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GEL FILTRATION MEDIUM DERIVED FROM GUAR GUM

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SUMMARY

Guar gum gels have been synthesised by cross-linking guar gum with epichlorhydrin in water–2-propanol. The degree of cross-linking was adjusted by controlling three factors: the concentration of guar gum, the amount of cross-linking agent and the water:2-propanol ratio. The preparation and chromatographic evaluation of the two gels, designated guar gel 5-X30 and 2-X10, are described. For these two gels, molecular weight exclusion limits of approximately 15,000 and 70,000, respectively, were observed for globular proteins.

INTRODUCTION

It is about 20 years since Lathe and Ruthven[1] and Porath and Flodin[2] first reported that gels can fractionate molecules on the basis of molecular size. Since then, several kinds of gel filtration media have been introduced. Andrews[3–5] found that the elution characteristics of proteins on these gels are uniquely determined by molecular weight. Gel filtration techniques, therefore, became widely used for fractionation and molecular weight determinations of macromolecules. The choice of soluble polysaccharides for producing cross-linked gels has to some extent been arbitrary and limited[6]. A detailed study on a wide variety of polysaccharides would be necessary in order to be able to generalize the factors on which the molecular sieving actions of such gels depend. As a wide range of gels based on dextran and agar are commercially available, there seems to be a reluctance among workers to develop new gels based on other polysaccharides.

In seeking new gels of this type, we have investigated the gel-filtration properties of cross-linked guar gum (the polysaccharide guaran from the seeds of *Cyanaposis tetragonolobus*, which is known to be galactomannan). Although cross-linked guar gum gels were prepared as early as 1954[7], their gel-filtration properties have not so far been studied. Guaran was considered to be a suitable choice for making porous gels as it has an almost straight chain of mannose units with equally spaced galactose units as grafts, and swells readily in water to form mechanically stable xerogels. The cost of production of the guaran gels is much lower than that of dextran-based gels. Further, the guaran gels can be prepared in any laboratory. This paper describes the preparation and chromatographic evaluation of two gels, guar gel 5-X30* and guar

gel 2-X10*, which were found to be effective in the fractionation of proteins over the pH range 4–10.

Apart from the molecular sieving action of the these gels, the presence of *cis*-diol groups helps in the reversible binding of borate in the alkaline pH range. The separation and concentration of borate on such a gel has been recently reported[8].

The column and gel parameters, flow properties and adsorption effects of certain low-molecular-weight aromatic and heterocyclic compounds on the gel, have been studied.

EXPERIMENTAL AND RESULTS

Reagents

All of the reagents employed were commercial products of high purity and were used without further purification. Bovine serum albumin (Cohn fraction V), egg albumin (ovalbumin) and phenol reagent (Folin–Ciocalteu reagent) were purchased from Centron Research Laboratories (Bombay, India). Trypsin (bovine pancreas) was obtained from Aldrich (Milwaukee, Wisc., U.S.A.). Zinc-free insulin was obtained from Boots (Bombay, India) and cytochrome *c* from Sisco Research Laboratory (Bombay, India). Guar gum was procured from a local factory.

Purification of guar gum

Commercial gum was purified by the usual method of precipitation with ethanol. A 1% aqueous solution of the gum was prepared by vigorous stirring. After standing for 2–8 h at room temperature, the gum was precipitated by addition of 50% ethanol. The white amorphous mass so obtained was washed with several volumes of ethanol and finally the precipitated gum was washed with diethyl ether and dried at room temperature.

Preparation of guar gel 5-X30

In a 2-l three-necked reaction flask, equipped with a 10-cm diameter semi-circular paddle stirrer, was placed 1 l of deionized water containing 20% (v/v) of 2-propanol. Nitrogen was passed through the mixture for 1 h, then the gas inlet tube was raised above the surface to maintain a nitrogen atmosphere in the reaction vessel. Next, 7.0 g (0.17 mole) of sodium hydroxide were added, and after dissolution was complete, 50 g of purified guar gum were added with vigorous stirring to prevent the formation of lumps. After rapid stirring for 15 min, 15 g (0.16 mole) of epichlorohydrin were added and stirred into the viscous gum solution. The reaction vessel was then placed in a water-bath at 40° and the mixture was stirred until the gel formed (2–3 h). The reaction was allowed to continue for 24 h and then the temperature of the bath was increased to 70°. After standing at this temperature overnight, the gel was allowed to settle and the supernatent liquid was decanted off. The gel was placed in 500 ml of distilled water, neutralised with hydrochloric acid and sedimented three times in an excess of water to remove fines and excess of acid. The soluble reaction products were then washed out on a filter. On the filter-paper the gel particles were

* The terms guar 5 and guar 2 imply that 5 and 2% guar gum solutions, respectively, were used, and X30 and X10 indicate the concentration of epichlorohydrin employed in the respective solutions.

dehydrated with ethanol (three 100-ml volumes) and finally dried overnight in an oven at 70°.

Preparation of guar gel 2-X10

The preparative procedure was the same as that employed for guar gel 5-X30, except that the water contained 15% of 2-propanol. 20 g of guar gum were taken and the amounts of sodium hydroxide and epichlorohydrin were 0.86 g (0.022 mole) and 2 g (0.02 mole), respectively.

A small amount of carboxyl groups (0.05–0.1 mequiv./g) was found to be introduced during the cross-linking of the gels.

Determination of column and gel parameters

After swelling to equilibrium in deionized water, the gels were stirred into a thick slurry and poured into a glass column (20 × 2 cm) already half-filled with deionized water. For each column, the void volume (V_0) was estimated[9] from the elution volume of Indian ink, making due corrections for the column fittings and tubular connections. The volume of water taken up by the gel particles (V_i) or the volume available to a totally included molecule was calculated by subtracting V_0 from the elution volume. With partially included solutes, the filtration behaviour was characterized by relating the elution volume, V_e, to the absolute distribution coefficient, K_d, using the Wheaton and Baumann[10] equation:

$$K_d = \frac{V_e - V_0}{V_i} = \frac{V_e - V_0}{aW_r}$$

where W_r and a are the water regain and amount of dry gel present, respectively, in the column. Owing to the uncertainty in the determination of V_i, the constant K_{av} (av = available) appears to be generally applicable for this purpose[11], and is given by the expression

$$K_{av} = \frac{V_e - V_0}{V_t - V_0}$$

where V_t is total bed volume of the swollen gel. Both K_d and K_{av} are independent of the geometry and packing density of the column.

Flow properties of guar gel

The guar gels were found to work well at moderate hydrostatic pressures (10–50 cmH_2O). A linear increase in flow-rate with pressure was observed for both guar gel 5-X30 and 2-X10 (100–200 mesh size) (Fig. 1). At higher pressures, the flow-rate did not increase proportionately.

Adsorption effects on guar gels

Although the separation of large molecules by gel filtration is based primarily on the exclusion principle, adsorption effects have been observed with certain low-molecular-weight aromatic and heterocyclic compounds using dextran gels[12,13]. Such adsorption effects have also been observed on guar gels. Elution studies with aromatic

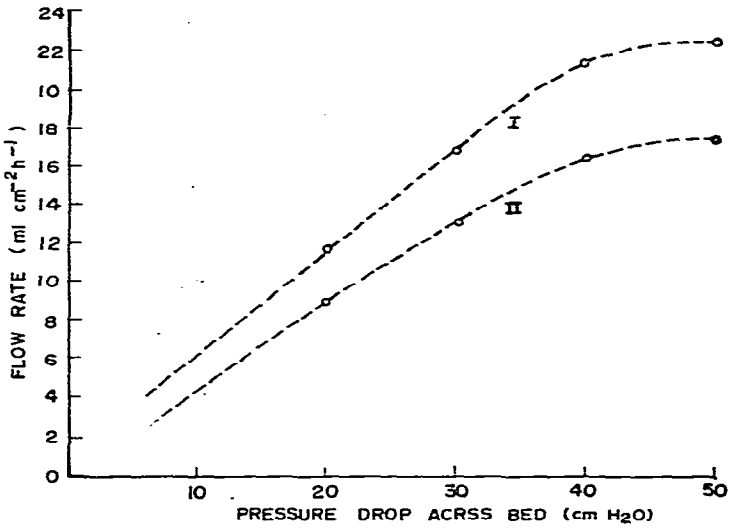

Fig. 1. Effect of hydrostatic pressure drop across bed on flow-rate through guar gel 2-X10 (I) and guar gel 5-X30 (II). Bed size: 2 × 20 cm.

amino acids carried out on columns of identical dimensions revealed that the adsorption effects are less pronounced (Table III).

Gel-filtration chromatography using guar gel

Desalting of proteins. The exclusion limit of guar gel 5-X30 for globular proteins was 15,000. Therefore, any protein having a molecular weight of 15,000 or above can be desalted easily. For desalting purposes, a column (20 × 2 cm) was prepared by pouring a thick slurry of the gel pre-swollen in deionized water. A 5-mg amount each of egg albumin and sodium chloride were dissolved in 2 ml of water and carefully applied on to the top of the gel bed, and the column was then eluted with distilled water. Fractions of 2 ml were collected at a flow-rate of 20 ml/h. The elution profile is shown in Fig. 2.

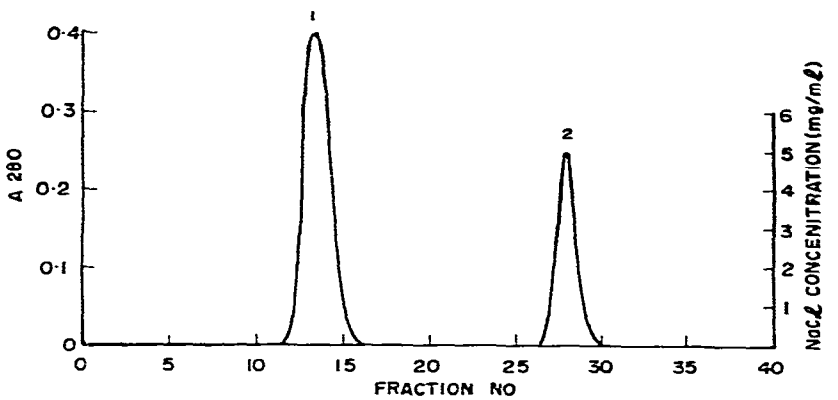

Fig. 2. Desalting of protein (egg albumin) using guar gel 5-X30. 1 = Egg albumin; 2 = sodium chloride.

Separation of proteins using guar gel 5-X30. Guar gel 5-X30 was found to be suitable for the fractionation of globular proteins in the molecular weight range 1000–15,000. A 20-g amount of the gel was suspended in 500 ml of Tris buffer (0.1 *M* Tris–HCl + 0.2 *M* sodium chloride, pH 8.0) for 1 h. The gel was allowed to sediment and the supernatant liquid was decanted. This procedure was repeated twice. The swollen gel was then suspended in a fresh volume (200 ml) of the same buffer, slurried into a column (50 × 1.5 cm) and permitted to pack under gravity. Final equilibrium was achieved by running 200–300 ml of the same buffer through the column. Amounts of 5 mg each of cytochrome *c* (molecular weight 13,000), insulin (molecular weight 5700) and DNP-glycine (molecular weight 241) were run through the column individually as well as in a mixture. The samples were diluted to 2 ml with the column buffer and applied on to the top of the gel bed through a small tube. Elution was carried out with the same buffer and fractions of 2 ml were collected at a flow-rate of 12 ml/h. Indian ink and DNP-glycine were routinely run through the column to check the void volume (V_0) and inner volume (V_i). The elution profile is shown in Fig. 3.

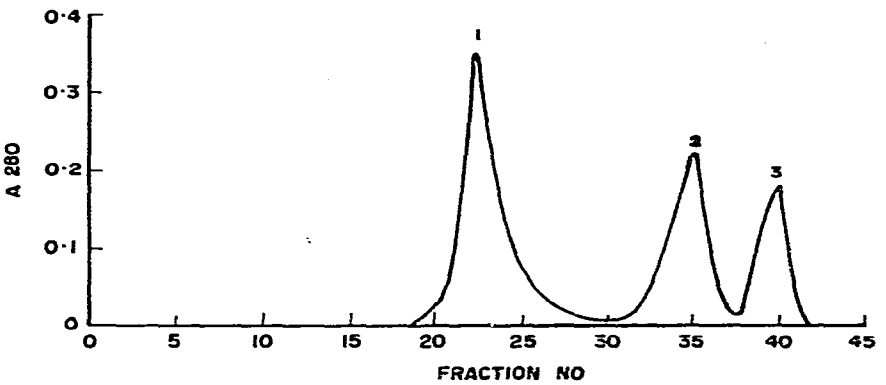

Fig. 3. Elution profile for the separation of a mixture of proteins on a guar gel 5-X30 column (50 × 1.5 cm). Rate of elution, 12 ml/h. Peaks: 1 = cytochrome *c*; 2 = insulin (zinc-free); 3 = DNP-glycine.

Separation of proteins using guar gel 2-X10. The fractionation range of guar gel 2-X10 was found to be 5000–70,000 for globular proteins. A column (75 × 1.5 cm) of the gel was prepared in the same buffer and same manner as described for guar gel 5-X30. Amounts of 5 mg each of the proteins, bovine serum albumin (molecular weight 69,000), egg albumin (molecular weight 45,000), trypsin (molecular weight 24,000) and insulin (molecular weight 5700) were run through the column individually and in a mixture. The void volume and inner volume were also checked. All of the experiments were carried out at room temperature. The elution profile of the proteins is shown in Fig. 4.

DISCUSSION

According to Flodin[6], the three factors on which the gel parameters depend are the concentration of soluble polysaccharides, the concentration of cross-linking

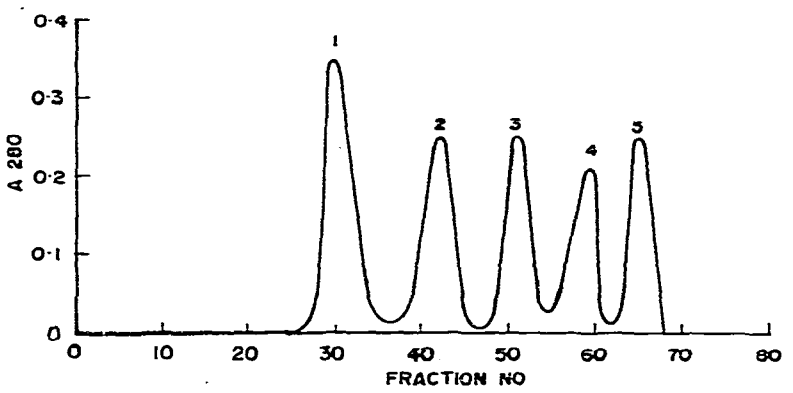

Fig. 4. Elution profile for the separation of a mixture of proteins on a guar gel 2-X10 column (75 × 1.5 cm). Rate of elution, 12 ml/h. Peaks: 1 = bovine serum albumin; 2 = egg albumin; 3 = trypsin; 4 = insulin; 5 = DNP-glycine.

agent and the molecular weight of the polysaccharides. We presume that the conformation of a linear polysaccharide in aqueous solution would be another factor influencing the gel parameters when the gels are synthesized from different polysaccharides.

With guar gum, the short side-chains (grafts) on the linear polysaccharide are expected to prevent helix formation and hence intermolecular interaction, and only gels with a high water regain were expected to be formed in aqueous solution. Expecting this, we employed water–2-propanol, which has been found to reduce the hydration of the polysaccharide chains and bring them together, producing a close-knit gel with a low water regain. Thus, it has been found that even with polysaccharides of very high molecular weight (220,000), gels with a reasonably low water regain and comparable to those obtained from dextrans, could be produced.

In contrast to starch, guar polysaccharide does not contain many reactive reducing groups and hence the gels formed are chemically and physically stable. During cross-linking reactions in alkaline medium, a large amount of acidic (carboxylic) groups were found to be introduced[6] in the gels. Whereas with dextran gels the formation of such ionic gels was kept low by using sodium borohydride, in the present work this was achieved by carrying out cross-linking under an atmosphere of nitrogen.

The use of water–2-propanol also permitted guar gum solutions of higher concentrations to be used during the cross-linking. In pure water, the viscosity of even a 1 % solution becomes too high for convenient handling. In fact, we have found that the water:2-propanol ratio is the most important factor determining the pore size and water regain properties of the gels.

Some typical characteristics of the gels are presented in Tables I–III.

All of the results presented were obtained over a period of 6 months. Each protein was passed through the column three or four times over this period, and no significant change in the elution pattern or elution position was noticed for any of the protein with either gel. Fig. 2 shows the elution profile during desalting of a protein. Here egg albumin elutes at 25.6 ml (V_e = 25.5 ml for egg albumin) and sodium chloride elutes at 49.5 ml. The egg albumin molecules are not capable of penetrating the gel grains, *i.e.*, they are eluted at the void volume ($V_e = V_0$).

TABLE I

APPROXIMATE VOLUMES OF 1 g OF GUAR GELS AFTER SWELLING IN WATER

Gel type	*Gel bed volume, V_t (ml)*	*Void volume, V_o (ml)*	*Inner volume, V_i (ml)*	*Density when swollen (g/ml)*
Guar 5-X30	5.1	2.1	2.4	1.20
Guar 2-X10	8.3	3.2	4.5	1.05

TABLE II

PROPERTIES OF GUAR GELS

Gel type	*Particle size when dry (mesh)*	*Water regain (ml/g)*	*Gel bed (ml/g)*	*Fractionation range (mol.wt.) for globular proteins*
Guar 5-X30	100–200	2.5	5.1	1000–15,000
Guar 2-X10	100–200	4.1	8.3	5000–70,000

TABLE III

ELUTION OF AROMATIC AMINO ACIDS ON GUAR GEL 5-X30 TO ILLUSTRATE THE ADSORPTION EFFECT

Column dimensions: 75 × 1.5 cm.

Amino acid	*Elution volume (ml)*
Glutamic acid	75
DNP-glycine	92
Phenylalanine	110
Tyrosine	112
Tryptophan	115

The following correlations are inferred from a comparison of the data in Fig. 2 and the values obtained for guar gel 5-X30 from Table I. The theoretical value of 12.5 × 5.1 = 63.75 ml for V_t is in good agreement with the experimental value of 65 ml. The volume of 25 ml for $V_o = V_e$ (egg albumin) is also in good agreement with the expected value of 12.5 × 2.1 = 26.25 ml. Only the theoretical value of 30 ml for V_i is significantly higher than the experimental value of 27 ml. It has been observed frequently that low-molecular-weight substances may be eluted earlier than expected. The explanation of this behaviour may be similar to that for dextran gels, as offered by Determann and Gelotte[14]. A portion of the solvent taken up during the swelling process is required for solvation of the polymer chains that form the gel. This volume is therefore not available for diffusion of the solute molecules.

During elution with distilled water, proteins may be retarted by interaction with the few carboxyl groups in the dextran gel and not separated from salt[15], and the same may apply to guar gel. The elution may also be carried out with volatile buffers and the protein isolated by lyophilization.

Fig. 3 shows the elution profile of the proteins cytochrome *c* and zinc-free insulin on column of guar gel 5-X30. Most evident are the sharp, symmetrical peaks and the resolving power of this gel. Fig. 4 shows the separation of a mixture of the proteins, bovine serum albumin, egg zlbumin, trypsin and insulin on guar gel 2-X10.

These results suggest that guar gel 5-X30 is most useful for the fractionation of proteins in the molecular weight range 1000–15,000, whereas guar gel 2-X10 is more suitable for fractionation of proteins in the molecular weight range 5000–70,000. The separation of cytochrome *c* and insulin is better on guar gel 5-X30 than guar gel 2-X10, although the proteins can be fractionated on either gel.

The adsorption effects are not readily understandable. If they are attributed to the presence of polar (hydroxyl) groups in the gel, then the less pronounced adsorption effect with guar gel could be due to the possible intramolecular hydrogen bonding of *cis*-diol groups. Although the aromatic ring-containing amino acids were eluted after glutamic acid, their separation from each other was not very distinct.

As for the flow properties, guar gels, like other polysaccharides[16], are satisfactory only at moderate pressures. They are, therefore, inferior to polyacrylamide and polystyrene gels, which can be used at high pressures.

Work on thin-layer gel filtration and molecular weight determination using these gels is in progress. We are also working on the development of other gels based on guar gum, including ion-exchanges resins.

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