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PREPARATION OF CYANOGEN BROMIDE-ACTIVATED AGAROSE GELS

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SUMMARY

By performing the cyanogen bromide activation of hydroxylic gels (*e.g.*, agarose) in alkaline phosphate solutions of very high buffer capacity, the pH control that has hitherto been necessary can be omitted. Strongly, moderately and weakly activated gels can easily be prepared in a simple and reproducible manner.

INTRODUCTION

Cyanogen bromide-activated water-insoluble polysaccharides[1], especially agarose gels in bead form[2], have come into general use in the preparation of adsorbents for bioaffinity chromatography[3,4] and for the preparation of immobilized enzymes[5]. Activation in this context means that the polysaccharide is substituted with highly reactive groups by treatment with cyanogen bromide under strongly alkaline conditions[1]. The organic chemistry of the activation process has been investigated in several laboratories[6–8]. The activated gel has the property to bind covalently in water systems constituents that contain primary or secondary amino groups. The conditions for the fixation reaction are mild and the technique is therefore particularly suitable for sensitive proteins.

EXPERIMENTAL

Agarose gels in bead form (Sepharose 4B and 6B) were purchased from Pharmacia, Uppsala, Sweden. Epichlorohydrin cross-linked agarose gel beads were prepared according to Porath *et al.*[9].

The concentration of the aqueous cyanogen bromide solution was 100 mg/ml.

The buffer systems were characterized by pH values measured with a GK2302C electrode (Radiometer, Copenhagen, Denmark). The phosphate buffer was prepared by dissolving 3.33 moles of K_3PO_4 + 1.67 moles of K_2HPO_4 per litre of solution (at 10-fold dilution, the pH was 11.9).

Procedure A: highly activated agarose gels (1–4% agarose)

The gel spheres were washed with distilled water and the interstitial water removed on a büchner funnel. Ten grams of p% gel were suspended in 2.5 p ml of cold (5–10°) potassium phosphate buffer (5 M) and the suspension was diluted with distilled water to a total volume of 20 ml. Cyanogen bromide solution (1.0 p ml) was added in small

portions during 2 min. The solution was stirred gently and the temperature was maintained at 5–10° for a reaction time of 10 min (which includes the time required for the addition of reagent). The product was washed on a glass filter with cold distilled water until neutral.

Procedure B: highly activated agarose gels (4–8% agarose)

Agarose gel was washed on a büchner funnel with 2 *M* phosphate buffer and then freed from interstitial buffer. Ten grams of gel spheres of *p* % agarose were suspended in 2.5 *p* ml of cold phosphate buffer and activated as under *Procedure A* with 1.0 *p* ml of cyanogen bromide solution.

Procedure C: moderately activated agarose gels (1–8% agarose)

The procedure was as under *Procedure A* but the volume of buffer was 0.5 *p* ml and the volume of cyanogen bromide solution was 0.2 *p* ml.

Procedure D: weakly activated agarose gels (1–8% agarose)

The procedure was as under *Procedure A* but the volume of buffer was 0.12 *p* ml and the volume of cyanogen bromide solution was 0.05 *p* ml.

Coupling of glycyl-leucine and chymotrypsin

The activated products were rapidly washed with 0.25 *M* sodium hydrogen carbonate solution (pH 9) and 2 g of gel were transferred to a cylindrical reaction vessel. Then 2 ml of 0.25 *M* sodium hydrogen carbonate solution were added, followed by 10 mg of chymotrypsin or 35 mg of glycyl-leucine. The coupling was performed by rotating the reaction vessel end-over-end at room temperature for 24 h. The products were washed in a small column connected to a peristaltic pump. The following solutions were used: 0.5 *M* sodium hydrogen carbonate (6 h); 0.1 *M* borate buffer–1 *M* sodium chloride, pH 8.5 (24 h); 0.1 *M* sodium acetate–1 *M* sodium chloride, pH 4.1 (12 h); and finally distilled water (6 h). The flow-rate was 10 ml/h.

The peptide or protein content of the products was determined by total amino acid analysis[5]. The samples were prepared for analysis by shrinking the gel by treatment with water–acetone mixtures of increasing acetone concentration, washing with acetone and drying over phosphorus pentoxide at 100° *in vacuo*.

RESULTS AND DISCUSSION

The cyanogen bromide activation process involves the very rapid production of hydrogen ions. The degree of activation is highly pH-dependent: the higher the pH, the more efficient is the introduction of reactive groups. In the original description of the reaction[1], the pH was kept constant by the continuous addition of concentrated sodium hydroxide solution. The introduction of reactive groups, and consequently also the introduction of ligand attachment, was regulated by means of the activation pH (9.5–11.5)[1,10]. In order to prevent pH gradients within the reaction mixture, vigorous stirring was needed. We have described above how the cyanogen bromide reaction can be carried out at pH 11–12 in a concentrated phosphate buffer. The introduction of active groups is governed by the concentration of cyanogen bromide in the reaction mixture. The recommended technique differs depending on the percen-

tage of polysaccharide present in the agarose gel. The activated gels obtained are assayed by their ability to bind covalently a small peptide (glycyl-leucine) and a protein (chymotrypsin). The results are summarized in Tables I and II. Two kinds of agarose gels were used: commercially available chromatographic agarose beads and epichlorohydrin cross-linked agarose gels[9]. Chemically cross-linked agarose gels are advantageous in the preparation of immobilized enzymes and biospecific adsorbents because of the increased stability of the matrix[11]. The results show, however, that the coupling capacity is decreased as a consequence of the cross-linking.

It should be mentioned in this connection that a high degree of ligand substitution is not always desirable. Protein ligands intended as constituents of biospecific adsorbents can become adsorption inactive at a high degree of substitution[11]. An enzyme ligand at a high degree of substitution is often hindered in its functioning by inadequate diffusional transport of substrate and product[10]. A general optimization of the cyanogen bromide activation process is therefore not possible and the conditions have to be adjusted to the particular problem being considered. As a guide for the selection of suitable conditions in the activation for a desired amount of fixed ligand, three types of procedure have been described above that lead to highly, moderately and weakly activated gels.

TABLE I

CHEMICAL ACTIVATION OF AGAROSE GELS BY MEANS OF CYANOGEN BROMIDE

Chemical fixation of glycyl-leucine and chymotrypsin.

Procedure	*Concn. of agarose in the gel*	*Amount of ligand (μmole/g dry conjugate)*	
		Glycyl-leucine	*Chymotrypsin*
A	4	250	2.5
B	6	300	3.0
C	4	140	0.76
D	4	105	0.44

TABLE II

CHEMICAL ACTIVATION OF EPICHLOROHYDRIN CROSS-LINKED AGAROSE GELS BY MEANS OF CYANOGEN BROMIDE

Chemical fixation of glycyl-leucine and chymotrypsin.

Procedure	*Concn. of agarose in the gel*	*Amount of ligand (μmole/g dry conjugate)*	
		Glycyl-leucine	*Chymotrypsin*
A	4	120	1.7
B	6	110	1.7
C	4	70	0.35
D	4	25	0.13

The technique of activating agarose gels with cyanogen bromide in concentrated phosphate buffer is simple and reproducible. Because only gentle stirring or shaking is needed, the mechanical destruction of fragile gels is prevented. The use of buffer has definite advantages in the activation of large amounts of gel in comparison with the procedure described originally[1]. It facilitates the homogeneous activation of large gel spheres. We have activated agarose membranes mounted in holders by this technique and also thin layers of agarose covering glass beads or the inner surface of glass tubes.

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