Journal of Chromatography, 132 (1977) 519–524

CHROM. 9590

MICROSCOPIC OBSERVATIONS ON AGAROSE BEADS

DEHYDRATION BY SOLVENT EXCHANGE AS AN ALTERNATIVE TO LYOPHILIZATION

T. C. J. GRIBNAU and C. A. G. VAN EEKELEN
Department of Organic Chemistry, Catholic University, Toernooiveld, Nijmegen (The Netherlands)
C. STUMM
Department of Microbiology, Catholic University, Toernooiveld, Nijmegen (The Netherlands)
and
G. I. TESSER
Department of Organic Chemistry, Catholic University, Toernooiveld, Nijmegen (The Netherlands)
(Received July 27th, 1976)

SUMMARY

Dehydration of agarose by means of solvent exchange, has been investigated as an alternative to freeze-drying. The effects of both methods on the structure of the gel beads, after drying and also re-swelling, have been compared by means of microscopy. The influence of the two drying procedures on the coupling capacity of cyanogen bromide-activated gels has been examined by coupling with L-cystine.

INTRODUCTION

The activation of polysaccharides (*e.g.*, agarose) by cyanogen bromide is the main method for ligand immobilization in affinity chromatography[1–3]. However, the limited stability of the isourea bond connecting the ligand to the matrix can lead to leakage[4]. In the development of alternatives for the CNBr activation[5,6] it may be necessary to use dry agarose, especially when anhydrous reaction conditions are required. Standardization of activation procedures is also easier with dry agarose.

Agarose, for chromatographic purposes, is commercially available as a suspension of swollen beads in distilled water containing sodium azide as a bacteriostatic agent (Sepharose, Bio-Gel A, Servachrom A, Ultrogel). Derivatized agarose, for application in affinity chromatography, is supplied as a freeze-dried powder. In the case of cyanogen bromide- or bis(oxirane)-activated agarose, water has to be absent in order to preserve the active groups. Dehydration by means of lyophilization requires previously freezing of a wet suspension, prolonged freezing can damage the matrix structure as we found in agreement with Lowe and Dean[7].

We investigated dehydration by solvent exchange as an alternative to freeze-drying. The effects of both methods on the structure of the gel beads, after drying

and also re-swelling, were compared by means of microscopic observation. The influence of the two drying procedures on the coupling capacity of CNBr-activated gels was examined by coupling with L-cystine. The degree of substitution was determined by elemental analysis of the sulphur content.

MATERIALS

Sepharose 4B (Lot no. 7135, 5448) and freeze-dried CNBr-activated Sepharose 4B (Batch no. 3581) were purchased from Pharmacia (Uppsala, Sweden); Ultrogel AcA 34 (Batch no. 3714) was a gift from LKB (Stockholm, Sweden) and Affi-Gel 10 (Control no. 13269) was obtained from Bio-Rad Labs. (Richmond, Calif., U.S.A.). Cyanogen bromide, L-cystine and polyethylene glycol (PEG; MW 1000) were obtained from EGA-Chemie (Steinheim/Albuch, G.F.R.), Aldrich (Milwaukee, Wisc., U.S.A.) and Koch-Light (Colnbrook, Great Britain) respectively.

Nomarski differential-interference-contrast photomicrographs were taken with the aid of a Leitz Ortholux microscope on 35-mm Agfapan 25 Professional film.

METHODS

The CNBr activation was performed essentially as described previously[1–3]. The Sepharose 4B, after washing with distilled water, was gently sucked dry on a sintered-glass funnel. The CNBr was dissolved in 15% (v/v) formic acid, prior to addition[8]. Two activations were carried out: A (2 g of CNBr per 20 g of Sepharose 4B); B (0.4 g of CNBr per 20 g of Sepharose 4B).

Coupling with L-cystine was performed in carbonate–bicarbonate buffer (0.5 *M*, pH 9.3) at room temperature (0.5 g of L-cystine in 30 ml of buffer per 5 g of CNBr–Sepharose). The coupling products were washed with coupling buffer, distilled water, 1 *N* HCl (briefly) and distilled water. Samples for elemental analysis* were prepared by washing the gels with acetone and drying *in vacuo* over concentrated sulphuric acid.

Unsubstituted matrices (Sepharose, Ultrogel) were washed with distilled water and lyophilized. The dried gels were re-swollen in distilled water. In the solvent-exchange procedure the gels were washed several times with acetone, tetrahydrofuran (THF) or methanol respectively, sucked dry and finally dried *in vacuo* overnight. Re-swelling in water occurred instantaneously for the acetone- and THF-treated gels. Photomicrographs were taken of the dried materials (suspended in liquid paraffin) and of the re-swollen beads. CNBr-activated Sepharose 4B was treated as described above. Lyophilization was also carried out in the presence of 1% (w/w) lactose or 25% (w/w) PEG in water. In the solvent-exchange procedure 25% (w/w) PEG in acetone was also used as an eluent.

RESULTS

The results of the microscopic observations are represented in Fig. 1 and Table I. In order to check the re-swelling of the dried agarose gels, the wet volumes

* Elemental analyses were carried out by the Element Analytical Section of the Institute for Organic Chemistry TNO (Utrecht, The Netherlands) under the supervision of Mr. W. J. Buis.

of Sepharose 4B before and after treatment were compared (the gels were filtered by gravity on a sintered-glass funnel, and the volumes marked). Acetone treatment as well as lyophilization resulted in dry materials, which regained 90% of their original volume after re-swelling, whereas with methanol-treated Sepharose 4B only 15% re-swelling occurred.

The influence of the two drying procedures on the coupling capacity of CNBr-activated Sepharose 4B was examined by coupling with L-cystine. One sample of CNBr–Sepharose (A, B; see Methods) was coupled with L-cystine immediately after activation. Two other samples were lyophilized or acetone-dried respectively, and after re-swelling were coupled with L-cystine. Elemental analyses showed only minor differences between the degrees of substitution of the different samples Table II).

DISCUSSION

There were remarkable differences between the results of the freeze-drying and solvent-exchange treatments. Treatment of unsubstituted Sepharose 4B with water-miscible, but rather apolar, solvents such as acetone or THF, according to the solvent-exchange procedure, resulted in dry, uniformly shrunken, beads (Fig. 1a). These beads kept their spherical appearance, and regained their original structure quickly, after re-swelling in water (Fig. 1b). Repeated swelling and drying in this way caused no damage to the matrix structure. Application of a more polar solvent such as methanol also led to uniform shrinkage, but with a severe loss of re-swelling capacity (Figs. 1c and d). Lyophilization from water led to a more deformed re-swollen structure than did dehydration by means of solvent exchange. Dry lyophilized beads seem to have more or less collapsed instead of having shrunk uniformly.

Ultrogel, a mixed polyacrylamide–agarose gel, consists of clear beads of uniform size, without vacuoles. Sepharose beads have a more variable diameter, and often are vacuolated[9]. The differences between the results of solvent exchange and freeze-drying were somewhat more pronounced for Ultrogel (Fig. 1e–h).

Both drying procedures applied to CNBr-activated Sepharose 4B led to minor re-swelling products. This was also found with Affi-Gel, a dehydrated substituted agarose. The tendency existed that the re-swelling capacity was inversely proportional to the degree of cross-linking. Neither of the two methods affected the coupling capacity of the activated gels.

Drying of activated agarose in the presence of additives led to better results; the additives prevent the beads from collapsing or shrinking too severely. CNBr-activated Sepharose 4B is usually freeze-dried in the presence of lactose and dextran[10] (Fig. 1i and j). We found that this material contained 10% CNBr–Sepharose and 90% additives. Lyophilization in the presence of PEG or treatment with PEG–acetone gave the same results with respect to bead structure. However, the solvent-exchange procedure is less time-consuming than freeze-drying. Moreover, the amount of PEG used in the acetone treatment is substantially less than in the freeze-drying procedure, since in the former method the excess of interstitial PEG is removed during filtration. Therefore, dehydration by means of solvent-exchange offers a good alternative to lyophilization.

TABLE I

MICROSCOPIC OBSERVATIONS ON DIFFERENTLY TREATED AGAROSE MATRICES

No.	*Matrix*	*Treatment*	*Structure*
1	Sepharose 4B	acetone-dried; dry	Fig. 1a; uniformly shrunken beads
2		acetone-dried; re-swollen	Fig. 1b; re-swollen beads as in untreated Sepharose 4B
3		THF-dried; dry	as no. 1
4		THF-dried; re-swollen	as no. 2
5		methanol-dried; dry	Fig. 1c; shrunken beads
6		methanol-dried, re-swollen	Fig. 1d; only minor re-swelling
7		lyophilized; dry	as no. 13
8		lyophilized-re-swollen	as no. 14
9		lyophilized (1% lactose); dry	as no. 13
10		lyophilized (1% lactose); re-swollen	as no. 14
11	Ultrogel AcA 34	acetone-dried; dry	Fig. 1e; uniformly shrunken beads
12		acetone-dried; re-swollen	Fig. 1f; re-swollen beads as in untreated Ultrogel
13		lyophilized; dry	Fig. 1g; collapsed beads
14		lyophilized; re-swollen	Fig. 1h; re-swollen, but damaged beads
15	CNBr–Sepharose 4B (Pharmacia)	lyophilized (lactose, dextran); dry	Fig. 1i; shrunken beads and flakes of additives
16		lyophilized (lactose, dextran); re-swollen	Fig. 1j; re-swollen, but deformed beads
17	CNBr-Sepharose 4B (A, B)	acetone-dried; dry	as no. 1
18		acetone-dried; re-swollen	only minor re-swelling
19		lyophilized; dry	as no. 13
20		lyophilized; re-swollen	only minor re-swelling
21	CNBr-Sepharose 4B (B)	(acetone + PEG)-dried; dry	as no. 1; beads had larger diameter
22		(acetone + PEG)-dried; re-swollen	as no. 16
23		lyophilized, (PEG); dry	as no. 1; beads had larger diameter
24		lyophilized, (PEG); re-swollen	as no. 16
25	Affi-Gel 10	dehydrated*; dry	as no. 16; beads were more deformed
26		dehydrated; re-swollen	as no. 16; minor re-swelling, beads were more deformed

* The Bio-Rad catalogue does not give details of the dehydration procedure.

TABLE II

INFLUENCE OF DEHYDRATION ON COUPLING CAPACITY OF 5 g CNBr-SEPHAROSE 4B

Activation system	*Treatment*	*Degree of substitution (%S)*
A	untreated	1.37
A	acetone, dried	1.14
A	lyophilized	0.99
B	untreated	0.76
B	acetone, dried	0.72
B	lyophilized	0.73

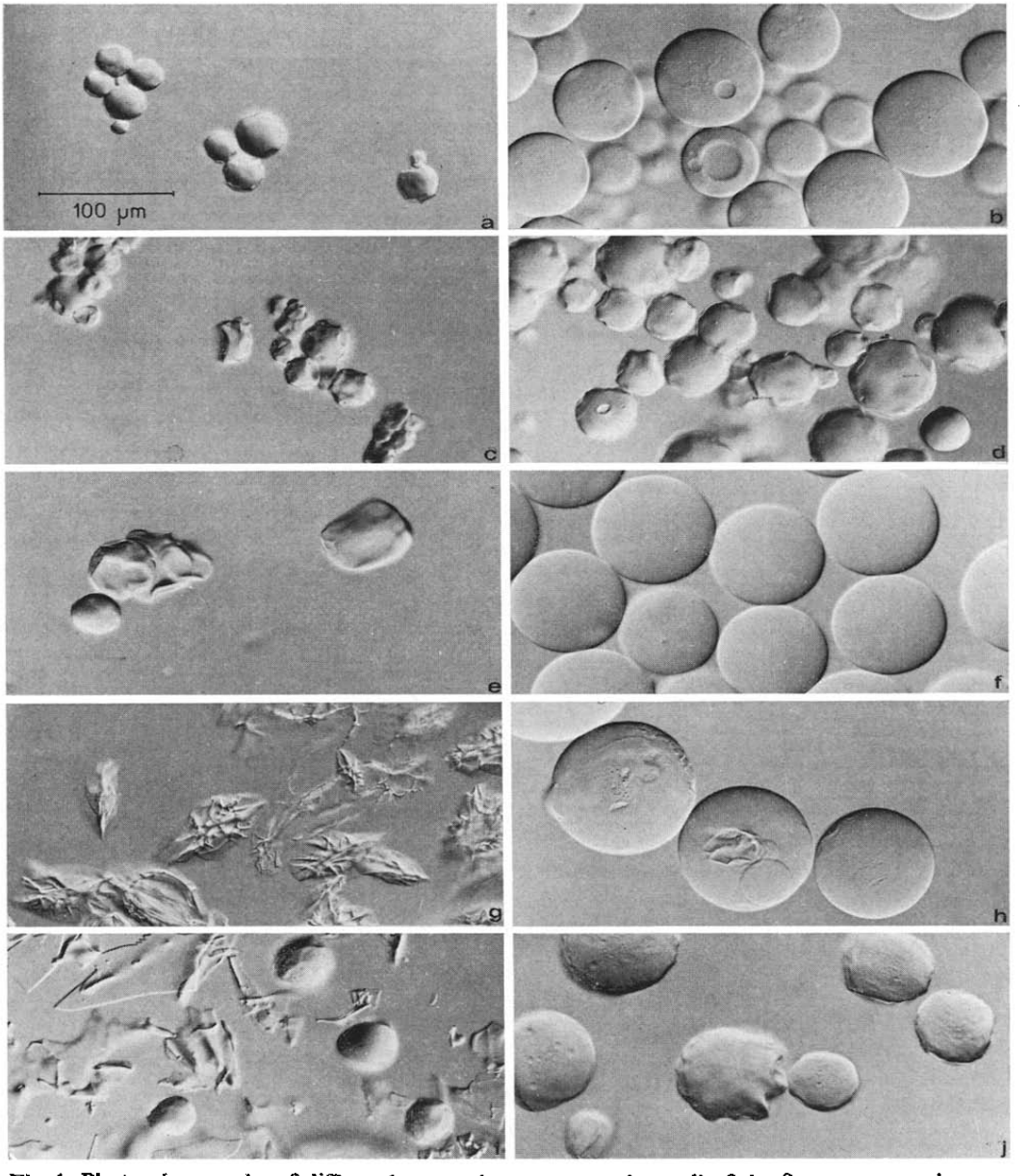

Fig. 1. Photomicrographs of differently treated agarose matrices; all of the figures are on the same scale. For details see Table I and text.

ACKNOWLEDGEMENTS

We thank Professor Dr. R. J. F. Nivard for his kind interest. The investigations have been carried out under the auspices of the Netherlands Foundation for Chemical Research (SON), and with the financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO).

REFERENCES

1 R. Axén, J. Porath and S. Ernback, *Nature (London)*, 214 (1967) 1302.
2 J. Porath, R. Axén and S. Ernback, *Nature (London)*, 215 (1967) 1491.
3 R. Axén and S. Ernback, *Eur. J. Biochem.*, 18 (1971) 351.
4 G. I. Tesser, H.-U. Fisch and R. Schwyzer, *Helv. Chim. Acta*, 57 (1974) 1718.
5 T. C. J. Gribnau and G. I. Tesser, *Experientia*, 30 (1974) 1228.
6 T. C. J. Gribnau and G. I. Tesser, *Chem. Weekbl.*, 71 (1975) 15 and 29.
7 C. R. Lowe and P. D. G. Dean, *Affinity Chromatography*, John Wiley, London/New York, 1974, p. 202.
8 P. J. Boon, Personal communication.
9 T. C. J. Gribnau, C. Stumm and G. I. Tesser, *FEBS Lett.*, 57 (1975) 301.
10 *Affinity Chromatography, Principles and Methods*, Pharmacia, Uppsala, 1974, p. 8.