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Note

Comparison of chromatographic characteristics of a series of homologous Bence–Jones proteins during size-exclusion chromatography by high-performance liquid chromatography and by Sephadex

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High-performance liquid chromatography (HPLC) has become popular for protein purification and analysis as a consequence of its high resolution capability. Most commonly, protein separations are effected by combined ion exchange and hydrophobic interactions between the protein and the chromatographic matrix[1,2]. However, the presence of such interactions has impaired the suitability of most porous HPLC matrices for size-exclusion protein fractionation on the basis of molecular weight[3–5].

Size-exclusion chromatography, which is commonly used for protein purification and molecular weight estimation, has also provided a means to study reversible interactions between proteins[6–8]. However, analytical interpretation of these experiments requires minimal "non-specific" interaction between protein and matrix, as is typically the case with polysaccharide or polymerized agarose gels. We have previously described the use of small zone gel filtration of immunoglobulin light chains (Bence–Jones proteins) to observe the interactions between these polypeptide chains[9]. Here we report the results of similar experiments with these proteins in which we used a commercially available HPLC matrix designed for separation of proteins on the basis of molecular weight.

MATERIALS AND METHODS

HPLC experiments

HPLC analysis was carried out with a Waters Assoc. liquid chromatograph. A column (60 × 0.75 cm I.D.) obtained prepacked from LKB and containing either TSK G2000SW or TSK G3000SW was used for gel filtration. A guard column (7.5 × 0.75 cm I.D.) containing TSK GSWP was inserted between the sample injection loop and the gel filtration column. Protein elution at 0.1 ml/min was monitored continuously at 280 nm.

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Protein samples

The Bence–Jones proteins were obtained as lyophilized samples of electrophoretically purified urinary light chains[9], and were used as received from Dr. A. Solomon, Department of Medicine, University of Tennessee, Knoxville, TN, U.S.A. The κ_I Bence–Jones protein Au was originally provided by Dr. N. Hilschmann. Typically 10-μl samples of protein at a nominal concentration of 1 mg/ml were used for each experiment.

Reagents and buffers

All buffers were made with Fisher reagent-grade chemicals and Fisher HPLC-quality water. The buffers were pH adjusted after sodium chloride was added and were sterile filtered. The buffer used was 0.12 *M* sodium phosphate (mono:dibasic, 1:1), 0.15 *M* sodium chloride (pH 6.7).

RESULTS AND DISCUSSION

The goal of these experiments was to evaluate the usefulness of the HPLC gel filtration system for the study of interactions between proteins. Immunoglobulin light chains were used as a representative model of an interacting protein system. The Bence–Jones proteins or immunoglobulin light chains, which are present in the urine of multiple myeloma patients, represent a series of structurally homologous monoclonal antibody components. The light chains from each individual patient are homogeneous but are distinct in sequence from light chains produced by another individual. Accordingly, each patient's light chain has a unique composition of amino acids that are conformationally positioned on the surface of the molecule; each chain from one individual is thus potentially unique in its interactions with the chromatographic matrix.

A Bence–Jones protein sample characteristically consists in varying composition of light chain monomer, light chain dimer, and occassionally fragment, the light chain variable domain[10]. Therefore, potentially three molecular weight species are resolvable by gel filtration. Since the molecular weight of light chain monomers is 22 000 and that of the dimer is 45 000, we chose bovine serum albumin (67 000), ovalbumin (43 000), and chymotrypsinogen (25 000) as reference proteins.

We restricted our choice of buffer to one of physiological composition. Although buffer formulations have been developed[11–14] to eliminate protein–matrix interactions and, accordingly, provide a reliable estimate of intrinsic molecular weight, protein–protein interactions would also be expected to be perturbed.

It has been reported that the elution behavior of most proteins on a TSK column is dependent upon the pH of the buffer[1–4,11]. We confirmed these observations with tests of the elution behavior of standard proteins with buffers ranging from pH 5.6 to 7.6 (data not shown). A pH of 6.7 was selected for our experiments because it provided a suitably linear relationship between log molecular weight and elution time for the reference proteins.

The covalent light chain dimer has a molecular weight of 45 000. In the absence of further aggregation or interaction with the chromatographic matrix, light chain dimers from different individuals, although distinct in primary structure, can be expected to migrate at uniform rates on a size-exclusion gel filtration column. Our

TABLE I

ELUTION BEHAVIOR OF κ_I BENCE–JONES PROTEIN ON HPLC AND SEPHADEX

Peaks I, II and III refer to covalent dimer, monomer, and fragment positions respectively. Light chain monomers are capable of non-covalent self-association and may exhibit apparent molecular weights between $22 \cdot 10^3$ and $45 \cdot 10^3$ in the absence of further aggregation. Numbers in parentheses indicate components present in trace amounts.

Protein	*Apparent molecular weight* ($\times 10^{-3}$)					
	HPLC			*G-75**		
	I	*II*	*III*	*I*	*II*	*III*
Und	43	31	–	45	22	–
Gal	–	36	–	(45)	22	–
Cro	38	30	–	45	23	–
Kin	35	24	–	45	26	–
Wat	–	34	18	(45)	27	13
Bus	(60)	43	–	(50)	27	–
Epp	41	32	–	45	30	–
Au	42	35	20	45	34	14
Joh	41	36	–	45	35	–
Cla	50	43	25	50	35	–
Hou	44	38	–	45	39	–
Ell	44	36	≈10	58	52	29

* Ref. 9.

previous observations showed this pattern[9]; out of 14 Bence–Jones proteins of the κ_I subgroup, only four presented atypical molecular weights for the dimer; two had apparent molecular weights of *ca.* 50 000; two others were clearly forming aggregates. In no case did a dimer appear to have less than the expected molecular weight.

A summary of results of the HPLC runs and the results of the previous study, in which a Sephadex G-75 column was used, are presented in Table I. Clearly these similar proteins show a heterogeneity of elution patterns in the HPLC runs that was not apparent in the Sephadex system previously used. The variation in apparent molecular weight values of the light chain dimers determined from HPLC profiles does not correspond to physical differences in the size of the molecules. The uniformity of the molecular weights for the covalent light chain dimers was previously demonstrated by sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis[9]. The constancy of the dimer elution position in the Sephadex G-75 study may be taken as evidence of (1) uniform Stokes radius among these proteins, and (2) uniform and minimal interaction between the proteins and the polysaccharide resin. Thus, this heterogeneity of HPLC mediated elution behavior among the homologous proteins appears to be related to the differences in surface amino acid interactions with the HPLC matrix.

In addition to the aberrant behavior of the covalent light chain dimers, we observed contradictory behavior of light chain monomers during HPLC. A notable example of this aberrant behavior can be seen in the elution pattern of the light chain monomer Gal (Table I). In the Sephadex study[9], the protein eluted at the position of a non-self-associating monomer, with an apparent molecular weight equivalent to that of the intrinsic monomer 22 000.

However, in the TSK3000 system, we observed an apparent molecular weight of 36 000, which if taken at face value would suggest significant reversible dimer formation. In general, the apparent molecular weights of the non-covalently linked light chains are higher by HPLC than had been observed in the Sephadex system. Moreover, there is little correlation between the monomer molecular weight observed in the two systems (Table I). The following hypothesis may accouant for these observations.

Repulsive forces may exist between the TSK matrix and many of the light chain monomers. The existence of repulsive forces has been shown[4,11,15] to effectively decrease the column volume available to the solute. The resulting earlier elution is interpreted as an increase in the apparent molecular weight of the molecule. On the other hand, some dimers (Cro, Kin) had a lower than anticipated apparent molecular weight. Thus, while the light chain dimer and light chain monomer are composed of polypeptides of identical sequence, it may be necessary to postulate that the dimer is attracted to the matrix, whereas the monomer is repelled. This is not impossible. In the dimer, the amino acids that constitute the interfacial surface no longer form part of the surface of the molecule, as they do in the light chain monomer. Therefore, if the interfacial surface is distinctive in its composition, the average surface properties of the dimer are necessarily different than those of the monomer from which it is formed.

To evaluate the usefulness of the HPLC column to study protein–protein interactions, we performed several experiments with protein Au, chosen because its self-association properties have been well characterized[16,17]. In triplicate experiments, we studied the effect of protein concentration and temperature on the chromatographic behavior; samples were run at concentrations 0.5, 1, 2, and 5 mg/ml (data not shown). Elution profiles of Au at 1 mg/ml concentration were also determined at 4, 20, and 37°C. As expected from our previous studies, the retention of covalent dimers was not affected by variation of either parameter, while the retention time of the monomer and of the fragment increased with decreasing concentration and decreasing temperature. The observed effect was systematic and reproducible. However, the magnitude of the response to the series of two-fold changes in concentration was very small, only two to three times the standard deviation of the measurements. The reproducibility of peak positions was within 0.5 to 1.0 min in sequential runs of the same protein samples. Data representative of the elution characteristics are illustrated by the temperature studies summarized in Table II.

TABLE II

Au TEMPERATURE STUDIES

Temperature of column (°C)	*Elution times (min)**		
	Dimer	*Monomer*	*Fragment*
4	220.3	240.3	281.8
25	219.2	236.8	275.1
37	218.8	235.0	273.5

* Values represent an average of two consecutive measurements. Elution positions differed by less than 1.0 min on consecutive runs.

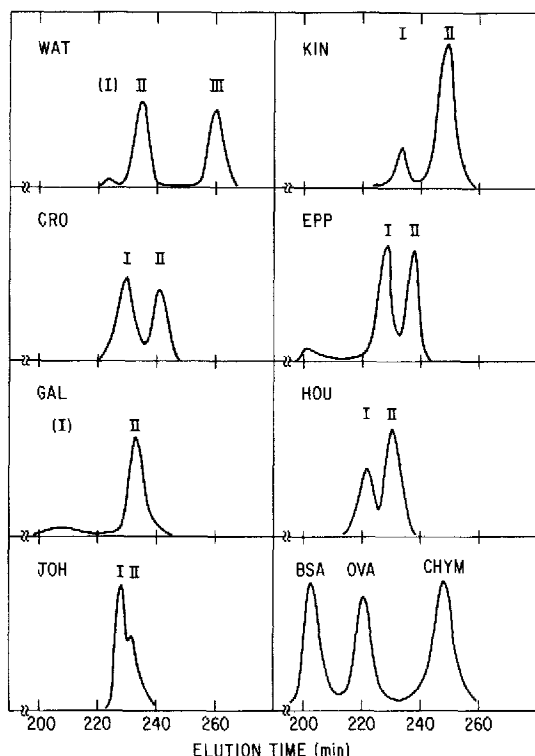

Fig. 1. Representative elution patterns of κ_I Bence–Jones protein on TSK G3000SW. Flow-rate, 0.1 ml/min; buffer, 0.12 *M* sodium phosphate (mono:dibasic, 1:1), 0.15 *M* sodium chloride (pH 6.7). Reference proteins: bovine serum albumin (BSA), ovalbumin (OVA), chymotrypsinogen (CHYM). Dimer, monomer, and fragment portions as indicated by I, II and III, respectively.

CONCLUSIONS

An HPLC system has several features appropriate for size-exclusion studies of interactions between proteins, most significantly the small sample size and short turnaround time. However, limitations are imposed by the presence of ionic and hydrophobic interactions between the proteins and the existing size-exclusion HPLC matrices. As illustrated by the survey of the Bence–Jones proteins, the comparison of apparent molecular weights of this series of structurally homologous proteins of the same size suggested heterogeneity of size, in contradiction with results of SDS electrophoresis or non-HPLC gel chromatography.

On the other hand, the possibility remained that within a series of experiments with the same polypeptide, the lack of experiment-to-experiment variability in the tertiary structure of the protein may control the protein–matrix variable. With the additional assumption that monomers and dimers of Au exhibit the same degree of interaction with the HPLC matrix, the system seems to provide a sensitive and efficient method for observing the qualitative effects of protein concentration, temperature and flow-rate on the elution characteristics of the immunoglobulin light chain. However, from our experiments with homologous light chains, it is clear that the

TSK resin must be used with caution in studies of protein–protein interactions. Recent progress in development of inert, microparticulate rigid agarose matrices[18,19] may provide a means to apply the attributes of HPLC to the study of the interactions between proteins. These studies demonstrate the need for such new matrices.

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