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A Novel Polyacrylamide-Type Support Prepared by *p*-Benzoquinone Activation

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Abstract

A new and simple method for the activation of polyacrylamide gels using *p*-benzoquinone is described. The optimal conditions of activation have been elaborated. The activated support could be successfully applied to the immobilization of ligands having nucleophilic groups active over a broad pH range.

Index Entries: Polyacrylamide-type support, prepared by *p*-benzophenone activation; support, polyacrylamide-type; *p*-benzophenone activation, of a polyacrylamide-type support; activation, of a polyacrylamide-type support by *p*-benzophenone.

Introduction

A great number of methods for the immobilization of biologically active macromolecules and ligands of low molecular weights have been reported in the last decade (*1–3*). Among supports used for immobilization, polyacrylamide gels have great practical importance because of their chemical and mechanical stability and their resistance to enzyme and microbial attack. These gels also show very

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slight nonspecific adsorption of most macromolecules. There exist many derivatization techniques to convert the amide group into chemically more reactive groups (*4–6*). In the post *p*-benzoquinone was used only as an activating agent for the activation of supports having hydroxylic groups (*7, 8*). In the present paper we present a method of activating polyacrylamide gels using *p*-benzoquinone and the application of these activated gels to the immobilization of proteins and small ligands.

Materials and Methods

Akrilex P-100, a polyacrylamide-type bead polymer (particle size 40–120 μm; pore size, max. 12 nm) was produced by the Reanal Factory of Laboratory Chemicals, Budapest, Hungary. Catalase (EC 1.11.1.6.) from beef liver, and peroxidase (EC 1.11.1.7) from horseradish were also obtained from the Reanal Factory. Bovine serum albumin was a product of Philaxia, Budapest, Hungary. Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12), adolase, fructose-1,6-bisphosphate, and D-glyceraldehyde-3-phosphate-lyas e (EC 4.1.2.13) were isolated and purified from pig muscle in our laboratory according to the procedure of Elódi et al. (*9*) and Taylor et al. (*10*). Glucose oxidase (EC 1.3.4.) from *Aspergillus* niger was purchased from Sigma Co., St. Louis, USA. Trypsin (EC 3.4.21.4.) from bovine pancreas was a product of Merck, Darmstadt, Germany. All other chemicals were purchased from Reanal.

Measurements of Ligand Concentrations

The amount of immobilized ligand was determined indirectly from the difference between the amount of ligand introduced into the reaction mixture and the amount present in the washing solutions after immobilization. The concentration of proteins was determined by measuring the optical density at 280 nm using calibration curves of given proteins. The concentration of γ-amino-butyric acid was determined by the ninhydrin method (*11*). The glucose concentration was determined in the following way: The reaction mixture contained 2.5 mL of 0.1*M* phosphate buffer at pH 7.5, 0.1 mg of peroxidase, 0.63 mg of glucose oxidase, and 0.17 mg of *o*-dianisidine. To this mixture a 0.1 mL of aliquot of glucose was added in the concentration range of 1×10^{-4} to 1×10^{-3} *M*. The reaction was allowed to proceed for 30 min at room temperature and then 0.2 mL of 4.0*M* HCl was added to stop the reaction. The optical density change of the mixture was measured at 400 nm. The molar extinction coefficient used was 9.88 cm^2/M.

Determination of Enzymic Activities

The activities of the immobilized enzymes were measured spectrophotometrically by observing the rate of optical density change in a continuously stirred reaction mixture at 298 K. The activity of glyceraldehyde-3-phosphate dehydrogenase was assayed by Warburg's optical test in 50 m*M* Tris-HCl buffer of pH 7.5. The reaction mixture of 1.8 mL contained 3.6 μmol of glyceraldehyde-3-phosphate, 3.6

μmol NAD, and 3.6 μmol AsO_4^{3-}. The activity of aldolase was determined with the help of glyceraldehyde-3-phosphate dehydrogenase. The reaction mixture of 1.8 mL contained 3.5 μmol fructose-1,6-diphosphate, 3.6 μmol NAD, 3.6 μmol AsO_4^{3-}, and 4.8 nmol glyceraldehyde-3-phosphate dehydrogenase. In the case of the above two enzymes, the reactions were followed by the detection of NADH formed measuring the optical density change at 340 nm. The activity of glucose oxidase was measured in 0.1*M* phosphate buffer of pH 7.0. The reaction mixture of 3.0 mL contained 0.5 mg of *o*-dianisidine, 0.25 mmol of glucose, 30μg of peroxidase, and 0.1 mL of soluble enzyme or 0.1 g of immobilized enzyme. The reaction was followed at 465 nm. The determination of catalase activity was carried out in 50 m*M* Tris-HCl buffer of pH 7.5 containing 25 m*M* H_2O_2. To 2.0 mL of reaction mixture wsa added 0.1 mL of soluble enzyme or 0.1 g of immobilized enzyme and the change in the optical density was recorded at 240 nm.

The activity of trypsin was determined with urea denatured casein as substrate prepared according to the method of Anson (*13*). The reaction mixture contained 0.9 mL of 0.8% denatured casein in a 0.1*M* phosphate buffer of pH 7.5 and 0.1 mL of soluble enzyme or 0.1 g of immobilized enzyme. The reaction mixtures were incubated for an appropriate time, generally 5, 10, and 15 min, respectively, and then the undigested protein was precipitated by the addition of 2.0 mL of 10% trichloroacetic acid. After 10 min the suspension was centrifuged and the optical density of supernatant was measured at 280 nm.

Results

Activation of Akrilex P-100 by p-Benzoquinone

We have found tht the Akrilex P-100, polyacrylamide-type bead polymer could be activated by *p*-benzoquinone (Fig. 1) with the best result in the following way: 0.1 g of Akrilex P-100 xerogel was swollen in 4.0 mL of 0.1*M* phosphate buffer at pH 8.0. To this was added 1.0 mL of 0.1*M* *p*-benzoquinone in dioxane. The reaction was allowed to proceed at 323 K for 24 h. When the reaction was complete, the gel was successively washed on a glass filter with 20% dioxane in water, water, 0.1*M* sodium acetate buffer at pH 4.0 containing 1.0*M* sodium chloride, 0.1*M* sodium bicarbonate solution at pH 8.5 containing 1.0M sodium chloride, and last with the coupling buffer. The settled volume of the swollen *p*-benzoquinone activated Akrilex P-100 gel was 1.5 mL.

General Method for the Coupling of Ligands to p-Benzoquinone-Activated Akrilex P-100

The 1.5 mL activated and washed gel samples were suspended in 2.0 mL of coupling buffer containing the ligand to be bound. The concentration of both glucose and γ-amino butytric acid was $1 \times 10^{-2}M$ and that of bovine serum albumin and enzymes was 20 mg/mL. The coupling buffer was generally 0.1*M* phosphate at pH 7.5. The coupling reactions were performed at 277 K for 24 h with gentle stirring. The products were then successively washed on glass filter with the coupling

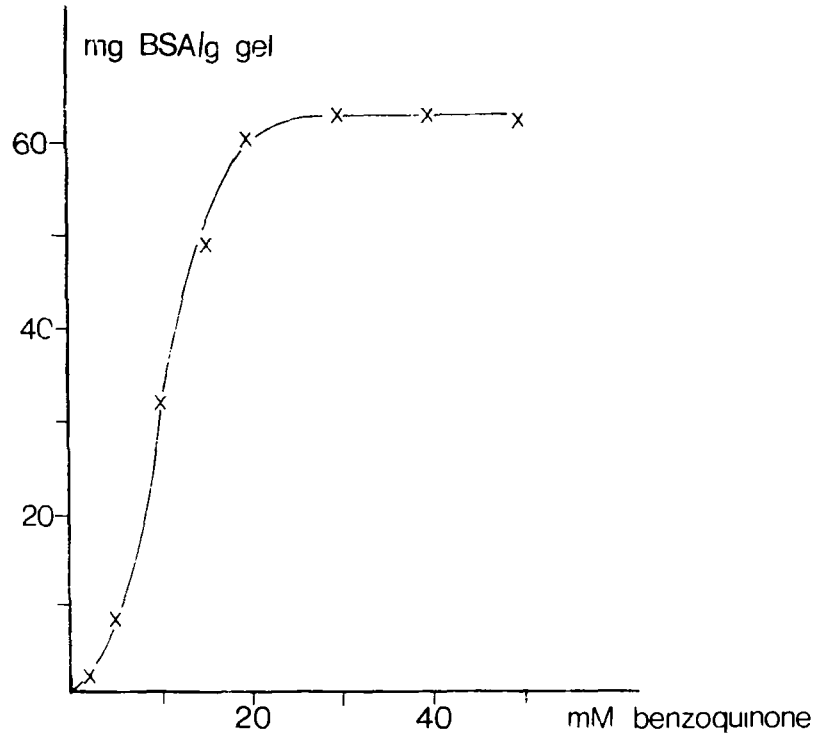

Fig. 1. The effect of the time of activation with *p*-benzoquinone on the binding capacity of activated Akrilex P-100. The activation was carried out in 0.1*M* phosphate buffer of pH 8.0 containing 20% dioxane and 20 m*M* *p*-benzoquinone at 323 K. The binding capacity was determined on the basis of bound bovine serum albumin amount. The immobilization of albumin was performed according to the general method described in the text.

buffer and then with 0.1*M* sodium acetate buffer at pH 5.0 containing 0.5*M* sodium chloride, 0.1*M* sodium bicarbonate solution at pH 8.5 containing 0.5*M* sodium chloride, and last with 0.1*M* sodium phosphate buffer at 7.5. The time curve and the pH dependence on coupling with activated gel are presented in Figs. 2 and 3.

Immobilization of Small Molecular Ligands

Glucose and γ-amino butyric acid could be fixed on *p*-benzoquinone activated Akrilex P-100 using the general method described above. The concentration of these ligands on the gel was found to be 60 and 40 μmol/g solid, respectively.

Immobilization of Proteins

The binding of bovine serum albumin on *p*-benzoquinone-activated Akrilex P-100 was also studied. The amount of protein bound on the gel varied as the function of pH of coupling medium (Fig. 4) and the concentration of albumin introduced into the reaction mixture (Fig. 5), respectively.

We have attempted to immobilize some different type of enzymes. The results are summarized in Table 1. As it can be seen the specific activity of the enzymes fixed on the gel varied over a wide range. The glyceraldehyde-3-phosphate dehydrogenase was totally inactivated.

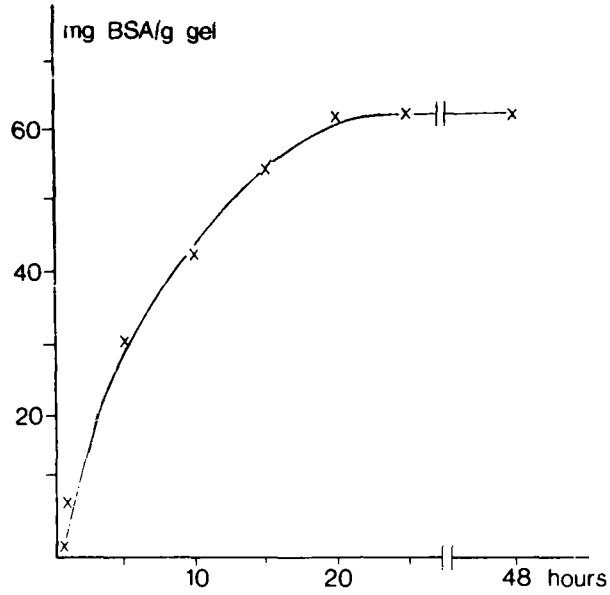

Fig. 2. The effect of pH on the activation of Akrilex P-100 by *p*-benzoquinone. The gel was activated in 20% dioxane containing 20 m*M* *p*-benzoquinone for 24 h at 323 K. Buffers used were the following: 0.1*M* sodium formate (pH 3.0–4.0), 0.1*M* sodium acetate (pH 4.0–6.0), 0.1*M* sodium phosphate (pH 6.0–8.0) and 0.1*M* sodium bicarbonate (pH 8.0–11.0). The binding capacity was determined on the basis of the amount of the bovine serum albumin. The immobilization of the albumin was performed according to the general method described in the text.

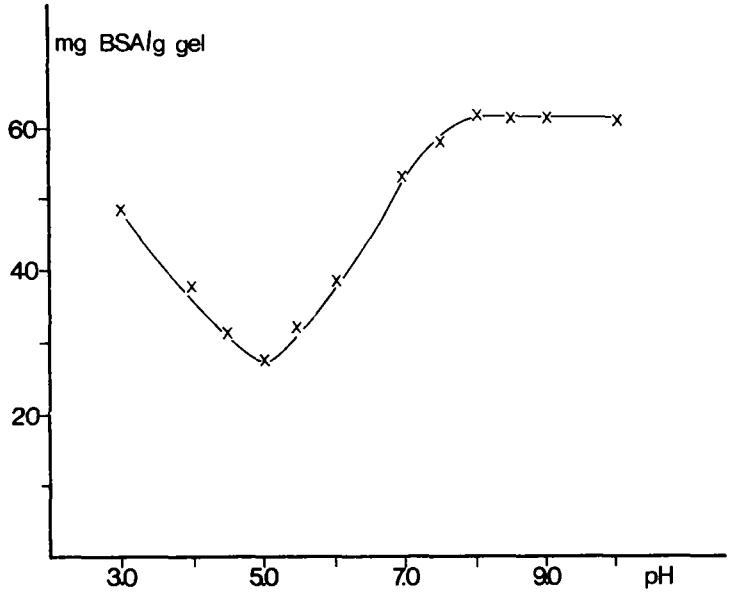

Fig. 3. The effect of the concentration of *p*-benzoquinone applied to the activation of activated Akrilex P-100 on its binding capacity. The reaction was carried out in 0.1*M* phosphate buffer of pH 8.0 containing 20% dioxane. The binding capacity was determined on the basis of the amount of the bound bovine serum albumin. The immobilization of the albumin was performed according to the general method described in the text.

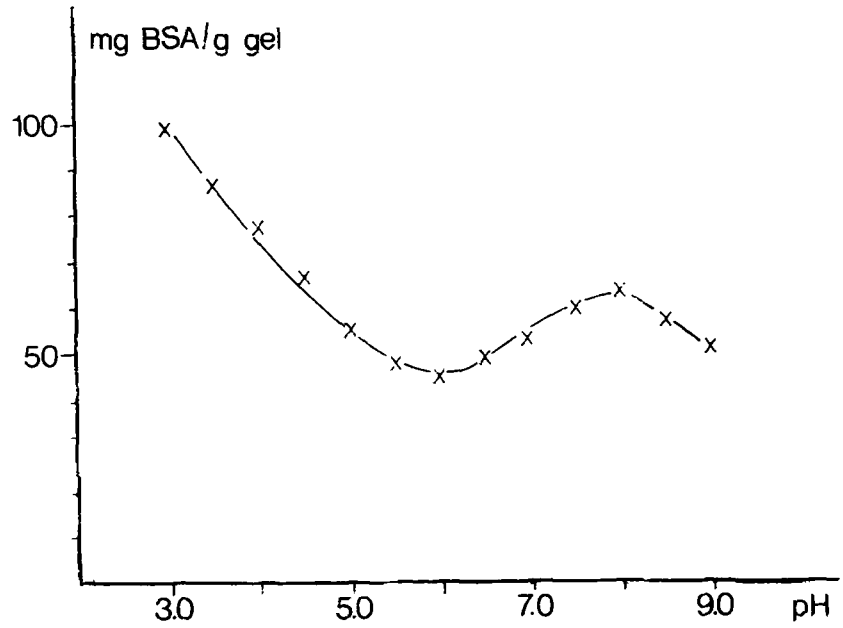

Fig. 4. The effect of the pH of coupling buffer on the binding of the bovine serum albumin to *p*-benzoquinone-activated gel. Buffers used were the following: 0.1*M* sodium formate (pH 3.0–4.0) 0.1*M* sodium acetate (pH 4.0–6.0), 0.1*M* sodium phosphate (pH 6.0–8.0), and 0.1*M* sodium bicarbonate (pH 8.0–11.0).

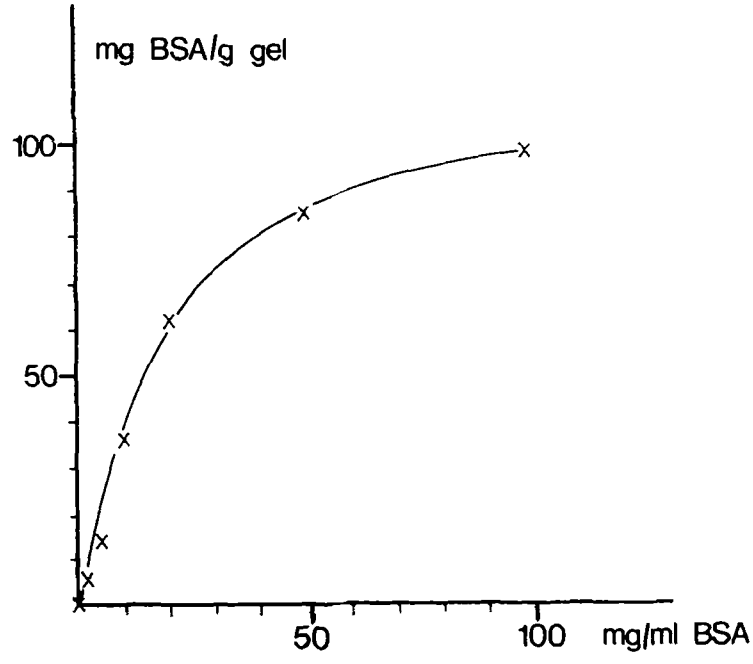

Fig. 5. Dependence of the amount of bound bovine serum albumin on the protein concentration in the reaction mixture. The immobilization experiments were performed according to the general method described in the text.

Table I
Balance Sheet on The Enzyme Immobilization Experiments[a]

Enzyme	Immobilized protein, %	Immobilized activity, %	Activity recovered in the filtrate and the washing solutions, %	Activity loss, %	Remaining specific activity on the gel, %
Aldolase	18.8	2.1[b]	61.5	36.4	11
Catalase	13.3	0.3[c]	82.5	17.2	2
Glucose oxidase	11.3	0.9[d]	80.0	19.1	8
Glyceraldehyde-3-phosphate dehydrogenase	15.5	0.0[e]	66.0	34.0	0
Trypsin	10.5	0.1[f]	41.0	58.9	1

[a]The gel samples were activated in 0.1*M* phosphate, pH 8.0, containing 20% dioxane and 20 m*M* *p*-benzoquinone. Enzymes were introduced in a concentration of 20 mg/mL.
[b]One unit is the amount of enzyme that converts 1.0 μmol of fructose-1,6-diphosphate to dihydroxyacetone phosphate and glyceraldehyde-3-phosphate per minute at pH 7.5 at 298 K.
[c]One unit is the amount of enzyme that catalyzes the decomposition of 1.0 μmol of H_2O_2 per minute at pH 7.5 at 298 K.
[d]One unit is the amount of enzyme that oxidizes 1.0 μmol of β-D-glucose to D-gluconic acid and H_2O_2 per minute at pH 7.0 at 298 K.
[e]One unit is the amount of enzyme that oxidizes and phosphorylates 1.0 μmol of D-glyceraldehyde-3-phosphate forming 1,3-diphosphoglycerate at pH 7.5 at 298 K.
[f]The activity was expressed in arbitrary units. One unit causes 0.1 optical density change in 1.0 mL of final volume using 0.1 mL aliquot of sample in 1 min at 280 nm at pH 7.5 at 298 K.

Discussion

Within the framework of our research program we looked for the most effective method for the transformation of polyacrylamide type gels into activated supports for affinity chromatography and enzyme immobilization. In preliminary experiments we had found that Akrilex P-100 gel could be activated by the method Brandt et al. (*7*) described for the activation of Sepharose 4B, but only with very low binding capacity. In our procedure the gel was reacted with *p*-benzoquinone at room temperature for 1 h. However, it was observed that *p*-benzoquinone activated Akrilex P-100 showed high binding capacity when the temperature and the time of incubation with *p*-benzoquinone had been elevated. It was found that after 24 h of incubation at 323 K there was no further increase in the binding capacity determined on the basis of coupled bovine serum albumin. The activation strongly depends on the pH (cf. Fig. 2). The pH profile of the activation has a minimum value at 5.0. It differs from the activation pH profile of both Sepharose 4B and hydroxyalkyl methacrylate gels (*7*, *8*), this difference in pH profile is probably a result of the pH dependence of reactivity of carboxamide groups toward *p*-benzoquinone. The mechanism of the reaction is not yet clear. Whether the am-

ide group can act as a nuclephilic agent on the 2-C carbon or could form a condensation product with the keto group of *p*-benzoquinone can be decided only by further studies.

The amount of bovine serum albumin fixed on the gel was somewhat lower than that found in the case of Sepharose 4B (*7*), but it was considerably higher than that in the case of hydroxyalkyl methacrylate gels (*8*).

The data related to the specific activities of enzymes immobilized on *p*-benzoquinone activated Akrilex P-100 (cf. Table 1) suggest that certain enzymes could be sensitive to this type of coupling procedure. The results presented here indicate that the *p*-benzoquinone-activated Akrilex P-100 can successively be applied to the immobilization of both proteins (including enzymes) and small ligands and as a new aid for the preparation of affinity sorbents. The advantage of this coupling procedure are high binding capacity and the high stability of the bonds formed. The low cost and low toxicity of the reagent are also important. Ligands having hydroxyl or amino groups can be attached to polyacrylamide gels by this method.

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