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ISOLATION OF AGAROSE AND GRANULATION OF AGAR AND AGAROSE GEL

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SUMMARY

A method has been developed for the isolation of agarose from "Difco" bactagar by means of ammonium sulphate and acetone. A modification of PHILIPSON's method was used for granulation of agar and agarose. A sprayer with removable discs allows one to prepare bead-shaped granules of any size required at gel concentrations of 2–7 %. The method may be a highly effective laboratory technique.

INTRODUCTION

Physicochemical properties and concentration of gel, on the one hand, and the size and shape of the granules, on the other hand, determine the effectivity and the conditions for application of the gel filtration method. Sephadex and Biogels are known not to fractionate macromolecules of molecular weight exceeding $4 \cdot 10^5$ (*e.g.*, macroglobulin, viruses, nucleic acids). POLSON[1] in 1961 suggested that granulated agar gel with large particles should be used for fractionating proteins of high molecular weight, but the carboxyl and sulphate groups of agaropectin present in the agar are responsible for extensive sorption and, consequently, separation on the agar column depends upon the ionic strength of the buffer[2]. HJERTÉN[3,4] succeeded in applying agarose gel, which is the natural component of agar, for separation of viruses and nucleic acids.

At present granulated agarose is commercially manufactored in Sweden ("Sepharose") and in the U.S.A. ("Sagarose")[5]. However, in practice, laboratory-made granulated agarose gel is often required. All the methods of isolation and granulation of agarose suggested so far have a common drawback: isolation of pure agarose requires costly reagents (*e.g.*, cetylpyridinium chloride) and granulation is time-consuming[1,3,6–8].

The present paper describes (1) a modification of the method of AJITSKY AND KOBOZIEV[9] used for preparation of agarose from bactagar "Difco" and (2) a modification of the method of PHILIPSON AND BENGTSSON[6], which is highly effective for granulating agar and agarose gel.

ISOLATION OF AGAROSE

Agarose was isolated from "Difco" bactagar by fractionation with ammonium sulphate (AS) (reagent-grade). Preliminary experiments revealed that complete precipitation of agar occurs between 0.18 and 0.47 saturation with AS. To fractionate the agar, 1 l of hot saturated AS solution was added to 2 l of hot 2 % agar with stirring (*i.e.* to 0.33 saturation) and left for 30 min in a hot water bath. Then the mixture was poured into preheated centrifuge tubes and centrifuged at 2500 r.p.m. for 15 min without cooling. After centrifugation the supernatant was separated; the dense sediment (Fraction I) was washed with cold water to remove residual gel and heated in 1 l of water till completely dissolved. Two volumes of acetone were then added to the resulting solution, and it was left standing overnight at 4°. The white friable flakes of the sediment were repeatedly washed with distilled water to remove AS completely. The disappearence of AS was estimated by quantitative reaction of the supernatant with $BaCl_2$. Fraction I, devoid of AS, was dehydrated with acetone and dried at room temperature. The supernatant remaining after Fraction I had been sedimented in the centrifuge was left standing overnight at 4° with three volumes of acetone. The sediment which remained on removal of the acetone was repeatedly washed with distilled water to remove AS (Fraction II). Washed Fraction II was also dehydrated with acetone and air-dried. Unlike Fraction I, Fraction II sediments less readily in cold distilled water; therefore, it was sedimented in the centrifuge at 2500 r.p.m. for 30 min. Both fractions isolated from agar after dehydration look like white powder and readily dissolve in water; however, a 1 % solution of Fraction II forms a weaker gel than Fraction I and agar.

To identify Fractions I and II, the degree of electroendosmosis in electrophoresis of rabbit serum in gels of French agarose (L'Industrie Biologique Française) was compared to that in "Difco" agar and our Fractions I and II. All these gels were tested simultaneously in the same apparatus in veronal buffer (pH 8.6 ± 0.05) at E = 8 V/cm for 1 h. After electrophoresis the gel was dried, fixed and stained with

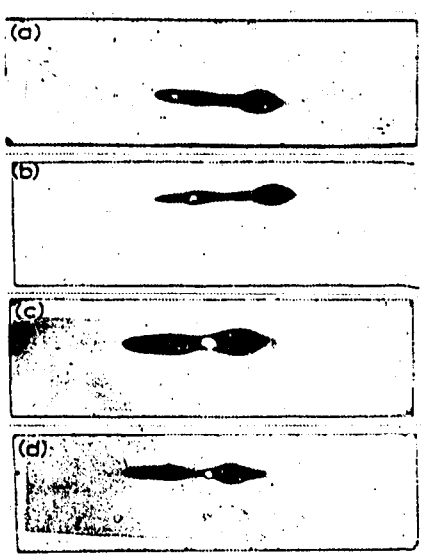

Fig. 1. Electrophoretograms of rabbit serum. (a) French agarose. (b) Agarose (Fraction I). (c) Agaropectin (Fraction II). (d) Difco agar.

Amino Black 10B. The electrophoregrams (Fig. 1) clearly show that in Fraction I the cathode displacement of protein is only slightly greater than in agarose whereas in the agar and, particularly, in Fraction II electroendosmosis is much more pronounced. Besides, Fraction II always shows worse protein separation as compared to agar and diffusion of proteins in the opposite direction of electrophoresis, which indicates strong sorption of proteins in Fraction II. The results of the above test indicate that probably Fraction I is agarose and Fraction II is ionisable agaropectin.

GRANULATION

A modification of the method of PHILIPSON AND BENGTSSON[6] was used for granulation of agar and agarose. A hermetically sealed 1-l metal tank (Fig. 2a) is attached to a holder fixed to a heavy slab*. The lower outlet of the tank is connected through a double-stroke valve to a sprayer** with removable brass discs which have a conical orifice. Compressed nitrogen, kept between 2–4 atm, is fed into the tank filled with melted agar or agarose which is then squeezed through the sprayer. The granulated gel enters a 20-l cylinder containing 15 l of a water–ether mixture (1:1) which has been precooled to 2°. The mixture is continuously blended by a stirring rod which is connected by a flexible shaft to a motor outside the hood***. After the granules have sedimented, the ether is removed and the granules are washed repeatedly to remove residual ether. They are then sieved through standard sieves under a strong stream of water.

This granulating device can be used to obtain granules from agar and agarose solutions at concentrations from 2 to 7 %. The granules sieved through 80–200 mesh appear bead-shaped in the phase-contrast microscope (Fig. 3). Depending on the size of granules required and on gel concentration, the diameters of the orifice of the brass discs fixed in the sprayer may be from 1 to 3.5 mm. The optimal diameter is decided

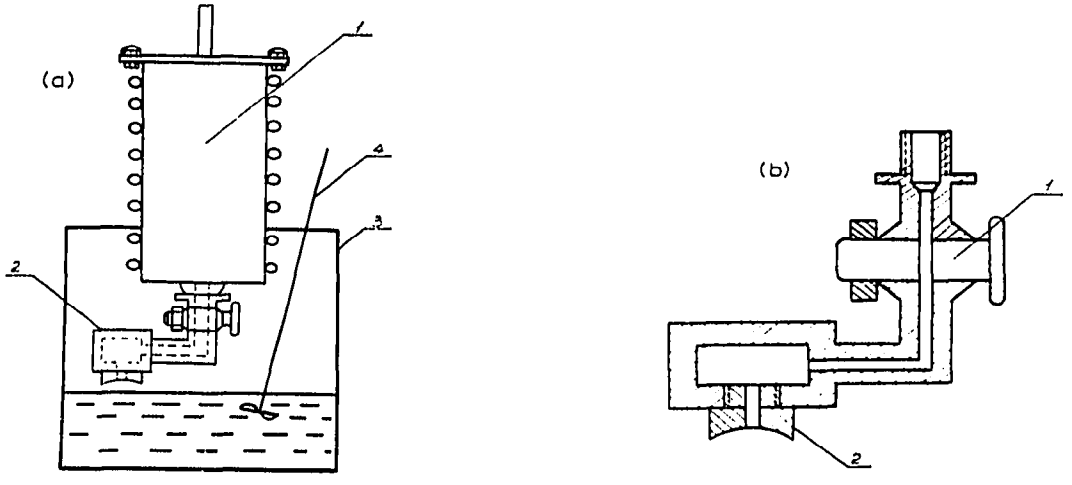

Fig. 2. (a) Device for making spherical agarose granules. 1 = hermetically sealed tank encircled with heating tube; 2 = sprayer; 3 = cylinder containing water–ether mixture; 4 = stirring rod. (b) Agarose sprayer. 1 = valve; 2 = removable brass discs with a conical orifice.

* The outer surface of the tank is encircled with a helical heating tube containing hot water.

** The design of the sprayer was suggested by S. B. KOOST.

*** Constant stirring is necessary to destroy the interfase to permit the granules to pass freely between the ether and water.

empirically as the size of granules at a given pressure and concentration decreases with the increase in the diameter of the orifice in the disc, except at some point at which the process of granulation ceases to give way to the liquid discharge of the gel. For more concentrated solutions, a bigger orifice is required.

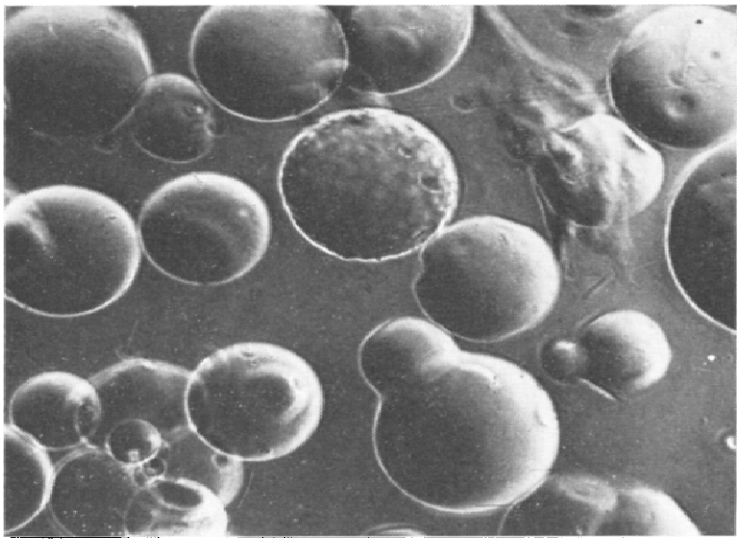

Fig. 3. Granules of 5% agarose, 80–200 mesh.

DISCUSSION

Agar is a mixture of two polysaccharides: agarose with a mol. wt. of 120000 and devoid of ionisable groups, and agaropectin with a mol. wt. of 12000 and containing acidic carboxyl and sulphate groups[10]. It is natural that gels of agarose and agaropectin display different behaviours in the column for gel filtration; the neutral agarose may serve as a "molecular sieve" whereas agaropectin is bound to have some undesirable properties and also accounts for considerable electroendosmotic flow in the direction of the cathode which is always present in electrophoresis in the agar gel. In the agarose gel no electroendosmosis is observed, which makes electrophoresis a convenient method of testing the purity of agarose.

Many methods of isolating agarose have been described. The procedure suggested by ARAKI[7] and modified by HJERTÉN[3] is based on agar acetylation by acetic anhydride in pyridine. Agarose prepared by this procedure has an admixture of agaropectin and has weaker gel-forming properties than agar because of partial hydrolysis of polysaccharide. Later HJERTÉN suggested the use of quarternary ammonium salts, particularly cetylpyridinium chloride, which sediments agaropectin by reacting with the acidic groups of the latter[4]. POLSON *et al.*[8] used polyethyleneglycol with a mol. wt. of 6000 to fractionate agar. Agarose obtained by the two methods mentioned above contains minimum quantities of sulphur and has the same gel-forming properties as agar; but unlike the latter, agarose gel shows practically no electroendosmosis. Both these methods require expensive reagents and are time-consuming. Another mode of separating polysaccharides is based on sedimenting agar by concentrated salt solutions and organic solvents[11]. AJITSKY AND KOBOZEV[9] fractionated commercial agar with ammonium sulphate. At 0.16 saturation, a dark-brown sediment, which

these authors believed to be agaropectin, precipitated from agar. By increasing the concentration of AS to 0.26, these authors succeeded in isolating the second fraction, agarose, from agar.

According to our data, sedimentation of polysaccharides from "Difco" bactagar starts only at 0.18 AS saturation. Agaropectin, as may be deduced from electroendosmosis (Fig. 1), sediments after agarose. This was to be expected because, on salting out polysaccharides with a component of higher molecular weight, agarose was first to precipitate. "Difco" bactagar differs from commercial agar in that the agarose isolated from it forms no large flakes at 0.33 AS saturation and that centrifugation is required to sediment it. It is important that the molten solutions should be treated with acetone to extract the yellow pigment.

As was mentioned above, in agarose obtained at 0.33 AS saturation, electroendosmosis is somewhat higher than in the French agarose preparation. However, no appreciable improvement in the electrophoretic properties of agarose was achieved when the precipitation range of Fraction I was limited to 0.24 AS saturation. On the other hand this procedure led to a twofold decrease in agarose yield. It should be noted that the concentration of AS required for agarose precipitation is determined experimentally for various kinds of agar[8] because of extensive differences in the content of agaropectin, which is the result of different methods of purification and various natural sources of agar.

The effectivity of separation of macromolecules in molecular sieves largely depends on the shape and size of granules. If the granules are spherical, the columns are packed more homogeneously, the buffer passes through at a greater rate and the substance in the columns becomes denser more slowly, which makes the columns fit for repeated use. On the other hand, the smaller the size of granules the less the void volume and the greater the total working surface of the granules are. Hence, when the separation of fractions of close molecular weight is required the smallest granules should be used[12].

The primary procedures of agar granulation described were based on mechanical grinding of gel in cold. But due to irregular shape of the particles, the column becomes dense very quickly and the flow rate of the buffer becomes very low. In 1964 HJERTÉN[4] and PHILIPSON AND BENGTSSON[6] independently suggested the method of emulgation for obtaining spherical agar granules. Under favourable conditions agar gel forms an emulsion of the water/oil type in non-polar organic solvents. According to HJERTÉN, a mixture of benzene, toluene and some polysaccharide emulsion stabilizer is added to a hot agarose solution; the solution is then stirred and gradually cooled in a high-speed mixer and spherical gel granules are formed. PHILIPSON AND BENGTSSON suggested that to obtain spherical granules the hot agar solution should be squeezed under pressure in the Zeitz filter through a needle into a mixture of water and ether. The latter procedure is more efficient as neither an expensive emulgator nor a complex mixer is required; the disadvantage of the method is that it is impossible to prepare small granules and to granulate agar at a concentration exceeding 5 % as at higher concentrations the agar congeals in the needles. In addition, any drops of cold ether on the point of the needle also cause agar to congeal in the needle. The second drawback of the method of PHILIPSON AND BENGTSSON is the condition that agar should be squeezed in small portions as the granules agglutinate the water–ether interface.

The system which has been suggested in this paper does not have the above

drawbacks. The sprayer with removable discs allows the preparation of granules of any required size at gel concentrations from 2 to 7 %. As the sprayer device is sufficiently large and the orifice is rather big, the spraying of 1 l of agar requires no more than 2 min, hence no cooling of the water–ether mixture is necessary and the gel does not congeal in the sprayer. Also in our device the pressure of nitrogen has less effect on the size of granules than does squeezing the solution through a needle. The large cylinder and constant stirring prevent the granules from agglutinating on the water–ether interface, thereby allowing large volumes of gel to be discharged into the mixture.

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