CHROM. 6191

Rapid analytical gel chromatography

II. Sensitivity of protein detection by ultraviolet absorbance at 220 nm

We have recently described a rapid analytical gel chromatography (RAGC) method for proteins and peptides by using Sephadex microbore columns[1]. The elution parameters of marker proteins and peptides, compared in terms of retention time rather than elution volume under constant flow rate conditions, obeyed the empirical relationship $-\log \sigma = R \times M^{2/3} + S$ derived by HJERTÉN[2].

The absorbance of eluted compounds recorded at 280 nm depends mainly on the content of aromatic amino acids. This imposes limitations in the detection of peptides that do not contain chromophoric groups, and also in the quantitative evaluation of polydispersed mixtures since each peptide and protein exhibits its own characteristic extinction coefficient. Several authors[3-6] have described the absorbance properties of proteins in the 190–240 nm region. Absorbance in this region is largely due to the peptide bond (with some contribution from aromatic amino acids depending on wavelength) and is more sensitive than absorbance measurements at 280 nm. The choice of the appropriate wavelength in this region is a compromise between instrumental limitations and selectivity of buffer systems that do not interfere with the analysis. We chose 220 nm since this was the minimum wavelength we could operate the Gilford spectrophotometer in our system. WRIGLEY AND WEBSTER[5] reported that absorbance of protein at 220 nm follows Beer's law up to 2.0, is virtually independent of pH between values of 3 and 11, and is almost equivalent to that of protein determined colorimetrically. The present report describes the improved sensitivity of detection obtained at 220 nm which allows for molecular weight determinations of as little as 0.5 μg of protein in 30 min.

Experimental

The apparatus employed in these experiments has been described previously[1]. It involves mainly the use of a Chromatronix microbore column (MB-3-500) equipped with Cheminert fittings and a sample injection tee. Constant flow rate is provided by a Milton Roy instrument minipump. The eluates from the column are monitored with a Gilford 2000 recording spectrophotometer equipped with a Beckman monochromator and a Gilford flow cell (10 mm path length).

Sephadex G-50 (fine) was obtained from Pharmacia. Ribonuclease was purchased from Calbiochem, and α-chymotrypsin was a product of Sigma.

Results and discussion

Superimposed elution patterns of 1.5 μg of α-chymotrypsin and ribonuclease on Sephadex G-50 (fine) monitored at 220 nm are shown in Fig. 1. Complete elution is achieved in approximately 30 min. The electronics of the Gilford spectrophotometer can successfully record very low levels of absorbance which contributes to the improved sensitivity of the present method. Comparative elution patterns of different amounts of ribonuclease recorded at 220 nm are illustrated in Fig. 2. Even 1 μg of the protein exhibits a clearly defined peak for measurement of retention time. The peak

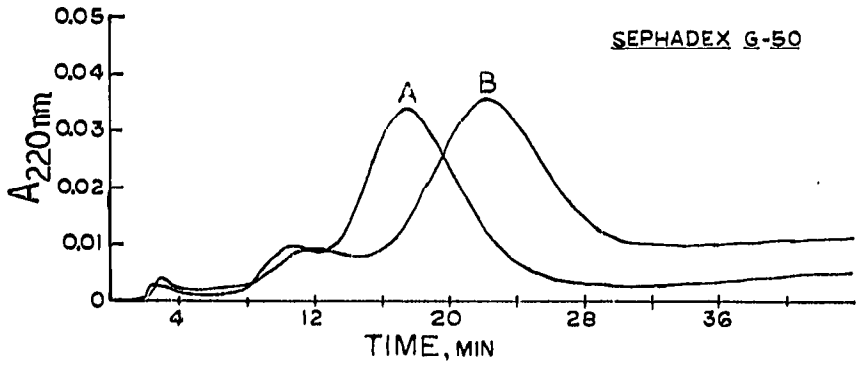

Fig. 1. Superimposed elution diagrams of 1.5 μg each of α-chymotrypsin (A) and ribonuclease (B) obtained by gel chromatography on a Sephadex G-50 microbore column and recorded at 220 nm. A pH 7.6 phosphate buffer (0.1 *M* in NaCl) was used for the elution at a flow-rate of 5 ml/h.

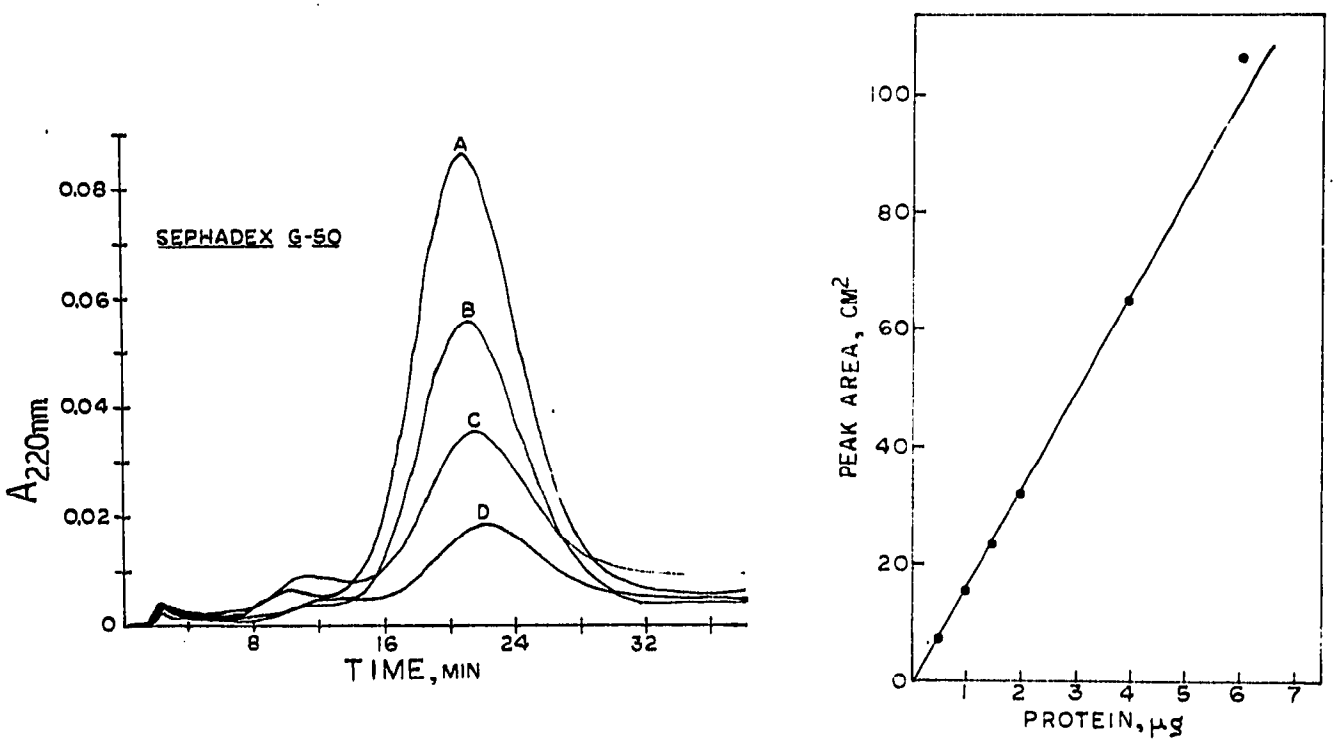

Fig. 2. Gel chromatography on Sephadex G-50 of different amounts of ribonuclease. A, 6 μg; B, 4 μg; C, 2 μg; and D, 1 μg. Experimental conditions as described in Fig. 1.

Fig. 3. Linear relationship between elution peak area and amount of ribonuclease applied to the Sephadex G-50 column. Experimental conditions as described in Fig. 1.

areas—corrected for angular baseline—have a linear relationship with the amount of the protein injected in the column (Fig. 3). Pronounced "wall effects" observed previously[1] which result in a trailing rear boundary have been significantly eliminated probably because of the smaller amount of sample used in the present studies.

The improved sensitivity of detection at 220 nm, elimination of pronounced "wall effects", and rapidity of the present method offer significant advantages for the micro-analysis of proteins and peptides by gel chromatography. It is hoped that the method can be extended to the use of other gels by employing high pressure

liquid chromatography equipment. It is also suggested that this method may be used for the quantitative micro determination of total protein in biological fluids by exclusion of low molecular weight UV absorbing compounds. Experiments in this respect are in progress.

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