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Separation of Proteins by Thin-Layer Gel Filtration

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A method for the separation of proteins by means of gel filtration applied as a thin layer chromatographic technique is described. Sephadex G-50 and G-75 are useful for the separation of relatively low molecular weight proteins whereas G-100 and G-200 permit separations of proteins with higher molecular weights, *i.e.* blood serum proteins. The method allows easy detection of enzymic activities after gel filtration.

The application of gel filtration as a thin layer chromatographic technique has previously been reported by Determan [1] and Johansson and Rymo.[2] The latter authors used Sephadex G-25 and Sephadex G-75 for the separation of various amino acids and proteins. With Sephadex G-75 a separation of proteins with molecular weights less than about 150 000 seems to be possible. In the present study the use of Sephadex G-100 and G-200 for the separation of proteins with higher molecular weights will be described, *i.e.* serum proteins. Some different applications of the thin layer gel filtration technique using the various Sephadex gels will also be presented.

EXPERIMENTAL

Materials

Sephadex gels. Sephadex gels were obtained from AB Pharmacia, Uppsala, Sweden. The following gels were used: G-25, fine (200–400 mesh, water regain 2.4); G-50, fine (200–400 mesh, water regain 4.7); G-75, fine (200–400 mesh, water regain 7.4) which are all commercially available. Sephadex G-75, G-100 and G-200 "superfine" (<400 mesh) were kindly supplied by Dr. B. Gelotte, AB Pharmacia.

Serum proteins conjugated with fluorescein isothiocyanate were prepared as described by Killander *et al.*[3] using Sephadex G-25 for the removal of excess dye.

Human serum albumin was kindly supplied by AB Kabi, Stockholm. Two preparations were used, one of which was lyophilized and the other a 20 % sterile solution. Crystalline bovine serum albumin was obtained from Nutritional Biochemical Corp. (Cleveland, U.S.A).

β-Lactoglobulin and *α-lactalbumin* from cows milk were prepared according to the method of Aschaffenburg and Drewry.[4]

Enzymic hydrolysates of γ-globulin and milk immune globulins were obtained by degradation with trypsin [5] or papain.[6]

Enzymes used for localization of enzymic activities were commercial preparations of *trypsin* and *intestinal alkaline phosphatase* (Worthington, Biochemical Corp.). A purified preparation of *β-glucuronidase* from human urine was kindly supplied by Dr. O. Hygstedt, Göteborg.

Pathological blood sera were obtained from patients suffering from multiple myeloma and macroglobulinemia Waldenström. *Reduction of γ-globulin* with β-mercaptoethanol was performed as described by Edelmann and Poulik.[7] It was performed in water solution or in 8 M urea.

Methods

The thin layer plates were prepared as described earlier.[2] Sephadex gel powder was swelled in a suitable buffer for an appropriate time (Table 1). In this communication the experiments were performed in a 0.02 M sodium phosphate buffer, pH 7.0, containing 0.2 M sodium chloride, if not otherwise stated. The gel was then spread over a glass plate with a Desaga applicator (C. Desaga, GmbH, Heidelberg, Germany). The thickness of the gel layer was kept at 0.5 mm in all experiments. Glass plates of the dimensions 20 × 30 cm, 20 × 40 cm or 20 × 50 cm were commonly used. After spreading the plates were placed in a flat box of acrylic plastic with the dimensions 22 × 55 × 4 cm. The box was covered with a sheet of acrylic plastic and leant against a buffer reservoir which was placed on a small table with changeable height in order to get an easily variable inclination of the plate. A piece of thick filter paper (Whatman No. 3 MM) applied on the upper end of the plate through a slit in the plastic box ensured contact between the gel layer and the buffer giving a liquid flow through the gel. Before a run it was found advisable to have a flow through the plates for at least one hour. The samples were then applied with capillary pipettes as round spots 2—4 mm in diameter corresponding to sample volumes of about 1—5 μl. The flow rate was adjusted by levelling the buffer reservoir with the guidance of the rate of movement of a suitable coloured marker substance. Suitable flow rates seemed to correspond to a movement of non-retarded substances of 1—2 cm/h. After interruption of the run the plates were either dried at 60°C in an oven with circulating air or (in the case of Sephadex G-100 and G-200) first covered with a filter paper (Schleicher and Schüll 2043 b) of the same dimensions as the plate and then dried in the same way. Care was taken when applying the filter paper to avoid trapping air between the paper and the gel.

The plates were dyed in a saturated solution of amido black 10 B in methanol-acetic acid-water, 80:10:10 (v/v), for 2 h and washed repeatedly with methanol-acetic acid-water, 70:5:25 (v/v), to remove excess dye. The plates were then dried at room temperature. The plates, covered with filter paper, were placed in a saturated solution of lissamine green in 5 % acetic acid for one hour. The filter paper loosened rapidly from the plate in the dye bath and the latter could be removed. The paper was washed several times in 5 % acetic acid. Removal of excess dye from the paper was much more readily accomplished when using lissamine green than amido black 10 B. No problems concerning the colour background were found by direct dyeing of the plates.

Lipoproteins were localized by dyeing the filter paper with a saturated solution of oil red O in 60 % ethanol and subsequent removal of the excess dye with ethanol.

Alkaline phosphatase activity was detected by spraying the plate with β-naphthyl phosphate in a sodium barbital buffer pH 8,6. After incubation for 30 min the plate was sprayed with a 2 % water solution of fast red TR. Reddish-purple spots indicated the site of phosphatase.[8] The enzymic activity could also be detected after transfer of the enzyme to a dry filter paper impregnated with the substrate. For detection of trypsin the last mentionede procedure was used with a filter paper impregnated with benzoyl DL-arginine-nitroanilide hydrochloride in a 0.05 M Tris—HCl buffer, pH 8.0.[9] β-Glucuronidase was localized after action of the enzyme on phenolphthalein-glucuronide and detection of phenolphthalein by alkalization with sodium carbonate.[10]

RESULTS AND DISCUSSION

Comments on the technical procedures. The swelling of the gel for a suitable time and the consistency of the gel slurry seems to be important for obtaining

Table 1. Proportions of gel-buffer and times of swelling for different Sephadex gels used for thin layer gel filtration.

Type of Sephadex	Particle size (mesh)	Amount of buffer (ml) to be added to 1 g of dry Sephadex	Swelling time, h
G-25, fine	200–400	5	2
G-50, fine	200–400	11	2
G-75, fine	200–400	15	5
G-75, superfine	<400	15	5
G-100, superfine	<400	19	24
G-200, superfine	<400	25	24–48

satisfactory gel plates. The appropriate consistency must be gained by experience as the proportions between gel and buffer may be unequal for different gel batches. In Table 1 is given the proportions between gel and buffer as well as suitable times of swelling for gels used by us. There were no striking differences in the results obtained by Sephadex G-75, fine and G-75, superfine. The only difference noted was a slight roughness of the surface of the gel after spreading of G-75, fine. Concerning gels with lower degrees of crosslinking (G-100 and G-200) experiments with the standard gels available for column gel filtration have given poor resolution of components in contrast to the "superfine" gels. By screening standard gels the fractions with particle size less than 325 mesh can be used. The yield of this fraction, however, is very low, only about 20 % of the unscreened material.

The use of larger plates than described earlier [2] has some advantages; the flow rates are more constant, better separations are obtained with longer running distances and a larger number of fractions and reference substances may be run on the same plate for comparative purposes. The rate of flow through the gel could be visualized by applying strongly coloured substances as references. Dinitrophenyl (DNP) amino acids were used in some experiments. The slow rates of movement of DNP-amino acids, however, made it desirable to use more rapidly moving substances. A number of coloured proteins were tested (coeruleoplasmin, haemoglobin, serum albumin with high bilirubin content) but none of these could be easily detected on the plates during the run. The most satisfactory results were achieved with fluorescein isothiocyante conjugated serum proteins, giving a pronounced green-yellow fluorescence in ultraviolet light. In the case of filtrations on Sephadex G-50 and G-75 it was most often possible to watch even uncoloured proteins during their migration as these formed distinct clear zones moving through the gel.

The drying of the plates and the protein dyeing procedure were easily performed after gel filtrations with Sephadex G-25, G-50 and G-75. Sephadex G-100 and G-200, however, most often showed an uneven surface after drying with a pronounced retraction of the gel. Dyeing of such plates gave results which were difficult to interpret. By covering the wet gels with filter paper after the gel filtration, the buffer with the separated substances was transferred to the filter paper, which could be dyed as after paper electro-

phoresis. This technique was also employed before detection of ninhydrin-positive substances as ninhydrin treatment of the plate always gave very faint spots. The use of substrate impregnated papers also made this technique useful for the detection of enzymes after thin layer filtration.

Applications of the method. Sephadex G-25 and G-50 are most suitable for separation of proteins from low-molecular contaminants and for a group separation of peptides. With Sephadex G-50 also a retardation of small protein molecules is obtained. Fig. 1 illustrates the separation of bovine serum

Fig. 1. Thin layer gel filtration on Sephadex G-50, fine. Bovine serum albumin (a). β-Lactoglobulin (b). α-Lactalbumin (c). A mixture of bovine serum albumin, β-lactoglobulin and α-lactalbumin (d). Shaded areas represent localization of DNP-aspartic acid.

Fig. 2. Gel filtration of a mixture of bovine serum albumin, β-lactoglobulin and α-lactalbumin on Sephadex G-75, fine.

albumin, β-lactoglobulin and α-lactalbumin on Sephadex G-50. A slight difference in the moving rates of serum albumin and β-lactoglobulin was found while α-lactalbumin moved considerably slower.

When using *Sephadex G-75* for the same proteins the separations of albumin and β-lactoglobulin was complete (Fig. 2) and furthermore two albumin components were obtained possibly due to the formation of dimers-polymers of the albumin molecules. The separation of albumin into two components was established in other experiments by running pure bovine serum albumin (Fig. 3 a). It might be noted that human serum albumin did not give such an evident separation. The separation of dimer and polymers from the albumin monomer has been accomplished by gel filtration in columns on Sephadex G-100.[11] On Sephadex G-75, using column technique, this separation is not obtained.

Sephadex G-75 has also been used for the separation of high molecular weight fragments after enzymic degradation of proteins. In Fig. 3 (b)—(e) is illustrated

Fig. 3. Gel filtration on Sephadex G-75, fine. Separation of bovine serum albumin (a), trypsin hydrolyzates of human γ-globulin and human milk immune globulin (b and c), papain hydrolyzates of milk immune globulin and γ-globulin (d and e).

Fig. 4. Separation of normal blood serum proteins on Sephadex G-100, superfine. Double amount of sample applied in (a) compared to (b).

the separation of fragments obtained by degradation of human blood serum γ-globulin and human milk immune globulin[12] with trypsin and papain. The similarity of the fragments of the two different γ-globulin hydrolyzates is evident as are the differences between the patterns of the γ-globulin and milk immune globulin hydrolyzates. These comparative analyses could be performed after degradation of less than 0.5 mg protein.

The results obtained by thin layer gel filtration on *Sephadex G-100* are rather analogous to those described for Sephadex G-75. The latter gel seems to be preferable in most cases due to technical reasons as described above. It may be noted, however, that blood serum proteins may be separated into two fractions on Sephadex G-100 (Fig. 4) in contrast to Sephadex G-75 where only one long-stretched zone was obtained. These two fractions contained grossly γ-globulin and albumin. Behind the albumin fraction a trace component was observed, especially after application of larger serum volumes on the plates.

With *Sephadex G-200* using column technique a separation of serum proteins into three main fractions has been obtained by Flodin and Killander.[13] By thin layer gel filtration a similar separation was found (Fig. 5) besides the appearance of a faint spot behind the albumin zone. Fig. 5 also illustrates the patterns of a few sera containing abnormal components in the electrophoretogram (paraproteins) from patients with multiple myeloma (b, c, d) and macroglobulinemia (e). The myeloma serum (b) was of γ_{ss}-globulin type and contained a component with the same gel filtration movement as γ-globulin. Serum samples shown in (c) and (d) were of γ_{1A}-globulin type and contained a few components with probably larger molecular size than γ-globulin. The presence

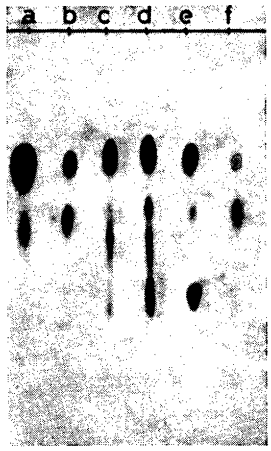

Fig. 5. Separation of blood serum proteins on Sephadex G-200, "superfine". Normal serum (a), myeloma serum of γ_{ss}-globulin type (b), two γ_{1A}-myeloma sera (c and d) and a serum containing a pathological macroglobulin (e). In (f) a sample of fluorescein isothiocyanate conjugated rabbit serum was applied. Encircled zones showed fluorescense in ultraviolet light. The most fast-moving component (in a) is not visible in the figure.

of a large component with a high rate of movement in (e) corresponded to a pathological macroglobulin. Lipoprotein detection has also been performed after serum thin layer filtration. In accordance with the results of Flodin and Killander,[13] all lipid stainable material was found in the most fast moving fraction.[14]

Detection of enzymic activity after thin layer gel filtration is possible either directly on the wet plate or after transfer of the enzymes to a filter paper. Filtration of a crude phosphatase preparation from calf intestinal mucosa on Sephadex G-75 revealed two poorly resolved components with activity towards β-naphthyl-phosphate (Fig. 6). β-Glucuronidase from human urine could be detected after thin layer filtration on Sephadex G-200. The localization of the activity indicated that the enzyme was only slightly retarded. The results obtained with crystalline trypsin preparation on Sephadex G-75 were also satisfactory.

The thin layer gel filtration technique might also be useful as a complementary method in the studies of protein "subunits". Separation of two components from γ-globulin, reduced with β-mercaptoethanol in water solution

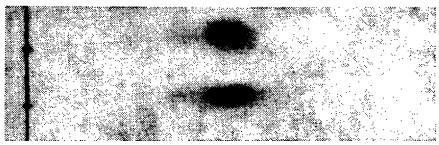

Fig. 6. Localization of phosphatase activity after thin layer filtration of a purified preparation of intestinal alkaline phosphatase. Separation on Sephadex G-75, fine.

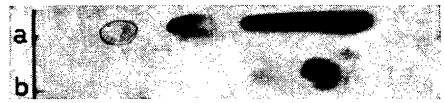

Fig. 7. Thin layer gel filtration on Sephadex G-75 in M HAc containing 0.1 M β-mercaptoethanol. Mixture of human serum albumin, α-lactalbumin and DNP-aspartic acid (a). Human γ-globulin reduced with 0.1 M β-mercaptoethanol in water solution (b). The shaded area in (a) represents the localization of DNP-aspartic acid.

and alkylated, may be performed in 1 M HAc by column filtration on Sephadex G-75.[15] The same separation was achieved by thin layer filtration on Sephadex G-75 (Fig. 7). This filtration was performed with reduced γ-globulin in 1 M HAc with 0.1 M β-mercaptoethanol included. In this connection it might be remarked that thin layer gel filtration in 8 M urea may be performed without difficulties. By making the experiments in urea solutions the changed swelling capacity of the gels must be taken into account.[16] It is also advisable to transfer the separated material to a filter paper after the runs to avoid the massive crystallization of urea on the plates. Experiments with separation of polypeptide chains with different molecular sizes from fully reduced γ-globulin on Sephadex G-75 in 8 M urea containing β-mercaptoethanol were, however, unsuccessful. All material moved in one elongated zone. The reason for this lack of separation is not clear and further investigations including experiments with other types of Sephadex gels in urea are needed.

CONCLUSIONS

Thin layer gel filtration is a simple and convenient method for separation of small amounts of material: quantities of 0.1 mg proteins may be used for the separation. The method is useful for the following purposes:

1. Approximate determinations of molecular sizes of substances available in small quantities.
2. Comparison of the gel filtration patterns of several similar substances in a single run.
3. Investigation of suitable gel type and conditions for gel filtration with column technique.
4. Determination of the resolution of the components obtained by gel filtration in a preparative scale.

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