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REGENERABLE AFFINITY CHROMATOGRAPHY SUPPORT

V. K. JOSHI and K. M. SHAHANI

Department of Food Science and Technology, University of Nebraska-Lincoln, Lincoln, Nebr. 68583 (U.S.A.)

A. KILARA

Department of Food Science, Pennsylvania State University, University Park, Pa. 16802 (U.S.A.)

and

FRED W. WAGNER*

Laboratory of Agricultural Biochemistry, University of Nebraska-Lincoln, Lincoln, Nebr. 68583 (U.S.A.)

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SUMMARY

A derivative of Sepharose 4B, *p*-(N-acetyl-L-tyrosineazo)benzamidoethyl Sepharose 4B, was synthesized and used for the selective immobilization of proteins. The azo bond of this derivative can be reduced to liberate *p*-aminobenzamidoethyl Sepharose 4B, which can be diazotized and recoupled to N-acetyl-L-tyrosine and subsequently to a fresh preparation of protein. This regenerable affinity chromatography support was regenerated successively through five cycles without detectable loss of functional groups.

INTRODUCTION

The use of immobilized proteins has become common, and a variety of methods exist for coupling proteins to solid supports. In our experience, variations in the method described by Cuatrecasas and Parikh[1] have been most advantageous as proteins are coupled principally via α-amino groups, minimizing the number of points of chemical attachment[1]. This method has been shown to have a minimal influence on biological activity of a variety of proteins immobilized in our laboratories, including lactase[2], papain[3], lipase[3], antibodies[4], leghemoglobin**, mitochondrial ATPase[5] and a mixture of enzymes co-immobilized to a single support[6].

Application of immobilized proteins and enzymes to industrial processes has received considerable attention recently[7]. One of the principal problems of using large-scale immobilized proteins is the high cost of the solid support, which is discarded after the biological activity of the ligand is lost.

* To whom all communications should be addressed.
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We have synthesized a novel spacer arm on a solid support for immobilizing enzymes and proteins, which offers all the advantages of the method of Cuatrecasas and Parikh[1], but also can be regenerated to remove denatured protein and reused to immobilize native protein of the same or different identity.

EXPERIMENTAL

Materials

Sepharose 4B was purchased from Pharmacia (Piscataway, N.J., U.S.A.). Special chemicals used included N-hydroxysuccinimide, dicylcohexylcarbodiimide (Eastman, Rochester, N.Y., U.S.A.), *p*-nitrobenzoyl chloride (Aldrich, Milwaukee, Wisc., U.S.A.), L-valine methyl ester, N-acetyl-L-tyrosine (Sigma, St. Louis, Mo., U.S.A.) and L-[^{14}C]valine (Schwartz/Mann, Orangeburg, N.Y., U.S.A.).

*Preparation of RACS**

PABS was prepared exactly by the method of Cuatrecasas[8] using 250 mg of cyanogen bromide per ml of packed gel. Briefly, cyanogen bromide-activated Sepharose 4B was allowed to react with ethylenediamine to form aminoethyl Sepharose 4B. The latter product was then allowed to react with an equal volume of 0.07 *M* *p*-nitrobenzoylazide in 40% dimethylformamide and 0.2 *M* sodium borate buffer pH 9.3 at room temperature for 1 h. The product was thoroughly washed with 50% dimethylformamide in the same buffer, then with 0.2 *M* sodium borate, pH 8.5, and incubated in 0.1 *M* sodium dithionite in 0.2 *M* sodium borate for 1 h to reduce the *p*-nitrobenzamidoethyl Sepharose 4B.

p-Nitrobenzoylazide was prepared by slowly adding 0.7 g of sodium azide dissolved in 2 ml of ice-cold water to an ice-cold solution of *p*-nitrobenzoyl chloride (2.45 g in 25 ml of acetone). The reaction was kept below 4° by incubating the solution of a bath of melting ice for 15 min. The product, *p*-nitrobenzoylazide, was obtained by adding 50 ml of cold distilled water to the reaction mixture. It was filtered and washed with water, then dried over P_2O_5. The yield of the product was 76%.

PABS (150 ml packed gel) was suspended in 250 ml of 0.1 *N* HCl and chilled to 4°, then 1.4 g of $NaNO_2$ was added and diazotization was allowed to proceed for 7 min[8]. The diazotized resin was filtered and washed with cold 0.1 *N* HCl. The filter cake was slowly added to 150 ml of 0.1 *M* $NaHCO_3$, pH 9.3, containing 10 μmol of N-acetyl-L-tyrosine per milliliter of packed gel. The azo coupling reaction was allowed to proceed for 3 h at room temperature. As the reaction progressed the resin became deep red. The reaction product, RACS, was washed with water and then dioxane, and stored at room temperature in dioxane until used.

Preparation of L-[^{14}C]valine methyl ester

L-[^{14}C]Valine (1.4 ml, 70 μCi, sp. act. 260 mCi/mol) was dried for 16 h over

* Abbreviations: RACS = regenerable affinity chromatography support; PABS = *p*-aminobenzamidoethyl Sepharose UB; ONPG = *o*-nitrophenyl-β-D-galactopyranoside; DCC = dicyclohexylcarbodiimide.

P_2O_5 under reduced pressure. The dried valine was dissolved in 1 ml of hot methanol and then diluted to 2 ml with diethyl ether. Methylation was performed using diazomethane, by the procedure of Schlenk and Gellerman[9]. The product, L-[^{14}C]valine methyl ester, was dissolved in methanol and added to 1.5 g of L-valine methyl ester in 5 ml of methanol. L-[^{14}C]Valine methyl ester of sp. act. 6.5 mCi/mol was crystallized by the slow addition of diethyl ether. The crystals were collected, washed with diethyl ether and dried (yield 99.8%). Thin-layer chromatography of the product gave one ninhydrin positive spot using methanol as the solvent, which resolves the methyl ester from the free acid.

Reduction and regeneration of RACS

The column containing the desired amount of RACS was washed at room temperature with 10 volumes of 0.1 *M* sodium dithionite in 0.2 *M* sodium borate buffer, pH 9.0. The color due to the azo bond disappeared after *ca.* 5 volumes of buffer had passed through the resin. The column was then washed with distilled water to remove excess dithionite. Reduction reconverted RACS into PABS. To regenerate the azo bond to N-acetyl-L-tyrosine, the resin was diazotized and treated as described above. The initial resin (150 ml packed gel) was recycled in this manner five times and a 20 ml packed gel sample was saved after each cycle.

Determination of available binding sites

L-[^{14}C]Valine methyl ester was coupled to RACS using DCC. Three ml of packed gel were suspended in 9 ml 1,4-dioxane containing 0.2 g of DCC and 30 μmol of L-[^{14}C]valine methyl ester. The reaction was allowed to proceed for 90 min at room temperature. The resin was washed in 2 volumes of dioxane, followed by 2 volumes of methanol and finally 4 volumes of dioxane. The product was dried and aliquots of the resin were assayed for radioactivity. Other methods of coupling used DCC in dimethylformamide and 1-ethyl-3,3-(dimethylaminopropyl)carbodiimide, but these agents were not as effective as DCC in 1,4-dioxane.

To study the effect of reaction time on the quantitative attachment of L-[^{14}C]valine methyl ester, aliquots were withdrawn from the reaction mixture at 20, 40, 70, 90, 120, 140 and 160 min using dioxane, as described above. They were washed, dried and assayed for radioactivity.

Coupling of Enzymes

Aliquots of RACS and recycled RACS were suspended in 2 volumes of dioxane and to them were added DCC and N-hydroxysuccinimide to bring their concentrations to 0.1 *M*. The reaction was allowed to proceed for 90 min and the resin was washed successively with dioxane, methanol and dioxane. The resin was air-dried for 5 min to evaporate residual dioxane then suspended in an equal volume of 0.1 *M* potassium phosphate buffer, pH 7.0, containing 40 mg of enzyme per milliliter of packed gel. The reaction was allowed to proceed for 16 h at 4° and then terminated by rendering the reaction mixture 0.2 *M* with glycine. The product was washed extensively with cold 0.1 *M* phosphate buffer, pH 7.0, until all surface-absorbed enzyme was eluted. Enzymes immobilized were lactase ('maxilact', Enzyme Development Corp., N.Y.), papain, beef heart mitochondrial ATPase and lipase. With the exception of the ATPase, which was a gift of Dr. Sheldon M. Schuster,

Department of Chemistry, University of Nebraska-Lincoln, these enzymes were obtained commercially.

Enzymes were assayed using ONPG as the substrate for lactase[3], casein as the substrate for papain[3], butter oil as the substrate for lipase[11] and ATP as the substrate for ATPase[5]. The amounts of enzyme immobilized were determined from the activity level of the resin and expressed as the corresponding weight of soluble enzyme. This correlation is justified and based on earlier work from these laboratories[2] which showed these procedures to yield similar coupling levels of lactase (0.5 mg/ml) based on the amino-acid composition of immobilized preparations. The soluble and immobilized forms of lactase were used to determine the pH optimum and the $K_{m(app)}$. The kinetic data were analyzed statistically by the method of Wilkinson[10].

RESULTS AND DISCUSSION

The structure of RACS and the chemical method used for its regeneration are shown in Fig. 1. The synthesis of the arm involved activation of Sepharose 4B and coupling of ethylenediamine, followed by reaction with *p*-nitrobenzoylazide. The *p*-nitrobenzamidoethyl Sepharose 4B was then reduced with dithionite to form PABS. Procedures for these syntheses are well documented[8]. The advantage of this arm is that it can be diazotized to form azo compounds with aromatic amines or phenolic compounds. Subsequent reduction of the azo bond regenerates PABS. The next section of the arm therefore had to be a phenolic or aromatic compound with a carboxylic acid function, which is necessary for the selective attachment of proteins primarily via α-amino groups[1]. N-Acetyl-L-tyrosine was selected, because it is inexpensive and readily available. On regeneration, its loss with the undesirable ligand is of no consequence (Fig. 1).

Resin containing *ca.* 2 μmol of N-acetyl-L-tyrosine per milliliter of packed gel was obtained by this synthesis, as evidenced by its ability to couple L-[^{14}C]valine methyl ester in the presence of DCC (Fig. 2). Other methods for coupling L-[^{14}C]valine methyl ester were not as effective as DCC in dioxane (Table I).

To test the efficacy of regeneration, 100 ml of RACS was packed into a column and washed with 0.1 *M* dithionite in 0.2 *M* sodium borate buffer, pH 9.0. Reduction of the arm could be followed visually as the loss of the red color of the resin due to to the presence of the azo linkage. The product, PABS, could be diazotized and recoupled to N-acetyl-L-tyrosine. Regenerated resin could then be used to couple L-valine methyl ester or protein. Table II shows the influence of recycling the resin on its ability to be activated and react with either L-valine methyl ester or "maxilact", a commercial preparation of lactase from *Saccharomyces lactis*. Recycling the resin five complete times did not affect its ability to react either with L-valine methyl ester in the presence of DCC or with lactase when the resin was activated with DCC and N-hydroxysuccinimide.

In earlier work[2] we have demonstrated that immobilizing lactase from *S. lactis* by the method of Cuatrecasas and Parikh[1] using succinylaminoethyl Sepharose 4B did not affect either the $K_{m(app)}$ or the pH optimum for the hydrolysis of ONPG. Use of RACS and this same coupling procedure yielded a resin containing *ca.* 0.35 mg of lactase bound per millilitre of packed RACS, regardless of the number of times the resin had been recycled. Fig. 3 shows the pH optimum and Lineweaver–

Burk plot for immobilized and soluble forms of lactase with ONPG used as substrate. Neither the pH optimum nor the $K_{m(app)}$ for the hydrolysis of ONPG was affected by the immobilization of lactase on RACS or by prior regeneration of the resin.

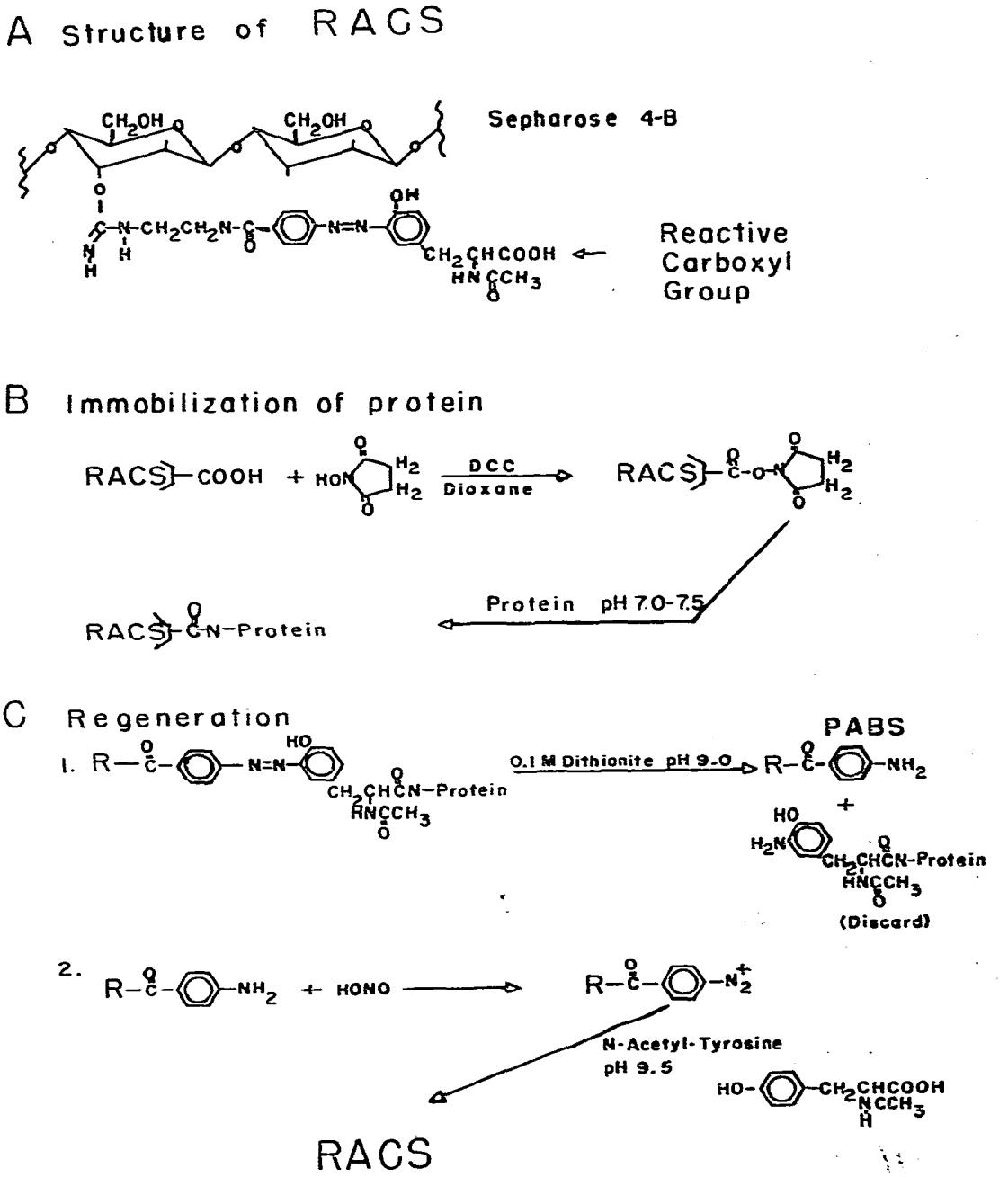

Fig. 1. Schematic representation of the regenerable affinity chromatography support (RACS). A, Structure of the arm; B, reactions involved to immobilize proteins[1]; C, reactions involved in removing denatured protein and adding new protein.

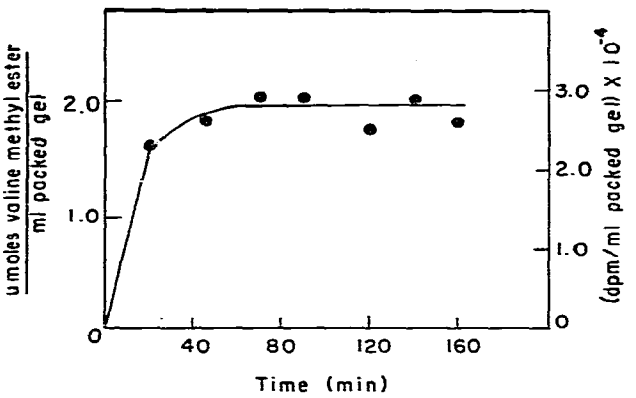

Fig. 2. Incorporation of L-[^{14}C]valine methyl ester into RACS. RACS (3 ml packed gel) was allowed to react in 6 ml of dioxane with 30 μmol of L-[^{14}C]valine methyl ester for the times shown. To stop the reaction, RACS was filtered and washed with dioxane, methanol and dioxane. To avoid variations in estimating the packed volume of RACS, the density of the packed gel was determined by weighing a dried sample of RACS from a known volume.

TABLE I

EFFECT OF THREE ACTIVATION PROCEDURES ON THE L-[^{14}C]VALINE METHYL ESTER REACTIVITY OF RACS

Activation procedure	*μmol L-[^{14}C]valine methyl ester per ml packed gel*
DCC in 1,4-dioxane	1.90
DCC in 50% dimethylformamide in methanol	0.45
DCC in 100% dimethylformamide	1.10
Water-soluble carbodiimide, pH 5.0	0.13

When RACS containing immobilized lactase was regenerated, fresh lactase could be immobilized at a concentration of *ca.* 0.35 mg per milliliter of packed gel.

In previous communications[2,3,5,6] we have reported the preparation of immobilized forms of papain, lactase and mitochondrial ATPase which were highly active and possessed the same kinetic properties as did the soluble forms of the enzymes. Immobilization of these enzymes to RACS gave gels with 0.25 mg of papain, 0.05 mg of mitochondrial ATPase and 0.4 mg of lipase per milliliter of

TABLE II

EFFECT OF REGENERATION ON THE ABILITY OF RACS TO REACT WITH L-VALINE METHYL ESTER OR LACTASE

Cycle No.	*μmol L-[^{14}C]valine methyl ester per ml packed gel*	*Lactase bound (mg)*
0	2.2	0.40
1	1.9	0.33
2	1.8	0.32
3	2.2	0.34
4	2.2	0.37
5	2.2	0.33

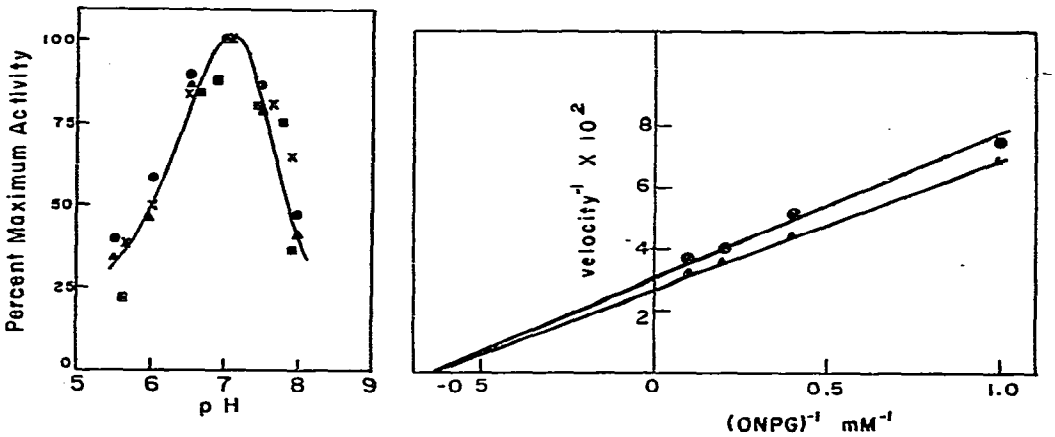

Fig. 3. Enzymatic parameters of lactase (*Saccharomyces lactis*) immobilized to RACS using ONPG as the substrate. ●—●, soluble lactase; ■—■, lactase immobilized to RACS recycled once; ▲—▲, enzyme immobilized to RACS recycled three times; ×—×, enzyme immobilized to RACS recycled five times. A, pH optimum; B, Lineweaver–Burk plot.

packed gel. Coupling of organic amines, in this case proteins, to N-hydroxysuccinimide-activated resin in aqueous solution is a competitive reaction between the amine acid and water. Thus amounts of protein coupled to the resin were a function not only of pH[1], but also of their molar concentrations. Higher levels should be obtained by using more concentrated solutions of proteins. Mitochondrial ATPase immobilized to RACS retained its ability to be activated by bicarbonate, as reported previously for the same enzyme immobilized to succinylaminoethyl Sepharose 4B[5]. Thus it appears that RACS has a broad applicability as a resin for the immobilization of proteins with minimal modification of biological activity.

The concept of RACS originated from a need for a reuseable affinity support principally for the preparation of large-scale immobilized enzyme processing applications. In such an endeavor, the cost of replacing the support when immobilized enzyme is denatured is often limiting. The synthesis of RACS using Sepharose 4B should be feasible using other supports, including controlled pore glass beads, Sephadex, polyacrylamide or any other support on to which a free aromatic amino group can be attached. This compound can be diazotized and coupled with a phenolic compound or aromatic amine. Moreover, regenerable resins with reactive groups other than the carboxyl group should be readily synthesized by judicious choice of the phenolic or aromatic amine to generate the azo linkage to the support. Finally, a single batch of RACS could be recycled to attach proteins of markedly different activity or function, depending on the immediate research demands.

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