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Immobilization of protein ligands with methyl vinyl ether–maleic anhydride copolymer

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ABSTRACT

Methyl vinyl ether–maleic anhydride copolymer (MMAC) is a water-insoluble polymer with an acid anhydride group which reacts with amino groups of ligands to form stable amide bonds. MMAC was used to immobilize protein ligands on two kinds of supports, the wells of plastic microtitre plates for enzyme-linked immunosorbent assay and related methods, and gels for affinity adsorbents. The wells were first coated with MMAC and then allowed to react with proteins. The immobilization of proteins by this method was efficient and occurred in a dose-dependent manner. Shodex Et123, a gel having amino groups, was incubated with MMAC, and then the activated Shodex was used to immobilize high concentrations of proteins. Concanavalin A–Shodex thus obtained had high affinities and was successfully used for the high-performance liquid affinity chromatography of sugar derivatives on a short column.

INTRODUCTION

Polymers having active groups have been effectively used in various biochemical methods, *e.g.*, immobilization of ligands for affinity chromatography [1,2], preparation of water-insoluble enzymes [3] and immobilization of proteins on a polyvinylidene-difluoride (PVDF) membrane [4]. Leakage of ligands from affinity adsorbents is a serious problem in the affinity chromatography of small amounts of protein. To prevent this problem, Wilcheck [1] reported the use of polyacrylhydrazide. When the polymer is coupled to agarose gel at various positions, it can serve a stable, long and multivalent spacer [1]. Goldstein [3] used an ethylene–maleic anhydride (1:1) copolymer to prepare water-insoluble derivatives of proteolytic enzymes.

The proteins to be analysed in enzyme-linked immunosorbent assay (ELISA) must first be immobilized in the wells of the microtitre plates. The immobilization is usually not very efficient and therefore gives low yields and sometimes does not proceed in a dose-dependent manner. This may be because the adsorption is due to non-covalent interactions between the proteins and the surface of the wells. If the proteins are coupled to the well via covalent bonds, the immobilization should be stable and efficient. However, we could not introduce the active groups efficiently to the well by convenient procedures. Therefore, we planned to coat the well with water-insoluble substances having active groups. In this paper, we report the successful use of methyl vinyl ether–maleic anhydride copolymer (MMAC) for this purpose. MMAC is a water-insoluble polymer with active groups of acid anhydride. MMAC reacts with amino groups of ligands to form stable multiple amide bonds. MMAC was also applied to the immobilization of affinity ligands on the gels for high-performance liquid affinity chromatography (HPLAC).

EXPERIMENTAL

MMAC (n = 263, relative molecular mass, M_r =

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41 000 was used for microtitre plates and $n = 320$, M_r 50 000 for affinity gel) were obtained from GAF (Linden, NJ, USA) and Shodex Et123 from Showa Denko (Tokyo, Japan). Horseradish peroxidase (HRP) was purchased from Toyobo (Osaka, Japan), bovine serum albumin (BSA) from Iwai Kagaku (Tokyo, Japan), anti-BSA antibody and concanavalin A (ConA) from Seikagaku Kogyo (Tokyo, Japan), methyl α-D-mannoside (Me α-D-Man) from Wako (Osaka, Japan) and *p*-nitrophenyl α-D-mannoside, *p*-nitrophenyl α-D-glucoside and *p*-nitrophenyl α-D-galactoside from Nakarai Tesque (Kyoto, Japan). HRP–BSA (BSA labelled with HRP) was prepared according to the method reported previously [5].

Immobilization of protein on microtitre plate

MMAC (0.5 mg) dissolved in 100 μl of dimethyl sulphoxide (DMSO) was added to each well of the microtitre plate (Immulon 200, C. A. Greiner & Sohne, Frickenhausen, Germany) and allowed to stand for 30 min at room temperature. The solution remaining in the wells was removed and the plates coated with MMAC were obtained. A 50-μl aliquot of HRP–buffer was added to each well of the coated plate and allowed to stand for 18 h at 4°C. After washing the wells three times with the same buffer, 100 μl of the substrate solution [0.4% *o*-phenylenediamine and 0.01% H_2O_2 in 100 m*M* citrate–phosphate buffer (pH 5.0)] were added to each well. After incubation for 5 min at room temperature, the reaction was stopped with 50 μl of 8 *M* H_2SO_4 and colour development was measured spectrophotometrically at 490 nm with a microplate reader (Bio-Rad Model 3550).

Anti-BSA antibody was diluted with 10 m*M* Tris–HCl buffer (pH 7.6) containing 150 m*M* NaCl (TBS) at various concentrations and a 50-μl aliquot of the antibody solution was added to each well of the MMAC-coated plate and allowed to stand for 18 h at 4°C. After washing with 10 m*M* Tris–HCl buffer (pH 7.5), 300 μl of 0.2% skim milk in TBS were added to each well to block the remaining protein-binding sites. A 50-μl aliquot of HRP–BSA (10 μg/ml of TBS) was added and incubated for 1 h at room temperature. After washing the wells three times with TBS, the colour reaction was performed as described above except that the substrate solution was incubated for 10 min. For inhibition assay, 50 μl of BSA solution were added just before addition of HRP–BSA solution.

Preparation of MMAC-activated Shodex

Shodex Et123 contains about 2.7 mmol amino groups per gram of gel. A 1-g amount of Shodex Et123 was suspended in 6 ml of 3% MMAC–DMSO at room temperature for 3 h with stirring. The gel was washed extensively with DMSO.

Immobilization of proteins on the activated gel

A 1-g amount of the MMAC-activated Shodex was suspended in 5 ml of 0.1 *M* acetate buffer (pH 3.5) containing 200 mg of BSA and incubated for 3 h at room temperature with stirring. Then the gel was washed extensively with TBS. With protein A and HRP, the immobilization was performed in 0.1 *M* acetate buffer (pH 4.5) and 0.1 *M* acetate buffer (pH 4.0), respectively. With ConA, 200 mg of ConA and 220 mg of Me α-D-Man were dissolved in 0.1 *M* acetate buffer (pH 6.5) containing 1 m*M* each of Ca^{2+}, Mn^{2+} and Mg^{2+} (buffer A). A 1-g amount of the MMAC-activated Shodex was suspended in this solution and the suspension was incubated for 3 h at room temperature with stirring. After washing with buffer A, the gel was incubated with 4 ml of 1% glutaraldehyde–buffer A containing 24 mg of $NaBH_3CN$ and 110 mg of Me α-D-Man. After washing with TBS, the gel was incubated with 4 ml of 1.0 *M* Tris–HCl (pH 7.8) containing 3 mg of $NaBH_3CN$ for 1 h at room temperature to block the remaining formyl groups. The gel was successively washed with 10 m*M* Tris–HCl buffer (pH 7.6) containing 1.0 *M* NaCl and 1 m*M* $CaCl_2$ and TBS containing 1 m*M* $CaCl_2$. The amount of protein immobilized on the gel was determined by difference analysis, monitoring the absorbance at 280 nm [6].

High-performance liquid affinity chromatography (HPLAC)

ConA–Shodex was packed into 10 × 4.6 mm I.D. and 50 × 8 mm I.D. columns by the slurry packing method at a flow-rate of 8 ml/min. An HPLC system with a high-pressure pump (Model DS-4, Showa Denko) and a spectromonitor (Model M-315, Showa Denko) were used. A 2-mg amount of HRP was dissolved in 1 ml of TBS containing 1 m*M* $CaCl_2$ (buffer B) and then an 80-μl aliquot was applied to a 10 × 4.6 mm I.D. column of ConA–Shodex (79 mg/g

gel) at a flow-rate of 0.2 ml/min. Maltose (0.2 *M*) in buffer B was used to elute HRP from the column. A 10-μl volume of *p*-nitrophenyl glycosides solution (9 mg each/ml buffer B) was applied to a 50 × 8 mm I.D. column of ConA–Shodex (110 mg/g gel) at a flow-rate of 1 ml/min.

RESULTS

Immobilization of proteins on MMAC-coated microtitre plate

Among the organic solvents tested, DMSO was the only solvent that can dissolve MMAC and does not damage the microtitre plates to cause an increase in turbidity of the transparent plates. HRP was used as a model protein to study the parameters that influence the immobilization of ligands to the microtitre plates. Optimum coating of the well was observed at a concentration of 5 mg MMAC/ml DMSO by judging the amounts of HRP immobilized.

The effect of buffers on the immobilization of HRP on the MMAC-coated microtitre plate is shown in Fig. 1. Tris–maleate buffers (pH 6.0 and 7.0) and Tris–HCl buffer (pH 7.6) (data not shown) gave better results on the immobilization of HRP than phosphate buffers (pH 6.0 and 7.0) and citrate–phosphate buffers (pH 6.0 and 7.0). The following experiments were performed in Tris buffer. The results of independent experiments on the immobilization are summarized in Fig. 2. HRP was efficiently immobilized on the MMAC-coated plate in a dose-dependent manner at protein concentrations between 0.5 and 100 μg/ml (Fig. 2A). The binding of HRP on the non-coated plate did not proceed in a dose-dependent manner, nor was it reproducible, as shown in Fig. 2B.

The results of the immobilization of anti-BSA antibody are shown in Fig. 3. The immobilization on the MMAC-coated plate occurred in a dose-dependent manner at protein concentrations between 1 μg/ml and 0.5 mg/ml and was more efficient than that on the non-coated plate. The binding of HRP–BSA and anti-BSA antibody on the MMAC-coated

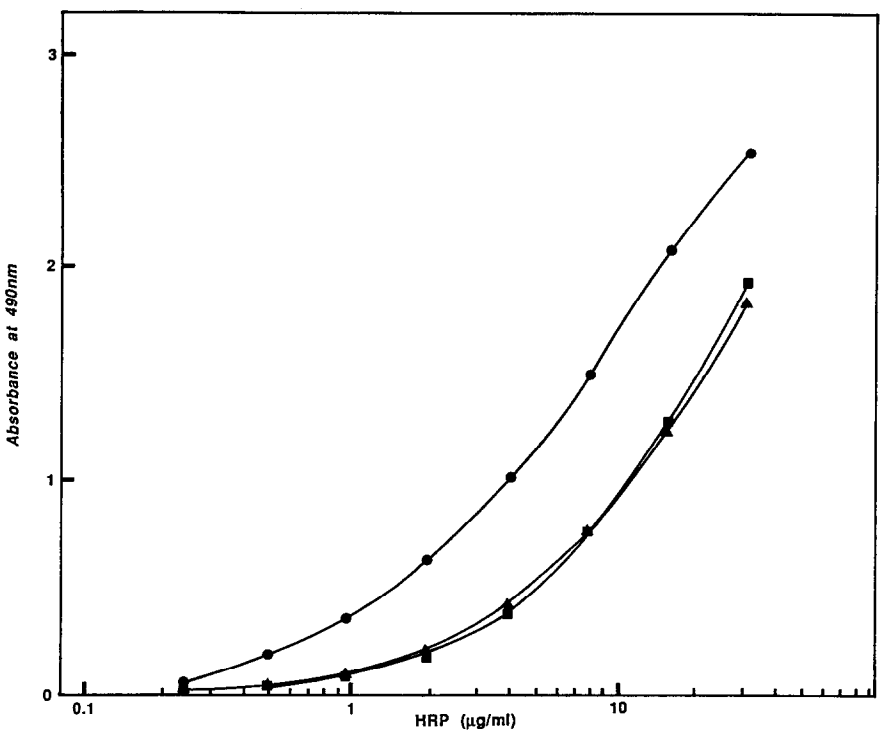

Fig. 1. Effect of buffer on immobilization of HRP. (●) Tris–maleate (pH 6.0 and 7.0), (▲) phosphate (pH 6.0 and 7.0) and (■) citrate–phosphate (pH 6.0 and 7.0) buffers were used as solvents.

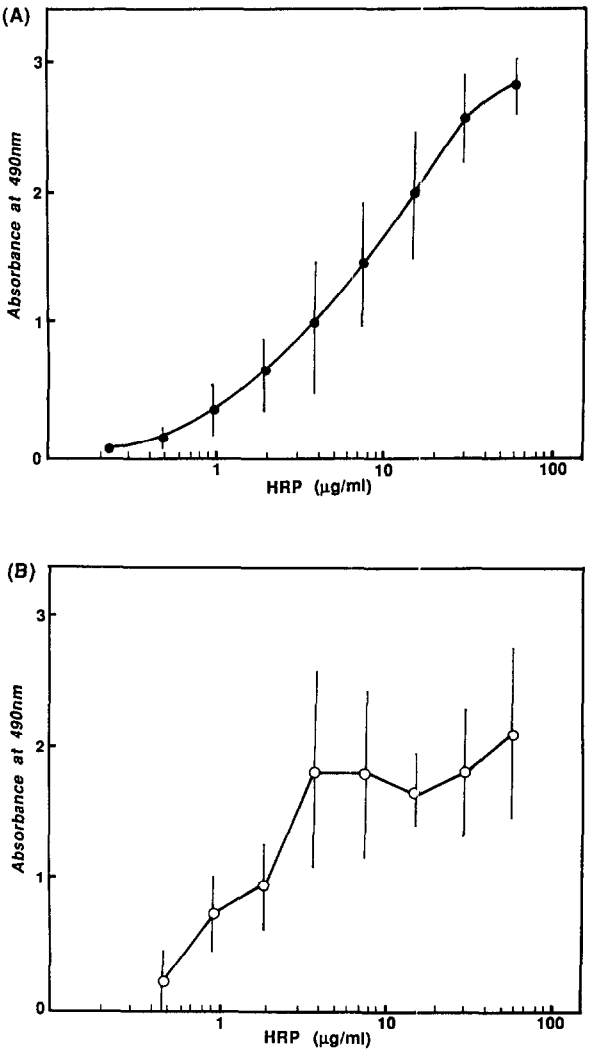

Fig. 2. Immobilization of HRP in Tris–maleate buffer. HRP was immobilized on the microtitre well (A) with MMAC and (B) without MMAC. Data are averages of (A) fourteen and (B) ten experiments.

plate was inhibited by BSA in a dose-dependent manner, and 5.5 μg/ml of BSA were required for 50% inhibition of the binding (data not shown).

Immobilization of proteins on the MMAC-activated Shodex

Very high concentrations of various proteins were immobilized on the MMAC-activated Shodex as shown in Table I. However, the yields of immobilization were not so high. The ConA–Shodex thus obtained was used for HPLAC without blocking the carboxyl groups produced in the Shodex gel.

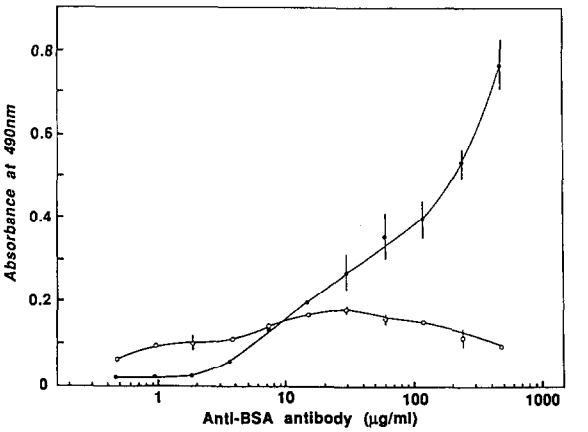

Fig. 3. Binding of HRP–BSA to immobilized anti-BSA antibody. Anti-BSA was immobilized on the microtitre well (●) with MMAC and (○) without MMAC. Determinations were done in duplicate.

HPLAC on ConA–Shodex

HRP is a glycoprotein having eight N-linked oligosaccharides which are receptors for ConA [7]. HPLAC of HRP was performed on a ConA–Shodex column of 1-cm length (Fig. 4). HRP was completely adsorbed on the column and specifically eluted with 0.2 *M* maltose.

HPLAC of *p*-nitrophenyl glycosides was also performed on a ConA–Shodex column. Although the specific sugar derivatives *p*-nitrophenyl glucoside

TABLE I

CONCENTRATIONS OF IMMOBILIZED PROTEINS

Protein	Concentration of immobilized protein (mg/g dry gel)	Yield of immobilization (%)
BSA	100	50
Protein A	100	40
HRP	61	29
ConA		
Preparation 1	79	56
Preparation 2	110	54
Preparation 3	112	56

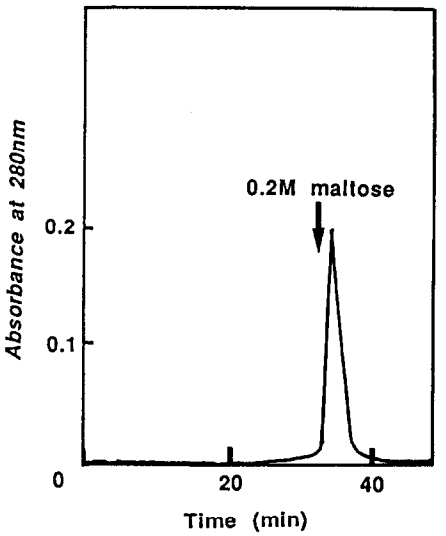

Fig. 4. HPLAC of HRP on ConA–Shodex. HRP solution (80 μl) was loaded on the column (10 × 4.6 mm I.D.) at a flow-rate of 0.2 ml/min. The column was washed with buffer B, and elution with 0.2 *M* maltose was started at the point indicated by the arrow.

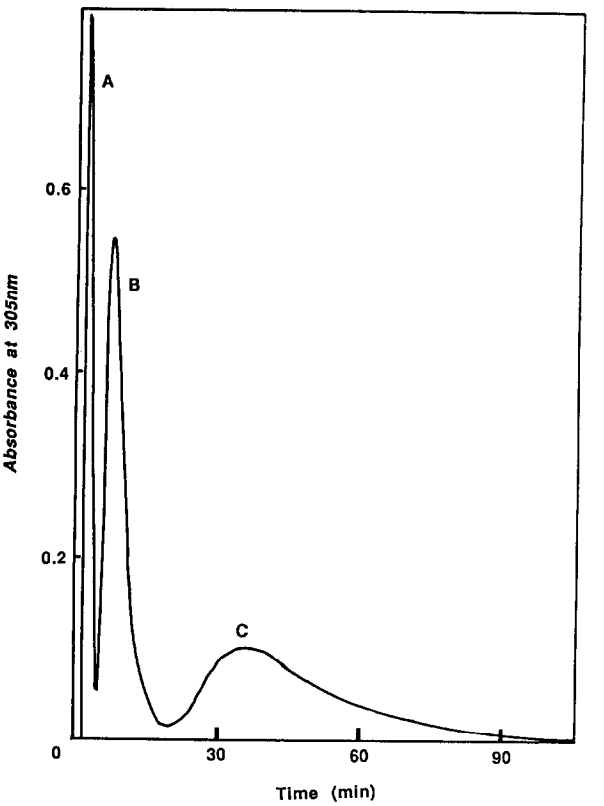

Fig. 5. Separation of *p*-nitrophenyl glycosides on ConA–Shodex. A mixture of *p*-nitrophenyl glycosides (10 μl) was loaded on the column (50 × 8 mm I.D.) at a flow-rate of 1 ml/min. TBS containing 1 m*M* $CaCl_2$ buffer was used as eluent. The glycosides were separated into three peaks: (A) *p*-nitrophenyl galactoside, (B) *p*-nitrophenyl glucoside and (C) *p*-nitrophenyl mannoside.

and *p*-nitrophenyl mannoside were not adsorbed on the column, they were retarded and separated from a non-specific *p*-nitrophenyl galactoside (Fig. 5). Further, the retardation of the specific glycosides was dependent on the affinity to ConA and the glucoside and the mannoside were eluted in the clearly separated peaks.

DISCUSSION

MMAC is highly hydrophobic and insoluble in aqueous solutions. Therefore, MMAC seems to bind with the surface of the wells of microtitre plates by hydrophobic interactions and the hydrophobic binding is expected to be stable in aqueous solutions. The hydrophobic interactions of protein ligands with the surface of the wells (Fig. 6a) seem to be far weaker than those of MMAC (Fig. 6b). Immulon 200, a plastic microtitre plate designed to have a high adsorption capacity for proteins, was used. To examine the optimum conditions for immobilization of proteins on the wells of microtitre plates treated with MMAC, HRP, which is widely used for ELISA, was chosen as a model protein ligand because it is directly detected by its enzymatic colour reaction.

First we thought that Tris buffer would not be a good buffer for immobilization of proteins, because the amino group of Tris will compete with amino groups of proteins for reaction with the acid anhydride groups of MMAC. Unexpectedly, however, Tris buffers gave better results on immobilization than phosphate and citrate buffers (Fig. 1). The unexpected results may be explained by an increase in the water solubility of protein–MMAC complexes because of the carboxyl groups produced (Fig. 6c). On reaction in Tris buffer, an acid anhydride group of MMAC gives one amide bond and one carboxyl group, and on hydrolysis it gives two carboxyl

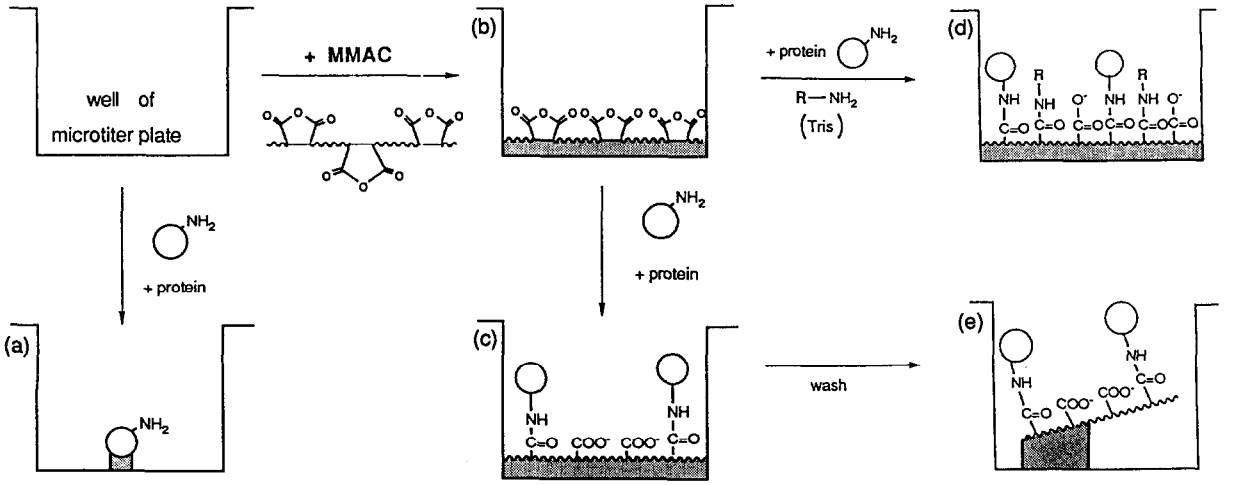

Fig. 6. Immobilization of protein ligands on the well of a microtitre plate. Hydrophobic interactions are illustrated with shading.

groups. With Tris buffer, protein–MMAC complexes maintain their water insolubility probably because of blocking of up to 50% of their carboxyl groups (Fig. 6d). With the other buffers, some of protein–MMAC complexes may be solubilized because essentially no carboxyl groups are blocked (Fig. 6e).

As shown in Table I, very high concentrations of proteins were conveniently immobilized on the MMAC-activated Shodex gel. The relatively low concentration of HRP immobilized may be because HRP is highly glycosylated and has fewer amino groups than the other proteins.

Affinity adsorbents with very high ligand concentrations have two advantages, extremely high adsorption capacities [8] and high ability to separate low-affinity substances. The high concentrations of carboxyl groups remaining on ConA–Shodex gel were not blocked and may cause non-specific adsorption of proteins, especially when this gel is used as a high adsorption capacity adsorbent for purification. In this study, however, we aimed to separate low-affinity sugar derivatives rather than to improve the adsorption capacity. For this purpose, the remaining carboxyl groups caused no problem.

ConA–Shodex obtained by this method exhibited very high affinities towards a glycoprotein and sugar derivatives. Only a 1-cm long column was required for the HPLAC of HRP on ConA–Shodex. Narayanan *et al.* [2] have reported a similar separation pattern of *p*-nitrophenyl glycosides using ConA–silica. With ConA–silica, it was necessary to use a 25-cm column, whereas a 5-cm column was sufficient to give the same separation with ConA–Shodex. This may be because ConA–Shodex contains a higher amount of ConA (110 mg/g gel) than ConA–silica (70 mg/g gel) and has a larger spacer. Although *p*-nitrophenyl mannoside was eluted in a broad retarded peak, the elution can be accelerated with a linear gradient of Me α-D-Man or maltose.

Amino derivatives of other gels such as amino–Sepharose [9] and amino–Toyopearl [10] can be activated with MMAC. Although we have reported the direct immobilization of proteins, the MMAC-activated gels are expected to provide various routes for the immobilization of a number of ligands, as shown for polyacrylhydrazide–agarose by Wilchek [1].

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