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CHARACTERISTICS OF IMMOBILIZED TANNIN FOR PROTEIN ADSORPTION

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SUMMARY

The adsorption of proteins on immobilized tannin has been found to fall into one of three classes: in the first the proteins are well adsorbed at neutral to weak acidic pH values, and the adsorption is not affected by salt concentration; in the second the proteins are well adsorbed at acidic or alkaline pH values, but the adsorption is affected by salt concentration; in the third class the proteins are only slightly adsorbed at all pH regions. On the basis of these results, a mixture of three different proteins has been separated using an immobilized tannin column.

INTRODUCTION

Previously, we have immobilized tannin by covalent binding to aminohexyl cellulose using cyanogen bromide or epichlorohydrin[1], and have also reported the characteristics of immobilized tannin prepared by the cyanogen bromide method[2]. Furthermore, we have reported the application of tannin for immobilization of enzymes[3,4] and continuous fining of saké[5,6]. Subsequently it was found[7] that the immobilized tannin prepared by the epichlorohydrin method was more suitable for continuous fining of saké than that obtained by the cyanogen bromide method.

In order further to develop this technique for separation, recovery and removal of proteins, we have now investigated the characteristics of immobilized tannin prepared by the epichlorohydrin method from the viewpoint of protein adsorption.

MATERIALS AND METHODS

Compounds

Filter pulp (No. 4) was purchased from Toyo Roshi (Tokyo, Japan), epichlorohydrin and Chinese gallotannin from Katayama Chemical Industries (Osaka, Japan). Crystalline α-amylase from *Bacillus subtilis* and alkaline protease from *Streptomyces griseus* were obtained from Sigma (St. Louis, MO, U.S.A.), crystalline lysozyme from egg and glucoamylase from *Rhizopus niveus* from Seikagaku Kogyo (Tokyo, Japan), crystalline ovalbumin and crystalline pepsin from porcine stomach from Worthington

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Biochemical (Freehold, NJ, U.S.A.) and bovine serum albumin (Fraction V) and crystalline trypsin from beef pancreas from Miles Labs. (Elkhart, IN, U.S.A.). Gelatin powder was the product of E. Merck (Darmstadt, G.F.R.). All other chemicals were of reagent grade.

Preparation of immobilized tannin

To 3 l of distilled water were added 200 g of filter pulp (No. 4) and the suspension was cooled to 4°C. Three litres of 6 *N* sodium hydroxide were added, and the mixture was gently stirred at 4°C for 3 min, then allowed to stand for 30 min at 4°C. To the alkali-treated cellulose, were added 12 l of distilled water and the suspension was stirred for 30 min at 60°C. Two litres of epichlorohydrin were added, and the mixture was stirred for 30 min at 60°C. The activated cellulose was collected by filtration, and washed with 10 l of distilled water. It was then suspended in 8 l of 0.625% hexamethylenediamine solution, and the mixture was stirred at 60°C for 120 min. After the reaction, the aminohexyl cellulose was collected and washed with 10 l of water. It then was suspended in 10 l of 0.25 *N* sodium hydroxide, 1 l of epichlorohydrin was added and the mixture was stirred for 30 min at 60°C. The activated aminohexyl cellulose was washed with 10 l of water, and then suspended in 8 l of 3% Chinese gallotannin aqueous solution adjusted to pH 7.0 with sodium hydroxide. After addition of 4.8 g of sodium borohydride, nitrogen gas was bubbled into the suspension with stirring for 150 min at 45°C. After the reaction, the immobilized tannin was collected and washed with 10 l of water. It was then suspended in 4.5 l of 30% aqueous acetone, and the pH of the mixture was adjusted to 2.0 with 3 *N* hydrochloric acid. The resulted suspension was stirred for 10 min at 25°C. Immobilized tannin was collected by filtration, washed with 10 l of water, three times with aqueous acetone and finally with 20 l of water. The resulting immobilized tannin preparation was suspended in 5 l of sodium citrate–hydrochloric acid buffer (pH 4.0). The immobilized tannin was then filtered off and stored at 4–10°C in a sealed vessel. The preparation was 1290 g in wet weight (*ca.* 4 l in volume), and contained 81% of water and 4.75% Chinese gallotannin.

Adsorption and desorption of proteins

The adsorption of proteins on immobilized tannin was carried out by shaking the adsorbent with protein dissolved in buffer solution for 30 min at 5°C. The mixture was then filtered, and the concentration of protein in the filtrate was determined.

The desorption of proteins from immobilized tannin was carried out by shaking the immobilized tannin–protein complex with selected desorbents for 15 min at 25°C. After filtration of the mixture, the concentration of protein in the resulting filtrate was determined.

Adsorption capacity of immobilized tannin for proteins

The adsorption capacity of immobilized tannin for proteins was measured by the column method as follows. One gram (wet weight) of immobilized tannin was packed into a column (45 × 15 mm, bed volume 8 ml), and the column was then equilibrated with a buffer solution. A 0.5% protein solution in the same buffer was applied to the column at a flow-rate of 13.8 ml/h at 10°C, until the protein concentration in the effluent became equal to that in the charged solution. Two millilitres of

each fraction were collected, and the concentration of protein in the fractions was determined. The adsorption capacity of immobilized tannin for a protein was calculated as the difference between the amount of protein in the effluent and that in the charged solution.

Chromatography

One gram (wet weight) of immobilized tannin was packed into a column (45 × 15 mm, bed volume 8 ml). After washing with water, the column was equilibrated with 0.05 *M* phosphate buffer, pH 7.5. Proteins dissolved in the same buffer solution were applied to the column at 10°C at a flow-rate of 13.8 ml/h. After washing the column with the same buffer, proteins adsorbed on the column were eluted with 0.05 *M* carbonate buffer, pH 9, and subsequently with 0.05 *M* acetate buffer, pH 4. Two millilitres of each fraction were collected, and the protein concentration and enzyme activity in the fraction were assayed.

Determination of protein

Protein was determined by the method of Lowry *et al.*[8], or by measuring the absorbance at 280 nm.

Determination of enzyme activity

Enzyme activities of trypsin and lysozyme were determined by the methods of Schwert and Takenaka[9] and Shugar[10], respectively.

Determination of ovalbumin

The concentration of ovalbumin was determined by measuring the content of carbohydrate with the phenol–sulphuric acid method.

RESULTS

Titration of immobilized tannin

The immobilized tannins prepared by the epichlorohydrin method and native tannin were titrated with hydrochloric acid and sodium hydroxide, and the resulting potentiometric titration profiles were compared. As shown in Fig. 1, immobilized tannin had two different titratable groups: one is acidic having a pK value (pK_1) of 5.0, the same as that of native tannin; the other is basic having a pK value (pK_2) of 8.5. As the titres for both groups were almost equal, *i.e.*, *ca.* 20 μmoles per gram adsorbent (wet weight), the isoelectric point of immobilized tannin is near neutral pH.

Adsorption of proteins on immobilized tannin

Effect of pH. Various proteins were shaken with immobilized tannin in 0.01 *M* buffer at pH 2–12. All the proteins used were adsorbed to various extents on the adsorbent as shown in Fig. 2a and b. However, the ratios of the extents of adsorption of proteins on immobilized tannin were affected by the pH of the protein solution, the optimum pH for adsorption depending on the protein used. Furthermore, some proteins such as trypsin and gelatin were only slightly adsorbed even at optimum pH.

Effect of salt concentration. In order to study the nature of the binding between proteins and immobilized tannin, the effect of salt concentration on the adsorption

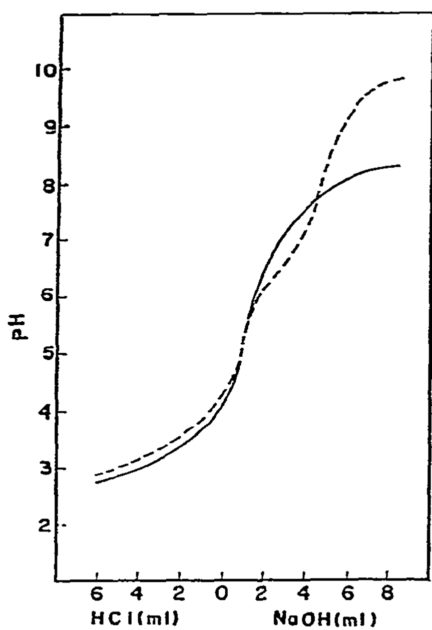

Fig. 1. Titration curve of immobilized tannin. One gram of wet immobilized tannin (--------) or 50 mg of native tannin (———) in 25 ml of water was titrated with 0.01 N NaOH or 0.01 N HCl using a potentiograph E 336 Metrohm.

of proteins was investigated at optimum pH for adsorption. As shown in Fig. 3, in the cases of ovalbumin and serum albumin having optimum pH at near neutral pH, the salt concentration had little effect on the adsorption. On the other hand, in the cases of pepsin and lysozyme having optimum pH at acidic or alkaline pH, the adsorption decreased with increasing salt concentration. Furthermore, the adsorption of gelatin increased with increasing salt concentration.

Recovery of ovalbumin

The results in Fig. 3 suggested that the binding between ovalbumin and immobilized tannin at the optimum pH for adsorption may not be ionic. In order to study the mode of binding in this case, the desorption of ovalbumin from immobilized tannin was carried out with different eluents. As shown in Table I, the recovery of protein was low when sodium chloride, sucrose, acetone or ethylene glycol was used as eluent. However, the recovery was high when a mixture of sodium chloride and other eluents was used. The desorptions were also carried out with some buffer solutions, and it was seen that buffer solutions of acidic or alkaline pH could also desorb ovalbumin from immobilized tannin.

Adsorption capacity of immobilized tannin for proteins

For practical use of immobilized tannin it is necessary to know its adsorption capacity for proteins. Therefore, pepsin, lysozyme, ovalbumin and trypsin were applied to the column at the respective optimum pH values for adsorption until the protein concentration in the effluent became equal to that in the charged solution;

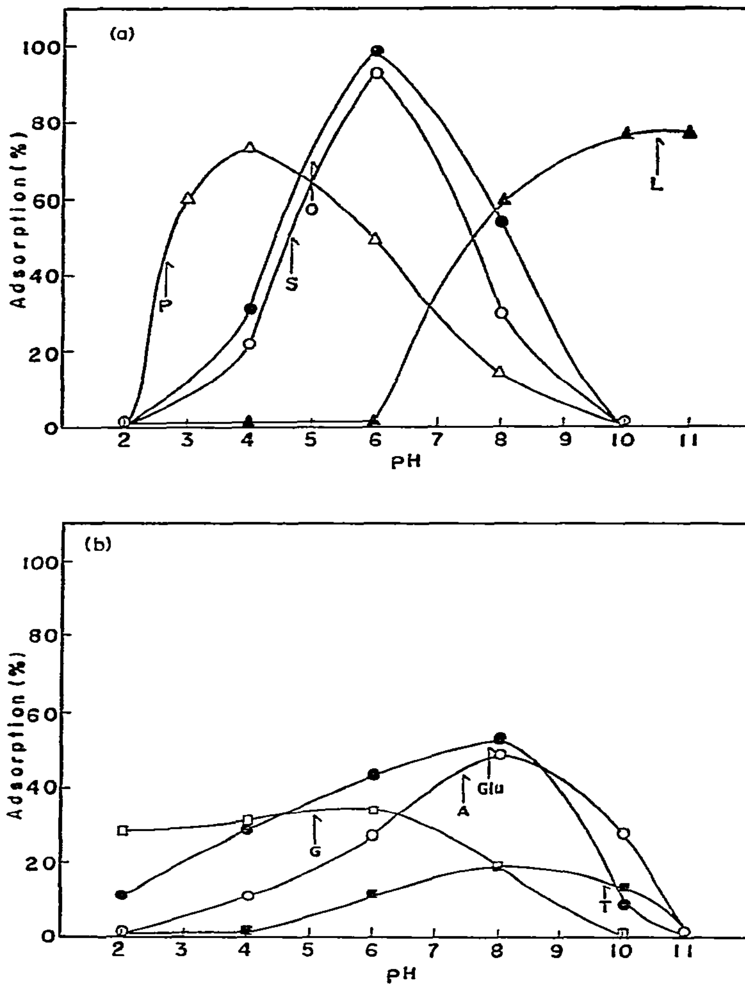

Fig. 2. Effect of pH on adsorption ratio for proteins. Adsorption was carried out by shaking 200 mg (wet weight) of immobilized tannin with 10 ml of 0.1% protein dissolved in the following buffer solutions: pH 2, 0.01 *N* HCl; pH 3–5, 0.01 *M* acetate buffer; pH 5–8, 0.01 *M* phosphate buffer; pH 9–11, 0.01 *M* carbonate buffer. The adsorption ratios were calculated from:

$$\text{Adsorption ratio } (\%) = \frac{(\text{protein used}) - (\text{protein not adsorbed})}{(\text{protein used})} \times 100$$

Other conditions are given in the text. Proteins: a, △, pepsin (P); ●, ovalbumin (O); ○, serum albumin (S); ▲, lysozyme (L); b, □, gelatin (G); ○, alkaline protease (A); ●, glucoamylase (Glu); ■, trypsin (T). Arrows indicate isoelectric points.

the adsorption capacities for these proteins were then determined. As shown in Fig. 4, pepsin, lysozyme and ovalbumin were well adsorbed, but trypsin was only slightly adsorbed. From the figure, the adsorption capacity per gram of immobilized tannin (dry weight) was calculated to be 950 mg for pepsin, 600 mg for lysozyme, 540 mg for ovalbumin and 68 mg for trypsin.

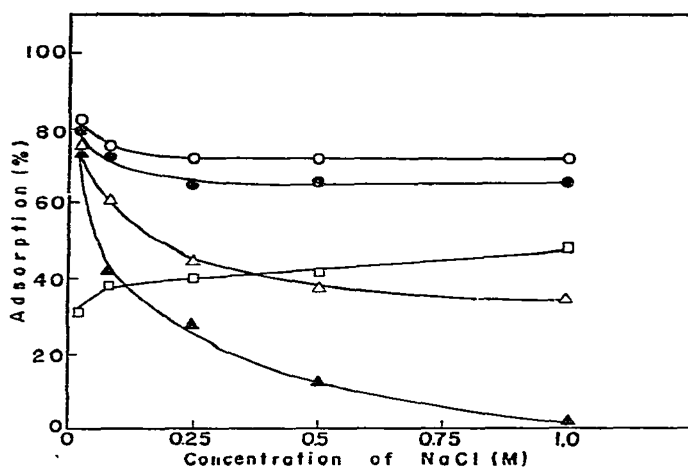

Fig. 3. Effect of salt concentration on the adsorption ratio for proteins. Adsorption was carried out by the method in Fig. 2 except that the pH and salt concentration of the protein solution was varied. Proteins: ●, ovalbumin; ○, serum albumin; □, gelatin; △, pepsin; ▲, lysozyme.

TABLE I

RECOVERY OF OVALBUMIN ADSORBED ON IMMOBILIZED TANNIN

Adsorption was carried out by shaking 35 g (wet weight) of immobilized tannin with 200 ml of 0.5% ovalbumin dissolved in 0.05 *M* acetate buffer solution, pH 6. Desorption was carried out by shaking 1 g (wet weight) with 10 ml of a buffer solution or a 0.05 *M* acetate buffer, pH 6, containing some desorbent. Other conditions are given in the text.

Solvent for desorption	*Recovery of protein (%)*
0.05 *M* Acetate buffer, pH 4	48.8
0.05 *M* Acetate buffer, pH 6	9.6
0.05 *M* Phosphate buffer, pH 8	73.7
0.05 *M* Carbonate buffer, pH 10	81.6
1 *M* Sodium chloride in acetate buffer, pH 6	17.6
50% Sucrose in acetate buffer, pH 6	17.6
30% Acetone in acetate buffer, pH 6	14.7
50% Ethylene glycol in acetate buffer, pH 6	17.3
1 *M* Sodium chloride and 50% sucrose in acetate buffer, pH 6	58.7
1 *M* Sodium chloride and 30% acetone in acetate buffer, pH 6	47.2
1 *M* Sodium chloride and 50% ethylene glycol in acetate buffer, pH 6	80.9

Separation of trypsin, ovalbumin and lysozyme on immobilized tannin

The separation of a mixture of these three pure proteins was tested using an immobilized tannin column. As shown in Fig. 5, trypsin was not adsorbed on the column at neutral pH, whereas the other two proteins were adsorbed and could be separately eluted with alkaline buffer and then acidic buffer without significant loss of protein.

DISCUSSION

In order to develop the separation, recovery and removal of proteins, we have investigated the characteristics of immobilized tannin for protein adsorption.

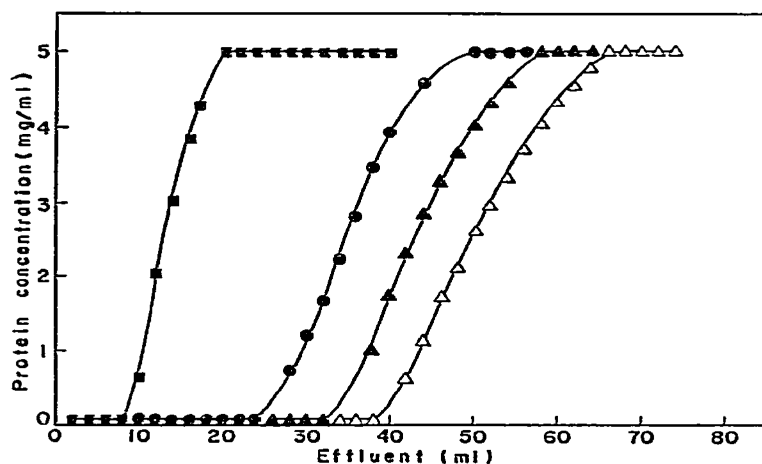

Fig. 4. Adsorption capacity of immobilized tannin for proteins. Buffer solutions for equilibration of the column were 0.05 *M* acetate buffer, pH 4, for pepsin; 0.05 *M* acetate buffer, pH 6, for ovalbumin; 0.05 *M* phosphate buffer, pH 8, for trypsin; and 0.05 *M* carbonate buffer, pH 10, for lysozyme. Other conditions are given in the text. Proteins: ■, trypsin; ●, ovalbumin; ▲, lysozyme; △, pepsin.

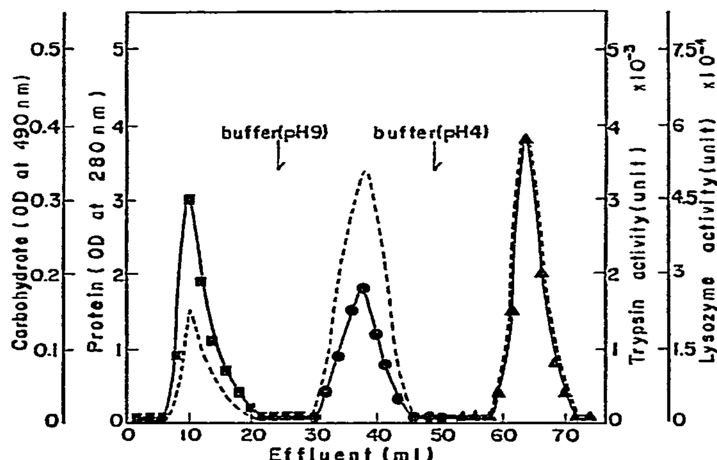

Fig. 5. Separation of a mixture of trypsin, lysozyme and ovalbumin on immobilized tannin. Two millilitres of protein solution (pH 7.5) containing 10 mg of trypsin, 10 mg of ovalbumin and 10 mg of lysozyme were applied to the column, and the proteins adsorbed were eluted with the specified buffer solutions. Other conditions are given in the text. ■—■, Trypsin activity; ●—●, carbohydrate; ▲—▲, lysozyme activity; – – –, protein.

It is well known that native tannin interacts with proteins mainly through hydrogen bonding[11]. However, as can be seen from Fig. 1, immobilized tannin has both acidic and basic groups. Therefore, it is suggested that ionic forces may also take part in the interaction between proteins and the adsorbent.

As the isoelectric point of immobilized tannin is at near neutral pH, the adsorption of proteins to immobilized tannin at neutral pH must be primarily due to hydrogen bonding, whereas ionic forces only assist the binding. This is borne out by the fact that ovalbumin adsorbed on immobilized tannin could not effectively be eluted by an eluent containing 1 *M* sodium chloride at neutral pH (Table I), and the

adsorption ratio of ovalbumin and serum albumin was not affected by salt concentration (Fig. 3).

On the other hand, immobilized tannin has positive and negative charges at acidic and alkaline pH values, respectively. Thus, in the pH region between the isoelectric point of a protein and that of immobilized tannin, ionic binding forces work in addition to hydrogen bonding and increase the adsorption ratio of proteins as observed in the case of pepsin and lysozyme. In other pH regions, where the charge of the protein is the same as that of immobilized tannin, ionic repulsion forces occur, and the adsorption decreases with increasing repulsion (Fig. 2 a and b). As ionic forces operate at acidic and alkaline pH values, the salt concentration also affects the adsorption of proteins such as pepsin and lysozyme, whose isoelectric points are far from that of immobilized tannin (Fig. 3).

Some proteins such as trypsin and gelatin were only slightly adsorbed on immobilized tannin at all pH regions. It is suggested that these proteins may have a structure which hinders the formation of hydrogen and ionic bonds with the immobilized tannin.

From the above results, proteins can be classified into three types on the basis of their interactions with immobilized tannin: first, proteins having low affinities for immobilized tannin, such as trypsin and gelatin; secondly, proteins having affinities for immobilized tannin through mainly ionic bonding, such as lysozyme and pepsin whose isoelectric points are far from that of immobilized tannin; thirdly, proteins having affinities for immobilized tannin through mainly hydrogen bonding, such as ovalbumin and serum albumin whose isoelectric points are near to that of immobilized tannin.

In order to confirm that the immobilized tannin can be utilized for the separation of proteins, a model experiment for chromatographic separation of different kinds of pure proteins was carried out. The proteins were well separated (Fig. 5). These results suggest that separation, recovery and removal of proteins using immobilized tannin may be carried out by selecting suitable conditions for adsorption and desorption.

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