disappearance of trypsin peak without formation of new peak.

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the void of the TSK-G3000SW column (Figure 4A) suggesting that the aggregation is even more extensive than with AT III.

Formation of a complex between $\alpha_1$-PI and trypsin is illustrated in Figure 4B. The peak due to free trypsin near 21 ml disappears when the enzyme is premixed with excess $\alpha_1$-PI. No new peak is apparent, suggesting that the enzyme-inhibitor complex elutes in the same position as the free inhibitor. The small peak eluting ahead of the main peak in the upper profile is

not large enough to account for more than a small fraction of the total amount of complex expected. This behavior is also similar to that seen with hCG/hCG-β and AT III/thrombin.

C1-Inactivator

C1-inactivator (C1-Ina) is another protease inhibitor involved in the regulation of the first component of the complement system. Partially purified preparations of this protein have been shown to be effective in the treatment of hereditary angioedema patients (16). HPLC has been useful in monitoring the heat-induced aggregation of C1-Ina as part of an effort to define conditions for pasteurization.

Figure 5A illustrates the manner in which HPLC was used for rapid purification of C1-Ina and one of its target enzymes, C1s, from crude concentrates of each protein. The upper two profiles represent the impure starting materials which gave multiple peaks. When antigen positive fractions were pooled and reapplied to the column, the two lower profiles were obtained indicating size homogeneity. A portion of each of the peak fractions was rechromatographed, separately, and after mixing, producing the profiles in Figure 5B. The upper profile of the mixture contained a new peak, well resolved from either constituent, presumably representing the enzyme-inhibitor complex. Residual material in the position of the individual components could be due to impurities of the same size as the components or to unreacted or inactive components.

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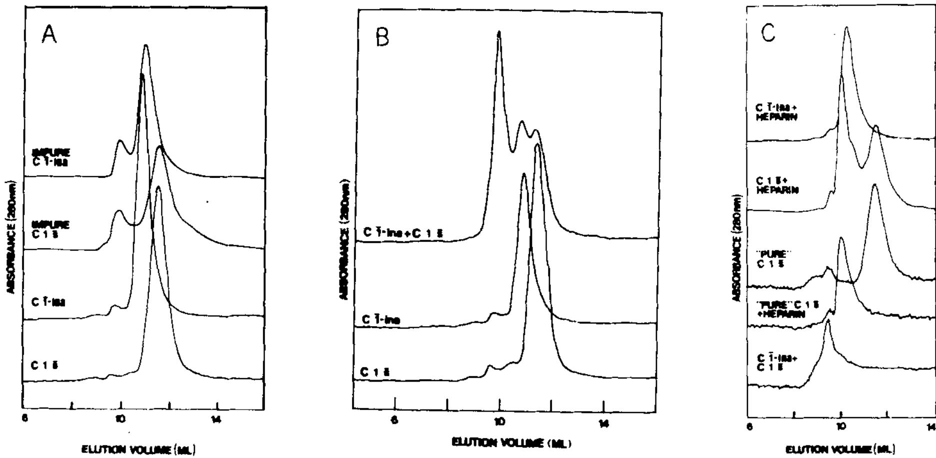


Figure 5 - Use of HPLC to further purify, characterize heparin binding, and demonstrate complex formation of C1s and C1-inactivator (C1-Ina). A: After injection of crude preparations of C1s and C1-Ina (top two profiles), and pooling the immunoreactive fractions, substantial purification of both proteins from larger molecular weight contaminants was achieved (bottom two profiles). B: Upon mixing the C1s and C1-Ina purified in 5A, followed by a 30 min incubation prior to injection into the HPLC, the formation of a complex could be demonstrated (top curve). C: When the purified C1s and C1-Ina from 5A were premixed with heparin before injection, the protein-heparin complexes eluted earlier than the proteins alone (compare top two curves with those in panel B). The profile of the C1s-heparin mixture (second from top) indicates the presence of a substantial amount of non-heparin binding protein contaminant which elutes in the same position as C1s alone. Further purification on heparin-Sepharose, gave material represented by the middle curve from which the non-heparin binding contaminant was eliminated, as shown by the second curve from bottom. A mixture of the purified C1s and C1-Ina, after prior incubation for 30 min, exhibited a single peak, presumably that of the enzyme-inhibitor complex (lower curve). The eluting buffer for these analyses was 0.033 M sodium citrate, 0.045 M NaCl, pH 7.

When C1-Ina was premixed with heparin, its elution shifted from 10.9 ml (Figure 5B) to about 10.3 ml (Figure 5C). When C1s was premixed with heparin, only part of the material shifted indicating the presence of impurities which fail to interact with heparin (Figure 5C). Therefore, the impure preparation of C1s was fractionated by affinity chromatography on heparin Sepharose. Gradient elution with NaCl produced a fraction which was more homogenous on HPLC and all of which was shifted toward the void when premixed with heparin (Figure 5C). When this material was mixed with C1-Ina (in the absence of heparin), a complex was formed which eluted earlier than either component alone with less evidence of residual material in the position of the individual components. The lower signal to noise ratio seen in the lower profiles of Figure 5C is due to the higher sensitivity range required on the samples which were now quite dilute because of the successive manipulations. These examples nicely illustrate the power of this tool in probing the purity and functional integrity of interacting proteins.

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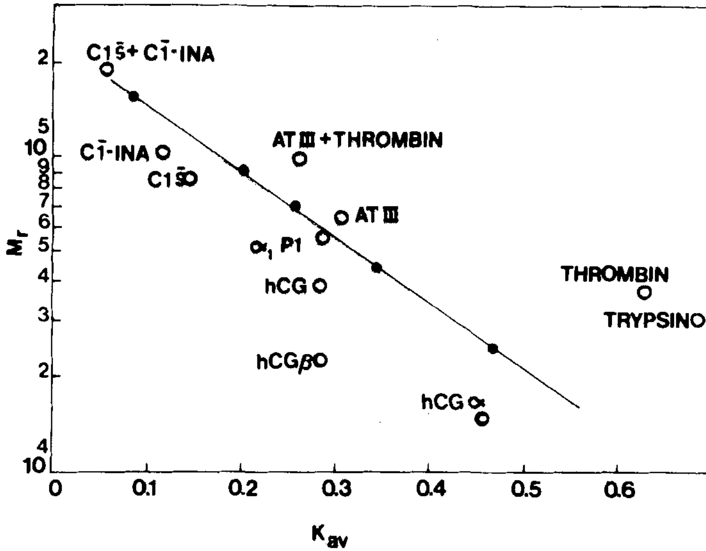
## DISCUSSION

The molecular weights and elution properties of the various proteins mentioned in this report are summarized in Figure 6. The filled circles represent globular protein standards, identified in the legend, which were chromatographed sequentially on the same column on a single day. The open symbols represent




<u>Figure 6</u> - Summary of the elution parameters for the various proteins used in these studies. The filled circles represent standard globular proteins in order of decreasing $\underline{M}_r$: phosphorylase, aldolase, albumin, ovalbumin and chymotrypsinogen. $K_{av}$ values were calculated as $(V_e - V_o)/(V_{total} - V_o)$.

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data gathered over a period of two years on three different columns of identical size. Thrombin and trypsin are both basic proteins which may tend to interact with the column accounting for their late elution. The relatively early elution of some of the other proteins may be due to the presence of large amounts of carbohydrate in their structures, a property which also affects the elution from conventional exclusion media (17). In our experience, the value of this tool for a particular application can best be determined by a few preliminary experiments with the

proteins involved. It is difficult to predict *a priori* whether a given protein will be amenable to analysis. For example, human plasma fibronectin, a large (440 Kd) glycoprotein interacted so strongly with the column that it could not be eluted in the absence of denaturants. However, with 6 M urea in the buffer, the column was useful for observing the degradation of fibronectin into smaller fragments. The high expense of these columns discourages extensive exploration of the use of denaturants. This is unfortunate since exclusion chromatography under dissociating conditions could provide a fast and sensitive alternative to SDS-PAGE. These and other applications are certain to emerge as improved and less expensive column materials become available.

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## ABBREVIATIONS

hCG, human chorionic gonadotropin; AT III, Antithrombin III; $\alpha_1$-PI, $\alpha_1$-proteinase inhibitor; C1-Ina, C1-inactivator or C1-inhibitor.

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