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SIZE-EXCLUSION CHROMATOGRAPHY OF DNA RESTRICTION FRAGMENTS

FRAGMENT LENGTH DETERMINATIONS AND A COMPARISON WITH THE BEHAVIOUR OF PROTEINS IN SIZE-EXCLUSION CHROMATOGRAPHY

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SUMMARY

Size-exclusion chromatography of double-stranded DNA restriction fragments on Superose 6® is shown to be an accurate method for chain length determination of unknown DNA. Ionic interaction was observed between DNA and the gel matrix but was easily prevented by the addition of 0.15–0.2 *M* sodium chloride to the eluent. Compared to protein, the selectivity curve of DNA fragments was found to be steeper reflecting the different chromatographic behaviour of rod-like and globular molecules. The relationships between the selectivity curves of DNA and protein were similar on Superose 6 and on Sephacryl® S-500.

INTRODUCTION

Size-exclusion chromatography (SEC) or gel filtration (SEC in aqueous phase) of DNA is widely used in molecular biology laboratories both in low-pressure[1–4] and in high-pressure liquid chromatographic systems[5–7]. Although generally offering lower resolution than ion exchange (IEC) and reversed-phase chromatography (RPC), SEC offers other advantages: easy instrumentation, isocratic elution, great freedom of buffer choice and elution in strict order of size. Up to now, applications of SEC in DNA research include the purification of, *e.g.*, vectors and linkers[8,9], restriction fragments[7,10–13], plasmids[4,14], RNA–DNA hybrids[15] and mitochondrial DNA[16]. The general use of SEC and other chromatographic techniques in molecular biology has been reviewed several times[17–24].

Due to the fact that DNA fragments in SEC are eluted in a strict order according to size, we have investigated the potential of SEC for DNA size determinations on Superose 6®, an agarose-based matrix. Since size determination requires constant and ideal size exclusion conditions, the effect of ionic strength was evaluated. We also

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wanted to explore in some detail the elution behaviour of DNA during SEC. To be able to discriminate between solute effects and support effects we chose two chromatography media (Superose 6 and Sephacryl® S-500) with similar overall separation properties but with completely different chemical compositions. We compared the elution behaviour of proteins and DNA on the two gels.

EXPERIMENTAL

DNA preparation

pBR 322 DNA and restriction endonucleases HaeIII and HinfI were supplied by Pharmacia LKB Biotechnology (unless otherwise stated, all chemicals and equipment were from this source). DNA was digested essentially according to the supplier's instruction (50 m*M* Tris–HCl pH 7.6 with 10 m*M* $MgSO_4$ and 1 m*M* dithiothreitol at 37°C for 3 h using a ratio of three units of endonuclease to 1 μg DNA). The mixture obtained by the HaeIII digestion contained fragments of 587, 540, 504, 458, 434, 267, 234, 213, 192, 184, 124, 123, 104, 89, 80, 64, 57, 51, 21, 18, 11 and 7 base pairs (bps). The HinfI digestion yielded fragments of 1631, 517, 506, 396, 344, 298, 221, 220, 154 and 75 bps.

The fragments were separated on Mono Q™ HR 5/5 (column dimensions 50 mm × 5 mm) anion-exchange media. Twentyseven out of the 32 fragments were purified to baseline separation with a linear gradient from 0.65 (buffer A) to 0.78 *M* (buffer B) salt (gradient slope 6.5 m*M* NaCl per ml buffer in 20 m*M* Tris–HCl pH 7.6)[25]. DNA fractions were precipitated with ethanol and analysed for purity by polyacrylamide gradient gel electrophoresis.

Chromatography

Separations were performed with a complete fast protein liquid chromatography (FPLC)® system (Pump P500, liquid chromatography controller LCC 500, UV monitor UV-M, recorder REC-482 and fraction collector Frac 100) with detection at 254 and 280 nm for DNA and proteins respectively. Size-exclusion chromatography was carried out on Superose 6 with 106 mm × 10 mm or 300 mm × 10 mm (prepacked Superose 6 HR 10/30) columns and on Sephacryl S-500 Superfine with a 89 mm × 10 mm column. Superose 6 consists of 13-μm particles derived from 6% cross-linked agarose[26,27], while Sephacryl is allyl dextran covalently cross-linked with N,N'-methylenebisacrylamide. The standard eluent was 20 m*M* Tris–HCl pH 7.6 containing 0.15 *M* sodium chloride.

Purified DNA restriction fragments (0.2–1.0 μg/ml) were typically chromatographed two or three at a time, at ambient temperature (*ca.* 24°C) with a flow-rate of 0.35 ml/min and a sample volume of 100 μl. The same conditions were used for protein (3 mg/ml) separations. The molecular weights of the proteins were in the range of 6500 to 669 000 (Pharmacia LMW and HMW calibration kits and from Sigma). When studying the effect of the ionic strength on the elution volume, the salt concentration was varied between 0 and 5 *M*.

Partition in the size-exclusion bed is expressed by the K_{av} value of a solute[28]

$$K_{av} = V_e - V_0/V_c - V_o \quad (1)$$

where V_e is the elution volume, V_0 is the void volume and V_c is the geometrical column volume. The void volume was measured with Blue Dextran 2000 for Superose 6 and with large bacteria (*Serratia*) for Sephacryl S-500.

RESULTS AND DISCUSSION

General chromatographic behaviour on Superose 6

Of the fragments studied only the largest (1631 bps) fragment was eluted in the void volume. From the calibration graph given in Fig. 1 the exclusion limit can be estimated to be ≈ 600 bps. A typical chromatogram is shown in Fig. 2.

One of the purposes of this study was to evaluate the suitability of the gel for molecular weight determinations of DNA restriction fragments. In an ideal gel filtration process the solute should not interact with the gel matrix. As an agarose-based medium, Superose 6 inherently contains small amounts of sulphate esters and carboxylic groups. In principle, there are two main types of possible interactions between a slightly charged solute and an agarose matrix. At low ionic strength, repulsive (in the case of negatively charged molecules such as DNA) or attractive (positively charged molecules) ionic forces may lead to decreased or to increased elution volumes, respectively. At high ionic strength the hydrophobic interaction increases and may be strong enough to cause delayed elution. In addition, the ionic strength may affect the size and shape of the solute.

To investigate the effect of ionic interactions we determined K_{av} values of DNA at different ionic strengths. At low ionic strengths the elution volumes decreased (lower K_{av} values) as predicted (Fig. 3). To avoid ionic interactions the addition of 0.15–0.20 *M* sodium chloride to the buffer solution seems appropriate. At higher ionic strengths the elution volumes tend to become more constant, an observation in agreement with studies made on other types of resins, *e.g.*, silica-based media[5]. This indicates that the hydrophobic interaction between DNA fragments and different matrices is very low even at an high ionic strength. The absence of marked hydrophobic interaction can be explained by the extremely hydrophilic nature of the DNA fragments since they are strong polyanions with a thick hydration shell protecting them from hydrophobic contacts with the gel[24].

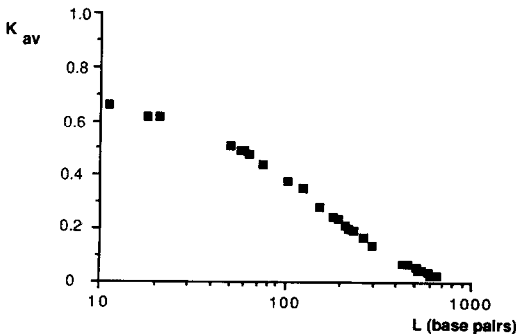

Fig. 1. Selectivity curve derived from SEC of DNA restriction fragments on Superose 6. DNA fragments were derived from a HaeIII/pBR 322 digest (and purified on a Mono Q anion-exchange column) and gel filtration was performed on a 106 mm × 10 mm column with 0.02 *M* Tris–HCl pH 7.6 containing 0.15 *M* sodium chloride as the eluent.

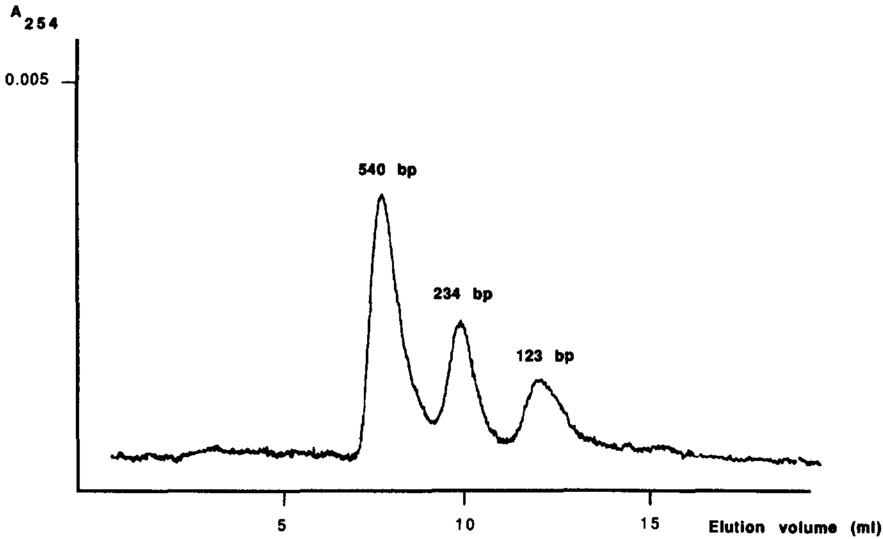

Fig. 2. Size-exclusion chromatography of three DNA restriction fragments on Superose 6. Conditions as described in Fig. 1.

Calibration graphs

When the logarithm of fragment length in base pairs was plotted against K_{av} for each purified fragment in the pBR 322/HaeIII digest a sigmoidal curve was obtained (Fig. 1). In the range of 60 to 450 base pairs the relationship was approximately linear. Using regression analysis in this linear region (K_{av} 0.05–0.5), the fragment length can be expressed as a function of K_{av}:

$$L = 2.7623 - 1.9861\ K_{av} \quad (2)$$

where L is the DNA fragment length as expressed by the logarithm of the number of base pairs and K_{av} is defined as in eqn. 1.

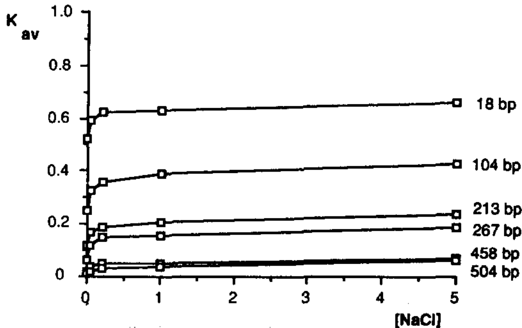

Fig. 3. Relationship between K_{av} and the amount of sodium chloride (*M*) added in the eluent (0.02 *M* Tris–HCl pH 7.6) in SEC of six DNA restriction fragments on Superose 6.

Interestingly, nearly every point in the calibation plot made a perfect fit to the line given by eqn. 2 (r = 0.998). This suggests SEC as a more accurate method for determination of the length of restriction fragments than IEC and RPC where the elution position is influenced by the base composition of the DNA[24,29,30]. To test the accuracy by which such determinations can be performed, we chromatographed another set of fragments originating from the pBR 322/HinfI digest. The K_{av} values for fragments of suitable lengths were applied to eqn.1 and the results are shown in Table I. It can be concluded that for the 75, 154, 220/221 and the 298 bp fragments, the estimated lengths differed by less than 4% from the true length.

Given this accuracy, it is obvious that gel filtration may be a useful alternative to gel electrophoresis for fragment length determinations. In fact, chromatography has some distinct advantages as compared to electrophoresis:

(1) Once the column is calibrated there is no further need for molecular weight markers.

(2) Accurate fragment length determinations can be completed within 30 min.

(3) While the base composition has been demonstrated to influence the migration velocity of DNA in gel electrophoresis[31], there is no demonstrable effect in SEC.

(4) Preparative SEC is a very harmless method while agarose electrophoresis has a serious drawback for preparative purposes in that the purified DNA is often contaminated with agarose impurities having enzyme-inhibiting properties.

It should be noted that the restriction fragments we used represented both fragments with blunt ends (the HaeIII digest) and fragments with 5′ overhang (the HinfI digest). In the molecular size range studied, this difference did not affect the chromatographic behaviour in contrast to the behaviour in RPC[32].

There are several reports of unexpected delayed elution of A/T-rich DNA fragments in IEC and RPC[24,29,30,33]. Two explanations have been suggested: (1) A/T-rich regions are less rigid (anomalous bending) and can therefore make more intimate contact with the matrix strengthening the interaction[24], and (2) the binding of counter ions depends on the bases[33]. Hypothesis 1 suggests that the hydrodynamic radius and hence K_{av} should depend on the base composition. When analysing the A/T

TABLE I

CHAIN LENGTH DETERMINATION OF DNA RESTRICTION FRAGMENTS ON A CALIBRATED SUPEROSE 6 COLUMN

The column was calibrated with DNA fragments originating from a HaeIII/pBR 322 digest. In the linear part of the selectivity curve derived with these fragments, the relationship between the fragment length and K_{av} was as in eqn. 2. Applying K_{av} data from a HinfI/pBR 322 digest to this relationship, fragment length estimates were obtained.

Actual length (bps)	*Estimate (bps)*
75	77
154	157
220/221	230
298	309
506	452
517	471

content of the fragments used in this study we did not observe any tendency for A/T-rich fragments to differ from expected K_{av} values, *i.e.*, fall outside the selectivity curve. Either the hypothesis is wrong or the effect of the different stiffness on interaction in IEC and RPC is greater than its effect in SEC since it is manifested through different mechanisms.

Evaluation of DNA and protein selectivity curves

In Fig. 4 the K_{av} values for both DNA fragments and proteins derived from Superose 6 have been plotted against the logarithm of the molecular weight. The molecular weights of the DNA fragments were calculated with an average value of 660 daltons per base pair. Examining Fig. 4, there are two features that differentiate the selectivity curves for the two types of macromolecules. First, the selectivity curve for the DNA fragments is much steeper than the curve established with standard proteins (the slopes of the DNA and the protein selectivity curves are -0.50 and -0.16 respectively). Secondly, while the selectivity curve for protein is linear up to a K_{av} value of 0.8, the shape of the selectivity curve for DNA fragments bends at a K_{av} value of approximately 0.5. We suggest that, at least partly, both these differences can be explained by the structures of the two macromolecules, proteins being roughly globular and larger DNA fragments being rod-like molecules. We will discuss the two differences separately.

Slope of the selectivity curves. It is well known that retention in SEC is strongly dependent on the shape of the solute as well as the size (see for example, refs. 28, 34 and 35). It has been demonstrated that the parameter governing the retention in SEC is the hydrodynamic volume of the solute. The molecular weight of a solute is related to the radius of gyration

$$R_g = kM^a \tag{3}$$

with $a = 1$ for rods, *ca.* 0.5 for flexible coils and 0.3 for spheres[36]. From this equation it follows that the gyration radius, R_g, (and the hydrodynamic volume) increases more rapidly with mass for rods (DNA) than for spheres (proteins). It can therefore be predicted to obtain a steeper selectivity curve for rod-like DNA than for proteins[28,36], in fact exactly as observed (Fig. 4).

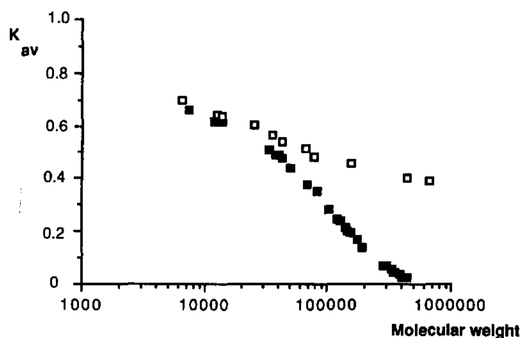

Fig. 4. Selectivity curve derived from SEC of DNA restriction fragments (■) and standard proteins (□) on Superose 6. Conditions as described in Fig. 1.

In practice this means that, with SEC, molecular weight (MW) values can be more accurately determined for DNA than for proteins since a small change in the molecular weight of DNA has a greater effect on the elution volume (K_{av}).

Shape of the selectivity curves. The linear region of the selectivity curve for DNA extends only between K_{av} of 0.1 and 0.5, in contrast to the curve for proteins which is linear at least from $K_{av} = 0.1$ to 0.8. We suggest that this relatively short linear region of the DNA curve also reflects the shape of the DNA molecules. Double-stranded DNA has a diameter of 20 Å with each base pair adding 3.4 Å in length. $K_{av} = 0.5$ where the bending of the DNA curve begins, corresponds to a molecular weight of *ca.* 12 000 or 18 base pairs. This molecule has the dimensions 20 × 60 Å, that is an axial ratio of 1 to 3. Obviously such a molecule is no longer a perfect rod and still smaller DNA fragments will be even closer to a spherical form. Consequently and according to the above discussion, the slope of the part of the selectivity curve for small DNA fragments will differ from the part for longer fragments. This should explain why the slopes of the selectivity curves for DNA and proteins tend to merge in the low-molecular-weight range.

On the other hand, fragments longer than the persistence length (for DNA, approximately 150 bps) will behave more and more like flexible coils. According to eqn. 3 the slope of a selectivity curve for very large DNA molecules should therefore approach a value intermediate between that of proteins and that of moderately sized, rod-like DNA molecules. Unfortunately the separation range of Superose 6 is too small for this phenomenon to be seen.

Relationship between selectivity curves for DNA and proteins

On the basis of the parameter K_{av} we have calculated the empirical relationship between the selectivity curve for proteins and the linear part of the selectivity curve for DNA fragments on Superose 6. In this way the molecular weight of a DNA fragment, MW_{DNA}, can be expressed as a function of a protein molecular weight

$$MW_{DNA} = 101.2\ MW_{prot}^{0.495} \quad (4)$$

and the protein molecular weight, MW_{prot}, is then the molecular weight giving the same K_{av} value as a DNA fragment. This relationship should be useful for molecular weight estimates of DNA fragments on Superose 6 columns calibrated with proteins.

Comparison of Superose 6 and Sephacryl S-500

To investigate whether the relationship between the separation behaviour of DNA and proteins (as expressed in eqn. 4) is specific for the separation media, we separated suitable test proteins and DNA fragments also on Sephacryl S-500 (Fig. 5). Disregarding the fact that the fractionation ranges do not overlap exactly, it is obvious that the general picture is the same.

To test the applicability of eqn. 4 on Sephacryl S-500, the elution positions of some of the proteins in Fig. 5 were taken as elution positions for hypothetical DNA fragments given molecular weights calculated with eqn. 4. The resulting K_{av} values were plotted together with the experimentally determined values of the real DNA fragments (Fig. 6). The values calculated by eqn. 4, which was derived using Superose 6, agree very well with the experimental results on Sephacryl S-500. Hence these results indicate that the relationship between the separations of DNA fragments and proteins

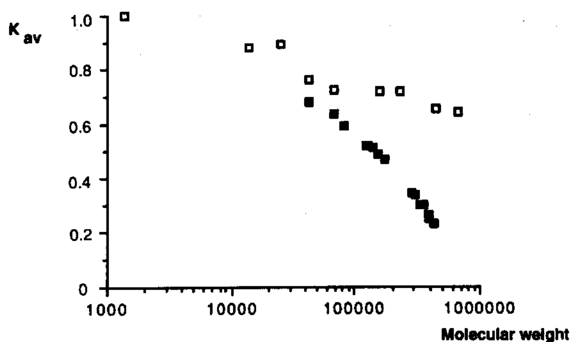

Fig. 5. Selectivity curve derived from SEC of DNA restriction fragments (■) and standard proteins (□) on Sephacryl S-500. Column dimensions: 89 mm × 10 mm. Other conditions as described in Fig. 1.

is governed by the properties of the solutes rather than by the chromatographic support. It should be emphasized, however, that eqn. 4 is applicable only in the region where DNA can be regarded as rods.

CONCLUSIONS

We have found that at low ionic strengths the elution volumes of DNA fragments on Superose 6 decrease. This indicates repulsion between the slightly negatively charged matrix and the DNA. To suppress ionic interaction, 0.15–0.20 *M* sodium chloride should be added to the buffer. Hydrophobic interactions seem to play little rôle in SEC of DNA on this support. Up to 5 *M* sodium chloride was added without significant effect on the elution behaviour. It is also shown that fragment length determinations can accurately be performed on Superose 6.

Furthermore, we have found that there is a span in molecular size where a linear relationship exists between K_{av} and log molecular weight of the DNA. It is suggested that this span reflects the size region where DNA fragments can be regarded as perfect rods. A less steep slope of the selectivity curve was observed in the low-molecular-

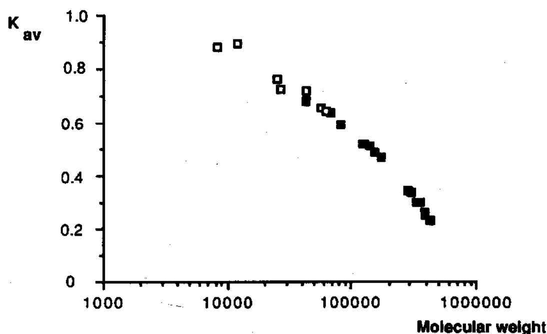

Fig. 6. Selectivity curve derived from SEC of DNA restriction fragments (■) on Sephacryl S-500. White dots (□) indicate transformed protein values according to the empirical relationship between proteins and DNA fragments established on Superose 6 and given in eqn. 4. For further details about this relationship, see text. Conditions as described in Figs. 1 and 4.

weight region where the hydrodynamic behaviour of shorter fragment approaches that of spheres. Consequently, the slope of this part of the DNA selectivity curve was similar to that of the protein selectivity curve.

An empirical relationship (eqn. 4) was derived that relates K_{av} for DNA and proteins on gel filtration media. Columns for fragment length determinations can therefore be calibrated with protein standards.

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