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POLYACRYLHYDRAZIDO-AGAROSE: PREPARATION VIA PERIODATE OXIDATION AND USE FOR ENZYME IMMOBILIZATION AND AFFINITY CHROMATOGRAPHY

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SUMMARY

A new method for the preparation of polyacrylhydrazido-agarose is described. The method is based on the periodate oxidation of Sepharose followed by reaction with polyacrylhydrazide. Enzymes, antibodies, lectins and small ligands were coupled to this column. High degrees of binding and activity were obtained. The bound enzymes exhibited increased stability to heat and 6 *M* urea compared with the respective soluble enzyme as well as enzymes coupled to cyanogen bromide-activated agarose. Antibodies and lectins were also found to maintain specificity for the appropriate antigens and glycoprotein receptors and were used effectively for purifications by affinity chromatography. Polyacrylhydrazido-agarose provides a carrier that combines the advantages of both agarose and acrylamide.

INTRODUCTION

Polyacrylhydrazido-agarose[1,2] (PAHA) has been used in recent years for immobilization of protein[1], purification of lectin receptors[1–3] and for the isolation of antigens[1,4] and antibodies. It is prepared by coupling polyacrylhydrazide to cyanogen bromide-activated Sepharose[5,6]. PAHA shows enhanced stability toward nucleophiles compared to monovalent hydrazides or amines coupled by the same method. However, some leakage could be observed during purification of antibodies on coloured antigen columns[1]. Also, in many cases, the PAHA became pink, which in itself did not interfere with its routine use, but gives an unpleasant appearance to the column.

Polysaccharides including Sepharose can be oxidized with periodate to form aldehyde groups[7]. These aldehyde groups can be used directly to couple proteins[7] or be modified further with different reagents including hydrazides[8] to give low capacity columns. The reason for the low capacity is the lack or absence of enough diols on the Sepharose available for oxidation and the small amount of aldehyde formed may be a result of primary hydroxyl oxidation.

In this paper we describe the coupling of polyacrylhydrazide to periodate-oxidized agarose giving columns containing high contents of free hydrazides available for further reactions. All the preparations yielded columns which were colourless and

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stable after reduction with sodium borohydride. These new PAHA columns could be used directly or after further modification with different reactive groups for protein immobilization and affinity chromatography[6,9].

MATERIALS AND METHODS

Materials

Sepharose 4B was obtained from Pharmacia (Uppsala, Sweden), α-chymotrypsin, ovalbumin and ribonuclease from Worthington (Freehold, NJ, U.S.A.). Trypsin, rabbit serum albumin, trinitrobenzenesulphonic acid (TNBS), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman reagent) were purchased from Sigma (St. Louis, MO, U.S.A.). Hydrazine hydrate, glutaraldehyde, succinic anhydride, N-acetylhomocysteine thiolactone, sodium borohydride and sodium periodate were from Fluka (Buchs, Switzerland), D-tryptophan methyl ester, aminocaproyl-D-tryptophan methyl ester and dinitrophenyl-rabbit serum albumin (DNP-RSA) were obtained from Miles-Yeda (Rehovot, Israel). Fetuin was obtained from Gibco (NY, U.S.A.); poly(L-lysine) (n = 80), acetyltyrosine ethyl ester and benzoylarginine ethyl ester were prepared by Israel Jacobson at the Weizmann Institute. Polyacrylhydrazide was prepared as described[1].

Methods

Periodate oxidation of Sepharose. Ten grams of washed Sepharose 4B were suspended in freshly prepared 0.25 M sodium periodate (30 ml). The suspension was slowly stirred at 24°C for 3 h in the dark. The oxidized Sepharose was washed with cold water to remove excess of $NaIO_4$. Freshly prepared oxidized Sepharose was used for further derivatization.

Preparation of polyacrylhydrazido-Sepharose (PAHOS). The oxidized Sepharose was suspended in three volumes of polyacrylhydrazide solution in water (0.1–0.5 %). The coupling was allowed to proceed overnight in the dark at 24°C with slow stirring. The conjugate was washed with 0.1 M sodium chloride until the washings showed no colour reaction with trinitrobenzenesulphonic acid. The conjugate was reduced with 0.3 M sodium borohydride in a strong buffer, *ca.* pH 8, for 3 h at room temperature. The reduced gel was washed with water on a sintered glass funnel. Derivatives containing 15–25 μmole hydrazide per ml of Sepharose were used for further substitution.

Preparation of poly-Lys-Sepharose. The oxidized Sepharose was suspended in 1 % poly(lysine) (n = 80) solution in 0.1 N $NaHCO_3$. The coupling was allowed to proceed overnight at 4°C with slow stirring. The conjugate was washed with 0.1 N $NaHCO_3$. The gel was reduced with 0.3 M sodium borohydride for 3 h at room temperature. Carriers containing 25 μmole of amine per ml of Sepharose were obtained.

Preparation of aldehyde-containing PAHOS. Aldehyde-containing PAHOS was prepared by suspending the resin in three volumes of 7 % glutaraldehyde in water, with slow stirring for 2 h at 24°C. Excess of glutaraldehyde was removed by washing the gel with cold water on a sintered glass funnel until no further reaction of glutaraldehyde with nitrophenylhydrazide was detected.

Preparation of carboxyl-containing PAHOS. Free-carboxyl-containing resins were prepared by acylation of the free hydrazide groups with an excess of succinic anhydride. The resulting succinylhydrazides provide carboxyl groups with long spacer arms. A 0.5-mmole amount of succinic anhydride was usually added per ml of packed hydrazido-Sepharose in an equal volume of water. The pH of the reaction mixture was maintained at 6.0 by addition of 1 *N* NaOH. The reaction was allowed to proceed at room temperature for 3 h with slow stirring. Completion of acylation was assayed with TNBS. The resin was washed with 0.1 *N* HCl and water.

Preparation of amino-containing PAHOS. Compounds containing primary amino groups were coupled either to the aldehyde resin at pH 7.0 for 16 h at room temperature or alternatively to the carboxyl derivatives at pH 5.0 in the presence of water-soluble carbodiimide. Coupling to carboxyl derivatives was also performed under anhydrous conditions using dioxane and dicyclohexylcarbodiimide. A large excess of diamine was used to decrease cross-linking in cases where free amino terminal groups were required. Such amino groups may be useful for further substitution with different ligands.

Preparation of sulphhydryl-containing PAHOS. Sulphhydryl groups were introduced by treating the resin with N-acetylhomocysteine thiolactone. One gram of N-acetylhomocysteine thiolactone was added to a cold suspension of 10 ml hydrazido-Sepharose in 20 ml 1 *M* $NaHCO_3$. The reaction mixture was stirred at 4°C for 16 h and the product was washed extensively with water and 0.1 *N* NaCl. It can also be prepared by the coupling of dithiodiglycolic acid in aqueous solution using water-soluble carbodiimide or in dioxan using dicyclohexylcarbodiimide as described above for carboxyl coupling. After reaction the disulphide resin was reduced with 0.2 *M* mercaptoethanol for 30 min.

Coupling of nucleotides and RNA to PAHOS. Nucleosides, nucleotide phosphate coenzymes and RNA possessing vicinal free hydroxyl groups were oxidized with periodate and were coupled to PAHOS as described earlier for other hydrazide columns[10].

Coupling of proteins and lectins. The aldehyde-containing resin obtained after glutaraldehyde treatment was suspended in two volumes of 0.1 *M* phosphate buffer, pH 7.5, containing the appropriate protein (enzyme, ovalbumin, DNP-RSA, lectins) at concentrations of 5–10 mg/ml with slow stirring for 16 h at 4°C. The peanut lectin (PNA) was coupled in 0.1 *M* $NaHCO_3$, 0.14 *M* NaCl and 0.1 *M* D-galactose. The conjugates were washed with cold 0.01 *M* phosphate buffer −0.14 *M* NaCl, pH 7.4, (PBS). The excess of aldehyde groups was removed by reduction, with 0.5 mg $NaBH_4$ per mol PBS for 2 h at 4°C, or by treatment with 0.05 *M* hydrazine hydrate solution (pH 8.0) for 2 h at 24°C.

Preparation of affinity columns. Coupling of D-tryptophan methyl ester and ε-aminocaproyl-D-tryptophan methyl ester to carboxy-PAHOS was performed in 30% dimethylformamide (DMF) in aqueous solution. The carboxyl gel (1 g wet resin) was suspended in 2.0 ml of the above solvent containing D-tryptophan methyl ester (15 mg/ml), the pH was brought to 5 and 12.5 mg water-soluble carbodiimide were added. The suspension was left for 16 h at room temperature with slow stirring. The conjugate was washed successively with 25% DMF in water, water, 0.1 *N* HCl and water. Coupling of D-tryptophan methyl ester and its ε-aminocaproyl derivative to aldehyde-PAHOS was performed in phosphate buffer as described for binding of

proteins. The conjugate was reduced with 0.03 *M* $NaBH_4$ for 2 h at room temperature. Gels containing between 15 and 20 μmoles of ligand per gram packed gel were obtained.

Characterization of insoluble derivatives. The amount of free hydrazides or free amino groups on conjugates was determined by reaction with an excess of TNBS. The quantity of unreacted TNBS was measured and subtracted from its initial concentration. Samples of washed gel (200 mg) were suspended in 1.0 ml of 1% TNBS to which was added 1.0 ml of saturated sodium tetraborate. The suspension was stirred for 30 min at 24°C then washed with 23 ml of 0.2 *M* NaCl. Aliquots (100 μl) of the washings were incubated with 1.0 ml of 0.2 *M* adipodihydrazide and 0.9 ml saturated borate for 15–20 min. The absorbance at 500 nm was recorded. A value of E^{M}_{500} 16,500 was used for calculating the quantity of TNBS that had reacted with adipodihydrazide.

The amount of free sulphhydryl on the resin was determined by using an excess of 5,5′-dithiobis(2-nitrobenzoic acid). Samples of washed gel (200 mg) were suspended in 1.0 ml of Ellman reagent (0.05 *M*) in 0.1 *M* phosphate buffer, pH 7.5, for 30 min at 24°C and then filtered. The absorbance of the filtrate at 412 nm was recorded. A value of E^{M}_{412} 13,600 was used for calculating the concentration of free sulphydryl on the resin.

Amino acid and protein contents were estimated either by UV spectroscopy or after acid hydrolysis (6 *M* at 110°C for 24 h) by an amino acid analyzer.

Nucleotide, nucleoside and RNA were estimated by UV spectroscopy. Enzyme activity was assayed with synthetic substrate at constant pH. The rate of hydrolysis was recorded using acetyltyrosine ethyl ester as substrate for α-chymotrypsin and benzoylarginine ethyl ester as substrate for trypsin. Assays were performed in 5.0 ml of substrate (0.01 *M*) in 0.1 *N* KCl at 24°C. Unit activity is defined as the amount of enzyme which will hydrolyze 1 μmole of substrate per minute at 24°C.

RESULTS AND DISCUSSION

The matrix

The method of preparing polyacrylhydrazido-polysaccharides including PAHOS and some derivatives is shown in Fig. 1. Column containing up to 125 μmole hydrazide per ml of Sepharose could be obtained. In most of our studies, columns containing 20–25 μmole/ml were used. The concentration of periodate used was $\leq$0.25 *M*. Lower concentrations of periodate had little effect on the capacity of the carriers since polyacrylhydrazides were coupled to the aldehydes and the amount of free hydrazide left was always high, independent of the amount of aldehyde formed. Higher concentrations of periodate may modify the polysaccharide and should not be used. This is in contrast to other hydrazide carriers prepared from periodate-oxidized polysaccharides and dihydrazide where the amount of free hydrazide available for reaction is dependent on the amount of aldehyde formed. Therefore more oxidant is required and one has to keep a balance between the destruction of the carrier and the coupling capacity.

After coupling of the polyacrylhydrazide the matrix was reduced with sodium borohydride. The reduction step was introduced to remove the excess of reactive aldehyde groups on the polysaccharide as well as to stabilize the hydrazone bond

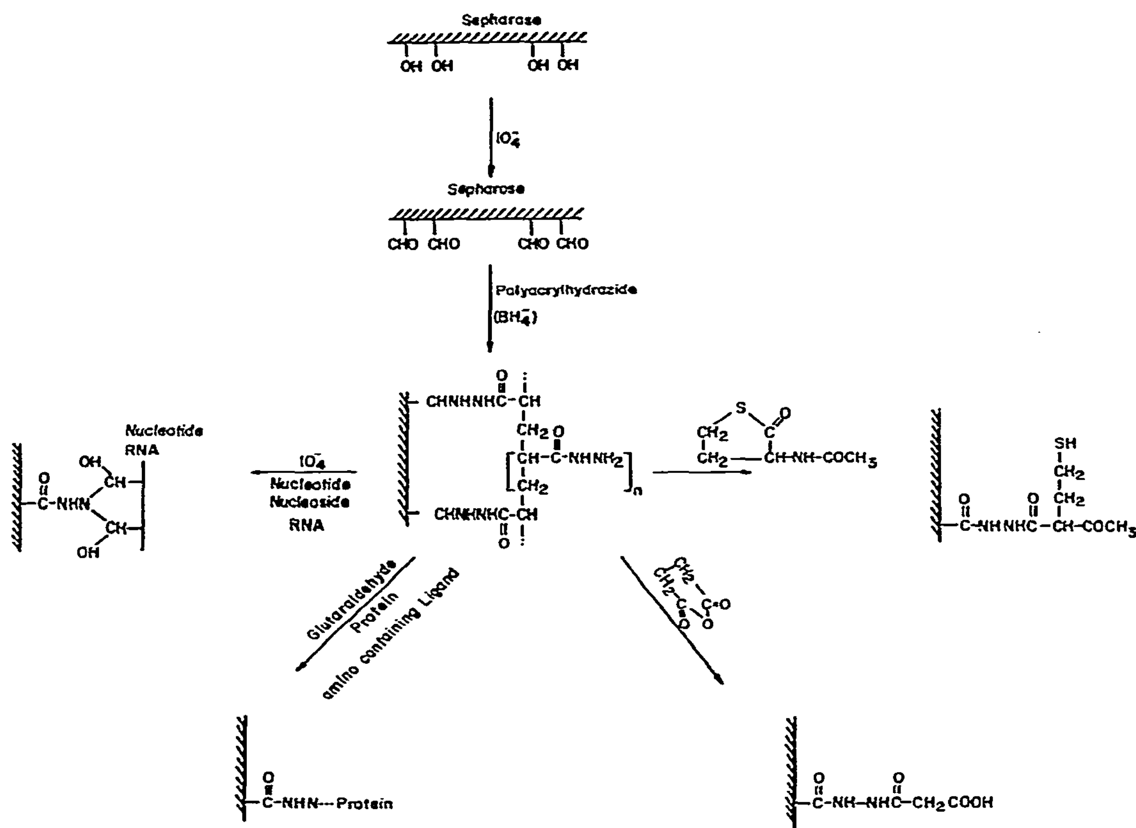


Fig. 1. Procedures for preparing derivatives of hydrazide agarose.

formed. The hydrazone bonds themselves do not need to be reduced as they are coupled by multiple bonds and thus are stable.

PAHOS prepared by this method has many advantages over PAHA prepared by the cyanogen bromide method. They are more stable, colourless and do not contain other functional groups such as carbamates and carbonates which are present on CNBr-activated polysaccharides. PAHOS kept for 2 years remained unchanged in binding capacity and other properties.

Matrix with spacers

PAHOS can be used directly for coupling of compounds containing aldehyde groups such as glutaraldehyde or periodate-oxidized nucleic acids, or can be modified further by reaction with different functional groups as described earlier for PAHA prepared by the CNBr method[2,9]. As can be seen in Fig. 1 and in Materials and Methods, carriers containing carboxyls, thiols, imidazoles, phenols and amines can be prepared. These reactive functional groups can be used to couple ligand and protein via amide bonds, thioesters, diazonium, etc. Carriers containing amino groups can be prepared in different ways either starting from PAHOS via several reaction steps such as succinylation followed by reaction with carbodiimides and amines, or directly from poly(lysine) coupled to the periodate-oxidized polysac-

charide, thus giving very stable and high capacity carriers containing amino groups[11]. The only disadvantage of using such columns is that they are charged and contain an excess of primary amino groups as well as secondary amino groups at the point of attachment to the polysaccharides, after borohydride reduction.

Coupling of proteins–enzymes

Proteins cannot be coupled directly to PAHOS. The PAHOS must first be treated with excess of glutaraldehyde thus incorporating free aldehyde groups that can be utilized for the coupling of proteins and other compounds containing amino groups. Glutaraldehyde-activated PAHOS can be stored for prolonged periods in NaN_3 (0.02%) and used when required. Several proteins were coupled to PAHOS; the amount coupled and conditions for coupling are given in Table I.

TABLE I

PREPARATION OF PROTEIN BOUND TO GLUTARALDEHYDE-ACTIVATED PAHOS

Ten milligrams of each protein were coupled to 1 g of activated gel in phosphate buffer (0.1 *M*), pH 7.5. Coupling was performed at 4°C overnight; binding of lectins was carried out in presence of the appropriate sugar.

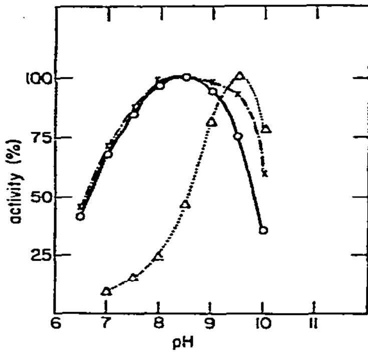
Protein	*mg protein bound per g resin*
α-Chymotrypsin	7.3
Trypsin	5.0
Ribonuclease A	9.8
Ovalbumin	3.5
DNP-RSA	10
Anti-DNP antibodies	10
Peanut lectin	10*
Concanavalin A	10*

* Coupling was done in presence of sugar.

A more detailed study was performed on α-chymotrypsin. The addition of 10 mg α-chymotrypsin to 1 g of wet glutaraldehyde-activated PAHOS resulted in the covalent attachment of 7.3 mg enzyme to the resin. The optimal pH for hydrolysis of acetyltyrosine ethyl ester by chymotrypsin did not change and remained as 8.5 (Fig. 2). Upon coupling the soluble enzyme to poly-Lys-Sepharose the optimum pH shift was from 8.5 to 9.5 (Fig. 2). The immobilized enzyme retained 70% of the specific activity of the soluble protease.

The α-chymotrypsin–PAHOS resin was stored at 4°C and 24°C in 0.02% NaN_3 for intervals up to 5 months, and the activity and protein content were determined periodically. The content of bound protein remained at 90–100%, and there was no decrease in specific activity. Parallel studies on enzyme bound to CNBr-activated Sepharose showed 50% leakage of protein over 5 months at 4°C.

The thermal stability of immobilized chymotrypsin was evaluated by heating in a 0.1 *M* phosphate buffer, pH 7.5, for 15 min at increasing temperature (25–70°C). The residual enzyme activity was determined by pH-stat titration. As seen in Fig. 3, immobilization of chymotrypsin on glutaraldehyde-derived PAHOS increases the thermal stability of the enzyme when compared with the soluble form. Reduction of



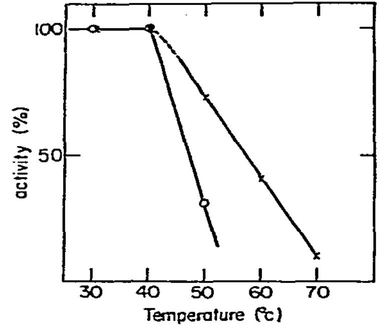


Fig. 2. pH–activity curves of α-chymotrypsin using acetyltyrosine ethyl ester as substrate. O—O, Native enzyme; ×—×, enzyme immobilized on PAHOS; △—△, enzyme immobilized on polylysyl-Sepharose. Assay conditions are described in Materials and Methods.

Fig. 3. Residual activity of free and immobilized chymotrypsin after heating for 15 min at different temperatures in 0.1 *M* phosphate buffer, pH 7.5. Activity was measured at pH 8.5, 25°C. O—O, Native α-chymotrypsin; ×—×, α-chymotrypsin bound to glutaraldehyde-activated PAHOS.

the chymotrypsin–PAHOS derivative with $NaBH_4$ results in some additional thermal stability for the enzyme–PAHOS complex.

Immobilization on PAHOS was found to stabilize chymotrypsin activity in the presence of 6 *M* urea. The immobilized chymotrypsin or the soluble enzyme was suspended in 6 *M* urea containing 0.1 *M* phosphate buffer, pH 7.5. At designated times, aliquots were removed from the reaction mixture and assayed at pH 8.5. As can be seen from Fig. 4, the enzymatic activity of chymotrypsin–PAHOS was maintained throughout the incubation period (20 h) whereas soluble chymotrypsin loses all its activity after 1 h.

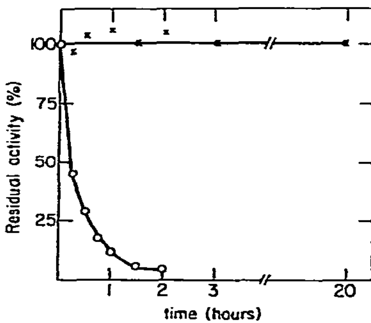


Fig. 4. Denaturation of α-chymotrypsin in 6 *M* urea. Fifty milligrams of immobilized enzyme were incubated in 1.0 ml of 6 *M* urea in 0.1 *M* phosphate buffer, pH 7.5, at 25°C. Samples of 0.1 ml were assayed at pH 8.5 for residual activity after incubation. Symbols as in Fig. 3.

Affinity chromatography

Coupling of lectins. Ten milligrams of peanut lectin were bound to 10 g of wet glutaraldehyde-activated PAHOS in the presence of 0.1 *M* D-galactose, 0.1 *M* $NaHCO_3$ and 0.14 *M* NaCl and reduced with $NaBH_4$. The activity of the reduced

PNA-PAHOS complex was ascertained by its ability to bind specifically asialo-fetuin. The adsorbed asialo-fetuin was released from the column with 0.5 *M* lactose in 0.1% Triton X-100. Other lectins could also be coupled similarly to PAHOS and used effectively to adsorb glycoprotein.

Coupling of antigens and antibodies. The purification of anti-DNP antibodies on dinitrophenyl-Sepharose columns[12] prepared by the CNBr methods yielded a yellow, partially inhibited antibody preparation containing up to 0.5 DNP residues per antibody. Binding DNP-rabbit serum albumin (DNP-RSA) to PAHOS resulted in 10 mg DNP-RSA bound per gram of wet resin. Serum containing anti-DNP antibodies was adsorbed batchwise on these columns. After incubation for 1 h the immunoadsorbent was washed with PBS until the absorbance at 280 nm was less than 0.02. The adsorbed antibodies were eluted with 0.1 *N* acetic acid at 24°C. The resulting clear, colourless and completely active antibody solution bound 1.5–2.0 hapten residues per antibody molecule, as determined by equilibrium dialysis[12]. The same DNP-RSA-PAHOS column was used several times for more than a year without loss of efficiency.

Goat anti-DNP antibodies were coupled to glutaraldehyde-activated PAHOS yielding 10 mg of coupled antibody per g of wet resin. The antibody column was used to purify DNP-containing peptides. Affinity chromatography of an enzymatic digest of DNP-labelled proteins[13] on the antibody PAHOS column resulted in a significant purification of the DNP-peptides. The ratio of the absorbance at 280 and 360 nm [$R_{(280/360)}$] for the peaks eluted with 6 *M* guanidine hydrochloride was 0.96, and was usually lower than the value for the applied tryptic digest sample, *ca.* 2.0. Chromatography of an identical sample of tryptic digest on anti-DNP antibodies coupled to CNBr-activated Sepharose with subsequent elution yields a peptide peak with $R_{(280/360)} = 2.05$. The increased ratio reflects leakage of antibody from the column under these elution conditions. The peptide eluted from the PAHOS column did not require any further purification other than removal of the guanidine.

α-Chymotrypsin. Low-molecular-weight compounds can also be coupled to PAHOS directly or after derivation and can be used for affinity chromatography. The purification of chymotrypsin was accomplished on D-tryptophan methyl derivatives of glutaraldehyde-activated or succinylated PAHOS. Application of commercially available samples of "pure" chymotrypsin to the above columns resulted in adsorption of 70–80% of the protein. The unbound protein was devoid of chymotryptic activity, while the adsorbed proteins, which were eluted with 0.1 *M* acetic acid, had three times the specific activity of the applied protein. Similar results were obtained for ε-aminocaproyl-D-tryptophan methyl ester bound to glutaraldehyde-activated or succinylated PAHOS. The chymotrypsin adsorption was specific as evidenced by the complete failure of trypsin and other proteins to adsorb to the resin.

CONCLUSIONS

PAHOS and its derivatives retain most of the properties of Sepharose, including minimal non-specific interactions with protein and good flow-rate. The derivatives also exhibit many of the properties of acrylamide, including absence of charged groups and a large number of modifiable groups. The combination of the agarose and acrylamide characteristics results in a resin with increased mechanical stability to

coupling and elution. The macromolecular polyacrylhydrazide spacers also provide greater separation of the ligand from the matrix than conventional spacers and enable the bound protein to retain the properties of the soluble protein. The uncharged, hydrophilic character of the polyacrylhydrazide results in the suppression of hydrophobic interactions by providing a polar environment for the immobilized protein. PAHOS prepared by periodate oxidation is also very stable, colourless and devoid of functional groups other than hydrazide and hydroxyl.

Although in this paper we have used mainly agarose as the polysaccharide backbone, other polysaccharides such as Sephadex and cellulose can be employed with high efficiency for immobilization of protein.

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