GEL FILTRATION OF PROTEINS ON SEPHACRYL® S-200 SUPERFINE IN 6 M GUANIDINE-HCl

Makonnen BELEW and Jan FOHLMAN
Institute of Biochemistry, Uppsala University, Box 576, 751 23 Uppsala

and

Jan-Christer JANSON
Pharmacia Fine Chemicals AB, Box 175, 751 04 Uppsala, Sweden

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1. Introduction

Gel filtration of reduced and alkylated or oxidized proteins in 6 M guanidine-HCl is an accurate and widely used method for estimating the polypeptide chain length and molecular weight of proteins [1]. Using Sepharose® 6B as a matrix, Fish et al. [1] have obtained an accuracy of 7% for peptides having molecular weights in the range of 10 000 to 40 000. The accuracy decreases, however, for peptides whose molecular weights lay outside these limits.

The purpose of this report is to show that Sephacryl® S-200 Superfine (a new chromatographic medium recently introduced by Pharmacia Fine Chemicals AB, Uppsala, Sweden) is a useful gel filtration medium for estimating the peptide chain length of proteins having molecular weights in the range of 1000 to 30 000. The peptide chain length of small peptides (less than 10 000 in molecular weight) can be estimated with an accuracy of better than 5%.

2. Materials and methods

The calibrating proteins used were: bovine serum albumin, chymotrypsinogen A, ribonuclease and *Naja naja naja* toxin 4 [2]. A mixture containing equal amounts of these proteins was reduced with DTE and S-carboxymethylated with iodo[^{3}H]-acetate [3]. As test proteins, the native and trypsin-modified inhibitors from chick pea [4] were used. These were also reduced and S-carboxymethylated as above. Sephacryl S-200 superfine was obtained from Pharmacia Fine Chemicals AB, Uppsala. Guanidine–HCl (purum) was bought from Fluka AG, Switzerland and was purified by treatment with charcoal according to Fohlman et al. [3].

2.1. *Preparation and operation of the column*

The pre-swollen gel was washed with distilled water to remove preservatives, excess liquid was removed by suction, and the gel was re-suspended in and equilibrated with 6 M guanidine-HCl, pH 5.0. The slurry was deaerated, poured in a column (1.6 × 100 cm) and allowed to settle under continuous flow. In order to obtain a tightly packed bed, the slurry was continuously fed into the column by means of a peristaltic pump. The flow rate was maintained at 30 cm/h and the packing was complete in about 3 h. When a bed height of 86 cm had formed, the upper plunger was introduced into the column and the bed was eluted overnight with 6 M guanidine-HCl at a flow rate of 10 cm/h.

To the equilibrated column was applied 100 μl of the calibration mixture or the test protein solution together with 100 μl of a 2% solution of blue dextran. The flow rate was maintained at 3 cm/h with a Gilson peristaltic pump and fractions of 1.0 g were

collected. The elution positions were determined by weight rather than by volume in order to obtain good reproducibility [1]. The distribution of material in the effluent fractions was determined by liquid scintillation counting performed according to Anderson [5].

2.2. *Treatment of data*

From the elution volume of each of the calibrating proteins, the distribution coefficient, K_d, was calculated using the expression:

$$K_d = \frac{W_e - W_o}{W_{re} - W_o}$$

where W_e, W_o and W_{re} represent, respectively, the elution 'weights' of the protein, blue dextran 2000 and excess reagents from the reduction and alkylation reaction. A calibration curve was then constructed by plotting log 100 K_d vs. $N^{2/3}$ (where N represents the number of amino acid residues in the calibrating protein) as described by Rydén [6]. The peptide chain length of the test proteins was computed by reference to this calibration curve.

3. Results and discussion

The elution profile obtained with the calibration mixture on the column of Sephacryl S-200 is shown in fig.1. Data concerning size, elution volume and the calculated K_d for each of the calibrating and test proteins are summarized in table 1.

The results show that Sephacryl S-200 superfine has good resolving power for proteins having molecular weights below 30 000. Larger proteins cannot be resolved since they elute too near the void volume (see fig.1). The calibrating proteins are well separated and elute as symmetrical peaks with little zone spreading. The results also show that this type of chromatographic analysis is suitable for testing the homogeneity of protein preparations. Thus, crystalline ribonuclease and a once chromatographically homogeneous *Naja naja naja* 4 toxin [2] were found to contain minor fractions of high molecular weight, probably aggregates.

The calibration curve relating K_d to the peptide chain length of the calibrating proteins is shown in fig.2. The relationship is linear in the molecular weight range of the calibrating proteins employed. However, since proteins having molecular weights exceeding 30 000 are eluted at the void volume, only the left

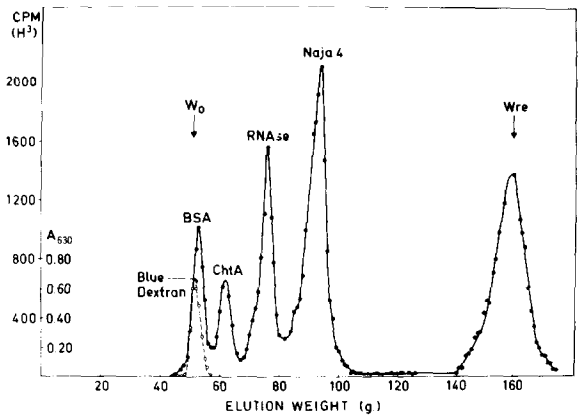

Fig.1. Gel filtration of: bovine serum albumin (BSA), chymotrypsinogen A (Cht.A), ribonuclease (RNAse) and *Naja naja naja* 4 toxin (Naja 4) on a column (1.6 × 86 cm) of Sephacryl S-200 Superfine. The column was equilibrated and eluted with 6 M guanidine-HCl, pH 5. The proteins were reduced and alkylated with iodo[^{3}H]acetate. The elution positions are expressed in terms of effluent weight. W_o and W_{re} represent, respectively, the elution 'weights' of Blue dextran 2000 and of excess reagent from the reduction and alkylation reaction.

Table 1
Peptide chain length and elution parameters of calibrating and test proteins chromatographed on Sephacryl S-200 in 6 M guanidine-HCl

Solute	No. of residues	K_d
Calibrating proteins:		
Bovine serum albumin	579	0.014
Chymotrypsinogen A	245	0.098
Ribonuclease	124	0.228
Naja naja naja 4	71	0.395
Test proteins:		
Native inhibitor	67 (66)[a]	0.410
Trypsin modified:		
(a) large fragment	52 (52)[a]	0.480
(b) small fragment	16 (14)[a]	0.78

[a] The figures in parentheses represent actual number of residues in the inhibitor fractions [4]

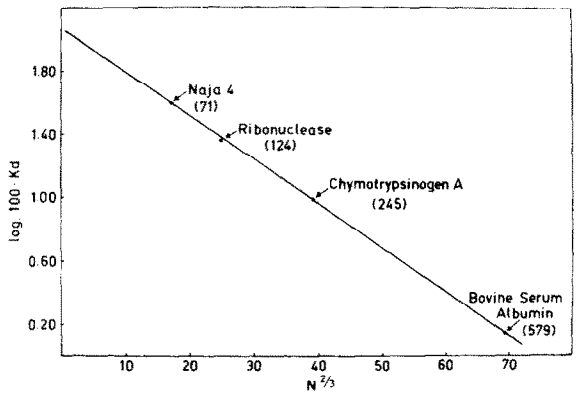

Fig.2. Plot of log 100 K_d vs. $N^{2/3}$ for the calibrating proteins shown in fig.1. N and the figures in parentheses represent the number of amino acid residues in the protein polypeptide chain.

half of the calibration curve can be used for determining the peptide chain length of an unknown protein chromatographed on the column under the conditions used here.

As shown in table 1, the number of amino acid residues determined by this method for the native inhibitor and for the two fragments derived from the modified inhibitor obtained from chick peas [4] are in good agreement with values found earlier. The accuracy of the method using these test proteins is better than 5% for the larger fragments and is about 10% for the smallest (14 amino acid residues) fragment. The analytical accuracy is thus comparable to that reported by Fish et al. [1] who used Sepharose 6B as matrix. The results presented here thus show that Sephacryl S-200 superfine is the matrix of choice for the chromatographic estimation of the peptide chain length of small peptides and proteins.

References

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