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Polyacrylamide Gels Copolymerized with Active Esters. A New Medium for Affinity Systems†

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ABSTRACT: A new and versatile method for linking biologically active ligands to a polyacrylamide matrix is reported. Active esters of acrylic acid (*N*-succinimidyl acrylate and *N*-phthalimidyl acrylate) were synthesized, then copolymerized with acrylamide and *N,N'*-methylenebisacrylamide. Displacement of the active ester in the gel thus formed by various ligands containing aliphatic amino groups resulted in the formation of stable amide bonds between the ligands and the polyacrylamide gel. The affinity gel thus prepared has the following advantages: (i) resistance to chemical and microbiological degradation, (ii) ease of control of ligand level and higher levels of ligand possible, (iii) ease of control of porosity, and (iv) total displacement of the active ester under suitable conditions. Efficacy of this system was tested by preparation of 6-aminohexyl 2-acetamido-2-deoxy-β-D-glucopyranoside derivative of polyacrylamide gel by the described method. It was found to be more effective for purification of wheat germ agglutinin than the previously published affinity chromatography systems and the wheat germ hemagglutinin was obtained in crystalline form. In addition, partial resolution of isolectins was obtained by elution from the affinity gel with a pH gradient.

Immobilization of biologically active components has become an important tool in biological research, especially in its application to affinity chromatography for purification of biological molecules. Some of the variables for such immobilization are the insoluble support used and the type of bonding of biologically active compounds to that support. The use and comparative advantages of agarose, cellulose, dextran, glass, and polyacrylamide as insoluble carriers have been reviewed (Silman and Katchalski, 1966; Cuatrecasas and Anfinsen, 1971; Scouten, 1974). The most popular among the systems currently in use are the polysaccharides (especially agarose beads) activated by cyanogen halide treatment to accept ligands with amino terminals. However, this system has the disadvantages that the polysaccharide is labile to attack by chemicals as well as microbes, and the bonds linking the polysaccharides and the biologically active compounds are alkali labile.

Some of these disadvantages were absent in the system developed by Inman and Dintzis (1969), in which commercially available preformed polyacrylamide beads (Bio-Gel) were modified to provide acyl azide and other active functional groups suitable for coupling ligands. Although this method led to high capacity of the carrier beads for the ligands, porosity was prohibitively reduced, greatly decreasing the effectiveness of the gel in macromolecule purification (Cuatrecasas, 1970; Steers et al., 1971).

In a preliminary investigation, we have successfully formed polyacrylamide gels containing desired ligands by first coupling acrylic acid to the amino terminal of the ligands, and then copolymerizing them with acrylamide and cross-linking reagent (Lee, 1973). This approach has an obvious advantage of allowing easy control of porosity as well as level of ligand incorporation. In addition, the total level of ligand that can be incorporated can be made considerably higher than by the use of CNBr-activated polysaccharides. In a similar approach that has been reported independently (Hořejší and Kocourek, 1974), allyl glycosides were copolymerized with acrylamide to form an affinity gel system useful for purification of plant hemagglutinins. Although these methods overcame most of the disadvantages discussed above, the necessity of synthesizing individual ligands containing a double bond discourages general application of this method.

The objectionable features of the previous methods were resolved in the present studies. The acrylic acid esters of *N*-hydroxysuccinimide and *N*-hydroxyphthalimide were synthesized and copolymerized with acrylamide and *N,N'*-methylenebisacrylamide. The resulting "active" gels reacted readily with ligands containing primary amino groups (Scheme I). Thus, the present method provides a new and versatile system of wider applicability than the earlier systems. The applicability of this system to affinity chromatography is demonstrated by efficient purification of wheat germ hemagglutinin on polyacrylamide gel in which 6-aminohexyl 2-acetamido-2-deoxy-β-D-glucopyranoside was incorporated by the new method.

Experimental Section

Materials. Acryloyl chloride and *N*-hydroxyphthalimide were purchased from Aldrich Chemical Co. and used with-

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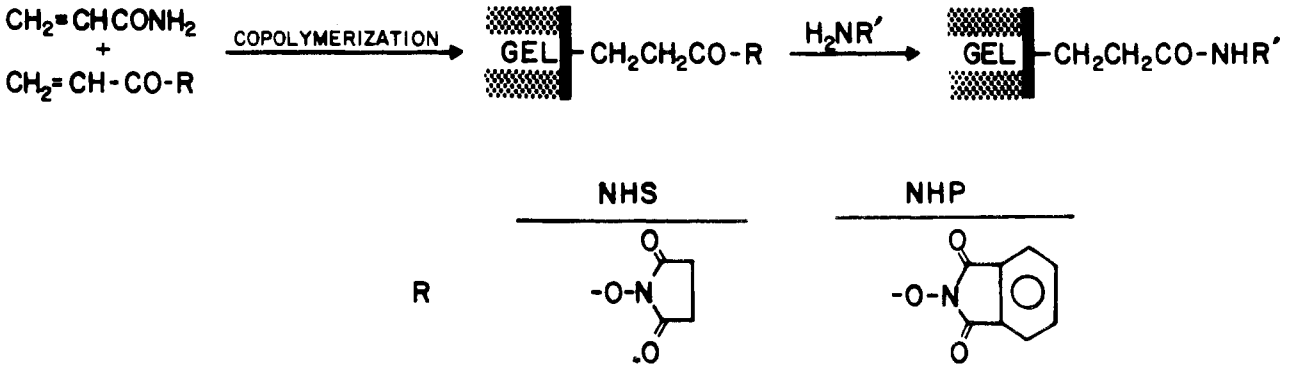
Scheme I



out purification. *N*-Hydroxysuccinimide was purchased from Pierce Chemical Co. and recrystallized twice from hot ethanol. 6-Aminohexyl 1-thio-β-D-galactopyranoside (Chipowsky and Lee, 1973) and 6-aminohexyl 2-acetamido-2-deoxy-β-D-glucopyranoside (Barker et al., 1972) were prepared by the described methods. 6-Aminohexyl β-D-galactopyranoside and *N*-(6-aminohexyl)-D-galactonamide were kindly supplied by Dr. Makoto Naoi, and 2-[6-(6-aminohexanamido)hexanamido]ethyl 1-thio-β-D-galactopyranoside (AHA-AHA-AES-β-Gal)[1] (Lee and Lee, 1974) was a gift from Dr. Reiko Lee. Commercial wheat germ was obtained locally.

Analytical Methods. Melting points (uncorrected) were determined on a Fisher-Johns apparatus. All evaporations were performed under diminished pressure at 30–45° with a rotary evaporator. Proton magnetic resonance (PMR) spectra were recorded with a JEOL NMH-100 spectrometer. Elemental analyses were performed by Galbraith Laboratories (Knoxville, Tenn.). Thin-layer chromatography (TLC) was performed with silica gel F-254 (Merck) precoated on aluminum sheets, using 6:1 (v/v) benzene–diethyl ether as a developing solvent. Components were detected on TLC by uv illumination (fluorescence quenching) and I_2 vapor.

Galactose was determined by a modified phenol–sulfuric acid method (McKelvy and Lee, 1969), *N*-(6-aminohexyl)-D-galactonamide by periodate oxidation followed by formaldehyde determination (Hanahan and Olley, 1958), and 2-amino-2-deoxy-D-glucose on an automated sugar analyzer (Lee, 1972). Protein was determined by the method of Lowry et al. (1951), using bovine serum albumin as the standard, or by absorbance at 280 nm on a Beckman DU spectrophotometer. Wheat germ agglutinin activity was determined as described previously (Shaper et al., 1973). Polyacrylamide gel electrophoresis at pH 3.8 using 7.5% gels was performed according to the procedure of Maurer (1971); protein bands were detected with Amido Black.

Results

Determination of N-Hydroxysuccinimide (NHS) and N-Hydroxyphthalimide (NHP). NHS and NHP were measured by either of two methods. The first is based on a method for determination of hydroxamate (Lipmann and Tuttle, 1945); 1 ml of sample solution (containing 0.1–2.0 μmol of NHS or NHP) was mixed with 1 *N* NaOH (0.1 ml) and incubated for 10 min at 60°; the reaction mixture was neutralized by addition of 1 *N* HCl (0.1 ml), and treated with 0.85 *N* HCl (1 ml) and 5% $FeCl_3$ in 0.1 *N* HCl (0.5 ml). After thorough mixing, the absorbance was immediately measured at 500 nm with a Bausch and Lomb "Spectronic 20" spectrophotometer. The absorbance resulting from the hydroxamate–ferric chloride assay was proportional to hydroxamate concentration in the range between 0.1 and 2.0 μmol. NHS yielded an absorbance of 0.37 per μmol, and NHP, 0.24 per μmol in a cuvet of 1-cm light path under these conditions (Figure 1A).

NHS and NHP could also be measured with a modified Folin method (Lowry et al., 1951). Sample solutions were adjusted to 0.1 *N* NaOH, and 0.5-ml aliquots (containing 0.1–1.0 μmol of NHS or NHP) were heated at 60° for 10 min. The alkaline copper reagent (5 ml) was added, and after 10 min at room temperature, 0.5 ml of Folin phenol reagent (diluted 1:1 with water) was added. Absorbance was determined at 700 nm on a "Spectronic 20" spectrophotometer.

Using the modified Folin method, the absorbance was proportional to hydroxamate concentration in the range from 0.1 to 1.0 μmol, and 1.0 μmol of NHS and NHP gave absorbance values of 0.76 and 1.03 (1-cm light path), respectively, under the described conditions (Figure 1B).

In either assay, longer base hydrolysis (up to 1 hr) did not appear to affect the color yield. Although the modified Folin method was 2–4 times more sensitive than the hydroxamate method, the latter was useful when interfering substances were present which prohibited use of the Folin reagent.

N-Hydroxyphthalimide degrades slowly in aqueous solution to substances not measurable by the above methods. Thus, when a fresh solution of NHP was stored for 1 and 4 days at room temperature, the NHP content decreased 20 and 80%, respectively. This phenomenon precluded the use of the assay method in experiments conducted with aqueous solvents over long time intervals. *N*-Hydroxysuccinimide, on the other hand, was stable under these conditions, and was measurable.

Synthesis of N-Succinimidyl Acrylate (NHS-acrylate). A mixture containing NHS (4.64 g, 40 mmol) and acryloyl chloride (18 ml, 220 mmol) was refluxed with vigorous stirring for 3 hr in an anhydrous atmosphere and the reaction mixture, a homogeneous solution, was evaporated to a syrup. Water (100 ml) was added to the syrup and the mixture was stirred for 30 min at 4°. Upon addition of chloroform (100 ml), the layers were separated, and the organic layer was extracted successively with water (100 ml each time) until the pH of the water layer was approximately 5.

[1] Abbreviations used are: AHA-AHA-AES-β-Gal, 2-[6-(6-aminohexanamido)hexanamido]ethyl 1-thio-β-D-galactopyranoside; NHS, *N*-hydroxysuccinimide; NHP, *N*-hydroxyphthalimide; NHS-acrylate, *N*-succinimidyl acrylate; NHP-acrylate, *N*-phthalimidyl acrylate; WGA, wheat germ agglutinin.

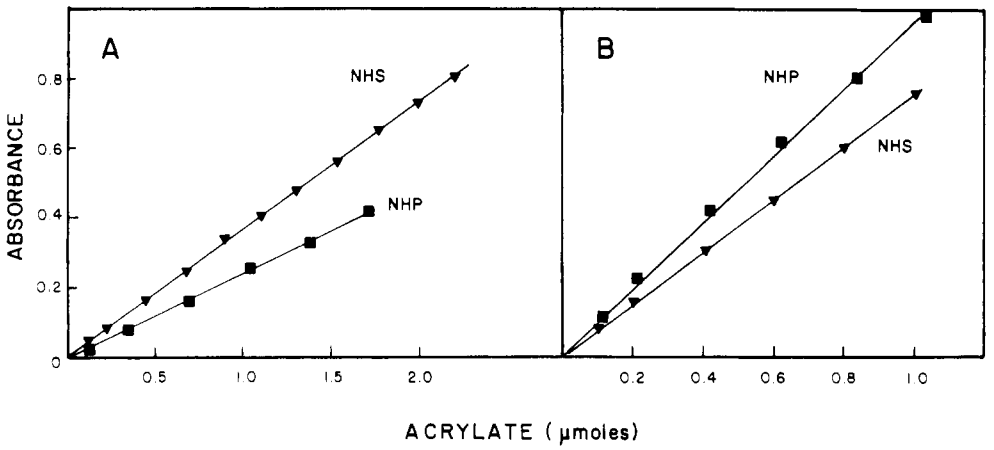


FIGURE 1: Determination of *N*-hydroxysuccinimide and *N*-hydroxyphthalimide by the hydroxamate-ferric chloride method (A) and by the modified Folin method (B), as described in the text.

The aqueous solutions were combined and extracted once with chloroform (50 ml); the chloroform solutions were combined, dried over sodium sulfate, and evaporated to a syrup. Crystals, obtained by storing the syrup overnight at −20°, were triturated with diethyl ether, and harvested by filtration. Recrystallization from absolute ethanol yielded 2.3 g (34%) of the desired product. Mp 60.5–62.0°, TLC showed a single spot (R_f 0.34). Anal. Calcd for $C_7H_7NO_4$: C, 49.71; H, 4.17; N, 8.28. Found: C, 49,56; H, 4.21; N, 8.23. PMR data (Me_2SO): δ 6.3–6.7 (m, 3, signals from CH_2=CH—CO) and 2.8–2.9 (m, 4, methylene protons).

Synthesis of N-Phthalimidyl Acrylate (NHP-acrylate). NHP (3.26 g, 20 mmol) was dissolved in tetrahydrofuran (120 ml). Acrylic acid (1.78 ml, 26 mmol) and dicyclohexylcarbodiimide (5.78 g, 28 mmol) were added to the solution, and the mixture was stored overnight at 0°. Dicyclohexylurea was removed by filtration, and filtrate was evaporated to a syrup, dissolved in chloroform (25 ml), and stored overnight a 4°, and additional dicyclohexylurea was removed by filtration. The filtrate was extracted with saturated sodium bicarbonate (25 ml), and evaporated to dryness, yielding a crystalline solid. The latter was recrystallized twice from ethanol, yielded 2.39 g (55%) of the desired product. Mp 119–121°; TLC showed a single spot; R_f 0.62. Anal. Calcd for $C_{11}H_7NO_4$: C, 60.83; H, 3.25; N, 6.45. Found: C, 60.73; H, 3.30; N, 6.39. PMR data ($CDCl_3$): δ 6.1–6.8 (m, 3, signals from CH_2=CHCO) and 7.7–7.9 (m, 4, aryl protons).

Polymerization with NHS-acrylate as a Comonomer. A solution of NHS-acrylate (1.15 g/20 ml of ethanol) was added to a mixture containing 15 g of acrylamide and 0.75 g of *N,N'*-methylenebisacrylamide in 37.5 ml of 0.1 *M* sodium phosphate buffer (pH 6.0). After thorough mixing, the solution was diluted with water (31.5 ml), and treated with 4% (v/v) tetraethylmethylenediamine (1.0 ml) and 5.6% (w/v) ammonium persulfate (10.0 ml). The solution was mixed vigorously, placed into a 100-ml graduated cylinder (2.5 cm diameter) and allowed to polymerize at room temperature for 20–30 min. The resulting gel was cut into 1–2-cm pieces, and homogenized with 4 volumes of cold water in a Virtis homogenizer for 15 min at medium speed. The gel particles were filtered and dehydrated by stirring with 10 volumes of dry dimethylformamide for 2 hr at 4°. The particles were filtered and the dimethylformamide treatment was repeated. The filtered particles were then stored under fresh, dry DMF at 4°. The "active" gel particles could be stored under these conditions for at least 6 months without detectable decrease in the concentration of active ester.

The level of copolymerized NHS was measured after alkaline hydrolysis and expressed in micromoles of active ester per milliliter of fully rehydrated gel. The dehydrated gel particles (ca. 50 mg) were filtered, accurately weighed, and treated with 0.5 *M* NaOH (1 ml) for 1 hr at 55°, and released NHS was measured by one of the methods described above. One gram of dehydrated gel particles corresponded to 5.7 ml of rehydrated gel. Under these conditions, 35 μmol of NHS was found to be present/ml of rehydrated gel.

Gels with higher level of incorporated active ester were synthesized by increasing the ratio of the active ester to the other acrylamide derivatives. For example, the gel formed from the following solution contained 130 μmol of active ester/ml of rehydrated gel: 30% (v/v) ethanol; 15% (w/v) acrylamide; 0.75% (w/v) *N,N'*-methylenebisacrylamide; 6.4% (w/v) NHS-acrylate; 0.56% (w/v) ammonium persulfate; and 0.05% (v/v) tetraethylmethylenediamine in 0.1 *M* sodium phosphate buffer (pH 6.0).

Polymerization with NHP-acrylate as a Comonomer. Gels containing NHP-acrylate were formed at 60° because of the low solubility of NHP-acrylate in aqueous solvents. The gel-forming solution contained the following ingredients in 0.1 *M* sodium phosphate buffer (pH 6.0): 40% (v/v) ethanol, 15% (w/v) acrylamide; 0.75% *N,N'*-methylenebisacrylamide; 4.0% (w/v) NHP-acrylate; 0.05% (v/v) tetraethylmethylenediamine; and 1.0% (w/v) ammonium persulfate. The resulting gel was homogenized, dehydrated, and a portion hydrolyzed as described above. Under these conditions, the gel contained 75 μmol of active ester/ml of rehydrated gel.

Incorporation of Ligands into the "Active" Gels in Dry N,N'-Dimethylformamide. The NHS gel, stored under *N,N'*-dimethylformamide, was filtered, washed with fresh *N,N'*-dimethylformamide, and suspended in a dimethylformamide solution containing the desired ligand. The mixture was stirred at 50° in a tightly capped vial, using a magnetic stirrer. During the reaction period, the solution was sampled periodically, and NHS released from the gel was determined. When the release of NHS had apparently ceased, additional ligand was added. After the reaction was complete as determined by the released NHS in the solution, the gel was removed from the reaction mixture by filtration or centrifugation. The gel was then washed by stir-

Table I: Incorporation of Ligands into Polyacrylamide Gels Copolymerized with Active Esters.

Ligands	Dehydrated Gels (g)	Dimethyl-formamide (ml)	Reaction Time (hr)	Total NHS (μmoles)	Ligands Incorporated (μmol)	Efficiency %
$NH_2(CH_2)_6$-*S*-β-Gal. (12.6 μmol)	0.049	1	10	5.05	5.08	101
$NH_2(CH_2)_6$-O-β-Gal (8.8 μmol)	0.043	1	10	4.55	4.36	96
$NH_2(CH_2)_6$-O-β-GlcNAc (850 μmol)	3.49	13	36	479	484	99
N-(6-Aminohexyl)-D-galactonamide (840 μmol)	3.49	90	152	700	716	102
AHA-AHA-AES-β-Gal (850 μmol)	3.49	32	96	535	440	75

ring in 10 volumes of 0.1 *N* HCl for 30 min, followed by centrifugation to remove the soluble components, and the washing procedure was repeated twice.

The levels of 1-thioglycosides incorporated into the gels were measured by determining the sugar released after cleavage. Gels (ca. 0.25 ml of rehydrated gel) containing 1-thioglycosides as ligands were incubated for 30 min with 2 ml of 0.1 *M* mecuric acetate in 0.1 *M* acetic acid. Gels containing *O*-glycosides (ca. 0.25 ml of rehydrated gel) were heated for 2 hr at 100° with 2 *N* HCl (2 ml). The free sugars were then measured as described in the Experimental Section. Results obtained with the gel containing NHS-acrylate are shown in Table I. The gel containing NHP-acrylate was tested with 6-aminohexyl β-D-galactopyranoside in a similar way and was found to incorporate the ligand quantitatively.

Incorporation of Ligands in Aqueous Solution. In each of a series of screw-capped test tubes (13 × 100 mm) was placed 40–50 mg (accurately weighed) of NHS gel stored under dry dimethylformamide. Solutions (1 ml) of 6-aminohexyl β-D-galactopyranoside (20% excess over the determined NHS level) in buffers ranging from pH 7.0 to 10.5 were added to the tubes, and the suspensions were stirred overnight at 4°. After centrifugation, aliquots of the supernatant fluids were removed for measurement of NHS released during the reaction. The gels were washed three times with 5 ml of 0.1 *N* HCl and then the galactose was released by incubation in 2 *N* HCl at 100° for 2 hr. An aliquot of the hydrolysis solution was removed and galactose determined. Results are expressed as the ratios between 6-aminohexyl β-D-galactopyranoside covalently bound and NHS released (Figure 2). The maximum ratio in aqueous systems under the above conditions (0.27 at pH 8.5) was far below that obtained in dimethylformamide (quantitative) when the same ratio of ligand to NHS was used. The gels containing NHP-acrylate did not exchange as efficiently in aqueous systems, giving an approximate maximum ratio of 0.12 at pH 8.0 under the same conditions.

To determine the effect of pH on hydrolysis of the *N*-hydroxysuccinimide ester, tubes were prepared as above with the exclusion of ligand. The tubes were centrifuged after 15 min and aliquots of the supernatant removed for measurement of NHS released. The results indicated that 1.7, 6.2, 9.8, and 57% of the NHS were released during this period at pH 7.1, 8.0, 9.0, and 9.9, respectively.

In an effort to compete more effectively for the active ester, addition of a larger excess of ligand to the reaction mixture was tested as follows. The reactions were done as above except that the buffer was always 0.05 *N* sodium barbiturate (pH 8.5), and the ratio of ligand to NHS was varied from 0.84 to 4.72. The results, shown in Figure 3, indicate that at the higher levels of ligand, the ratio of incorporated galactoside ligand to NHS released ("efficiency"), approached 0.70. By further increasing the ratios of ligand/active ester to 21:1 and 54:1, the efficiency was increased to 0.77 and 0.92, respectively.

Application of the Active Gel—Purification of Wheat Germ Agglutinin. Usefulness of the new gel system described here was tested by its application to purification of wheat germ hemagglutinin (WGA). All operations were carried out at 4°. Raw and untreated wheat germ (500 g) was extracted twice with petroleum ether (bp 40–60°, 2 l. each), then twice with acetone (2 l. each). The residue was air dried and pulverized in a Waring Blendor, and the powder was extracted with water (5 l.) overnight. The crude extract (3.82 l.) was obtained by filtering the mixture through nylon mesh followed by centrifugation (5,000*g*, 15 min). The extract was mixed with solid ammonium sulfate (306 g, 15% of saturation); the mixture was gently stirred for 2 hr and centrifuged at 10,000*g* for 15 min to remove precipitate. The resulting supernatant fluid (3.96 l.) was then mixed with additional ammonium sulfate (939 g, 55% of saturation); the solution was stirred for 2 hr and centrifuged at 10,000*g* for 15 min. The pellet thus obtained was suspended in cold water (750 ml) and insoluble material was removed by centrifugation at 10,000*g* for 20 min.

Affinity chromatography was also performed in the cold. The supernatant fluid (760 ml) was mixed with 4.7 ml of polyacrylamide gel containing 6-aminohexyl 2-acetamido-2-deoxy-β-D-glucopyranoside (see Table I), and the mixture was stirred overnight, after which no agglutinating activity could be detected in the solution. Celite (4 g) was added to the suspension and the gel–Celite mixture was collected by centrifugation, resuspended in 1 *M* NaCl containing 0.01 *M* sodium phosphate (pH 6.7) (100 ml), and packed into a column (2.5 cm i.d.) over a 0.5-cm layer of washed Celite, the final column height being 3.5 cm. The column was washed with 1 *M* NaCl containing 0.01 *M* sodium phosphate buffer (pH 6.7) at a flow rate of 35 ml/hr, until the absorbance at 280 nm decreased to a background

[2] Hexosaminidase assay was carried out by incubating purified WGA (200 μg), *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (3.6 m*M*), and sodium citrate, pH 4.0 (5.5 m*M*) in a final volume of 0.55 ml at 37° for 2 hr. Absorbance at 400 nm was measured after addition of 0.2 *M* sodium carbonate (2 ml).

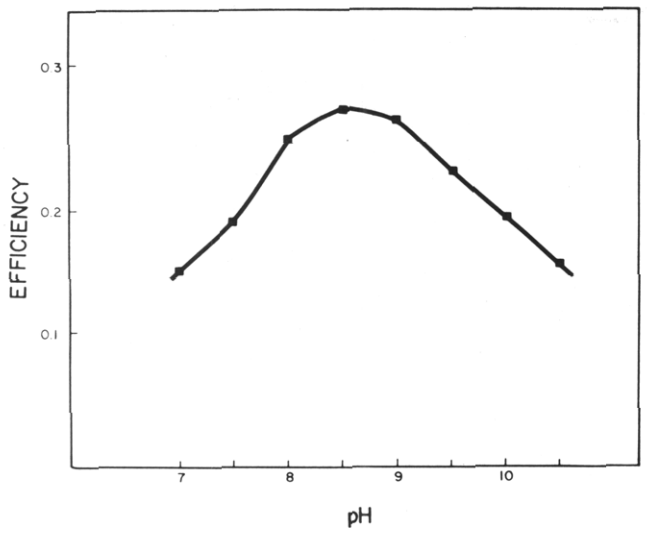


FIGURE 2: Ligand incorporation into NHS-acrylate polyacrylamide gel in aqueous solutions as a function of pH. The ligand, 6-aminohexyl β-D-galactopyranoside (20% excess), was dissolved in the following buffer solutions: pH 7.0 and pH 7.5, 0.1 *M* sodium phosphate; pH 8.0, pH 8.5, and pH 9.0, 0.05 *M* sodium barbiturate; pH 9.5, pH 10.0, and 10.5, 0.1 *M* sodium carbonate. "Efficiency" is defined as the ratio of micromoles of galactose covalently bound to the gel to micromoles of NHS released during the reaction.

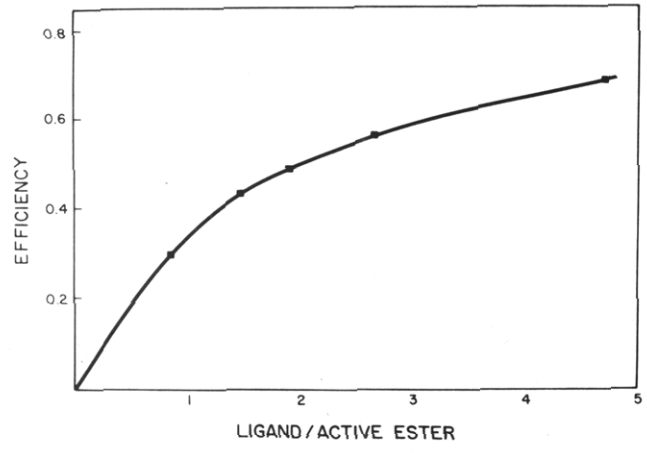


FIGURE 3: Ligand incorporation as a function of ratios of ligand/active ester. The ligand concentration ranged from 8.4 to 36.2 m*M*. The level of active ester was the amount NHS released from the gel upon alkaline hydrolysis. Ligand used and "efficiency" are described in the legend to Figure 2. Conditions are given in the text.

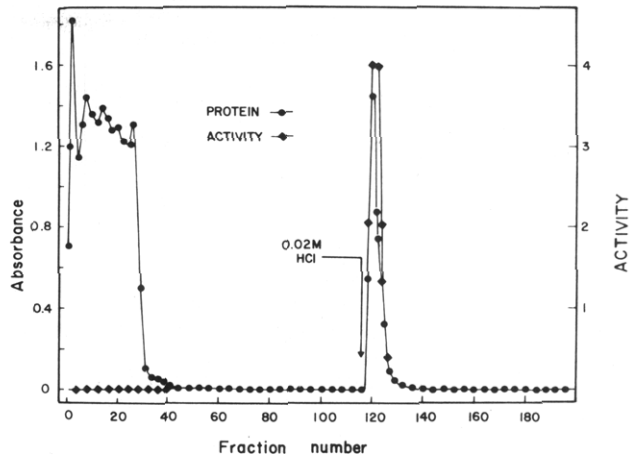


FIGURE 4: Chromatography of wheat germ agglutinin (WGA) on polyacrylamide gel containing 6-aminohexyl 2-acetamido-2-deoxy-β-D-glucopyranoside. The undialyzed ammonium sulfate (15–55% saturation) fraction (Table II) was adsorbed batchwise on the gel by mixing, and the gel was packed into a column (2.5 cm × 3.5 cm). The column was washed with 1 *M* NaCl in 0.01 *M* sodium phosphate buffer (pH 6.7). Elution of the WGA was commenced with 0.02 *M* HCl (arrow). Fractions (7.3 ml/fraction) were pooled and neutralized, and used for further characterization.

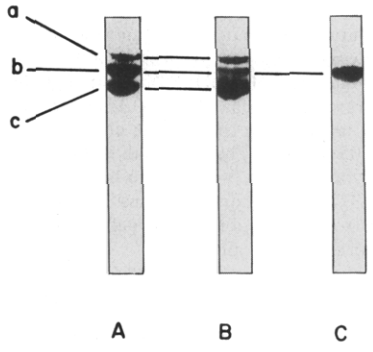


FIGURE 5: Polyacrylamide gel electrophoresis using 7.5% gels of wheat germ agglutinin at pH 3.8. Electrophoresis proceeded for 1.5 hr, 5 mA/tube (ca. 300 V), after which protein bands were stained with Amido Black stain (1% in 7% acetic acid) for 1 hr then destained in 7% acetic acid. (A) Preparation from elution of affinity column with 0.02 *M* HCl (Figure 4); (B) peak A from pH-gradient elution (Figure 5); (C) peak B from pH-gradient elution (Figure 5).

level. WGA was then eluted with 0.02 *N* HCl (Figure 4). The active fractions were pooled and carefully neutralized with NaOH. The preparation thus obtained was free of hexosaminidase activity as measured with *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside.[2] Crystals (monoclinic, 1–2 mm long) of pure WGA formed upon standing at 4°. WGA could alternatively be eluted from the affinity column by a solution of 2-acetamido-2-deoxy-D-glucose (0.2 g/ml), yielding crystalline material after dialysis. The agglutinin could not be eluted from the column at lower concentrations of 2-acetamido-2-deoxy-D-glucose (Table II).

Separation of Isolectins. WGA prepared by the method described here, as well as WGA prepared by the ovomucoid-Sepharose column (Marchesi, 1972), showed only one band on standard gel electrophoresis at pH 8.9. However, when the preparations were electrophoresed at pH 3.8, both showed multiple bands (Figure 6). The possibility that these were isolectins (Allen et al., 1973) was investigated by partial resolution of the different bands. WGA purified as above was adsorbed onto the affinity column. After washing the column with water, the column was eluted with a pH gradient. The results are shown in Figure 5. The first peak (fractions 2–10 was found not to be protein by the Lowry protein assay and Amido Black staining, and was discarded. Peak A, upon polyacrylamide gel electrophoresis as above, represented an enrichment for the fastest moving major band. Peak B corresponded to the second fastest moving major band (Figures 6 and 7). The two peaks were separately pooled, desalted by readsorption on an ovomucoid-Sepharose column (Marchesi, 1972), eluted from the affinity column with 0.02 *M* HCl, neutralized, and lyophilized. Agglutinating activity (the least amount of protein (μg) capable of causing agglutination) of the pooled peak A and peak B material was 0.77 and 1.18 μg, respectively.

Table II: Purification of Wheat Germ Agglutinin.

Steps	Vol (ml)	Protein (mg)	Titer[a]	L.E.L.[b]	Units[c] × 10^3	Recovery (%)	Purification (fold)
Crude extract	3840	48,000	1/8	156	307	100	1
Ammonium sulfate	760	19,500	1/32	80.4	253	82.4	1.94
Affinity chromatography	79	261	1/275	1.2	217	70.8	130

[a] The maximum level of dilution at which agglutination can be ovserved. Serial dilutions were made with a factor of 2. [b] The least amount of protein (μg) capable of causing agglutination. [c] The total amount of protein (μg)/L.E.L.

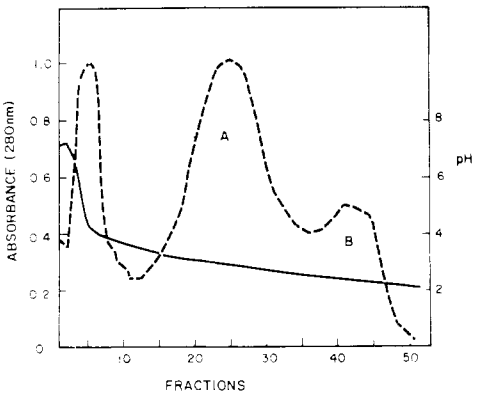


FIGURE 6: Elution of wheat germ agglutinin from a 6-aminohexyl 2-acetamido-2-deoxy-β-D-glucopyranoside polyacrylamide gel column with a pH gradient (pH 4.0–pH 2.0). A three-chamber (167 ml each) pH gradient was applied: chamber 1, 0.1 *M* citric acid adjusted to pH 4.2 with solid monosodium phosphate; chamber 2, 0.2 *M* citric acid; chamber 3, 0.05 *M* HCl. The first peak at the void volume (fractions 2–10, 10 ml/fraction) was found not to be protein and was discarded. Fractions 11–37 (peak A) and fractions 37–48 (peak B) were pooled and characterized by polyacrylamide gel electrophoresis as shown in Figure 7. Absorbance (---); pH (—).

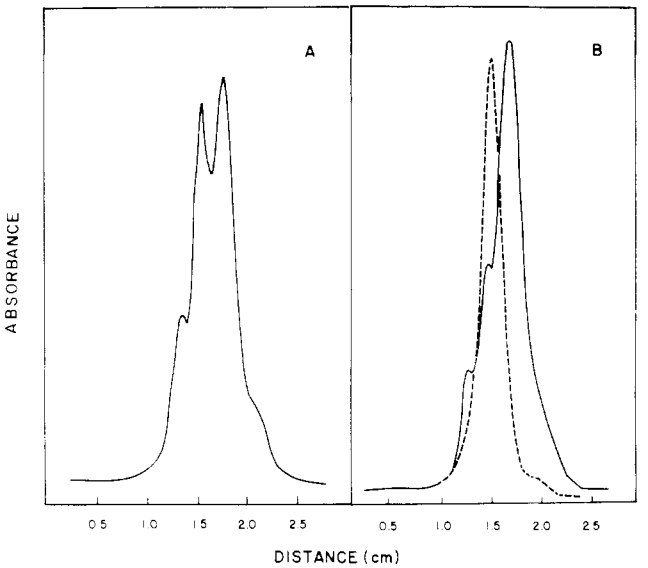


FIGURE 7: Densitographs of the gel electrophoreses of WGA preparations. (A) Preparation from affinity chromatography (Figure 4, Figure 6A). (B) (—) peak A, Figure 5 (Figure 6B); (----) peak B, Figure 5 (Figure 6C). Densitographs were recorded on a Beckman DU spectrophotometer equipped with a Gilford gel scanning device.

Discussion

The usefulness of NHS (Anderson et al., 1964) and NHP (Nefkens and Tesser, 1961) active esters for peptide synthesis in polar, as well as nonpolar solvents, is well established. NHS esters were used to "activate" carboxyl groups of spacer arms in affinity beads of agarose or glass (Cuatrecasas and Parikh, 1972). We have chosen to prepare acryloyl esters of NHS and NHP and to use them as comonomers in polyacrylamide gel formation.

The development of polyacrylamide gel systems containing active esters (NHS-acrylate or NHP-acrylate) provides a great degree of versatility in designing affinity systems. The porosity and other physical properties of the gel can be varied by varying the concentrations of acrylamide and bisacrylamide. The level of activation can be varied simply by varying the amount of NHS-acrylate or NHP-acrylate included in the polymerization mixture. The gels can be generated to fit molds of varying size and shape.

In addition, the "active" gels can be stored under dry dimethylformamide in the cold without any detrimental effect for at least 6 months. Further, unlike agarose or other polysaccharide gels, polyacrylamide gels per se are more immune to chemical and microbial degradation upon storage, and the ligands incorporated via the active esters described here are bound by stable amide linkages, thus rendering them less liable to loss while in use.

Ligand incorporation is essentially quantitative in dimethylformamide, using a relatively small excess (1.2–2-fold) of a variety of ligands containing a primary amino group. The level of free carboxylic acid groups generated on the gel surface (owing to hydrolysis) is reduced to the lowest levels when this method of linkage is used. Ligand incorporation into the gel system can be readily quantitated by determining the NHS or NHP liberated.

When the nature of the ligand (e.g., enzymes) precludes the use of organic solvents, ligand incorporation can be carried out in aqueous systems. Our work, as well as previous work in application of *N*-hydroxysuccinimide esters of derivatized agarose beads (Cuatrecasas and Parikh, 1972), demonstrated lability of the ester to hydrolysis in aqueous media. This side reaction resulting in the generation of carboxylic acid groups is undesirable. This problem can be largely circumvented by use of a large excess of ligand, a 50-fold excess resulting in greater than 90% efficiency in ligand replacement of active ester. However, where applicable, nonaqueous systems are preferable, since quantitative insertion of ligand can be accomplished using only a slight excess of ligand.

During the course of these studies, a report appeared in which *p*-nitrophenyl acrylate was used as a comonomer in polyacrylamide gel for immobilization of biologically specific molecules (Dattagupta and Buenemann, 1973). Polymerization was carried out in dimethyl sulfoxide in evacuated, sealed capsules. Our preliminary results indicated that *p*-nitrophenyl acrylate was too readily hydrolyzed in aqueous solutions, and was considered less effective for our use,

and thus abandoned. Dattagupta and Buenemann (1973) also noted the same shortcoming of their gel.

Usefulness of the present system for affinity chromatography was demonstrated by purification of WGA. WGA is a protein of 17,000 daltons (Nagata and Burger, 1974), capable of specifically binding molecules containing *N*-acetylglucosamine residues (Nagata and Burger, 1974; Burger and Goldberg, 1967). It has been used as a probe for mammalian cell surface changes (Lis and Sharon, 1973). This protein has been previously purified and obtained in crystalline form by conventional chromatographic methods (Nagata and Burger, 1974). Although affinity chromatography of WGA has been reported, using Sepharose columns containing ovomucoid (Marchesi, 1972), 6-aminohexyl 2-acetamido-2-deoxy-β-D-glucopyranoside (Shaper et al., 1973), or on a column of crushed, partially hydrolyzed chitin (Bloch and Burger, 1974), no crystallization of WGA has been reported from preparations obtained by these procedures.

The high concentration of ligand in the system reported here (about 30 μmol/ml of gel as compared to 6–7 μmol/ml in the Sepharose system) resulted in the following advantages. Even using relatively low porosity gels (15% acrylamide, 5% cross-linked), it allowed for a greater capacity (at least 56 mg of WGA/ml of gel) compared to the other affinity system published (7–12 mg of WGA/ml of gel, Bloch and Burger, 1974; Marchesi, 1972; Shaper et al., 1973). It also showed stronger binding of WGA. The agglutinin was easily adsorbed by a small quantity (4.7 ml) of the gel by merely stirring in a large volume (760 ml) of protein solution, where only 1.5% of the protein present was WGA. The strong binding of WGA to the gels described here was also manifested in the conditions of WGA elution from the gel. High salt concentration (1 *M* NaCl) did not release any activity from the gel. A low concentration of GlcNAc (0.05 g/ml), used for the Sepharose system (Shaper et al., 1973), was ineffective in our system, which required a near-saturation concentration of GlcNAc (0.2 g/ml) for WGA elution.

For practical purposes, acid elution was found to be more convenient. Elution of WGA with 0.02 *N* HCl followed by neutralization with NaOH gave a WGA preparation of high purity in a solution low in salt concentration, and crystals were obtained from the neutral solution. The column was reconditioned for further use simply by washing with 0.01 *M* sodium phosphate buffer (pH 7.2).

Isolectins of WGA have been previously reported (Allen et al., 1973), three wheat germ agglutinin activity peaks having been eluted by a salt gradient at pH 3.8. Although only one band was seen by electrophoresis at pH 8.9, as others have reported (Nagata and Burger, 1972), the WGA isolated by affinity chromatography reported here showed three major and one minor band by polyacrylamide gel electrophoresis at pH 3.8.

We have investigated the possibility of resolving isolectins using our affinity column. Using a pH gradient, partial resolution of these WGA components was obtained (Figures 5 and 6). The high and uniform specific activity throughout the fractions tends to confirm that they are, in fact, isolectins. However, final proof that every band is an isolectin must await complete resolution and isolation of the other protein components.

The high yield, rapid purification of WGA by chromatography on polyacrylamide gel containing a high level of ligand (6-aminohexyl 2-acetamido-2-deoxy-β-D-glucopyranoside) prepared by the present system attests to the usefulness of this approach, and demonstrates some advantages over other, similar systems.

Acknowledgments

We are grateful to Dr. Saul Roseman for his enthusiasm and valuable suggestions. We thank Dr. Agnes T. Wu for measurement of PMR spectra, and Dr. Mark Krantz for preparation of the ovomucoid-Sepharose column.

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