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POLYACRYLHYDRAZIDO–AGAROSE AS A SUPPORT FOR IMMOBILIZATION OF ENZYMES, LECTINS, ANTIBODIES, AND SMALL LIGANDS

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Enzymes, antibodies, lectins, and small ligands were coupled to glutaraldehyde-activated polyacrylhydrazido–agarose. High yields of binding and activity were obtained. The bound enzymes exhibited increased stability to heat and 6 M urea when 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 affinity chromatography purifications. Polyacrylhydrazido–agarose provides a new carrier that combines advantages of both agarose and acrylamide in one resin.

INTRODUCTION

Of the many methods available for immobilization of biologically active compounds (1), the most widely used is the cyanogen bromide method for activation of polysaccharides (2). However, the coupling of amines to the activated agarose introduces *N*-substituted isourea bonds that are not stable, especially in the presence of buffers containing nucleophiles (3). These columns can be stabilized by coupling the ligand through multiple sites to the resin. Recently we have applied this idea and have coupled polylysine to agarose, producing stable, high-capacity resins (4).

In order to overcome the inherent problems encountered by incorporating polycations, such as polylysine, into a resin, we prepared a noncharged, hydrophilic, and stable matrix with high capacity by coupling polyacrylhydrazide to cyanogen bromide-activated agarose (5).

In this study we demonstrate the activation of the polyacrylhydrazido–agarose (PAHA)[1] with glutaraldehyde and the coupling of enzymes, lectins,

[1] The abbreviations used are: PNA, peanut agglutinin; PAHA, polyacrylhydrazido–Sepharose (agarose); PBS, 0.01 M sodium phosphate buffer, pH 7.2, containing 0.9% NaCl; DNP-RSA, dinitrophenyl-rabbit serum albumin; TNBS, trinitrobenzene sulfonic acid.

antibodies, and small ligands to the columns. Their use in affinity chromatography and in solid-phase enzyme catalysis studies is described.

MATERIALS

Sepharose 4B was obtained from Pharmacia. Glutaraldehyde (50%), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl, cyanogen bromide, and succinic anhydride were purchased from Fluka D.G. Fetuin and [^{14}C]formaldehyde were obtained from Gibco and New England Nuclear, respectively. Trypsin and chymotrypsin were purchased from Worthington.

Dinitrophenyl–rabbit serum albumin (DNP-RSA) was prepared according to Eisen et al. (6). Goat, anti-DNP antibodies were prepared as described by Strausbauch et al. (7). Acetyltyrosine ethyl ester, benzoyl arginine ethyl ester, and tryptophan methyl ester were prepared by Israel Jacobson at the Weizmann Institute.

METHODS

Preparation of Polyacrylhydrazido–Agarose (PAHA)

Preparation of Polymethyl Acrylate. Sodium laurylsulfate (0.25 g), freshly redistilled methylacrylate (5 ml), 1% thioglycolic acid (1 ml), and ammonium persulfate (0.125 g) are added successively to 50 ml of distilled water under magnetic stirring (operated in a hood). The emulsion is refluxed for 2.5 h in a water bath (80°C) until the odor of methylacrylate nearly disappears. After polymerization, the emulsion is poured with stirring into 100 ml of ice cold water, to which 100 ml of cold 2 N HCl is added. The product, which coagulates in the form of white flakes, is kept for 30 min in the cold, and then washed by decantation with large amounts of cold water.

Preparation of Polyacrylhydrazide. Polymethylacrylate (5 g) is dispersed into 70 ml of hydrazine hydrate (98%). The reaction mixture is stirred vigorously and heated to 100°C in a boiling water bath. After 3 h a clear viscous solution is obtained. The reaction mixture is cooled to room temperature, the insoluble particles are filtered through gauze, and the filtrate is poured with constant stirring into 500 ml ice cold methanol containing 1 ml glacial acetic acid. The precipitate is filtered off, washed with cold methanol–acetic acid (500:1) and redissolved in 150 ml water. The insoluble particles are removed by filtration. This solution can be used immediately for coupling to Sepharose; can be washed with methanol followed by precipitation with ether; or can be freeze-dried, after care is taken to remove any trace of moisture to prevent cross-linking to the product.

Preparation of Polyacrylhydrazido–Agarose. Sepharose 4B is activated with cyanogen bromide. The activated Sepharose is suspended in three volumes of the above cold polyacryl hydrazide solution in water or 0.1 N $NaHCO_3$. The reaction is allowed to proceed overnight at 4°C with slow stirring. The conjugate is washed with 0.1 M sodium chloride, until the washings showed no color on reaction with trinitrobenzenesulfonic acid (TNBS). Columns containing up to 120 μmol of available hydrazide per milliliter agarose were obtained. Derivatives containing an average capacity of 15–25 μmol available hydrazide per milliliter of agarose were used for further substitutions.

The amount of hydrazide coupled to agarose was determined by reaction with an excess of TNBS. The quantity of unreacted TNBS was then measured and subtracted from its initial concentration. Samples (200 mg) of washed PAHA were suspended in 1.0 ml of TNBS solution (10 mg/ml) to which was added 1.0 ml of saturated tetrasodium borate solution. The suspension was reacted for 30 min at room temperature with stirring and then washed with 23 ml of 0.2 M NaCl. Aliquots (25, 50, and 100 μl) of the wash were incubated with 1.0 ml of adipic dihydrazide (0.1 M) + 1.0 ml of saturated tetrasodium borate for 15–20 min and the $A_{500\,nm}$ recorded. An extinction coefficient of 1.65×10^4 was used for calculating the quantity of TNBS that reacted with adipic dihydrazide.

Coupling of Proteins

The polyacrylhydrazido–agarose was derived with glutaraldehyde by suspending the resin in three volumes of 10% glutaraldehyde with slow stirring for 4 h. The resin was then washed with cold H_2O until no further odor of glutaraldehyde was detected or no further reaction with dinitrophenyl hydrazine was detected. The washed glutaraldehyde PAHA was then suspended in two to three volumes of 0.1 M sodium phosphate buffer, pH 7.5, containing the appropriate ligand (enzymes, DNP-RSA, and antibodies) at protein concentrations of approximately 5–10 mg/ml for 16 h at 4°C. The PNA lectin was coupled to the glutaraldehyde PAHA resin in 0.1 M $NaHCO_3$ + 0.9% NaCl + 0.1 M D-galactose. After slow stirring for 12 h at 4°C, the ligand–PAHA complex was washed with cold PBS. Reduction of ligand–PAHA complexes (only when indicated) was carried out in 1.5 volumes of PBS containing 0.5 mg/ml of $NaBH_4$ for 6 h at 4°C.

Determination of Bound Protein

Quantification of covalently bound protein to PAHA resin (wet weight samples) was performed on an amino acid analyzer after acid hydrolysis in

6 N HCl for 24–48 h at 110°C in vacuo. Quantities of bound peanut lectin were determined by measurement of unbound lectin absorbance at 280 nm in the washes.

pH Stat Enzyme Activity Assay

A pH stat assay of chymotrypsin activity at pH 8.5 and 10 was performed by addition of 100 μl aliquots of soluble enzyme (20 μg enzyme) or 100 μl of insoluble enzyme (5 mg insoluble enzyme complex/100 μl or 50 μg enzyme/100 μl) to 5 ml of acetyltyrosine ethyl ester (12 mM) in 0.2 M KCl at 25°C (flushed with nitrogen) using 0.1 M NaOH as a titrant.

The pH stat assay of trypsin activity was as above, utilizing benzoyl arginine ethyl ester as a substrate.

Preparation of [^{14}C]Asialofetuin

Terminal sialic acid residues were removed from fetuin following the method of Spiro and Bhoyroo (8). The asialofetuin was labeled with [^{14}C]formaldehyde utilizing the reductive methylation procedure of Rice and Means (9), and yielding $1–2 \times 10^6$ cpm/mg protein.

Coupling of D-Tryptophan Methyl Ester to Succinylated Polyacrylhydrazido–Agarose

Preparation of succinylated PAHA was performed according to Wilchek and Miron (5). Succinylated PAHA (1 g wet resin) was suspended in 2.0 ml water containing 10 mg D-tryptophanmethyl ester. To this suspension 125 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl was added. The pH was maintained at 5.0 while stirring for 20 h at 24°C. The resin was then washed successively with H_2O, *p*-dioxane:H_2O (1:1), and H_2O on a sintered glass funnel.

RESULTS

The Matrix

Polyacrylhydrazido–agarose can be derived by a number of different methods (5). Reaction with excess glutaraldehyde successfully incorporates free aldehyde groups into PAHA that can be utilized for the coupling of proteins and other compounds containing amino groups. The glutaraldehyde-activated PAHA can be stored for prolonged periods in NaN_3 (0.02%) and utilized when required for the coupling of ligands.

Coupling of Enzymes

The addition of 10 mg of chymotrypsin to 1 g of wet glutaraldehyde-activated PAHA resulted in the covalent attachment of 8.8 mg of chymotrypsin to the resin. The optimal pH for hydrolysis of acetyl tyrosine ethyl ester by chymotrypsin increased from 8.5 to 9.5 after coupling the soluble enzyme to PAHA. The immobilized enzyme maintained 70% of the specific activity of the soluble protease.

The chymotrypsin PAHA resin was stored at 4° and 24°C in 0.02% NAN_3 for intervals up to 5 months with periodic activity and protein determinations. Bound protein remained at 90–100% with no decrease in specific activity. In parallel studies on enzyme bound to Sepharose utilizing the CNBr method (2), the leakage of protein reached 50% over the 5-month period 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 temperatures (25–70°C) with the residual enzyme activity determined by pH stat titration. As seen in Fig. 1, immobilization of chymotrypsin on both CNBr-activated Sepharose and glutaraldehyde-derived PAHA increases the thermal stability of the enzyme when compared with the soluble form. Reduction of the chymotrypsin–PAHA derivative with $NaBH_4$ provides additional thermal stability for the enzyme–PAHA complex. Heating of the unreduced chymotrypsin–PAHA derivative was accompanied by a loss in the number of lysine residues (Table 1) without a loss of protein from the resin. This may indicate further reaction of the enzyme amino groups with free aldehyde groups on the resin at the elevated temperatures. The increased cross-linking of the protein with the resin may be the reason for the reduced enzymatic activity observed at elevated temperatures. The chymotrypsin–Sepharose prepared by the cyanogen bromide method probably lost its activity as a result of leakage of the protein from the matrix, since

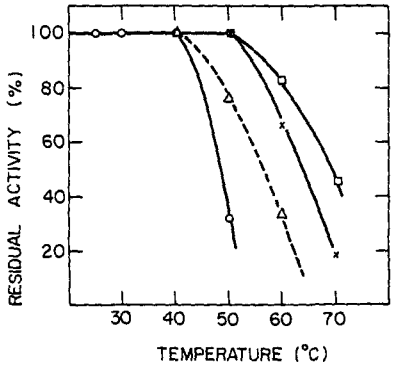


FIG. 1. Residual activity of free and immobilized chymotrypsin after heating for 15 min at different temperatures in 0.1 M phosphate buffer, pH 7.5. Activity was measured at pH 8.5 and 10.0 and 25°C. ○ denotes native α-chymotrypsin; □, α-chymotrypsin coupled to cyanogen bromide-activated Sepharose; △, α-chymotrypsin bound to glutaraldehyde-activated PAHA; ×, α-chymotrypsin bound to glutaraldehyde-activated PAHA and reduced with sodium borohydride.

TABLE 1. **Modification of Lysyl Residues on Heating of Unreduced Chymotrypsin Coupled to Glutaraldehyde-Activated PAHA**[a]

Chymotrypsin derivative	Lysyl residues per immobilized enzyme		Valyl residues per immobilized enzyme		Enzyme bound per ml carrier (mg)	
	Before heating	After heating	Before heating	After heating	Before heating	After heating
Chymotrypsin–Sepharose CNBr	13.5	14[b]	23	21	5.1	3.7
Unreduced chymotrypsin–PAHA	8.2	5.5	22	22	7.0	6.6
Reduced chymotrypsin–PAHA	8.2	8	22	22	7.0	6.7

[a] The heating conditions were 0.1 M phosphate buffer, pH 7.5, for 15 min at 70°C.
[b] Native α-chymotrypsin contains 14 lysine and 23 valine residues.

the number of lysine per milligram of immobilized enzyme did not change, but the quantity of bound protein was reduced by 30% (Table 1). Chymotrypsin, which leaks from the column like soluble enzyme, is immediately inactivated (Fig. 1).

Immobilization on PAHA was also 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 and 10. As can be seen from Fig. 2 the enzymatic activity of chymotrypsin–PAHA at pH 8.5 undergoes an initial activation upon incubation with 6 M urea. The enhanced enzymatic activity was maintained throughout the incubation period (40 h). The initial enzyme activation may be due to the exposure of hidden active sites by 6 M urea. When assayed at pH 10, the chymotrypsin–PAHA also exhibited a small initial activation. However, at this elevated pH the insoluble enzyme suffered a 30% activity loss during the first 60 min of incubation. The ratio of enzyme activity at pH 8.5 and 10.0 after 60 min of incubation in 6 M urea is the same for the chymotrypsin–PAHA complex as the ratio of native soluble enzyme. Apparently incubation of immobilized chymotrypsin in 6 M urea restores the pH activity profile of the insoluble enzyme to that of the soluble enzyme while maintaining the enhanced thermal stability and resistance to denaturation provided by immobilization. Chymotrypsin coupled to cyanogen bromide-activated Sepharose loses some of its activity, and the soluble chymotrypsin loses all its activity after 1.5 h.

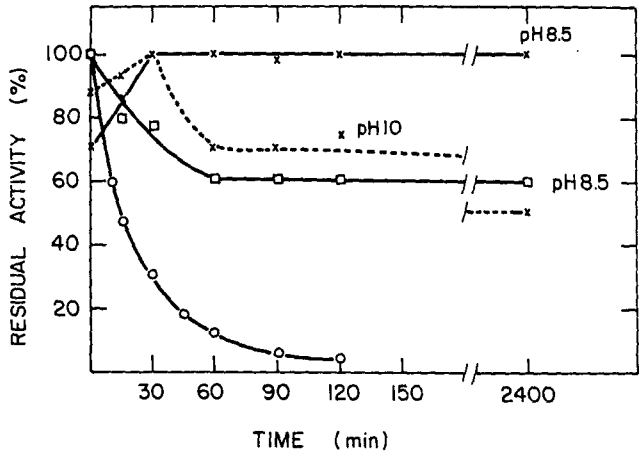


FIG. 2. Denaturation of α-chymotrypsin in 6 M urea. Fifty milligrams of immobilized enzyme was incubated in 1 ml of 6 M urea, 0.1 M phosphate buffer, pH 7.5, at 25°C. Samples of 0.1 ml were assayed at pH 8.5 and 10.0 for residual activity after incubation. ○ denotes native enzyme; □, enzyme coupled to cyanogen bromide-activated Sepharose; ×, enzyme coupled to glutaraldehyde-activated PAHA.

The incubation of 10 mg of trypsin per gram of wet glutaraldehyde-activated PAHA resulted in coupling of 8.0 mg of the enzyme to the resin. The pH of maximal activity was 10, which is 2.5 pH units higher than the pH of the enzyme in solution. The trypsin–PAHA complex had increased thermal stability and was stable to 6 M urea.

Coupling of Lectins

The lectin isolated from peanuts (10, 11) has been shown to bind galactose, lactose, and asialofetuin, but not the native sialylated fetuin. When 15.0 g of wet glutaraldehyde-activated PAHA was incubated with 100 mg of peanut lectin (PNA) in the presence of 0.1 M D-galactose and reduced with $NaBH_4$, approximately 95% of the added lectin coupled to the resin (6.4 mg lectin per milliliter of wet resin).

The activity of the PNA–PAHA complex was ascertained by the ability of the resin to specifically bind and release asialofetuin. A column of 15 ml of PNA–PAHA resin (Fig. 3A) adsorbed 61% of the applied [^{14}C]asialofetuin but did not bind or retard native fetuin. Application of 0.5 M lactose specifically released 50% of the bound [^{14}C]asialofetuin. Rechromatography of the bound and specifically eluted [^{14}C]asialofetuin (after dialysis to remove lactose) resulted in 96% retention of the applied material (Fig. 3B).

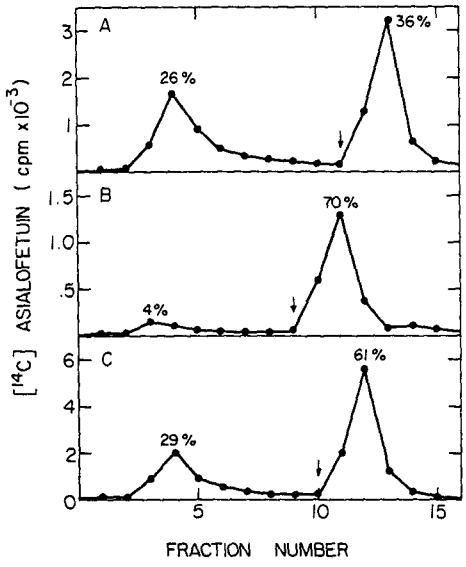


FIG. 3. Affinity chromatograph of [^{14}C]asialofetuin on PNA–PAHA. (A) A sample of [^{14}C]asialofetuin (20 ml PBS containing 2.0 mg specific activity, 1.5×10^6 cpm/mg) was applied to a column (1.8×9.5 cm) containing 15 ml of $NaBH_4$-reduced PNA–PAHA (6.4 mg PNA/ml resin) in PBS. The column was washed with PBS, affording a peak of radioactivity. Application of 50 ml of 0.5 M lactose in PBS (↓) specifically eluted a second peak of radioactivity. An aliquot (50 μl) from each fraction (10.0 ml) was counted by liquid scintillation. (B) The peak eluted with lactose from graph A was dialyzed against H_2O and then reapplied to the same column after the column was washed with PBS. The column was developed as above. (C) After washing the column with PBS containing 0.5% Triton X-100 a sample of [^{14}C]asialofetuin (1.0 ml of PBS–0.5% Triton X-100 containing 1.0 mg) was applied and the column developed as above, except all solutions contained 0.5% Triton X-100. Aliquots (100 μl) each fraction were counted. The radioactive content of each peak is recorded in terms of the percentage of the total radioactivity applied to the column.

The inability of lactose to give quantitative specific elution of [^{14}C] asialofetuin from the PNA–PAHA column may in part reflect the relatively low affinity of the lectin for the lactose haptene (10). However, the recovery of 88% of the bound [^{14}C]asialofetuin was obtained by specific elution with 0.5 M lactose in the presence of 0.1% Triton X-100, a nonionic detergent (Fig. 3C). The detergent by itself had no detectable ability to elute the receptor, nor did it interfere with asialofetuin adsorption to the column. The ability of Triton X-100 to increase receptor recovery has been observed by Adair and Kornfeld (12) and may reflect a contribution of hydrophobic forces to the specific interaction between the glycoproteins and the immobilized lectins. The PNA–PAHA columns retained lectin binding activity after multiple usage and storage for minimal periods of 6 months at 4°C, even in the presence of 0.5% nonionic and zwitterionic detergents. After elution of the columns with lactose, washing with PBS or PBS containing 0.5% detergent was sufficient for regeneration.

Coupling of Antigens and Antibodies

The purification of anti-DNP antibodies on dinitrophenyl–Sepharose columns (7) 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 PAHA resulted in 16 mg of DNP–RSA bound per gram of wet resin. Serum containing anti-DNP antibodies were adsorbed batchwise on this column. After a 1-h incubation the immunoadsorbent was washed with PBS until the adsorbance at 280 nm was less than 0.05. The adsorbed antibodies were eluted with 0.1 N acetic acid at 24°C or with 0.2 M NH_4OH. The resulting clear, colorless, completely active antibody solution bound 1.5 to 2.0 haptene residues per molecule, as determined by equilibrium dialysis (7). The same DNP-RSA–PAHA column was used several times for more than a year without loss of efficiency.

The goat anti-DNP antibodies (12 mg) were incubated with 1 g of wet glutaraldehyde-activated PAHA, yielding 10 mg of coupled antibody. This antibody column could be used to purify DNP-containing compounds efficiently; e.g., affinity chromatography of a tryptic digest of DNP-labeled ribonuclease (13) on the antibody–PAHA column resulted in a significant purification of DNP peptides. The ratio of adsorbance at 280 and 360 nm [R(280/360)] of the peak eluted with 6 M guanidine HCl (0.96) was lower than the R(280/360) of the applied tryptic digest sample (1.96). Chromatography of an identical sample of tryptic digest material on anti DNP-antibodies coupled to CNBr-activated Sepharose with subsequent elution yields a peptide peak with $R(280/360) = 202$. The increased ratio

reflects leakage of antibody from the column under these eluting conditions. The peptide eluted from the PAHA column was pure and did not require any further purification other than removal of the guanidine.

Coupling of Small Ligands

Low-molecular-weight compounds can also be coupled to PAHA directly or after derivation. Several derivatives of PAHA with different functional groups were prepared (5). The latter were the same as those prepared from adipic dihydrazide–Sepharose and aminoalkyl–Sepharose (5, 14), and were used for affinity chromatography studies. The purification of chymotrypsin was accomplished on D-tryptophan methyl derivatives of glutaraldehyde-activated or succinylated PAHA. 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 twice the specific activity of the applied protein (Table 2). The same commercial enzyme preparation purified on ε-aminocaproyl-D-tryptophan methyl ester–Sepharose (15) showed only a 40% increase in specific activity over that of the starting material. The chymotrypsin adsorption was specific, as evidenced by the complete failure of trypsin, ribonuclease, and subtilisin to adsorb to the resin.

DISCUSSION

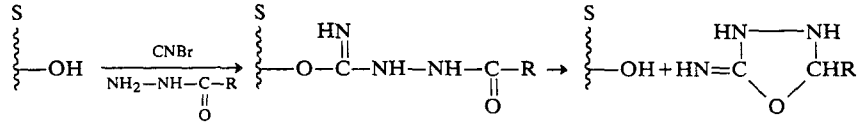
The introduction of monovalent hydrazido groups into agarose columns eliminates the undesirable effects of charge and hydrophobicity due to the formation of *N*-substituted isourea groups during CNBr activation of

TABLE 2. **Affinity Chromatography of α-Chymotrypsin**[a]

Sepharose derivative	Adsorbed (%)	Eluted (%)	Specific activity (units/mg protein)	Purification factor
Sepharose	0	—	33	1.0
Sepharose-ε-aminocaproyl-D-Trp methyl ester (CNBr)	90	100	46	1.4
PAHA-D-Trp methyl ester (glutaraldehyde)	70	100	65	2.0

[a] One gram of each adsorbent was filled into a Pasteur pipette. The column was equilibrated with 0.05 M Tris-HCl buffer, pH 8.0. One milligram of α-chymotrypsin was applied in the same buffer. One-milliliter fractions were collected and assayed. α-Chymotrypsin was eluted with 0.1 M acetic acid.

Sepharose. However, the monovalent hydrazide derivatives of agarose still suffer from leakage problems; both amines and hydrazides give rise to slow leakage of ligand when coupled through one group to the resin. This leakage effect, which is most pronounced in hydrazides coupled to Sepharose, possibly proceeds through a cyclization mechanism:



The coupling of polyamines, such as polylysine (4), proteins (16), or polyhydrazides (5), to agarose through multisite attachments followed by derivation of the polyamino or polyhydrazido agarose successfully overcomes most leakage problems. However, leakage of polymeric amines and hydrazides may occur upon excessive substitution of the Sepharose, resulting in polymers coupled through one reactive group. This effect is most prevalent when small ligands are coupled to the column. A further stabilization of proteins coupled to Sepharose is achieved by cross-linking with glutaraldehyde. Thus, chymotrypsin and trypsin coupled to glutaraldehyde-activated PAHA were completely stable (90–100% retention of bound protein and specific activity) for periods up to 5 months, while a 50% loss of enzyme protein was detected for resins prepared by the CNBr method over the same time period. Similarly, peanut lectin and antibody coupled to the glutaraldehyde derative of PAHA was found to be stable to nonionic and zwitterionic detergents and guanidine HCl, respectively, even after *6 months* of storage and repeated usage.

We observed that additional stabilization of protein coupled to PAHA through the glutaraldehyde linkage could be obtained by sodium borohydride reduction of the protein–resin complex. The stabilization is not only due to reduction of the Schiff base formed, but also to the elimination of excess aldehyde groups that may react with unmodified amino groups of the protein. The continued modification of the proteins can result in loss of activity due to denaturation or possible reaction with active-site amino acid residues. Unreduced chymotrypsin–PAHA complexes lost proteolytic activity at elevated temperatures with a parallel loss in the number of lysine amino groups (Table 1), while the reduced complex was found to be more stable to heat denaturation, with no reduction in the number of free lysine.

An increase in the pH required for optimal enzyme activity has been observed for a number of different immobilized enzymes (17). We also observed this effect after coupling of trypsin and chymotrypsin to PAHA. The initial shift in the pH of optimal proteolytic activity from 8.5 to 10.0 upon coupling of chymotrypsin to PAHA was maintained after months of

storage. However, treatment of chymotrypsin or trypsin coupled to glutaraldehyde-activated PAHA or CNBr-activated Sepharose with 6 M urea for a few hours permanently lowered the pH of optimal activity back to the soluble enzyme requirements. The reason for this urea-induced lowering of the pH optimum is not understood, but it is advisable to incubate immobilized enzymes that are stable to urea with urea in order to duplicate assay conditions of the soluble enzymes. At elevated pHs there can be significant hydrolysis of ester substrates by basic catalysis.

In summary, the PAHA derivatives retain most of the properties of Sepharose, including minimal nonspecific interactions with proteins 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 the conditions of 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 maintain the properties of the soluble protein. The noncharged, hydrophilic character of the polyacrylhydrazide results in the suppression of hydrophobic interactions by providing a polar environment for the immobilized protein.

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