

STUDIES ON POLYVINYL ALCOHOL CARRIERS

III. ACTIVE AMIDES AND ESTERS DERIVED FROM POLYVINYL ALCOHOL AND THEIR APPLICATION IN AFFINITY CHROMATOGRAPHY

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ABSTRACT

Polyvinyl alcohol carriers bearing active amide and active ester groups were prepared by acetalization of granular macro-reticular water-insoluble PVA with glyoxylic acid or p-formylbenzoic acid and further activation reaction with benzotriazole or N-hydroxysuccinimide using DCCI as condensation agent. The active amides or active esters obtained were easy to react with amines such as n-butylamine or proteins under mild condition. Ovomucoid, an inhibitor of trypsin was immobilized on the above-obtained reactive PVA carrier to give effective affinity adsorbent for the separation or purification of trypsin.

Affinity chromatography, as a powerful separation or purification method in biology and medicine fields, is now in the ascendant in recent years^[1]. This technique exploits the unique procedure of binding a ligand which shows the specific affinity towards certain biosubstances to an insoluble support and using this insoluble adsorbent for separation or purification of the biosubstances we interested. The often used insoluble supports such as cellulose derivatives, agarose gels, dextran gels, etc., often show some disadvantages such as difficulty to forming or beading, susceptible to the attack of acid or metabolism of microorganism, poor flow property, etc. Furthermore, some of these natural matrices exhibit undesirable non-specific absorption for proteins or other biosubstances.

Total synthesized polymer matrices may overcome some of the inherent defects of these natural supports by proper choosing the back-bone of the polymer matrices and the further molecular modification in the light of molecular design. The one-step preparation of macro-reticular spheric polyvinyl alcohol (PVA) carrier, the activation of these supports and the immobilization of proteins with these activated carriers have been reported in our previous papers^[2,3]. The present article would describe the introduction of various spacer arms with terminal carboxy group onto these inert matrices, the activation of these supportants with benzotriazole and N-hydroxysuccinimide, and the preparation of affinity adsorbents for the separation or purification of trypsin.

EXPERIMENTAL

Preparation of macro-reticular spheric polyvinyl alcohol (MR-PVA), measurement of the

specific surface area, the pore diameter and the pore volume were described as before^[2]. The packing density of MR-PVA mentioned in this article was 0.40–0.50 g/ml.

Reaction of MR-PVA with *p*-formylbenzoic acid (FBA) and glyoxylic acid (GA): To 40 ml dimethylformamide (DMF) which dissolved 1.2×10^{-2} mole of aldehyde compounds, 10 ml MR-PVA, 2 ml concentrated H_2SO_4 and 4g Na_2SO_4 were added. After the acetalization reaction was carried out for 4 hours at 65–70°C, the gel was filtered and washed with distilled water to neutral.

Preparation of active amides and active esters: The obtained MR-PVA beads bearing carboxy group were suspended in DMF and benzotriazole were added. After the solution was cooled below 0°C, dicyclocarbodiimide (DCCI) were added partially. The reaction was kept at this temperature for 2 hours. Filtered, the product was washed with methanol and tetrahydrofuran to remove the formed dicyclohexyl urea thoroughly. These activated supports were kept in anhydrous dioxane for further reaction (abbreviated as MR-PVA-FBA-BT and MR-PVA-GA-BT).

Preparation of active esters of *N*-hydroxysuccinimide were carried out as mentioned above (abbreviated as MR-PVA-FBA-NHS and MR-PVA-GA-NHS).

Reaction of PVA-active amides and active esters with amines or alcohols^[3,4]: 0.5 g of PVA-FBA-BT were dissolved in 20 ml DMF, 2 ml *n*-butylamine or allyl alcohol and 1 ml triethylamine were added. After the exchange reaction was carried out for 20 hours at room temperature, the mixture was poured into large amount of water to precipitate the product.

Immobilization of protein with MR-PVA-R-NHS: 10 ml of activated matrix and 0.25 g of chicken ovomucoid (CHOM) was coupled in 30 ml borate buffer of pH 7.5 at room temperature for 24 hours and then filtered. The obtained affinity adsorbent was washed with 0.2 *N* formic acid and distilled water for four times. The amount of immobilized protein was measured by UV method.

Affinity chromatography^[5]: CHOM-PVA adsorbent was charged into a column of 10 mm × 70 mm. After the column was equilibrated with Tris buffer of pH 7.5, newly activated bovine trypsin was dissolved in small amount of the buffer and then, applied to the column. Washed with the Tris solution until no protein was detected in the fraction, the demanded trypsin was eluted with 0.1 *M* potassium formate–0.5 *M* potassium chloride solution of pH 2.5.

RESULTS AND DISCUSSION

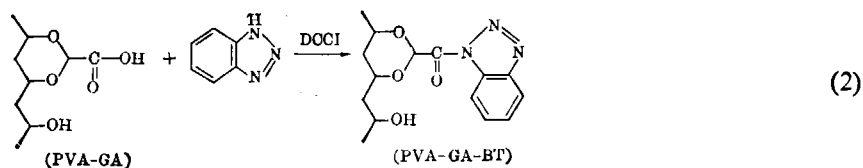
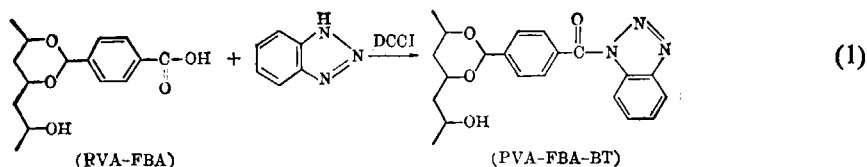
Macroreticular insoluble polyvinyl alcohol matrices prepared with glycol homologues as diluents and *p*-phthaldehyde as crosslinking agent possess satisfactory mechanical strength and flow property. The influence of diluents on the pore diameter, pore volume and specific surface area of the MR-PVA supports are showed in Table 1. It can be seen that the pore diameter of MR-PVA of tetraglycol reached 4000Å and this can be used as macroreticular resin.

For affinity chromatography support, the space or arm between the backbone of the matrix and the ligand must possess appropriate length. Furthermore, the accessibility of the matrix, the rigidity and the hydrophilic or hydrophobic property of the spacer arm, all

Table 1 Pore diameter, pore volume and specific surface area of PVA matrices

Diluent	Specific surface area $\Delta S(\text{m}^2/\text{g})$	Pore volume $V_p(\text{cm}^3/\text{g})$	Pore diameter $\bar{R}(\text{\AA})$
Diglycol	8.0	0.76	2400
Tetraglycol	7.3	1.16	4000

exert remarkable influence on the amount and the retained activity of the immobilized ligand. MR-PVA matrices possess abundant hydroxy groups and can be modified or functionalized easily by introducing various spacer arms with terminal carboxy groups onto the backbones and then activated as follows:



These activated MR-PVA supports can be ammonolyzed by compounds containing amino group such as *n*-butylamine at room temperature or alcoholized by compounds containing hydroxy group such as allyl alcohol in the presence of triethylamine at somewhat higher temperature.

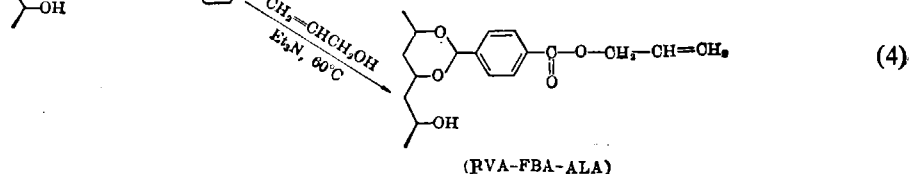
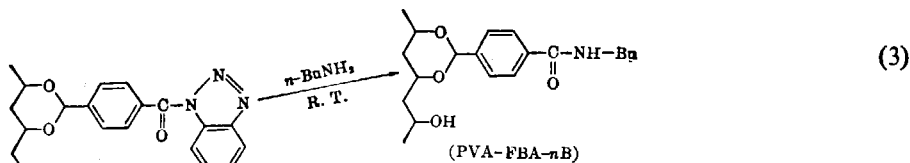


Fig. 1 shows the IR spectra of the exchange reaction products of functionalized PVA. In the spectrum of PVA-FBA, the $\nu_{C=O}$ absorption band of carboxy group appeared around 1680 cm^{-1} and ν_{C-H} absorption bands of aromatic ring appeared around 750 cm^{-1} . After reacted with BT, the absorption band near 1680 cm^{-1} weakened, instead, the $\nu_{C=O}$ band and ν_{C-N} of the imide group appeared around 1650 cm^{-1} and $1300\text{--}1400\text{ cm}^{-1}$, respectively. As PVA-FBA-BT was alcoholized with allyl alcohol (ALA), the absorption band of the carbon-carbon double bond appeared around 1620 cm^{-1} , whereas ammonolyzed with *n*-butylamine, the amide I band and amide III band of the amide group appeared near $1650\text{--}1700\text{ cm}^{-1}$ and 1300 cm^{-1} , respectively. This shows us that these activated MR-PVA supports may be reactive to the immobilization of proteins.

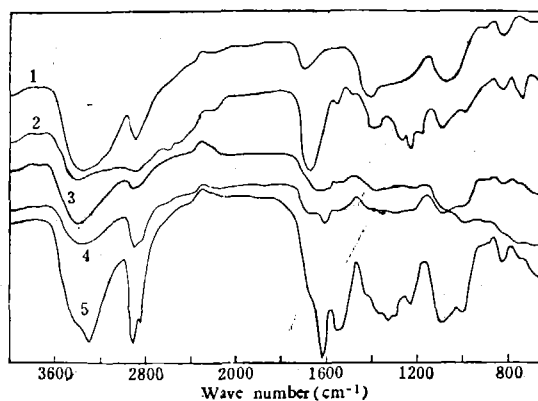


Fig. 1 IR spectra of PVA and its derivatives

1. PVA; 2. PVA-FBA; 3. PVA-FBA-BT; 4. PVA-FBA-ALA;
5. PVA-FBA-*n*Bu.

Chicken ovomucoid (CHOM), a natural inhibitor of trypsin, was coupled onto these activated MR-PVA supports with various spacer arms. The amount of coupled enzyme and its retained activity were affected remarkably by the length and rigidity of the arms between the ligand and the backbone of the matrix. The inhibition reactivity of the bound ovomucoid protein were showed in Table 2.

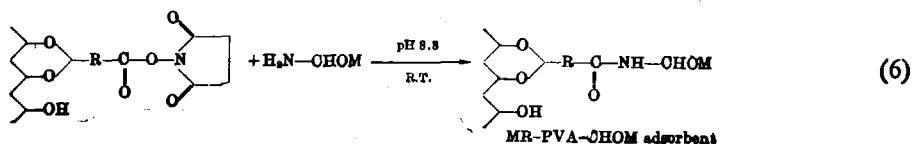
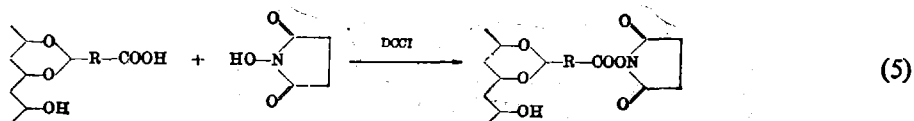
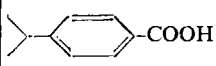
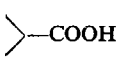
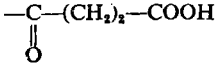


Table 2 Relationship of specific activity of immobilized protein and the ligand spacer arm

Arm			
—COOH amount (mmole/10ml carrier)	4.05	0.65	0.12
Immobilized protein (mg/mmmole —COOH)	8	104	268
Retained activity (BAEE unit/mg)	2000	4000	4000

It can be seen that when p-formylbenzoic acid was used as the spacer arm, due to the hydrophobic and steric effect of the aromatic ring, the amount and the retained activity of the bound protein, in spite of the rigidity of the arm, were much lower. This is different from some results reported in literature^[7]. Matrices with longer spacer arm possessed satisfactory coupling ability and retained protein activity.

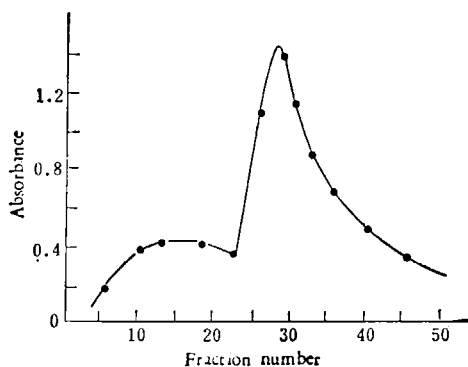


Fig. 2 Affinity chromatography of trypsin on PVA-CHOM affinity column

Column volume: 10 mm×70 mm;

Eluant: HCOOK-KCl buffer of pH 2.5;

Elution rate: 1.5 ml/min; Room temperature.

After the PVA-CHOM affinity adsorbent was packed in a column of 10 mm×70 mm and equilibrated with 0.1 M Tris solution of pH 7.5, the newly activated bovin trypsin was dissolved in the mentioned buffer and applied to the column. Active trypsin was then eluted with 0.10 M potassium formate-0.50 M potassium chloride solution of pH 2.5. The chromatography is showed in Fig. 2. This demonstrates that the affinity adsorbent prepared from the total synthesized MR-PVA matrix may be used in the separation or purification of trypsin effectively.

When used in the separation of C-reactive protein from rabbit serum, it was found that MR-PVA supports showed milder non-specific absorption in comparison with the conventional Sepharose matrix.

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