Affi-Gel® 10 and 15 Activated Affinity Media

High-Efficiency Coupling for All Primary Amino Group Ligands

Affi-Gel 10 and Affi-Gel 15 are complementary affinity media that offer rapid, high-efficiency coupling for all ligands that have a primary amino group — including proteins with any isoelectric point (pI) and low molecular weight ligands. Both Affi-Gel 10 and 15 are N-hydroxysuccinimide esters of a derivatized crosslinked agarose gel bead support (Figure 1), and both couple to ligands spontaneously in aqueous or nonaqueous solution.

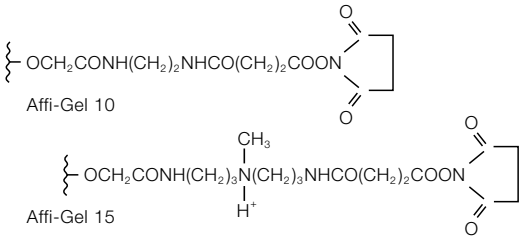

Fig. 1. Chemical structures of Affi-Gel 10 and Affi-Gel 15.

Affi-Gel 10, which contains a neutral 10-atom spacer arm, has been used to couple a variety of materials in affinity chromatography, immunoadsorption, and other techniques (see applications sections below). Affi-Gel 15 contains a positive charge in its 15-atom spacer arm, which significantly enhances coupling efficiency for acidic proteins at physiological pH. Both Affi-Gel 10 and Affi-Gel 15 offer the following additional advantages:

High Stability

Ether bonds link the spacer arm to chemically crosslinked agarose gel beads (Bio-Gel® A-5m support). Amide bonds couple the protein ligand to the terminal carboxyl of the spacer arm. Excellent resistance to urea, guanidine HCl, heat, solvents, acid, and base (pH 2–11) virtually eliminates ligand leakage during storage and use (Conn et al. 1981, La Porte et al. 1977).

Rapid, Gentle Coupling

Proteins are coupled with high efficiency within 4 hr at 4°C. The N-hydroxysuccinimide active ester method is highly selective for primary amino groups of ligands under physiological conditions.

Easy to Use

Wash Affi-Gel support with cold deionized water, add to buffered protein solution, and gently agitate. Low molecular weight ligands can be coupled in either aqueous or anhydrous solutions. Affi-Gel 10 and Affi-Gel 15 are supplied fully swelled and solvent-stabilized in isopropyl alcohol. The active ester content of both media is 15 µmol/ml.

Coupling Chemistry

Ligands with free alkyl or aryl amino groups will couple spontaneously with Affi-Gel 10 or 15 in aqueous or nonaqueous solution (Figure 2). Upon addition of ligand, the N-hydroxysuccinimide is displaced and a stable amide bond is formed. Since the reactive ester is immobilized on the gel and is highly selective for primary amino groups, spurious side reactions with the ligand (i.e., crosslinking or other modification in free solution) are eliminated. Among functional groups other than primary amines, free sulfhydryls are known to complete for coupling. In aqueous solution at neutral pH, Affi-Gel 10 will undergo gradual hydrolysis to yield a terminal carboxyl group.

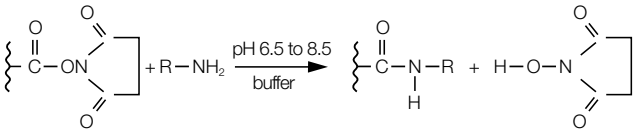

Fig. 2. Coupling reaction of Affi-Gel with ligand containing free amino groups.

Affi-Gel 10 and Affi-Gel 15 are well suited for coupling low molecular weight ligands. This can be done in aqueous solution or, when solubility of the liquid permits, in organic solvent.

Aqueous Coupling Conditions

pH

A major advantage of Affi-Gel 10 and 15 is the range of mild conditions that will permit coupling. This is particularly advantageous in applications that involve sensitive enzymes or other proteins that irreversibly lose biological activity when exposed to conditions outside of their physiological range. Useful coupling can be achieved with Affi-Gel 10 and 15 in the pH range of 3.0–10.0.

In order to maintain pH control, a minimum buffer strength of 0.01 M is recommended. Suitable buffers include MES, MOPS, HEPES, POPSO, acetate, and bicarbonate. Buffers such as Tris or glycine, which contain primary amino groups, will couple to the gel, as will any primary amine-containing compound that contaminates the ligand preparation.

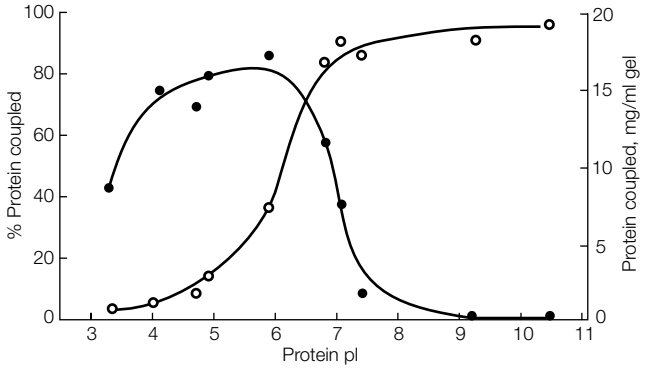

Fig. 3. Protein coupling with Affi-Gel 10 and Affi-Gel 15 at pH 7.5. Coupling conditions: Each protein solution (40 mg protein in 3 ml of 0.1 M MOPS, pH 7.5) was combined with 2 ml of Affi-Gel. The gel slurry was mixed at 4°C for 2 hr, then stripped with 7 M urea containing 1 M NaCl. The uncoupled protein was determined using published $E^{1\%}_{280}$ (Twining and Brecher 1975) by dilution of an aliquot of the urea effluent into 0.1 N HCl and measurement of the absorbance at 280 nm (○—○, Affi-Gel 10; ●—●, Affi-Gel 15). Proteins listed in Table 1.

Table 1. Protein Coupling to Affi-Gel 10 and Affi-Gel 15 at pH 7.5.

Protein	pI	Coupling Efficiency, % Affi-Gel 10	Affi-Gel 15
Fetuin	3.3	3.0	43
α-1-antitrypsin	4.0	5.0	76
Ovalbumin	4.7	8.5	70
Bovine serum albumin	4.9	14	80
Human transferrin	5.9	36	87
Bovine hemoglobin	6.8	83	59
Human globulin	5.8–7.3	90	39
Myoglobin	6.8–7.8	85	10
Cytochrome c	9.3	90	0
Lysozyme	10–11	95	1

Affi-Gel 10 couples proteins best at a pH near or below their pI, and Affi-Gel 15 couples proteins best near or above their pI. This is shown for coupling at pH 7.5 in Figure 3 and Table 1. Therefore, when coupling at neutral pH (6.5–7.5), Affi-Gel 10 is recommended for proteins with a pI of 6.5 to 11 (neutral or basic proteins), and Affi-Gel 15 is recommended for proteins with a pI below 6.5 (acidic proteins).

The difference in coupling efficiency of Affi-Gel 10 and Affi-Gel 15 for acidic and basic proteins can be attributed to interactions between the charge of the protein and charge on the gel. Hydrolysis of some of the active esters during aqueous coupling will impart a slight negative charge to Affi-Gel 10. This negative charge will attract positively charged proteins (proteins buffered at a pH below their pI) and enhance their coupling efficiency. Conversely, the negative charge will repel negatively charged proteins (proteins buffered at a pH above their pI) and lower their coupling efficiency. Affi-Gel 15, due to the tertiary amine incorporated into its arm, has a slight overall positive charge, and the effects are reversed.

In addition to its effect on coupling, the slight charge associated with each gel may sometimes be exploited in the affinity separation itself — for example, it may be used to enhance binding of weakly adsorbed materials, or elution of strongly adsorbed materials. In such cases, it may be preferable to use Affi-Gel 10 to couple an acidic protein, or Affi-Gel 15 to couple a basic protein. Coupling efficiency can then be enhanced by manipulating the coupling conditions in either of two ways.

Table 2. Coupling Efficiency of Acidic and Neutral-to-Slightly-Basic Protein under Various Coupling Conditions.

Coupling Buffer	Coupling Efficiency, % Affi-Gel 10	Affi-Gel 15
Bovine serum albumin, pI 4.9		
0.1 M MOPS, pH 7.5	14	80
0.1 M MOPS, pH 7.5 + 80 mM $CaCl_2$	90	—
0.1 M MOPS, pH 7.5 + 0.3 M NaCl	22	47
0.1 M MES, pH 4.8	90	38
Human globulin, pI 7.0 (average)		
0.1 M MOPS, pH 7.5	83	40
0.1 M MOPS, pH 7.5 + 0.3 M NaCl	69	70
0.1 M $NaHCO_3$, pH 8.5	80	70

Select the coupling pH so that the protein has a charge opposite that of the gel, or add salt to the coupling buffer to minimize charge interaction (80 mM $CaCl_2$ is recommended for coupling acidic proteins to Affi-Gel 10, and 0.3 M NaCl is recommended for coupling basic proteins to Affi-Gel 15). Examples of these manipulations are shown in Table 2. The more basic or more acidic the protein, the larger the observed effects will be.

Temperature

Coupling at 4°C is recommended whenever possible. The slower reaction rate at this temperature will afford a greater measure of control. Although the reaction will proceed at a faster rate at 20°C, the reaction efficiency is comparable and many ligands are more stable at 4°C.

Time

Coupling to Affi-Gel 10 and 15 is rapid. As shown in Figure 4, for Affi-Gel 10, about 75% of the maximum binding achieved with γ-globulin at pH 8.0 takes place within 30 min at 4°C. In 1 hr, 80% of the maximal coupling is achieved, and within 4 hr the reaction is complete.

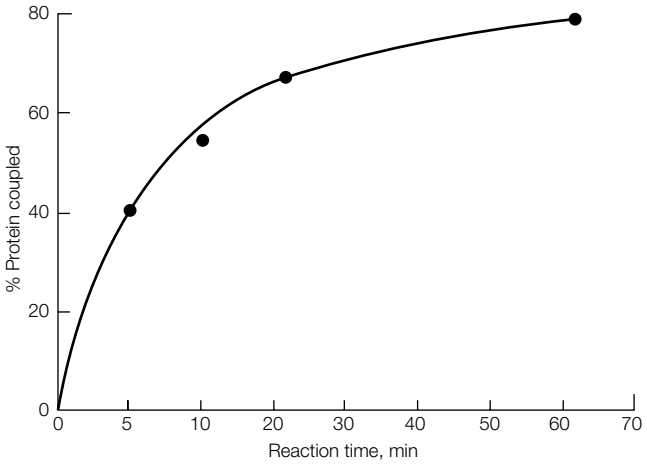

Fig. 4. Effect of time on protein coupling to Affi-Gel 10. A similar relationship is observed with Affi-Gel 15. Human γ–globulin (15 mg/ml of gel) was added in 0.1 M HEPES, pH 8.0 at 4°C.

Ligand Concentration

The amount of protein coupled is proportional to the amount of protein added to the gel, up to about 30 mg coupled/ml of gel (Figure 5). The efficiency of coupling will vary with the protein and conditions of coupling (see Figure 3). Above 30 mg protein/ml gel, more protein may be coupled, but efficiency will taper off. When maximum capacity is desired, a high concentration of ligand should be chosen (50–60 mg protein per ml of gel). When maximum efficiency is the goal, as would be the case with ligand preparation of limited quantity, the ligand concentration should be in the range of 25 mg protein/ml of gel. The sample that remains after coupling may be recovered and reused since it will not be subject to chemical modification by any reaction other than attachment to the gel. The optimum coupling efficiency is achieved when the total reaction volume is between 1.5 and 4.5 ml per ml of gel bed. The optimum ligand concentration for antibody applications is usually 5–10 mg antibody per ml of gel. The minimum concentration is 1 mg/ml.

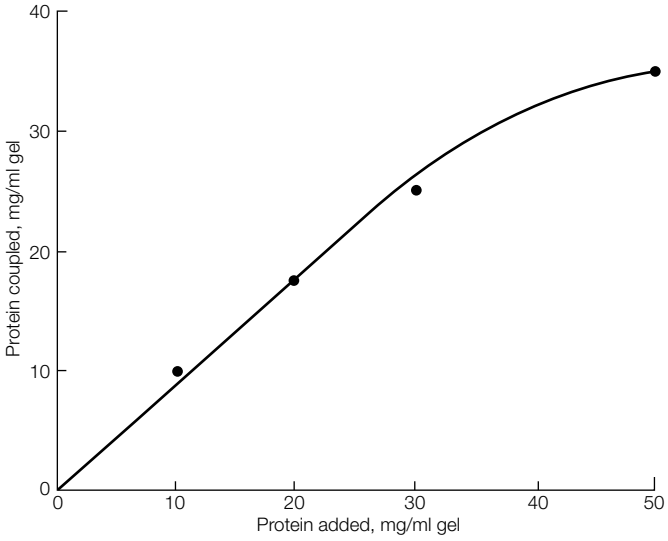

Fig. 5. Effect of amount of protein added on protein coupling to Affi-Gel 10. Human γ-globulin in 0.1 M MOPS, pH 7.5. A similar coupling capacity is observed with Affi-Gel 15.

Recommended Storage Conditions

Store Affi-Gel 10 in a freezer (-20°C) to retain 80% of original activity for at least one year. Store at -70°C to extend shelf life well beyond one year.

General Instructions

Aqueous Coupling

Shake the vial, and observe that a uniform suspension exists in the vial. Transfer the desired quantity of slurry to a small Buchner funnel or glass fritted funnel. Drain the supernatant solvent, and wash the gel with 3 bed volumes of cold (4°C) deionized water. The wash can be facilitated, particularly when working with larger amounts of gel, by applying a vacuum. Care should be taken, however, not to allow the gel bed to go dry. For optimum coupling of ligands, the washing procedure should be completed and the gel combined with the ligand solution within 20 min.

Transfer the moist gel cake to a test tube or flask and add the cold ligand solution. Add at least 0.5 ml of ligand solution/ml of gel, and agitate sufficiently to make a uniform suspension. Continue gentle agitation of the gel slurry on a rocker, shaker, or wheel for 1 hr at room temperature or 4 hr at 4°C.

If the coupling time is short, or if the gel is to be used immediately, we recommend a precautionary blocking of any active esters that might remain. This can be accomplished by adding 0.1 ml of 1 M glycine ethyl ester (pH 8) or 0.1 ml of 1 M ethanolamine-HCl (pH 8) per ml gel. Allow 1 hr for completion of the blocking reaction. Transfer the gel to a column and wash with water or coupling buffer until the gel is free of reactants detected by A_{280}. Wash the gel with other solvents that will be used subsequently to elute substances specifically bound to the column. The column is now ready for equilibration with starting buffer and application of sample. If the column is not to be used immediately, store it at 4°C or with the gel equilibrated in a solution containing 0.2% sodium azide.

Monitoring Protein Coupling

Soluble (unbound) protein remaining in the coupling and wash buffers can be quantitated using the Bio-Rad protein assay (catalog #500-0006) or by measuring its absorbance at 280 nm. If absorbance at 280 nm is preferred, the pH of the sample should be lowered by diluting in 0.01 N HCl. At neutral or basic pH the N-hydroxysuccinimide released during the coupling will absorb at 280 nm. N-hydroxysuccinimide will also interfere with the Lowry protein assay.

Anhydrous Coupling

Coupling under anhydrous conditions is the preferred method when suitable for the ligand. Since active esters are not hydrolyzed in the absence of water, the only reaction will be that of the ligand with the gel. To obtain a quantitatively substituted gel with low molecular weight ligands, it is necessary to add only a slight excess of ligand (about 15 µmol of active ester are available per ml of gel) in the solvent in which the gel is equilibrated. Suitable solvents include alcohols, dimethylsulfoxide, dioxane, acetone, dimethyl-formamide (grades that are contaminated with free amines should be avoided), or mixtures of these solvents. In the absence of hydrolysis, other factors such as time, concentration, and temperature are less important considerations. The reaction can be carried out in any convenient volume at room temperature for several hours. Any unreacted groups that remain can be blocked by addition of a slight excess of ethanolamine. The resulting support will have the lowest possible residual charge.

Affi-Gel 10 Applications

Table 3 lists typical applications of Affi-Gel 10, and specific application examples are shown for phosphodiesterase (Figure 6) and for prolactin receptors (Figure 7).

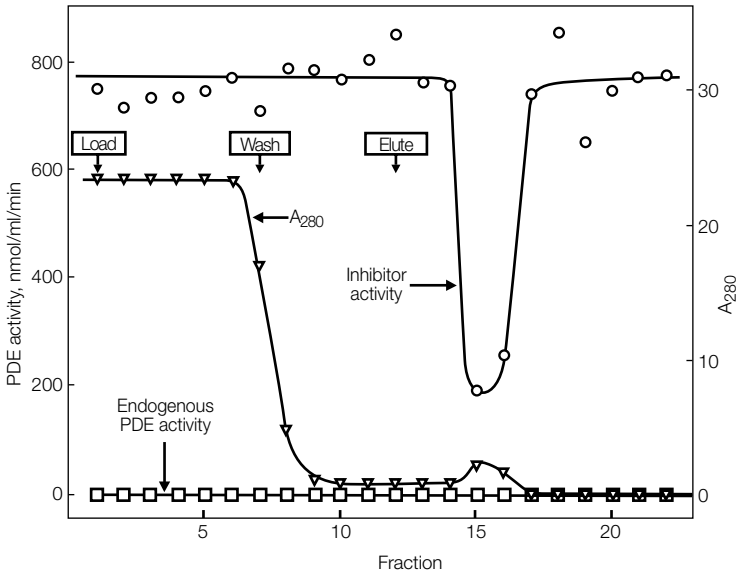

Fig. 6. Activator-agarose affinity column (calmodulin-agarose)* chromatography. Pooled phosphodiesterase (PDE) containing fractions from an Affi-Gel Blue column were adjusted to 1.5 mM $CaCl_2$, mixed with 15 ml of activator-agarose, and stirred gently overnight. The suspension was poured into a column (2.5 x 10 cm), washed with 60 ml of 50 mM Tris-HCl (pH 7.8), 3 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1 mM dithiothreitol, and then eluted. Fractions of 10 ml were collected; an aliquot of each was assayed for inhibitor (○—○) and PDE (□—□) activities. For more information, refer to Wallace et al. (1979, 1980).

* Calmodulin coupled to Affi-Gel 10.

Table 3. Applications of Affi-Gel 10.

Ligand Bound to Affi-Gel 10	Application	Reference
Enzymes		
Chymotrypsin	Isolation of inhibitors	Twining and Brecher 1975
Gentamicin C_{1a}	Purification of acetyltransferase	Williams and Northrup 1976
Plasminogen	Immobilized protease	Chibber et al. 1974
D-tryptophan methyl ester	Purification of α-chymotrypsin	Wilkinson et al. 1976
Lactoperoxidase	Solid-phase radioiodination of CEA	Tsao and Kim 1978
Soybean trypsin inhibitor	Removal of plasmin from plasminogen	Bouma et al. 1980
Immunoadsorption		
Fibrinogen	Sorption on antibodies	Chen and Shurley 1975
Antibody	Isolation of microtubule protein	Ikeda and Steiner 1976
Antibody	Isolation of brush-border aminooligopeptidase	Maze and Grey 1980
p-phenylazotyrosine (arsenic) hapten	Antihapten antibody	Kanellopoulos et al. 1979
Monospecific IgG	Purification of *Chlamydia trachomatis*–specific antigen	Caldwell and Kuo 1977
Anti-CEA antibody	Solid phase radioimmunoelectrophoretic assay of CEA	Saravis et al. 1974
Cell Membrane Receptors		
hGH	Purification of prolactin	Shiu and Friesen 1974
hCG	Purification of hCG receptors	Dufau et al. 1975
Insulin	Purification of human insulin receptors for production of specific antibodies	De Pirro et al. 1979
GnRH	Cellular gonadotropin release	Conn et al. 1981
Other		
Amino acids	General usage	Cuatrecasas and Parikh 1972
Polymyxin	Immobilized growth inhibitors to *E. coli*	La Porte et al. 1977
Castor bean lectin	Affinity chromatography of tyrosine transfer RNA	Garcia and Singhal 1979
Bovine serum albumin	Preparative purification of mouse immune interferon	Osborne et al. 1979
Hemagglutinin neuraminidase	Study of response of cytolytic thymus-dependent lymphocytes	Guertin and Fan 1980
L-tryptophyl-L-tryptophan	Human interferon purification	Zoon et al. 1979
Gelatin	Fibronectin purification	Coller 1980
Calmodulin	Isolation of calmodulin-binding protein	Wallace et al. 1979, 1980

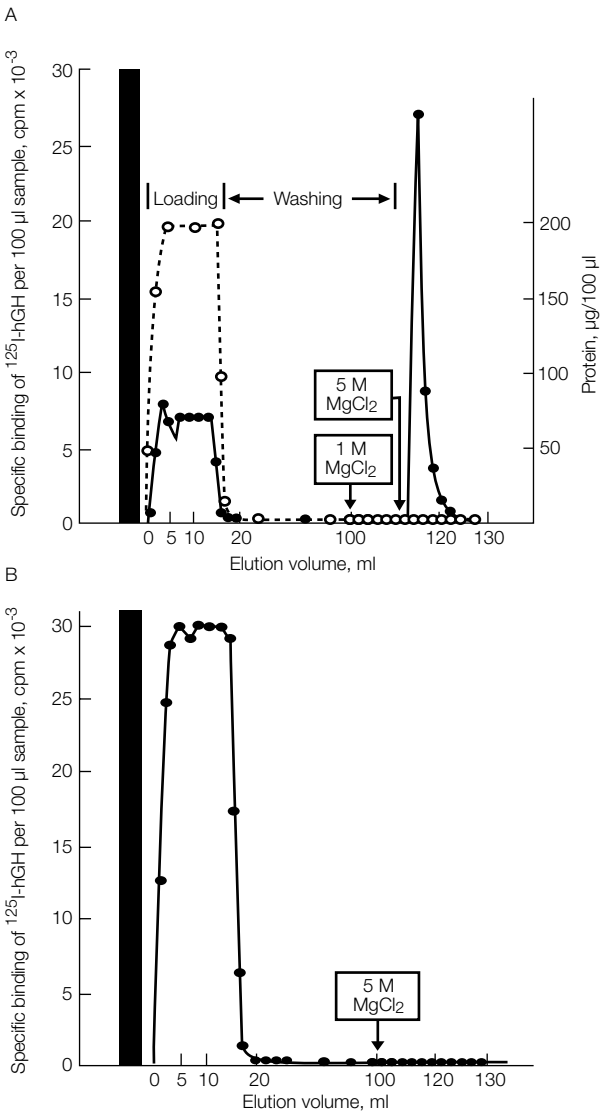

Fig. 7. Purification of prolactin receptors by Affi-Gel 10 chromatography. A 7.5 ml Triton X-100 extract of membrane (2 mg/ml) from mammary tissue was applied to 5 ml columns packed with Affi-Gel 10 coupled to either hGH (A) or BSA (B).(●—●), specific binding; (○—○), amount of protein. Solid bar represents receptor activity in 100 µl of the extract.

Affi-Gel 15 Applications

Immobilization of Purified Con A and Purification of Horseradish Peroxidase (HRP)

A highly effective method for preparing immobilized concanavalin A (Con A) and for purifying horseradish peroxidase was developed in our laboratories using ovalbumin, jack bean meal, and Affi-Gel 15 (Figure 8).

A solution of ovalbumin (40 mg/ml in 0.1 M HEPES, pH 7.5) was combined with an equal volume of Affi-Gel 15 and mixed at 4°C for 2 hr. Coupling efficiency was 62% (23 mg ovalbumin bound/ml of gel).

Crude jack bean meal was then extracted with phosphate-buffered saline (PBS) and the extract applied to the ovalbumin-agarose gel. After contaminants were washed out with PBS, the bound Con A was eluted with PBS containing 0.3 M α-methylmannoside. Capacity was 10 mg Con A/ml of ovalbumin-agarose.

The purified Con A (10 mg/ml in PBS-α-methylmannoside) was added to an equal volume of Affi-Gel 15 and mixed for 2 hr at 4°C. The coupling efficiency was 91% (9.1 mg of Con A coupled/ml of gel).

Impure HRP, 9 mg (70 U/mg), was applied to 10 ml of Con A-agarose prepared above. After a PBS wash the HRP was eluted with 0.1 M α-methylmannoside in PBS. The specific activity was increased 2.7-fold to 190 U/mg with a recovery of 90% (Figure 9). The RZ number (A_{403}/A_{275}) increased 3.1-fold, from 0.76 to 2.36.

The purified HRP is suitable for conjugation to antibody and the Con A–Affi-Gel 15 column can be used for purification of the conjugate by the method of Arends (1979).

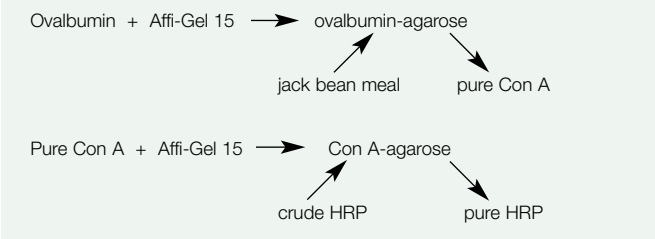

Fig. 8. Overview of HRP purification method.

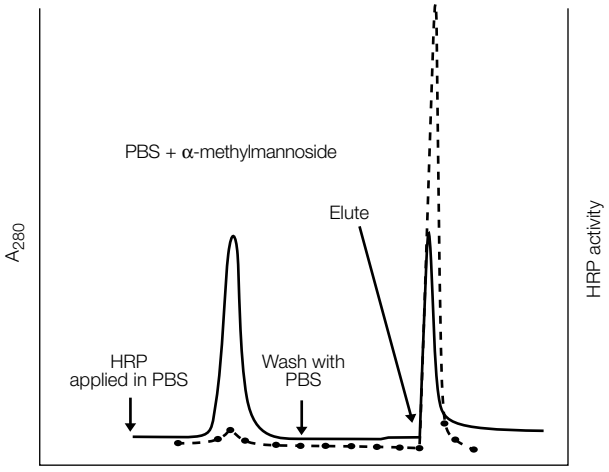

Fig. 9. Purification of horseradish peroxidase (HRP) on Con A–Affi-Gel 15. The solid line represents the absorbance at 280 nm. The broken line (---) represents HRP activity.

References

Arends J, Purification of peroxidase-conjugated antibody for enzyme immunoassay by affinity chromatography on concanavalin A, J Immunol Methods 25, 171–175 (1979)

Bouma BN et al., Human plasma prekallikrein. Studies of its activation by activated factor XII and of its inactivation by diisopropyl phosphofluoridate, Biochemistry 19, 1151–1160 (1980)

Caldwell HD, and KuomCC, Purification of a *Chlamydia trachomatis*–specific antigen by immunoadsorption with monospecific antibody, J Immunol 118, 437–441 (1977)

Chen JP and Shurley HM, A simple efficient production of neoantigenic antisera against fibrinolytic degradation products: Radioimmunoassay of fragment E, Thromb Res 7, 425–434 (1975)

Chibber BA et al., Immobilized human plasmins: preparation and enzymatic properties, Prep Biochem 4, 315–330 (1974)

Coller BS, Interaction of normal, thrombobasthenic, and Bernard-Soulier platelets with immobilized fibrinogen: defective platelet-fibrinogen interaction in thrombasthenia, Blood 55, 169–178 (1980)

Conn DPM et al., Stimulation of pituitary gonadotropin release does not require internalization of gonadotropin-releasing hormone, J Biol Chem 256, 1098–1100 (1981)

Cuatrecasas P and Parikh I, Adsorbents for affinity chromatography. Use of N-hydroxysuccinimide esters of agarose, Biochemistry 11, 2291–2299 (1972)

De Pirro R et al., Specificities of rabbit anti-human insulin receptor antibodies, J Clin Lab Immunol 2, 27–30 (1979)

Dufau ML et al., Gonadotropin receptors. Solubilization and purification by affinity chromatography, J Biol Chem 250, 4822–4824 (1979)

Garcia CM and Singhal RP, Affinity chromatography of tyrosine transfer RNAs, Biochem Biophys Res Commun 86, 697–703 (1979)

Guertin DP and Fan DP, Stimulation of cytolytic T cells by isolated viral peptides and HN protein coupled to agarose beads, Nature 283, 308–311 (1980)

Ikeda Y and Steiner M, Isolation of platelet microtubule protein by an immunosorptive method, J Biol Chem 251, 6135–6141 (1976)

Kanellopoulos J et al., Preparative isolation of the cell receptor for immunoglobulin E, J Biol Chem 254, 7691–7697 (1979)

La Porte DC et al., Inhibition of *Escherichia coli* growth and respiration by polymyxin B covalently attached to agarose beads, Biochemistry 16, 1642–1648 (1977)

Maze M and Gray G, Intestinal brush border aminooligopeptidases: cytosol precursors of the membrane enzyme, Biochemistry 19, 2351–2358 (1980)

Osborne LC et al., Large-scale production and partial purification of mouse immune interferon, Infect Immun 23, 80–86 (1979)

Saravis CA et al., Solid-phase radioimmunoelectrophoresis: an assay for carcinoembryonic antigen, J Natl Cancer Inst 53, 921–926 (1974)

Shiu RP and Friesen HG, Solubilization and purification of a prolactin receptor from the rabbit mammary gland, J Biol Chem 249, 7902–7911 (1974)

Tsao D and Kim YS, Glycoproteins from human colonic adenocarcinoma. Isolation and characterization of cell surface carcinoembryonic antigen from a cultured tumor cell line, J Biol Chem 253, 2271–2278 (1978)

Twining SS and Brecher AS, Isolation and identification of a1-antitrypsin as a component of normal and malignant human breast and other tissues, Proc Soc Exp Biol Med 150, 98–103 (1975)

Wallace RW et al., Purification and characterization of an inhibitor protein of brain adenylate cyclase and cyclic nucleotide phosphodiesterase, J Biol Chem 254, 377–382 (1979)

Wallace RW et al., High levels of a heat-labile calmodulin-binding protein (CaM-BP80) in bovine neostriatum, Biochemistry 19, 1831–1837 (1980)

Wilkinson TJ et al., Affinity chromatographic separations of chemically modified α-chymotrypsins from α-chymotrypsin, Sep Sci 11, 385 (1976)

Williams JW and Northrup DB, Purification and properties of gentamicin acetyltransferase I, Biochemistry 15, 125–131 (1976)

Zoon KC et al., Purification and partial characterization of human lymphoblast interferon, Proc Natl Acad Sci USA 76, 5601–5605 (1979)

Ordering Information

Catalog #	Description
153-6046	Affi-Gel 10, 4 x 25 ml
153-6052	Affi-Gel 15, 4 x 25 ml
153-6098	Affi-Gel 10/15 combination, 2 x 25 ml each

BIO-RAD

Bio-Rad Laboratories, Inc.

Life Science Group

Web site *www.bio-rad.com* ***USA*** *(800) 4BIORAD* ***Australia*** *02 9914 2800* ***Austria*** *(01)-877 89 01* ***Belgium*** *09-385 55 11* ***Brazil*** *55 21 507 6191* ***Canada*** *(905) 712-2771* ***China*** *(86-21) 63052255* ***Czech Republic*** *(420) 2-4141 0532* ***Denmark*** *45 44 52-1000* ***Finland*** *358 (0)9 804 2200* ***France*** *01 47 95 69 65* ***Germany*** *089 318 84-177* ***Hong Kong*** *852-2789-3300* ***India*** *(91-124) 6398112/113/114, 6450092/93* ***Israel*** *03 951 4127* ***Italy*** *39 02 216091* ***Japan*** *03-5811-6270* ***Korea*** *82-2-3473-4460* ***Latin America*** *305-894-5950* ***Mexico*** *52 5 534 2552 to 54* ***The Netherlands*** *0318-540666* ***New Zealand*** *64-9-4152280* ***Norway*** *47-23-38-41-30* ***Poland*** *(48) 22-8126 672* ***Portugal*** *351-21-472-7700* ***Russia*** *7 095 721 1404* ***Singapore*** *65-2729877* ***South Africa*** *00 27 11 4428508* ***Spain*** *590 5200* ***Sweden*** *46 (0)8-55 51 27 00* ***Switzerland*** *061 717-9555* ***United Kingdom*** *0800-181134*

Bulletin 1085 US/EG Rev E *02-169 0602 Sig 0402*