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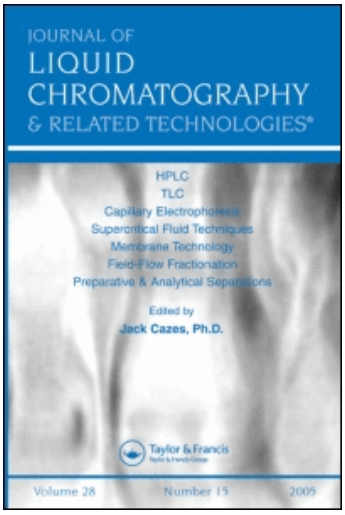

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NON-IDEAL SIZE EXCLUSION CHROMATOGRAPHY OF EUKARYOTIC PROTEIN SYNTHESIS INITIATION FACTOR 2

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ABSTRACT

Eukaryotic protein synthesis initiation factor 2 (eIF-2), a heterotrimer, was chromatographed on high performance (Superose 6) and standard (Ultrogel AcA 34 and Sephacryl S-300) size exclusion chromatography (SEC) media. The retention volume of eIF-2 on the Superose 6 column was higher than expected from its M_r and elution of a $\beta\gamma$ eIF-2 dimer was seen. Conversely, on AcA 34 and Sephacryl S-300 most of the eIF-2 was eluted at its expected position, considering its ellipsoidal structure, as the heterotrimer. Elution of the $\beta\gamma$ heterodimeric eIF-2 may therefore be promoted by Superose 6 high performance SEC which therefore provides a means for its rapid preparation for studies in protein synthesis initiation.

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INTRODUCTION

Size-exclusion chromatography (SEC), also known as gel filtration and gel permeation chromatography, is an established technique for the separation and characterization of biopolymers. In general, mild conditions for the mobile liquid phase are employed so as to preserve the structural and functional integrity of the molecules. In ideal, or "pure", SEC, solute molecules are separated solely on the basis of size by equilibrium partitioning via diffusion between the mobile phase and gel pores.[1] The non-ideal behaviour of biopolymers and other substances in size-exclusion chromatography (non-ideal SEC or nSEC) has been characterized for many situations[2-7] and has been effectively utilized to improve the separation of biomolecules.[8] It therefore has considerable potential for exploitation in the chromatographic purification and analysis of biopolymers.

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In this study the behaviour of eukaryotic protein synthesis initiation factor eIF-2, a heterotrimer ($\alpha\beta\gamma$) of M_r 122,000, on different size-exclusion chromatographic media was examined. It is shown that the chromatographic behaviour on the high performance medium Superose 6 differs markedly from that on standard media (Ultrogel AcA 34 and Sephacryl S-300), the latter, but not the former behaviour, being in accord with that expected for eIF-2 on SEC. On Superose 6 the interactions between solute and gel matrix responsible for this may have facilitated, together with the high separation efficiencies obtainable with this gel, the elution of a $\beta\gamma$ dimer form of eIF-2. Superose 6 high performance SEC thus affords a means for the rapid and efficient preparation of eIF-2 deficient in the α subunit, i.e. the $\beta\gamma$ dimer.

MATERIALS AND METHODS

The pre-packed Superose 6 column (1.0 x 30 cm) for high performance SEC and Sephacryl S-300 (pre-swollen) were from Pharmacia Fine Chemicals AB, Uppsala, Sweden. The AcA 34 Ultrogel (pre-swollen) was obtained from LKB (Bromma, Sweden). Protein molecular mass markers were from Sigma Chemical Company. Lactate dehydrogenase (pig heart), pyruvate kinase (rabbit muscle), glucose oxidase (*Aspergillus niger*) and hexokinase (yeast) were all acquired from Boehringer Mannheim. The initiation factor, eIF-2, was obtained from rabbit reticulocyte lysate by purification through the phosphocellulose step of a conventional purification procedure,[9] but with the substitution of DEAE-Sephacel for the DEAE-cellulose step since this improved the resolution of eIF-2.

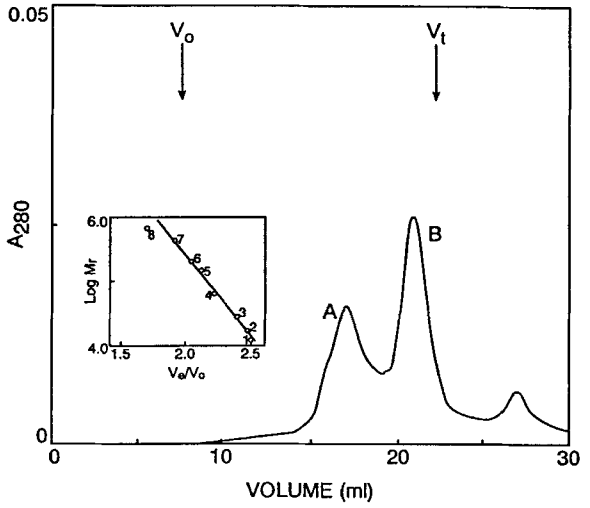

Figure 1. Superose 6 chromatography of eIF-2. The eIF-2 (100 μg in a loaded volume of 0.2 mL), purified through the phosphocellulose step of the conventional purification (9), was applied to the Superose 6 column. The column was equilibrated and developed, at a flow rate of 0.3 mL/min, with the following buffer: 0.1 M KCl; 10 mM potassium phosphate, pH 6.8; 7 mM 2-mercaptoethanol; 10% glycerol. The two major peaks (A and B) eluted from the column were analyzed by SDS-PAGE (Fig. 3). The chromatography was performed another three times and a virtually identical elution pattern was obtained on each occasion. The apparent M_r of peak A was 60,000 and that of peak B 5,000-13,000. These values were obtained by reference to the M_r calibration curve (inset) obtained from chromatography of the following protein M_r markers (M_r values in brackets) on the Superose 6 column: (1) cytochrome c (12,600), 0.3 mg; (2) myoglobin (17,500), 0.3 mg; (3) carbonic anhydrase (29,000), 0.4 mg; (4) bovine serum albumin (68,000), 0.8 mg; (5) alcohol dehydrogenase (150,000), 0.4 mg; (6) β-amylase (200,000), 0.3 mg; (7) apoferritin (443,000), 0.4 mg; (8) thyroglobulin (669,000), 0.6 mg. A very similar plot was obtained when the calibration was repeated.

For standard SEC, AcA 34 (1.6 x 28 cm) and Sephacryl S-300 (1 x 13 cm) columns were used. To maintain a smooth, even and reproducible flow of eluent through the columns the LKB 2150 HPLC pump was employed. The effluent was also monitored continuously with the LKB 2158 Uvicord detector set to measure absorbance at 280 nm.

For all the columns equilibration and development was with the following buffer: 0.1 M KCl, 10 mM potassium phosphate, pH 6.8, 7 mM 2-mercaptoethanol, 10% glycerol. The flow rate was 0.30 mL/min for the

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Superose 6 column. For the AcA column the flow rate was 0.17 mL/min and for the Sephacryl S-300 column it was 0.10 mL/min. The chromatography was conducted at 4 °C. The void volumes, V_o, of the columns were determined using blue dextran. The total permeation volumes, V_t , were determined with acetone.

For analysis of column effluent, sodium dodecyl sulphate-polyacrylamide gel electrophoresis was performed according to a modified Laemmli method.[10]

RESULTS

High Performance SEC of eIF-2

On the application of eIF-2 to the Superose 6 column, two A_{280} peaks were eluted (Fig. 1). The apparent M_r of each of the peaks was determined from the M_r calibration curve for Superose 6 as indicated. The apparent M_r of 5,000-13,000 (elution close to cytochrome c, but before V_t) for the larger peak and that of 60,000 for the other prominent peak were less than those expected for eIF-2. The recovery in terms of A_{280} units was 60%. Since the actual M_r of eIF-2 is 122,000[11,12] and it elutes from gel filtration columns with a higher apparent M_r than this owing to its ellipsoidal structure,[12-14] the peaks of low apparent M_r obtained for it on Superose 6 chromatography suggest retardation on the column and/or subunit dissociation.

High Performance SEC of other Proteins

Several different oligomeric proteins were chromatographed on the Superose 6 column to determine if any of them underwent clearly detectable subunit dissociation. Individually applied to the column were hexokinase, lactate dehydrogenase, glucose oxidase and pyruvate kinase. On elution a single symmetrical peak was obtained for each protein.

Hexokinase and glucose oxidase eluted in accord with their reported M_r values (Table 1), but the apparent M_r of 85,000 of lactate dehydrogenase was less than its reported M_r of 109,000. For pyruvate kinase the apparent M_r of 114,000 was about half its reported M_r[15] of 237,000. It should be noted that these proteins were loaded at 5 times the concentration of eIF-2.

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Table 1

Apparent M_r of Oligomeric Proteins on Superose 6 SEC*

Protein	M_r ($x10^{-3}$)	Apparent M_r on Superose 6 ($x10^{-3}$)
Hexokinase	104	101
Lactate dehydrogenase	109	85
Glucose oxidase	186	166
Pyruvate kinase	237	114

* The proteins (0.5 mg of each in a loaded volume of 0.2 mL) were chromatographed on Superose 6 and their apparent M_r values determined by reference to the M_r calibration curve for Superose 6 (inset to Fig. 1).

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Standard SEC of eIF-2

On AcA 34, eIF-2 eluted as two A_{280} peaks. The larger (peak A) was eluted with an apparent M_r of 200,000 and the smaller (peak B) of 68,000 (Fig. 2). The ellipsoidal structure[12-14] of the eIF-2 explains its elution at an apparent M_r value greater than the true M_r of 122,000. SDS-PAGE analysis of the eluted fractions confirmed the elution of the eIF-2 heterotrimer in peak A (not shown). The tailing of the first peak (A) was due, from the SDS-PAGE analysis, to some proteolysis of the β subunit which is a well-documented phenomenon.[16] The occurrence of the second peak (B) was due to a 67 kDa polypeptide present in the eIF-2 preparation (see below). Similar results were obtained on Sephacryl S-300. Care was taken in comparing the behaviour of eIF-2 on the three different SEC media used to ensure that the results on Superose 6 were not due to a lower ratio of amount loaded to column volume compared with the ratios for the AcA 34 and Sephacryl S-300 columns.

SDS-PAGE Analysis of eIF-2 Fractions Eluted on Superose 6 SEC

The two peaks, A and B of the experiment of Fig. 1, were each collected and concentrated by Millipore ultrafiltration. After lyophilization, the peaks were subjected to SDS-PAGE (Fig. 3). Electrophoresis was stopped before the bromophenol blue had run off the gel in order to search for the presence of any low M_r peptides. On staining the gel with Coomassie Blue only very faint bands could be detected for each of the peaks so the portion of the gel on which

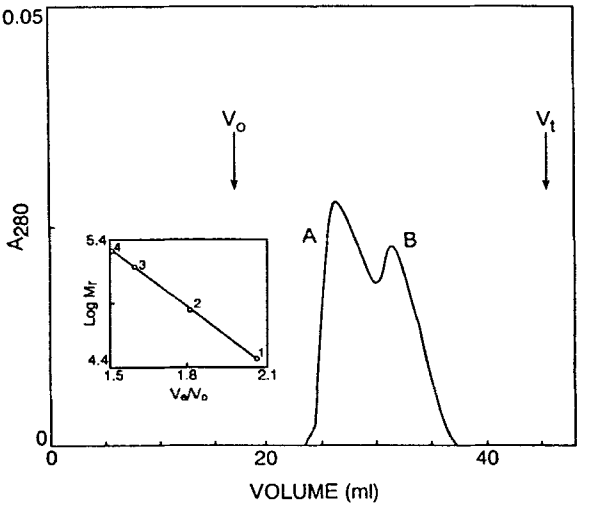

Figure 2. AcA 34 chromatography of eIF-2. The eIF-2 (100 μg in a loaded volume of 0.5 mL), purified through the phosphocellulose step, was chromatographed on AcA 34 (1.6 x 28 cm) at a flow rate of 0.17 mL/min. The column buffer was as for Superose 6 (legend to Fig. 1). The chromatography was repeated twice and similar results were obtained in each case. The apparent M_r of peak A was 200,000 and that of peak B 68,000 by reference to the M_r calibration curve for AcA 34 (inset) constructed using the following protein M_r markers (M_r values in brackets): (1) carbonic anhydrase (29,000), 3 mg; (2) bovine serum albumin (68,000), 6 mg; (3) alcohol dehydrogenase (150,000), 4 mg; (4)β-amylase (200,000), 4 mg. A similar plot was obtained when the calibration was repeated. On chromatography of the eIF-2, 2 mL fractions were collected, concentrated, lyophilized and analyzed by SDS-PAGE (not shown). Peak A consisted of the eIF-2 heterotrimer and peak B a 67 kDa polypeptide. This analysis was also undertaken on the fractions eluted for each of the two repeat chromatographic runs and the results were very similar.

the peaks were run was separated from the portion containing eIF-2 as a marker and the former silver stained. Comparing the bands obtained for each of the peaks with the subunit bands of the marker eIF-2 (Fig. 3), for peak A the β and γ subunits of eIF-2 were clearly evident, but not the α subunit. That loss was of the α subunit was confirmed by measurement of the migration distances of the polypeptide bands relative to that of bromophenol blue, i.e. the R_f values (legend to Fig. 3).

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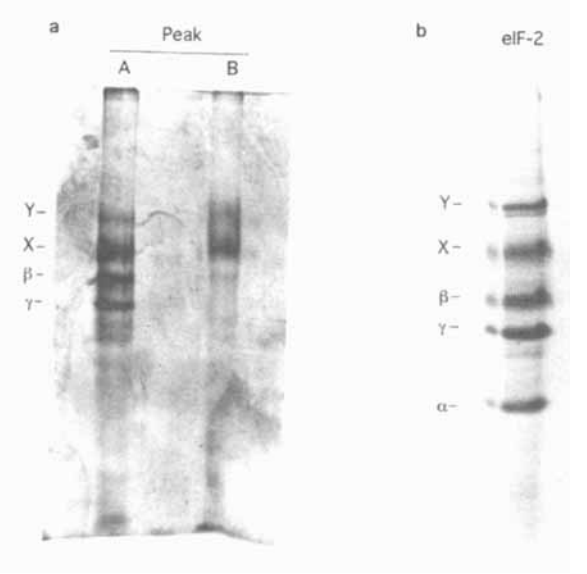

Figure 3. Analysis of peaks eluted on Superose 6 chromatography of eIF-2. The peaks A and B eluted on Superose 6 chromatography of eIF-2 (Fig. 1) were concentrated, lyophilized and then subjected to SDS-PAGE. Thirty μL SDS-PAGE sample buffer was added to each lyophilized peak. The portion of the gel containing the peaks was silver stained (a), and the portion containing marker eIF-2 stained with Coomassie Blue (b). Because of the different staining conditions, the latter was more swollen than the former. R_f values for the bands were as follows: (a) Y=0.30, X=0.37, β=0.44, γ=0.50. (b) Y=0.23, X=0.31, β=0.40, γ=0.45, α=0.58. Similar results were obtained on analysis of the peaks eluted on a repeat Superose 6 SEC of eIF-2.

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Two contaminating peptides, X and Y, of apparently higher M_r on the gel than eIF-2β were in the eIF-2 preparation. X could have been the 67 kDa noted previously[17,18] with a presumed function in protein synthesis initiation.[19] Very little could be detected in peak B (Fig. 3) except for the two relatively high M_r contaminating polypeptides, X and Y. Their positions on the gel implied that each had an M_r >60,000, whereas the apparent M_r of peak B from its elution position was 5,000-13,000. Retardation on the column must therefore have occurred. Retardation of eIF-2 was implied also by the apparent M_r of peak A being 60,000 (Fig. 1) whereas if the β and γ subunits eluted in this peak (Fig. 3) were still bonded together a higher apparent M_r (>90,000) would have been obtained. Retardation may have also been implicated in the loss of α subunit from the peak A eIF-2 (Fig. 3).

Peptides, as degradation products, eluted from the Superose 6 column would be expected to be largely lost during concentration by Millipore ultrafiltration since the nominal M_r of the filter unit was 30,000. Free α subunit may have also been lost on concentration because of its M_r, 32,000, being close to the nominal M_r of the filter unit.

DISCUSSION

The deviation of nSEC from "pure" SEC is generally due to one or more of the following effects: (i) electrostatic interactions between the solute molecule and charged groups of the gel matrix; (ii) hydrophobic interactions between solute and stationary phase; (iii) hydrogen bonding between solute and resin; and (iv) steric effects which are dependent on the shape of the solute molecules, i.e. whether spherical, ellipsoidal or rod-shaped, for example.

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Assessment of the different chromatographic behaviours of eIF-2 requires consideration of the compositions of the gel matrices. Superose 6 is agarose-based with a 6% agarose composition.[20] AcA 34 is a mixed polyacrylamide and agarose gel. Sephacryl S-300 is a cross-linked copolymer of allyldextran and N, N'-methylenebisacrylamide.

For explanation of the behaviour of eIF-2 on Superose 6, this gel is known to contain small amounts of negatively charged carboxyl and sulphate groups.[20] Superose media in most situations would therefore behave as weak cation-exchange resins.[6] Hydrophobic[5] and aromatic[4] interactions also cannot be ruled out. While the gel hydrophobicity parameter value, which is able to define the relative hydrophobicity of a gel,[21] of 0.084 for Superose 6 may seem relatively low, it is much higher than those of 0.015 and <0.01 for the polyacrylamide-based Biogel A-50M and Sephadex G-100 dextran, repectively.[21] Coulombic and hydrophobic attractions between solute molecules and Superose media are difficult to suppress.[5,22] Retarding aromatic interactions have been claimed to induce the unexpectedly late elution of different hybridoma antibodies when chromatographed on a Superose column.[4] Aromatic amino acids and hydrophobic peptides have been found to undergo retarding interactions with the matrix of a Superose 12 column during SEC of milk protein hydrolysates.[7] The potential for agarose gels to bind biopolymers has been demonstrated by the adsorption of ribosomal RNA,[23] specifically 28 S RNA,[24,25] to Sepharose 4B. Rat liver 60 S ribosomal subunits adsorb to the agarose gels, Sepharose 4B and Biogel A-15M, which could be as a result of interaction between the gel and the 28 S RNA of the subunits.[26] That the binding, in each case, was promoted by conditions of high salt and lower temperatures suggests that the interaction in each instance is hydrophobic.

Notwithstanding the influence of nSEC effects for specific biopolymers, the maximum selectivity and high efficiency of Superose is reported to make it very effective in the resolution of solutes differing in molecular mass by as little as 20%.[20,27] HPLC gel filtration has also been used to study the concentration-dependent association-dissociation of bovine and rat liver glutamate dehydrogenase.[28] It seems likely that in trimeric eIF-2 there is a dynamic equilibrium between the three subunits such that loss of a temporarily dissociated α subunit into a gel pore will lead to further α subunit dissociation to restore equilibrium of the reaction $\alpha\beta\gamma \Leftrightarrow \alpha + \beta\gamma$ in the interstitial volume. Repetition of sequestration of the α subunit by the gel will lead to progressive formation of the $\beta\gamma$ dimer. The much lower surface contact between gel and solution in the AcA and Sephacryl gels results in a much less effective sequestration of the α subunit. Superose 6 nSEC interactions may have enhanced sequestration of the α subunit with this matrix.

Elution of the $\beta\gamma$ dimer on Superose 6 chromatography of eIF-2 suggests that in addition to enabling rapid isolation of this dimer, the technique may be a very useful tool for study of the association-dissociation equilibrium between α subunit and the $\beta\gamma$ dimer in the same way that high performance gel filtration has been used to study the association-dissociation behaviour of bovine and rat liver glutamic dehydrogenase.[28]

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