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GEL FILTRATION IN SODIUM DODECYL SULPHATE OF HYDROPHOBIC MUSCLE PROTEINS ON SEPHACRYL S-400 SUPERFINE

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SUMMARY

Gel filtration has been applied to muscle proteins in the presence of the detergent sodium dodecyl sulphate (SDS). This detergent not only solubilizes a variety of proteins but also increases their hydrodynamic volumes, which is a disadvantage for the process of gel filtration. Nevertheless, appropriate choice of gel type, in this case Sephacryl S-400 Superfine, and optimal elution conditions make SDS gel filtration a useful tool in the separation of heterogeneous protein mixtures in the molecular weight range of 20,000–200,000.

INTRODUCTION

Proteins within a single biological sample may show considerable differences in solubilities. Although such differences are often used for the preparative isolation of a single protein or a class of proteins, there may be reasons for the use of a separation method in which all proteins are treated in the same way. This is especially important when there are several protein components of interest and when knowledge of their mutual contamination is important for the interpretation of the results. In such cases a single-step separation procedure for all proteins will be preferred over a time-consuming method for the isolation of each individual protein.

Lobley and Lovie[1] discussed these problems in studies of the relative turnover of muscle proteins. They stated that the differences between the specific radioactivities of the proteins, in particular actin, purified by various methods, have to be attributed to the loss of newly synthesized material of high specific radioactivity during the initial extraction procedures. This means that a separation method is required which ensures a minimal loss of protein.

Therefore we have developed a method for gel filtration of muscle proteins in the presence of sodium dodecyl sulphate (SDS), which, in our opinion, can also be applied to other types of heterogeneous protein samples.

SDS interacts in a constant ratio of 1.4:1 (w/w) with a variety of proteins and the subsequent denaturation changes their hydrodynamic properties[2,3]. Our earlier reports[4,5] indicate that SDS–protein complexes have smaller elution volumes than

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the native ones, suggesting an increase of their hydrodynamic volumes. Therefore the use of SDS in gel filtration has two important implications:

(1) The fractionation range of the gel must match the size of the SDS–protein complexes rather than the size of the native proteins.

(2) Relative to the native proteins, SDS–protein complexes will show less pronounced differences in size, which tends to lower the resolution. A reduction of the resolution, however, can partly be compensated by improving the elution conditions.

The present paper describes a procedure for the elution of a total muscle sample on Sephacryl S-400 Superfine in the presence of SDS. Although there is a drastic shift of the selectivity curve of the gel under these elution conditions, a good resolution of the major muscle proteins is obtained.

MATERIALS AND METHODS

Column preparation

A column (94 × 2.6 cm) of Sephacryl S-400 Superfine (Pharmacia, Uppsala, Sweden) is first packed in SDS-free eluent buffer in accordance with the prescribed packing procedure. The column is ready for use after elution at the operational flow-rate with three bed volumes of eluent buffer containing SDS. The eluent buffer is the same as used for SDS electrophoresis, only complemented with 0.02% sodium azide.

Sample application

The standard column (Pharmacia) is modified in a way that the column inlet tube ends 2–3 cm above the net at the top of the gel bed. This modification has the advantage that a fixed sample volume, stored between two air-bubbles in a sample applicator loop, can be applied during normal running of the column with little or no disturbance. For this purpose the density of the sample must be high relative to the density of the eluent. The volume of the sample applicator loop is 1 ml.

Monitoring and fractionation

The eluent of the column (24 ml/h) is monitored at 280 nm and collected in 200-drop fractions (*ca.* 5 ml). Samples of the column fractions can directly be subjected to SDS gel electrophoresis because the same buffer is used in both procedures.

Sample preparation

Samples of the Musculus longissimus dorsi of a rabbit are homogenized for 1 min in three volumes of water (IKA X-10, homogeniser). The homogenate is mixed with an equal volume of a 12% SDS solution and 2-mercaptoethanol (2%, v/v) is added to reduce disulphide bridges. After standing for 90 min at 60°C with incidental shaking, the mixture is centrifuged for 30 min at 3000 *g* to remove the fat. The solution obtained is opalescent but clear with a fat layer on the top and occasionally with a small pellet of probably unsolved connective tissue. The solution contains about 30 mg protein per ml and is applied to the column in a five-fold dilution.

Gel electrophoresis

Gel electrophoresis is performed in SDS by standard methods using 5% slab gels (130 × 150 × 3 mm) of Cyanogum'41 (BDH, Poole, Great Britain) at 100 V and

60 mA for 4 h. The fixing, staining and destaining procedures are carried out as prescribed by LKB[6]. The buffer used is 0.2% SDS in Tris–borate–EDTA (10.75 g/l:5.04 g/l:0.93 g/l, pH 8.35) as described by Pharmacia[7]. An HMW calibration kit (Pharmacia) is used for the estimation of the molecular weights. Volumes of 40 and 20 μl are used for the samples and the calibration kit, respectively. The total muscle sample (TMS) is applied in a fifteen-fold dilution.

RESULTS

The void volume of the column (elution volume of Dextran blue 2000) is determined as 37% of the gel bed volume.

The elution pattern of a total muscle sample, monitored at 280 nm, is shown in Fig. 1. In contrast to our experience with Sephacryl S-300 Superfine[5] there are no components which elute with the void volume. This means that all protein components are fractionated on this column. The profile consists of a protein and a non-protein part (see below). The protein part (peaks I–V) can easily be reproduced. In the non-protein part (peaks VI–VIII) some fluctuations may occur. The two small peaks are not always completely separated and the large peak at the end increases on standing of the sample. This increase is probably due to the presence of 2-mercapto-ethanol which oxidizes to its dimer with a higher extinction coefficient. The K_{av} values corresponding to the elution peaks are given in Table I.

SDS gel electrophoresis of the peak fractions is used to determine the molecular weight of the constituent proteins and moreover to show the degree of purity of the fractions. Fig. 2 shows the electrophoretic pattern of the total muscle sample

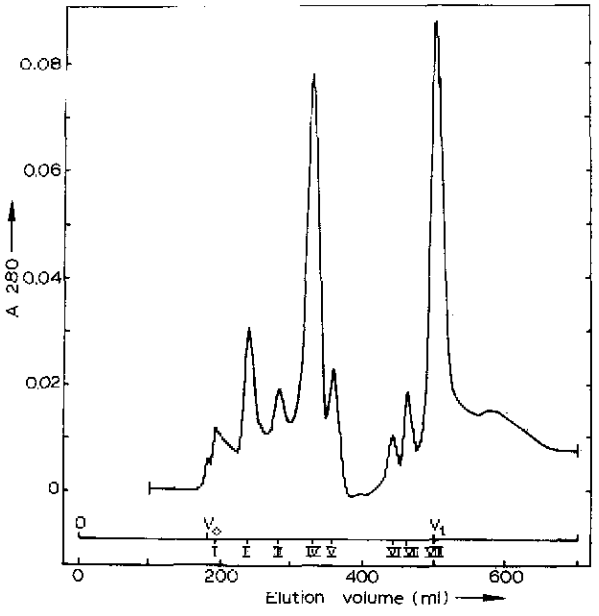

Fig. 1. Elution pattern of a total muscle sample on Sephacryl S-400 Superfine in the presence of SDS, monitored at 280 nm. V_0 and V_t are the void volume and the total bed volume of the column, respectively.

TABLE I

PARAMETERS OF MUSCLE COMPONENTS IN GEL FILTRATION AND ELECTROPHORESIS

Elution peaks are numbered as indicated in Fig. 1. K_{av} values are calculated from Fig. 1; $K_{av} = (V_e - V_0)/(V_t - V_0)$. R_F values are calculated from Fig. 2, relative to the smallest calibration protein. MW = Molecular weight of protein components.

Elution peak	K_{av}	R_F	*MW*
I	0.02	0.01	>330,000
II	0.23	0.12	≈250,000
III	0.30	0.34	110,000
IV	0.45	0.65	43,000
		0.69	39,000
		0.74	35,000
V	0.60	0.92	21,000
		1.00	18,500
VI	0.83	–	
VII*	0.89	–	
VIII	1.00	–	

* Not applied on this gel because of its capacity.

(trace 1), seven elution peaks (traces 2–8) and of the calibration proteins (trace 9). This gel as all others (not shown) indicates that the first elution peaks consist of components which show hardly any reaction with the staining agent, suggesting that little or no protein is involved. This suggestion is supported by the protein estimation of Lowry *et al.*[8]. The peaks II and III are relative pure, whereas peaks IV and V contain three and two components, respectively. Peaks VI and VIII do not contain components which are detectable on the gel, suggesting that only non-protein components of low molecular weight are involved. For each fraction we have calculated the R_F value for the major protein components on the gel. The values for muscle proteins and calibration proteins, calculated relative to the smallest calibration protein, are shown in Tables I and II, respectively.

In Fig. 3 we have plotted the calibration curve for gel electrophoresis in accordance with the R_F values of the calibration proteins in Table II. Using this calibration curve, the approximate molecular weights of the protein components in the peak fractions can be derived from their R_F values. The results are shown in Table I.

TABLE II

R_F VALUES OF CALIBRATION PROTEINS IN GEL ELECTROPHORESIS

R_F values are calculated from Fig. 2 (trace 9), relative to the smallest calibration protein.

MW	R_F
330,000	0.06
220,000	0.21
67,000	0.44
60,000	0.53
36,000	0.71
18,500	1.00

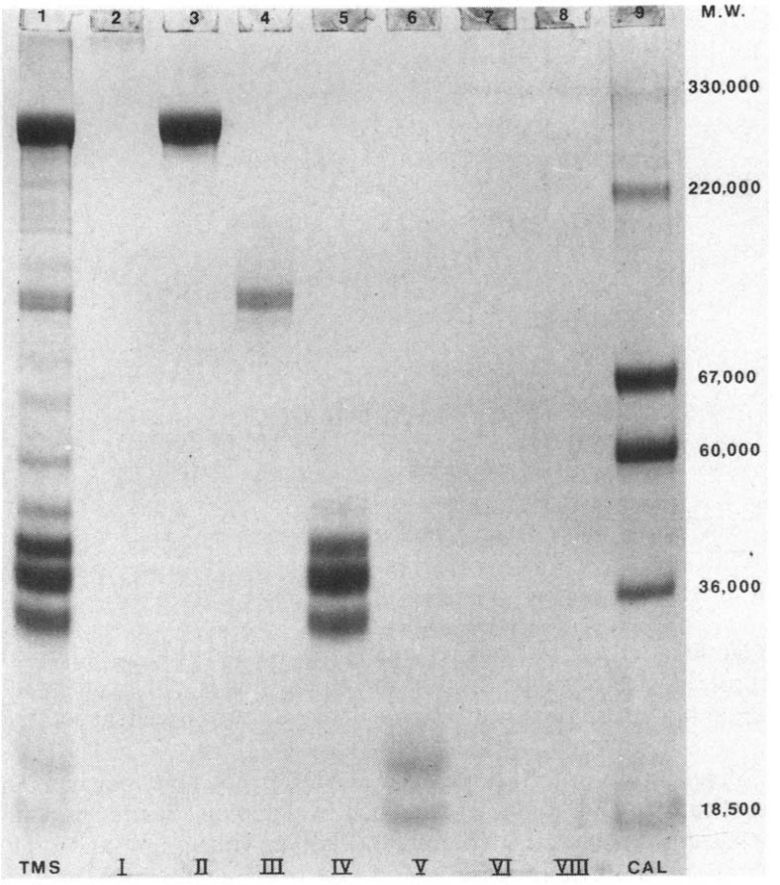

Fig. 2. SDS gel electrophoresis. Traces: 1 = total muscle sample (TMS); 2–8 = seven elution peaks (I–VI, VIII) as indicated in Fig. 1, 9 = calibration proteins (CAL) with corresponding molecular weight, M.W.

A plot of the molecular weights of the protein components against the corresponding K_{av} values results in the selectivity curve for Sephacryl S-400 Superfine in the presence of SDS (Fig. 4). For comparison we have also shown the selectivity curve without the use of SDS, as given by Pharmacia in the product information. It is clear that the elution volumes of the proteins are considerably smaller in the presence than in the absence of SDS, suggesting an increase of the hydrodynamic volumes of the proteins due to denaturation by SDS.

DISCUSSION

The method of gel filtration in the presence of SDS, described in this paper, is developed for application in our studies on the relative turnover of muscle proteins. In these studies we compare the incorporation of a labelled amino acid in various muscle proteins. From the work of Lobley and Lovie[1], it can be derived that such studies are best served by a single-step separation of the proteins with the highest at-

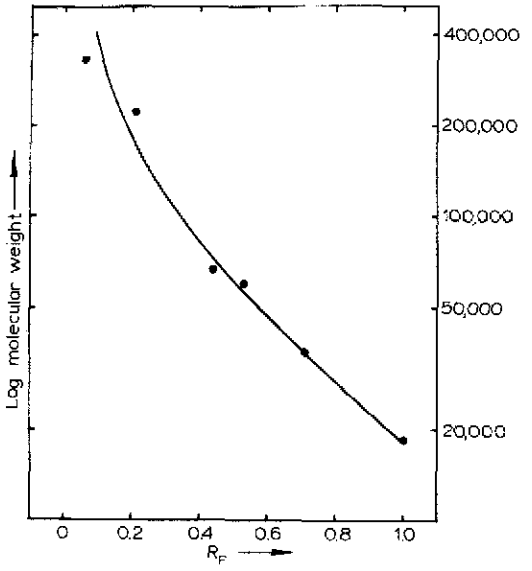

Fig. 3. Calibration curve for SDS gel electrophoresis.

tainable recovery. Gel filtration, an obvious technique for this purpose, is only suitable if the muscle proteins can be solubilized in the eluent buffer. This can be attained by use of sodium dodecylsulphate, an anionic detergent with excellent solubilizing properties[2,3].

The concept of gel filtration in the presence of SDS is not new, but has not found large scale application. This could be explained by some disadvantages compared with other separation techniques. The method is time-consuming and often gives only poor separation since in many cases most of the relative large SDS–protein complexes elute with or near the void volume of the column. Moreover, in the long

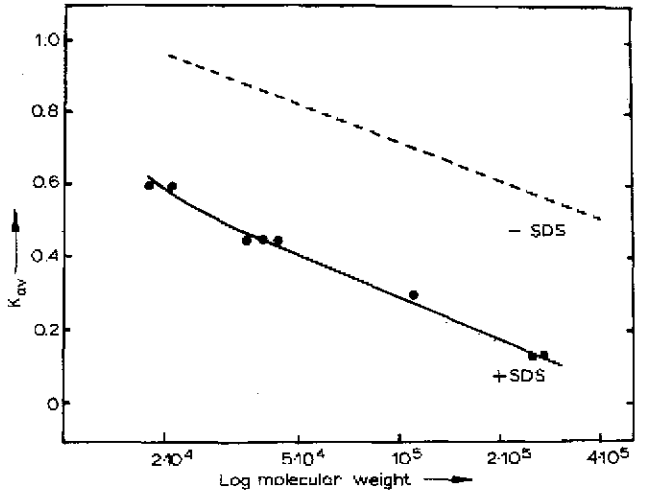

Fig. 4. Selectivity curve of Sephacryl S-400 Superfine with (+) and without (–) SDS. The latter is taken from the product information of Pharmacia.

term, many gels do not withstand this aggressive detergent. Nevertheless, this method has been used to establish the protein composition of biomembranes[9], and to estimate the molecular weights of polypeptides[9–17]. Due to its use in high-performance silica gel chromatography, the technique may find more applications for analytical purposes[16,17]. For preparative purposes, on the other hand, the new Sephacryl gels may give good prospects.

We have used Sephacryl S-300 Superfine for more than a year in the presence of SDS without any damage or decrease in resolution. This gel, however, has an exclusion limit too low for a good separation of all SDS–protein complexes in our muscle samples. In particular, the resolution of the first three elution peaks (see Fig. 1) is considerable lower on Sephacryl S-300 Superfine[5]. The Sephacryl S-400 Superfine described in this paper has a high exclusion limit and a large fractionation range. Our results show that proteins of $2 \cdot 10^4$–$2 \cdot 10^5$ dalton complexed by SDS elute within this fractionation range.

The shift of the selectivity curve due to the use of SDS roughly indicates that, in gel filtration, the apparent molecular weights of SDS denatured proteins are 10–20 fold higher than the molecular weights of the native proteins. This increase can be attributed partly to the binding of the SDS (1.4:1, w/w) and partly to a change in conformation due to denaturation by SDS. It is unclear whether this shift is also influenced by an "ionic exclusion" effect due to negative charges on the gel matrix, as described for silica gel[17].

The increase of the hydrodynamic volume of the proteins in the presence of SDS seems to lower the resolution. In part this may be due to a decrease of the relative differences between their hydrodynamic volumes when the proteins form a complex with SDS. The choice of a Sepharyl gel for the separation can also help to overcome this problem. Sephacryl, a very rigid gel, is developed to give under normal circumstances a good resolution even at high flow-rate. However, in the elution of SDS–protein complexes a high flow-rate only gives a poor separation. Good resolution can only be attained by decreasing the flow-rate. In this respect about 5 ml cm^{-2} h^{-1} has been shown to be optimal. Generally, the resolution is further determined by well known factors such as the composition, size, concentration and application mode of the sample. The best compromise between the various conflicting elution conditions will usually depend on the ultimate aim of the separation.

In the present case we have tried to attain the best separation of the muscle proteins under conditions where the proteins as a whole are baseline separated from the low-molecular-weight amino acids. This is a prerequisite for turnover studies since a contamination of labelled proteins with their precursor amino acids will give faulty results. Therefore this method, although time-consuming, is fast and relatively easy compared to the laborious isolation procedure for total protein described by Nicholas *et al.*[18]. Moreover, this method ensures a minimal loss of protein, also very important in the study of protein turnover[1].

It may be concluded that hydrophobic proteins in the molecular weight range of 20,000–200,000, solubilized in SDS, may satisfactorily be separated by gel filtration on Sephacryl S-400 Superfine. If only smaller proteins are involved, Sephacryl S-200 or S-300 Superfine will be more adequate. Moreover, the size of SDS–protein complexes may, more or less, be adapted to the fractionation range of the gel by changing the ionic strength of the elution buffer[16,17].

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