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STUDIES ON LECTINS

LIV. AFFINITY ELECTROPHORESIS OF LECTINS AND TRYPSIN ON AFFINITY GELS PREPARED FROM SEPHADEX DERIVATIVES INCORPORATED INTO A POLYACRYLAMIDE GEL MATRIX

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SUMMARY

Affinity gels were prepared by incorporation into a polyacrylamide gel matrix of either unmodified or ligand-substituted Sephadex G-100, G-200 or Sephacryl S-200 beads (*i.e.*, *p*-aminophenyl glycosides or *p*-aminobenzamidine coupled to periodate-oxidized beads). These gels were used for affinity electrophoresis of lectins of trypsin, respectively, including evaluation of the dissociation constants of the lectin-free sugar complexes.

INTRODUCTION

Affinity electrophoresis of proteins in polyacrylamide gel is based on their specific retardation in the gel which contains immobilized ligands capable of complex formation with the proteins. The applicability of the method depends essentially on an effective immobilization of the ligand in the polyacrylamide gel[1]. Several techniques have been used for this purpose[1]: (i) covalent attachment of the ligand to the polyacrylamide gel matrix; (ii) incorporation of a suitable water-soluble macromolecular derivative of the ligand into the gel; (iii) incorporation of a molten agarose derivative containing a ligand into the gel[2]; (iv) incorporation of ligand-substituted beaded agarose or polyacrylamide gels into the polyacrylamide gel matrix[2].

The last method seems to offer several advantages, especially its simplicity and the possibility to use the same materials both for preparative affinity chromatography and affinity electrophoresis. The concentration of immobilized ligand in Sepharose derivatives, obtained by coupling of the ligand to periodate-oxidized Sepharose, is relatively low, *ca.* 1 μmol/ml. In an attempt to increase the degree of substitution we have used other solid supports. In the present communication we describe the preparation of Sephadex or Sephacryl derivatives containing *p*-aminophenyl glycosides or *p*-aminobenzamidine (PAB) and their use in affinity electrophoresis of lectins and trypsin.

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MATERIALS AND METHODS

Sephadex G-50, G-100, G-200 and Sephacryl S-200 were obtained from Pharmacia (Uppsala, Sweden). *p*-Aminophenyl glycosides were prepared by hydrogenation of the corresponding *p*-nitrophenyl glycosides in 50% ethanol on a 10% palladium–carbon catalyst under atmospheric pressure. PAB was purchased from Serva (Heidelberg, G.F.R.).

Trypsin was obtained from Léčiva (Dolní Měcholupy, Czechoslovakia). Concanavalin A and lectin from seeds of *Lens esculenta* were isolated by affinity chromatography on Sephadex G-150[3,4]. Lectins from seeds of *Ricinus communis* and *Erythrina indica* were isolated on O-α-D-galactosyl polyacrylamide gel[5]. For isolation of lectins from seeds of *Glycine soja* and *Arachis hypogaea*, O-α-D-galactosyl Separon was used[6].

Preparation of Sephadex derivatives

Sephadex oxidation. To 50 ml of a swollen and centrifuged (700 × *g*, 4 min) suspension of Sephadex G-100, G-200 of Medium or Superfine grade were added 50 ml of 0.02 *M* $NaIO_4$. The suspension was gently shaken at 20°C for 2 h and then washed with 300 ml of distilled water and 200 ml of 0.5 *M* phosphate buffer pH 6.0. The same conditions were used for oxidation of Sephacryl S-200.

Coupling of p-aminophenyl glycosides to oxidized Sephadex. To 5 ml of a centrifuged suspension of oxidized Sephadex equilibrated with 0.5 *M* phosphate buffer pH 6.0 were added 1–30 mg of *p*-aminophenyl glycoside dissolved in 3 ml of the same buffer and, after mixing, 1 mg of solid $NaCNBH_3$; the total concentration of the ligand in suspension was 0.5–14 m*M*. The reaction mixture was gently shaken at 20°C for 72 h. Then the non-bound ligand was removed and the substituted gel washed by repeated centrifugation. The same conditions were used for coupling of PAB and ethanolamine to Sephadex gel and *p*-aminophenyl glycosides to Sephacryl.

The content of coupled ligands in the Sephadex derivatives was determined by two methods: (i) from the recovery of non-coupled ligand in washing solutions; (ii) spectrophotometrically. Packed Sephadex gel (0.1–0.3 ml) was suspended in 3 ml of 50% glycerol and UV spectra were scanned in the 200–350 nm range (Specord UV-VIS; Carl Zeiss Jena, Jena, G.D.R.). The content of immobilized ligand was estimated from the absorbance at the wavelengths of absorption maxima (246 nm for *p*-aminophenyl glycosides and 304 nm for PAB), using the corresponding free ligands dissolved in 50% glycerol as standards. The spectrophotometric method cannot be used in the case of Sephacryl derivatives.

Affinity electrophoresis

Affinity gels composed of unmodified or ligand-substituted Sephadex or Sephacryl beads embedded in a polyacrylamide gel matrix were prepared as described earlier[2]. As Sephadex derivatives containing higher amounts of bound *p*-aminophenyl glycoside or PAB partially inhibited polymerization of the polyacrylamide gel, these derivatives were mixed 1:1 with Sephadex beads containing bound ethanolamine and this mixture was used for affinity electrophoresis. For qualitative purposes (*i.e.*, detection of ligand-binding proteins) the height of the affinity layer was adjusted tot *ca.* 10–15 mm; in the case of the determination of dissociation constants of the

complex lectin-free sugar, the affinity gel layer formed more than 80% of the total length of the gel rod.

Affinity electrophoreses of lectins on polyacrylamide gels containing Sephadex or Sephacryl were performed in discontinuous alkaline[7] or discontinuous acidic[8] buffer systems according to the standard procedure (omitting large-pore gel layers). For affinity electrophoresis of trypsin, the Tris–diethylbarbituric acid buffer system[9] was used. A 50-μg protein sample in 20 μl of 20% glycerol solution was applied to each tube (5 × 75 mm). Electrophoresis in an alkaline buffer system[7] was run at 4 mA per tube until bromophenol blue reached the bottom of the gel, and in an acidic buffer system[8] at 7 mA per tube for 1.5 h. Electrophoresis of trypsin was run at 4 mA per tube (migration direction from anode to cathode) for 2 h.

Dissociation constants, K, or the lectin-free sugar complexes were determined using the simple equation[10]

$$\frac{d}{d_0 - d} = \frac{K_i}{c_i}\left(1 + \frac{c}{K}\right)$$

where d = migration distance in the affinity gel at free sugar concentration c, d_0 = migration distance in the control (non-interacting gel), the K_i/c_i value (determined by the nature of the affinity gel and the strength of its interaction with the lectin) was taken as an empirical constant. Control gels for the estimation of d_0 were prepared either from the beads containing coupled non-interacting ligand (*e.g.*, ethanolamine), or contained a high concentration (0.12 M) of free sugar which almost completely prevented the interaction with the immobilized sugar residues. The concentration of free sugar in the affinity gels was varied in the range $1.8 \cdot 10^{-4}\ M < c < 8.2 \cdot 10^{-2}\ M$.

RESULTS

Preparation and properties of Sephadex and Sephacryl derivatives

For coupling of ligands, Sephadex oxidized with 0.01 M $NaIO_4$ for 2 h was found to be most suitable. Milder and shorter periods of oxidation resulted in a very low amount of ligand bound to Sephadex, while prolonged oxidation or an increased sodium periodate concentration caused destruction of Sephadex particles and solubilization of the gel.

The following ligands were coupled to periodate-oxidized Sephadex of different types: p-aminophenyl glycosides, PAB and ethanolamine. The amount of ligand coupled was dependent on the initial ligand concentration in the reaction mixture and on the type of Sephadex. The dependence of the amount of coupled p-aminophenyl β-D-galactoside on its initial concentration in the reaction mixture is shown in Fig. 1. A very similar dependence was obtained when the amount of coupled ligand was calculated per g dry weight of the dextran gel. Greater differences between the amounts of coupled glycosides were found between Sephadex Medium and Superfine grades than between Sephadex G-100 and G-200. Similar dependences were observed when p-aminophenyl α-D-mannoside was used.

The same conditions for oxidation and coupling were used in the preparation of modified Sephacryl S-200. Sephacryl S-200 was much less sensitive to "over-oxidation" by periodate than Sephadex gels.

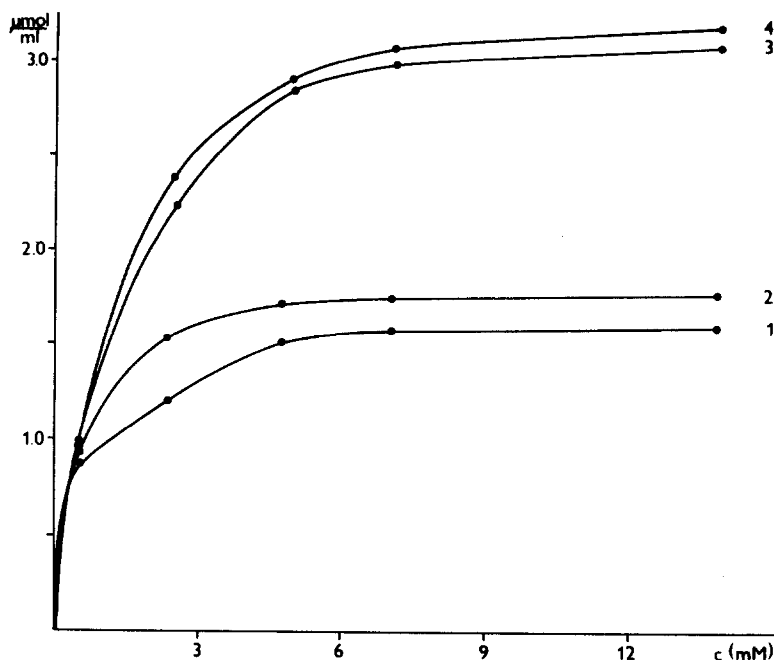

Fig. 1. Dependence of the amount of *p*-aminophenyl β-D-galactoside coupled to different types of Sephadex on its initial concentration in the rection mixture. Curves: 1 = Sephadex G-100; 2 = Sephadex G-200; 3 = Sephadex G-100 Superfine; 4 = Sephadex G-200 Superfine.

Affinity electrophoresis of lectins and trypsin on polyacrylamide gel containing substutited Sephadex or Sephacryl.

Specific interaction with immobilized β-D-galactosyl residues was observed in the case of lectin from *Glycine soja* and lectins from *Ricinus communis* seeds (Fig. 2); agglutinin of *R. communis* seeds interacted more strongly than the toxin. Lectins isolated from seeds of *Arachis hypogaea* and *Erythrina indica* did not interact with the Sephadex containing coupled *p*-aminophenyl β-D-galactoside either in alkaline or acidic buffer systems. Similar results were obtained when Sephacryl containing coupled *p*-aminophenyl β-D-galactoside was used.

The interaction of lectins with Sephadex or Sephacryl derivatives entrapped in polyacrylamide gel is inhibited in the presence of free specific sugar in the gel and the extent of inhibition is dependent on the concentration of free sugar, as in the case of electrophoresis on homogeneous affinity gels[10]. Affinity electrophoresis on polyacrylamide gels containing Sephadex with coupled *p*-aminophenyl β-D-galactoside was used to determine the dissociation constants of the complex *R. communis* agglutinin–free lactose; the determined *K* value, $4.6 \cdot 10^{-4}$ *M*, is very close to that obtained by affinity electrophoresis on homogneous polyacrylamide gels containing immobilized α-D-galactosyl residues[10].

As shown in our previous communication[2], polyacrylamide gel containing unmodified Sephadex or Sephacryl could be used for affinity electrophoresis of lectins which interact with D-glucose. These affinity gels containing in addition to beaded dextran gel also free D-mannose in different concentrations were used for the determi-

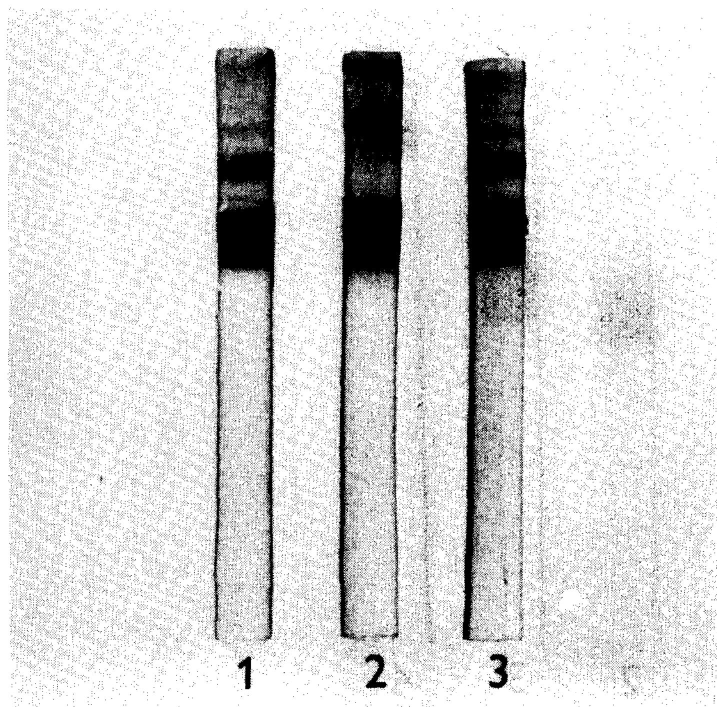

Fig. 2. Affinity electrophoresis of lectins from *Ricinus communis* seeds on polyacrylamide gel containing Sephadex substituted with *p*-aminophenyl β-D-galactoside. 1 = Control gel containing unmodified Sephadex G-200; 2 = affinity gel containing Sephadex G-200 substituted with *p*-aminophenyl β-D-galactoside; 3 = as gel 2, except that free lactose was added to the gel (c = 0.12 *M*). Electrophoresis was carried out in the alkaline buffer system[7].

nation of dissociation constants of the lentil lectin–free D-mannose ($K = 3.0 \cdot 10^{-3}$ *M*) and concanavalin A–free D-mannose ($K = 1.3 \cdot 10^{-4}$ *M*) complexes. These values are in good agreement with those obtained using homogeneous affinity gels containing immobilized α-D-mannosyl residues[10].

Fig. 3 shows the affinity electrophoresis of trypsin on polyacrylamide gel containing Sephadex with coupled PAB.

Incorporation of Sephadex of Sephacryl beads into the polyacrylamide gel had only moderate effect on the mobility of non-interacting proteins; a slight decrease of mobility was presumably due to a decrease in the effective porosity of these gels. The incorporation of periodate-oxidized (*i.e.*, unsubstituted with amino-ligand) beads into the polyacrylamide gel led to a very strong, apparently non-specific interaction of all proteins studied with these gels; the interaction was significantly stronger in the alkaline buffer system.

DISCUSSION

In our previous communication[2] the preparation of heterogeneous polyacrylamide affinity gels containing ligand-substituted agarose or polyacrylamide gel beads

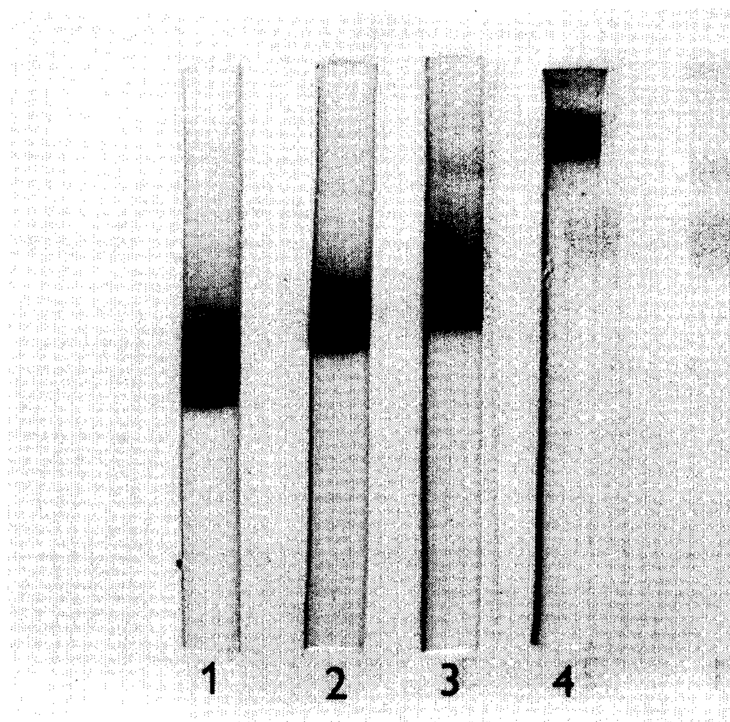

Fig. 3. Affinity electrophoresis of trypsin on polyacrylamide gel substituted with *p*-aminobenzamidine. 1 = Control gel containing no beaded gel; 2 = control gel containing Sephadex G-200 substituted with ethanolamine; 3 = control gel containing Sephadex G-200 substituted with *p*-aminophenyl β-D-galactoside; 4 = affinity gel containing Sephadex G-200 substituted with *p*-aminobenzamidine. Electrophoresis was carried out in the Tris–diethylbarbituric acid buffer system[9].

was described. The ligands were bound either to periodate-oxidized or cyanogen bromide-activated agarose or to hydrazide- or glutaraldehyde-activated polyacrylamide gel. The amount of coupled ligands was in the range 1.0–1.5 μmol per ml of the packed gel. In the present study we have used agarose gel dextran beads instead for binding different ligands; we have also investigated the conditions under which the degree of substitution could be increased. Under the conditions described, the amount of ligands coupled to oxidized Sephadex gel was 2–3 times higher than that of ligands coupled to periodate-oxidized Sepharose.

However, in comparison with Sepharose, the Sephadex gels are much more sensitive to periodate oxidation and it was necessary to find conditions of oxidation and coupling such that the structure of the Sephadex gel was not damaged. Under the oxidation conditions described (0.01 M $NaIO_4$, for 2 h), the structure of Sephadex gel remained stable and no solubilization was observed; also, determination of the dry weight of the prepared Sephadex derivatives showed that the water regain of these derivatives did not significantly differ from unmodified Sephadex. The reaction conditions (borate or carbonate buffer, pH 8–9)[11–13] usually applied for binding of ligands containing a primary amino group to periodate-oxidized polysaccharide carriers

of different types proved unsuitable for Sephadex; in this case the dextran gel was partially solubilized, the suspension was not homogeneous and the particle structure was altered. Best results were obtained when ligands were coupled in phosphate buffer pH 6.0 (ref. 14). Sephacryl S-200 was less sensitive to damage by periodate oxidation than Sephadex gels.

As in the case of affinity gels containing beaded Sepharose 4B derivatives[2], with Sephadex derivatives no interfering effects of the presence of beads in polyacrylamide gel on the quality of electrophoretic separation of proteins were observed. Non-specific interactions of proteins, especially in alkaline pH media, were observed on gels containing oxidized unsubstituted (non-reduced) Sephadex. This interaction was probably caused by the interaction of aldehyde groups of the oxidized dextran with the protein, which resulted in a non-specific retardation of protein zones.

In our previous communication[2] we showed that, in affinity isoelectric focusing, affinity gels must be prepared only from materials with very low electroendosmosis; thus the commercially available beaded agarose gels like Sepharose 4B are of limited use. On the other hand, the Sephadex derivatives described here might be useful for this purpose.

Although the type of affinity gels described would be employed mostly for qualitative purposes, *i.e.*, the detection and identification of ligand-binding proteins and for rapid testing of carriers potentially applicable to affinity chromatography, we also demonstrated their use for quantitative evaluation of the strength of the protein–ligand interaction. The values of the dissociation constants of lectin–sugar complexes determined in the present study are very close to those found earlier using other, more homogeneous types of affinity gels. Thus, our results indicate that the Sephadex–polyacrylamide gels behave as functionally homogeneous and that the extent of their structural heterogeneity may be considered as negligible.

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