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PREPARATION OF IMMOBILIZED TANNINS FOR PROTEIN ADSORPTION

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Preparation and adsorption specificity of tannins immobilized by covalent binding on water-insoluble matrices were investigated. Immobilized tannins were prepared by condensing cyanogen bromide activated tannins with aminohexyl derivatives of several kinds of matrices. The most suitable matrix for the immobilization of tannin was alkali-treated cellulose powder. The concentration of sodium hydroxide solution for alkali treatment influenced the adsorption capacity of immobilized tannin for a protein, but temperature and time for alkali treatment did not. Immobilized tannins having spacers of long chain length exhibited high adsorption capacity for a protein. Chinese gallotannin was the most favorable ligand for protein adsorption. The immobilization of tannin on aminohexyl matrices was also possible by using epichlorohydrin instead of cyanogen bromide. The maximum adsorption capacity of the immobilized tannin for a protein was about 50 mg/ml of the absorbent. Immobilized tannin adsorbed proteins specifically but did not absorb low molecular weight compounds.

INTRODUCTION

As protein adsorbents, there are many inorganic compounds, such as alumina, charcoal, clays, glass and silica, and many organic compounds such as starch, ion-exchange celluloses and ion-exchange resins. They have been used for recovery of proteins (1), for removal of proteins (2), for purification of proteins (3), and for immobilization of enzymes (4). However, they do not necessarily satisfy all the basic requirements for these uses, and this has certainly inhibited their use. For example, they are not specific for proteins but adsorb various organic and inorganic compounds together with proteins. Accordingly, proteins recovered from an aqueous solution by those adsorbents are usually contaminated with impurities other than proteins. Further, when those adsorbents are used for the removal of unfavorable proteins in

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an aqueous solution such as Japanese *saké* or beer, various useful compounds related to taste, flavor, and color are also removed, and the quality of the solution is lowered.

Therefore, we feel that if an adsorbent that specifically adsorbed proteins could be prepared, it would be a great advantage in many fields. To design this adsorbent, we observed that tannin has been used as a protein-precipitating agent for many years. We chose this compound as our ligand. That is, we felt that the immobilized tannin prepared by chemically binding tannin to a water-insoluble matrix would be a most suitable and specific adsorbent for proteins.

In this paper the procedures preparation for immobilized tannins and their adsorbent capacities for α-amylase and glucoamylase, which are known as unfavorable precursors of turbid material in Japanese *saké*, are presented. In addition, adsorption specificity of immobilized tannin is also described.

MATERIALS AND METHODS

Materials

Sepharose 4B and Sephadex G-100 were purchased from Pharmacia Fine Chemicals Co. (Uppsala, Sweden). Cellulose powder A (100 ~ 200 mesh), C (300 mesh), D (40 ~ 100 mesh), cotton linter pulp 5M-2400 and filter paper No. 7 were purchased from Toyo Roshi Co., Ltd. (Tokyo, Japan). Cellulose powder microcrystalline, Cellulose powder CC31, Cellulose powder Avicel, and PAB-cellulose were purchased from E. Merck (Darmstadt, Germany), Whatman (New Jersey, USA), Asahi Kasei Co., Ltd. (Tokyo, Japan) and Seravac Laboratories (Berks, USA), respectively. Enzacryl AA, Bio-Gel CM-2, Encor and Amberlite IRC-50 were purchased from Koch-Light Laboratories (Buckinghamshire, England), BIO · RAD Laboratories (California, USA), Corning Glass Co. (Massachusetts, USA) and Rohm and Haas Co. (Pennsylvania, USA), respectively. α-Amylase from *Bacillus subtilis* was purchased from sigma chemical Co. (St. Louis, USA), and glucoamylase was the product of Tanabe Seiyaku Co., Ltd. (Osaka, Japan). Crystalline asparaginase was prepared by the method of Tosa, Sano, Yamamoto, Nakamura, and Chibata (5). Crystalline lysozyme and crystalline trypsin were purchased from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan). Cyanogen bromide, sodium borohydride and chinese gallotannin were purchased from Katayama chemical Industries Co., Ltd. (Osaka, Japan). Persimon juice was a gift from Konishi Schuzo Co., Ltd. (Kobe, Japan), and epichlorohydrin and diaminoalkane were

purchased from Tokyo Kasai Kogyo Co., Ltd. (Tokyo, Japan). All other chemicals were of reagent grade quality.

Preparation of Immobilized Tannins

The general method for immobilization of tannin is shown in Figure 1.

Aminohexylation of Matrices. (1) *Cyanogen bromide procedure.* 1 g (dry weight) of a matrix was steeped in 200 ml of water at 25°C for 16 h. The swollen matrix was collected, washed with water, and suspended in 50 ml of a 0.1 M sodium bicarbonate solution. The suspension was adjusted to pH 11.5 with 5 N sodium hydroxide. To the suspension, 0.5 g of cyanogen bromide was added and the mixture gently stirred at 20 to 25°C for 10 min. During the reaction, the pH was maintained at 11.0 ~ 11.5 with 5 N sodium hydroxide. The treated matrix was collected by filtration, and washed

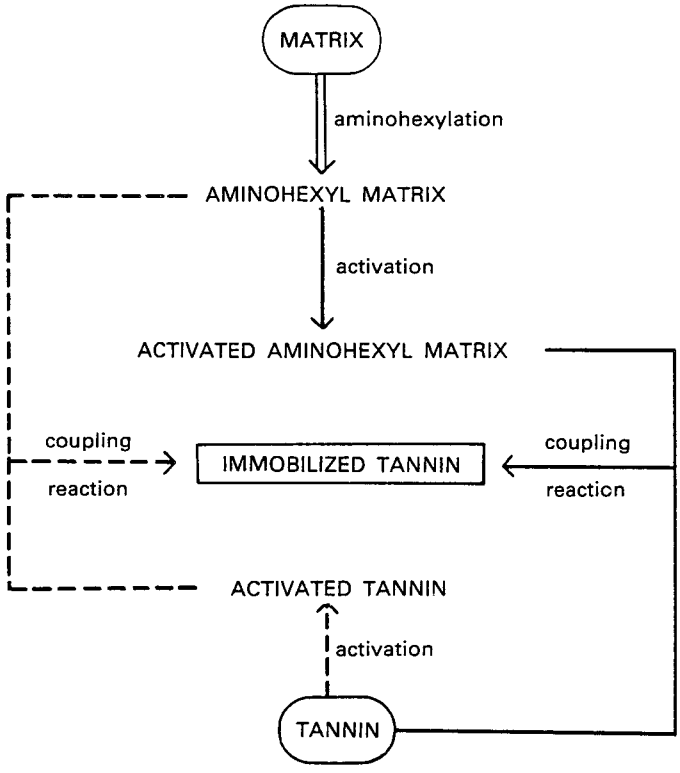

FIG. 1. The general method for the immobilization of tannin. The broken line represents the cyanogen bromide method; the solid line, the epichlorohydrin method.

quickly with 200 ml of cold distilled water (4°C) and 200 ml of 0.1 M sodium bicarbonate solution. The aminohexylation of the activated matrix was carried out by the method of Cuatrecasas (6) as follows. The activated matrix was suspended in 30 ml of 0.6 M solution of hexamethylenediamine previously adjusted to pH 9.5 with 6 N hydrochloric acid. The mixture was shaken at 25°C for 20 h. After the reaction, the aminohexyl matrix was collected and washed with 200 ml of distilled water and 200 ml of 0.1 M sodium bicarbonate solution.

(2) *Epichlorohydrin procedure.* Polysaccharides were activated with epichlorohydrin according to the method of Porath and Fornstedt (7) as follows. 1 g (dry weight) of a matrix was steeped in 200 ml of water at 25°C for 16 h. The swollen matrix was collected, washed with water, and suspended in 100 ml of 1 N sodium hydroxide. To the suspension, 2.5 ml of epichlorohydrin was added, and the mixture was vigorously stirred at 60°C for 30 min. The activated matrix was collected and washed thoroughly with distilled water until no epichlorohydrin was detected. The resulting matrix was suspended in 30 ml of 0.6 M solution of hexamethylenediamine previously adjusted to pH 11 with 6 N hydrochloric acid. The suspension was shaken gently at 60°C for 2 h. The aminohexyl matrix was collected by filtration, and washed with 200 ml of water and 200 ml of 0.1 M sodium bicarbonate solution.

(3) *Diazotization procedure.* Diazonium derivatives of *p*-aminobenzyl cellulose, Encor (arylaminoglass) and Enzacryl AA were prepared according to the method of Inman and Dintzis (8) as follows. 1 g (dry weight) of a matrix was washed with water, and suspended in 25 ml of cold 2 N hydrochloric acid (4°C). To the suspension 5 ml of 14% sodium nitrite solution were added dropwise at 4°C with stirring, and the mixture was stirred at 4°C for 1 h. The diazotized matrix was collected by filtration, and washed with 0.1 M sodium bicarbonate solution and water. The resulting matrix was suspended in 30 ml of 0.1 M borate buffer solution (pH 8.0) containing 500 mg of hexamethylenediamine. The suspension then was adjusted to pH 9.5 with 5 N hydrochloric acid and stirred at 25°C for 20 h. The resulting *p*-aminohexylazobenzyl matrix was collected by filtration and washed with 200 ml of water and 200 ml of 0.1 M sodium bicarbonate solution.

(4) *Carbodiimide condensation.* According to the method of Koshland (9, 10), matrices containing the carboxyl group were coupled to hexamethylenediamine in the presence of carbodiimide as follows. 1 g (dry weight) of a matrix was suspended in 30 ml of 0.1 M hexamethylenediamine and 200 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide were added. The suspension was adjusted to pH 6.0 with 2 N hydrochloric acid, and stirred at 25°C for 20 h. The resulted aminohexyl matrix was collected by filtration and washed with 0.1 M sodium bicarbonate solution and water.

Immobilization of Tannins on Aminohexyl Matrices. (1) *Cyanogen bromide method.* In 50 ml of 0.1 M sodium bicarbonate, 1.3 g of chinese gallotannin were dissolved. The solution was adjusted to pH 11.5 with 5 N sodium hydroxide, and 200 mg of cyanogen bromide were added thereto. The mixture was stirred at 25°C for 8 min. During the reaction, the mixture was kept at pH 11.0 ~ 11.5. By this procedure, activated tannin solution was prepared. This solution was adjusted to pH 10 with 6 N hydrochloric acid and then added to 1 g (dry weight) of aminohexyl matrix. The mixture was shaken at 25°C for 4 h. After the reaction, immobilized tannin was collected by filtration, and thoroughly washed with 20% acetone (v/v) and water until no tannin was detected in the washing solution.

(2) *Epichlorohydrin method.* 1 g (dry weight) of the aminohexyl matrix was added to 50 ml of 0.25 N sodium hydroxide solution and 5 ml of epichlorohydrin. The mixture was shaken at 60°C for 30 min. After the reaction, the epichlorohydrin activated aminohexyl matrix was collected by filtration and washed thoroughly with water until no epichlorohydrin was detected. The activated matrix was added to 50 ml of tannin solution in which 1.3 g of chinese gallotannin and 25 mg of sodium borohydride was dissolved (pH of the solution was adjusted to 7.0 with 5 N sodium hydroxide). The mixture was shaken at 30°C for 4 h. After the reaction, immobilized tannin was collected by filtration, and thoroughly washed with 20% acetone (v/v) and water until no tannin was detected in the washing solution.

Determination of Tannin

Tannin was determined by the colorimetric method using ammonium molybdate (11). The amount of tannin coupled to the matrix was calculated from the difference of tannin concentrations in the reaction mixture; between, before, and after, the reaction with the aminohexyl matrix.

Determination of Amino Residue

The amount of aminoalkylenediamine coupled to the matrix was determined by Kjeldahl method.

Measurement of Adsorption Capacity of Immobilized Tannin for Proteins

The adsorption capacity of immobilized tannin for proteins was measured by the column method as follows. Immobilized tannin was packed into a column (5 × 50 mm, bed volume 1 ml), and the column was equilibrated with acetate buffer (pH 4.3, 1 mmho). α-Amylase or glucoamylase

was dissolved in acetate buffer (pH 4.3, 1 mmho) at a concentration of 0.1%, and the specific conductivity of the solution was adjusted to 3 mmho by the addition of sodium chloride after adjusting the pH. The α-amylase or glucoamylase solution was continuously passed through the column until the protein concentration in the column effluent became equal to that in the charged solution. The column was washed with acetate buffer (pH 4.3, 1 mmho), and α-amylase or glucoamylase adsorbed to the column was eluted with sodium carbonate buffer (0.1 M, pH 10, adjusted to 50 mmho with the addition of sodium chloride). The recoveries of adsorbed protein were more than 95% in all experiments. The concentration of protein was determined by Lowry's method (12). The adsorption capacity of immobilized tannin for a protein was expressed by the amount of α-amylase or glucoamylase recovered.

The adsorption capacity of the aminohexyl matrix for a protein was also determined by the same method as that of immobilized tannin.

Determination of Low Molecular Weight Compounds

Concentration of amino acids was measured by ninhydrin methods (13), and that of sugars was measured by the method of Somogi (14). Fumaric acid and malic acid were measured by the method of Bock and Alberty (15) and that of Goodman and Stark (16), respectively. Nucleic acid related compounds were measured by optical density at 260 nm.

RESULTS

Selection of Matrix Suitable for Preparation of Immobilized Tannin

Chinese gallotannin was immobilized on various aminohexyl matrices, and the adsorption capacities of immobilized tannins for α-amylase were determined. The results are shown in Table 1. Although the aminohexyl matrices showed very low adsorption capacities for the protein, the capacities of immobilized tannins were much higher. Among matrices tested, alkali-treated cellulose was the most favorable matrix for the preparation of immobilized tannin having a high adsorption capacity for the protein. Both cyanogen bromide and epichlorohydrin methods for the aminohexylation of alkali-treated cellulose resulted in immobilized tannins with the same adsorption capacity for protein.

Optimum Conditions for Preparation of Immobilized Tannin

As it became apparent that alkali-treated cellulose was the most favorable matrix for the immobilization of tannin, we investigated the

TABLE 1. **Immobilization of Tannin on Various Matrices**[a]

Matrix	Procedure for aminohexylation	Adsorption capacity for α-amylase (mg/ml of adsorbent)	
		Aminohexyl matrix	Immobilized tannin
Sepharose 4B	CNBr	0.3	7.7
Sephadex G-100	CNBr	0.1	0.9
Cellulose powder C	CNBr	0.9	9.3
Alkali-treated[b] cellulose powder C	CNBr	8.1	28.1
Sephadex G-100	Epichlorohydrin	0.1	0.7
Cellulose powder C	Epichlorohydrin	0.9	7.2
Alkali-treated[b] cellulose powder C	Epichlorohydrin	6.7	27.3
PAB-cellulose	Diazotization	0.2	1.5
Encor	Diazotization	0.4	4.9
Enzacryl AA	Diazotization	2.4	4.5
Bio-Gel CM-2	Carbodiimide	1.4	10.8
Amberlite IRC-50	Carbodiimide	0.4	4.2
Wool	Carbodiimide	0.1	2.1

[a]The method of preparing aminohexyl matrices was described in the test. The immobilization of chinese gallotannin on aminohexyl cellulose were carried out by the cyanogen bromide method described in the text.
[b]Alkali treatment for cellulose was carried out as follows. 1 g (dry weight) of cellulose powder C was immersed in 20 ml of 25% sodium hydroxide solution, and stood at 25°C for 30 min. The alkali-treated cellulose was collected by centrifugation (2900g, 10 min) and thoroughly washed with water to remove sodium hydroxide.

optimum conditions for the preparation of immobilized tannin having a high adsorption capacity for proteins. Factors influencing adsorption capacity were: conditions for alkali-treatment, kinds of cellulose, chain lengths of diaminoalkane spacer, kinds of tannin, and immobilization methods of tannin on aminohexyl cellulose. These were tested as follows.

Conditions for Alkali Treatment. To alter the crystalline structure of cellulose and increase the reactivity for reagents, the alkali treatment was carried out under varying conditions as shown in Table 2. The table shows that the concentration of sodium hydroxide for alkali-treatment for cellulose strongly influenced the adsorption capacity of the resulting immobilized tannin. The adsorption capacities of immobilized tannins were enhanced about four times by the alkali treatment with 25% sodium hydroxide as compared to no treatment. The adsorption capacity of immobilized tannin was not affected by the temperature and time of the alkali treatment except for the case of treatment at 45°C for 30 min.

Kinds of Cellulose. The relationship between the kind of cellulose and adsorption capacity of the immobilized tannin for α-amylase and

TABLE 2. **Conditions for Alkali Treatment of Cellulose on the Immobilization of Tannin**[a]

Condition for alkali treatment			Adsorption capacity for α-amylase (mg/ml of adsorbent)
Concentration of sodium hydroxide (%)	Temperature (°C)	Time (min)	
0	25	30	7.2
7	25	30	11.4
14	25	30	20.3
25	25	30	28.4
25	25	240	28.6
25	10	30	27.4
25	10	240	28.1
25	45	30	21.9
25	45	240	27.6

[a] Alkali treatment for cellulose was carried out by the same method as given in Table 1 except for the varying of the specified temperature, time, and the concentration of the sodium hydroxide solution for treatment. The resultant celluloses were aminohexylated by the epichlorohydrin procedure. Then the resultant aminohexyl celluloses were used for the immobilization of chinese gallotannin according to the cyanogen bromide method described in the text.

glucoamylase was tested. The results are shown in Table 3. Cellulose powders were suitable matrices for the immobilization of tannin, but cotton linter pulp, filter paper, gauze, and absorbent cotton were not.

Chain Lengths of Spacer. Effect of chain lengths of the spacer on adsorption capacity of immobilized tannin was investigated for glucoamylase. Data shown in Table 4 indicate that the capacity increased with increase of the chain length of spacer and reached a plateau when the number of methylene residues was six. The low adsorption capacity of immobilized tannin with short chain lengths may be due to the steric hindrance of the matrix to the protein. Further, low contents of tannin in the adsorbents may also be considered to be one of the reasons for a low adsorption capacity.

Kinds of Tannin. To prepare immobilized tannin advantageously several kinds of tannin were coupled to aminohexyl cellulose or aminohexyl Sepharose 4B. Then the adsorption capacities of immobilized tannin for glucoamylase were compared. The results are shown in Table 5. When hydrolyzable tannins such as chinese gallotannin and nutgalls-tannin were immobilized as a ligand on aminohexyl cellulose, the resulting immobilized tannins showed higher adsorption capacities. However, when these tannins were immobilized on aminohexyl Sepharose 4B, the adsorption capacities of the resulting immobilized tannins were low. On the other hand, when condensed tannin such as the tannin of persimmon juice was used, the

TABLE 3. **Immobilization of Tannin on Various Celluloses**[a]

Cellulose	Adsorption capacity (mg/ml of adsorbent)	
	for glucoamylase	for α-amylase
Cellulose powder A	16.1	6.9
Cellulose powder C	45.8	29.4
Cellulose powder D	47.2	29.9
Cellulose powder CC31	—	21.3
Cellulose powder Avicel	—	21.0
Cellulose powder Microcrystalline	—	28.2
Absorbent cotton[b]	0.4	—
Cotton linter pulp 5M-2400[c]	3.2	—
filter paper No. 7[c]	0.2	—
Gauze[b]	0.1	—

[a] Alkali treatment for cellulose was carried out by the same method given in Table 1. The aminohexylation of the resultant cellulose was carried out by the epichlorohydrin procedure and the immobilization of chinese gallotannin on aminohexyl cellulose was carried out by the cyanogen bromide method.
[b] Absorbent cotton and gauze commercially available were used in the form of short fiber.
[c] Cotton linter pulp and filter paper No. 7 were used in the form of fibrous powder.

TABLE 4. **Effect of Chain Length of Spacer on the Adsorption Capacity of Immobilized Tannin for Glucoamylase**[a]

Diamino alkane spacer	Coupled amount (μmol/g of adsorbent)		Adsorption capacity for glucoamylase (mg of protein/ml of adsorbent)
	Diamino alkane	Tannin[c]	
$NH_2(CH_2)_2NH_2$	281	76	16.0
$NH_2(CH_2)_3NH_2$	—	84	17.2
$NH_2(CH_2)_4NH_2$	—	113	21.3
$NH_2(CH_2)_6NH_2$	230	133	48.8
$NH_2(CH_2)_7NH_2$[b]	—	137	45.6
$NH_2(CH_2)_8NH_2$[b]	241	145	51.2
$NH_2(CH_2)_{10}NH_2$[b]	—	129	47.9
$NH_2(CH_2)_{11}NH_2$[b]	196	130	49.7

[a] Alkali treatment for cellulose was carried out by the same method given in Table 1. Aminohexylation and immobilization of chinese gallotannin on various aminohexyl celluloses were carried out by the same method as given in Table 3 except that various kinds of aminoalkylenediamine were used.
[b] Dissolved in 50% ethanol.
[c] Molecular weight of tannin was taken as 2600.

TABLE 5. **Immobilization of Various Tannins on Aminohexyl Cellulose and Aminohexyl Sepharose**[a]

Kind of tannin	Aminohexyl matrix	Adsorption capacity for glucoamylase (mg of protein/ml of adsorbent)
Chinese gallotannin	Cellulose powder C	54.8
	Sepharose 4B	6.6
Nutgalls-tannin	Cellulose powder C	49.7
	Sepharose 4B	7.9
Tannin of persimmon juice	Cellulose powder C	39.8
	Sepharose 4B	46.4

[a] Alkali treatment and aminohexylation were carried out by the same method given in Table 1. Nutgalls-tannin was activated by the same method as chinese gallotannin. Tannin of persimmon juice was activated as follows. With 5 N sodium hydroxide solution 50 ml of persimmon juice was adjusted to pH 11.5, and 200 mg of cyanogen bromide were then added. The mixture was stirred at 25°C for 8 min. During the reaction the mixture was kept at pH 11.5.

resulting immobilized tannins showed higher adsorption capacities in both cellulose and Sepharose 4B.

Immobilization Methods of Tannin on Aminohexyl Cellulose. Immobilized tannins prepared by two immobilization methods of tannin on aminohexyl cellulose were compared with their adsorption capacities for a protein. As shown in Table 6, no difference was observed with either method. Therefore, it became apparent that epichlorohydrin could also be used for the immobilization of tannin on aminohexyl cellulose.

Adsorption Specificity of Immobilized Tannin

To clarify the adsorption specificity of immobilized tannin, various proteins, amino acids, monosaccharides, organic acids, and nucleic acid

TABLE 6. **Immobilization of Chinese Gallotannin on Aminohexyl Cellulose by the Cyanogen Bromide and Epichlorohydrin Methods**[a]

Immobilization method	Coupled amount of tannin[b] (μmol/g of adsorbent)	Adsorption capacity for glucoamylase (mg/ml of adsorbent)
Cyanogen bromide	130	49.4
Epichlorohydrin	138	57.1

[a] Alkali treatment and aminohexylation of celluloses were carried out by the method given in Table 1. Immobilization of chinese gallotannin on aminohexyl cellulose was carried out by the cyanogen bromide and epichlorohydrin methods described in the text.
[b] Molecular weight of tannin was taken as 2600.

TABLE 7. **Adsorption Specificity of Immobilized Tannin for Various Organic Compounds**[a]

Compound tested	Adsorption capacity	
	mg/ml of adsorbent	μmol/ml of adsorbent
Asparaginase	41000	0.34
Lysozyme	11000	0.73
Trypsin	28000	1.17
Alanine	2.3	0.03
Arginine	1.5	0.01
Aspartic acid	3.7	0.03
Histidine	1.9	0.01
Isoleucine	1.1	0.01
Leucine	2.0	0.02
Proline	1.9	0.02
Tryptophan	2.3	0.01
Valine	4.9	0.04
Fructose	0	0
Glucose	0	0
Ribose	0	0
Fumaric acid	0	0
Malic acid	0	0
Adenine	0	0
Cytosine	0	0
Guanine	0	0
Uracil	0	0

[a] Immobilized tannin was packed into a column (bed volume 1 ml), and equilibrated with 0.01 M sodium acetate buffer (pH 4.3, 1 mmho). The enzyme (20 mg) or low molecular weight compound (2 mg) was dissolved in 20 ml of the same buffer as was used for the equilibration of the column, and the conductivity of the solution was adjusted to 1 mmho by the addition of sodium chloride. The solution was charged into the column at a flow rate of 1 ml/h. The adsorption capacity of immobilized tannin for a low molecular weight compound was calculated from the difference between the amount of compound in the charged solution and that in the solution combining column effluent and washings. In the case of enzyme, adsorption capacity was calculated from the amount of protein eluted with 0.1 M sodium carbonate buffer (pH 10, 50 mmho).

related compounds, respectively, were charged through a column packed with tannin immobilized by cyanogen bromide on aminohexyl cellulose. The adsorption capacity for each compound is shown in Table 7. Except for proteins, the compounds tested had little affinity for the column. These results suggest that this absorbent can be advantageously used for specific adsorption for proteins.

DISCUSSION

In this paper, we intended to prepare an adsorbent having a high specificity and a high adsorption capacity for proteins. As a ligand for

specific protein adsorption, we chose tannins and investigated the immobilization methods of them on water-insoluble matrices. In order to prepare an adsorbent having a high adsorption capacity, the correct choice of matrices (Table 1 and 3), the chain lengths of spacer (Table 4), and kinds of tannin (Table 5) were carried out.

As the most favorable matrix for the immobilization of tannin, alkali-treated cellulose was selected. Alkali treatment of cellulose known as "marcerization" is effective for increasing the supply of chemical groups that allow covalent attachment of the ligand to cellulose. That is, by this treatment, the crystalline region of cellulose decreases, and the degree of substitution of the ligand in the cellulose increases (17). It is known in studies on affinity chromatography that accessibility of macromolecules toward ligand increases with the extention of the spacer arm. In the case of immobilized tannin, the maximum adsorption capacity for protein was observed with chain lengths greater than that of diaminohexane (Table 4). Therefore, as a spacer, we chose diaminohexane, which is commercially available at low cost.

The kinds of tannins did not influence the adsorption capacity of immobilized tannin when cellulose was used as a matrix. So, chinese gallotannin was chosen as a ligand (Table 5) as it was commercially available in pure form at low cost.

To immobilize tannin on the water-insoluble aminohexyl matrix we used cyanogen bromide and epichlorohydrin as coupling reagents. In both cases, covalent binding was considered to be formed between tannin and aminohexyl cellulose. To confirm the binding situation, the immobilized tannin was titrated with hydrochloric acid and sodium hydroxide, and the resulting titration curve was compared with those of aminohexyl cellulose and alkali-treated cellulose. As shown in Figure 2, immobilized tannin was more acidic than aminohexyl cellulose due to the binding of tannin molecules. Immobilized tannin, prepared by condensing tannin to aminohexyl cellulose by the cyanogen bromide method, had positivity charged groups as do adsorbents derived by the cyanogen bromide method, which is known to introduce positively charged groups into the adsorbent matrix (18). Further, tannin did not leak out from immobilized tannin by the contact with 0.01 N hydroxhloric acid and 0.01 N sodium hydroxide. Therefore, the binding between tannin and aminohexyl cellulose is considered to be covalent, but details of the binding situation are not clear.

As shown in Table 5, the immobilized tannin prepared under optimum conditions adsorbs about 50 mg of glucoamylase/ml of the preparation. This value is almost the same as that of conventional protein adsorbents such as ion-exchange celluloses. Further, this immobilized tannin has high

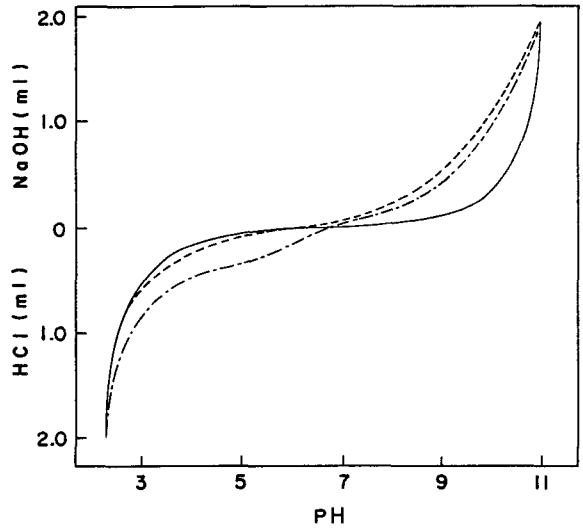

FIG. 2 The titration curve of immobilized tannin. 4 g (wet weight) of alkali-treated cellulose, aminohexyl cellulose, or immobilized tannin prepared by the cyanogen bromide method as given in Table 6 was suspended in 20 ml of water and then titrated with 0.25 N sodium hydroxide solution or 0.25 N hydrochloric acid solution by using potentiometer (Potentiograph E336, Metrohm). ———, Alkali-treated cellulose; -----, aminohexyl cellulose; —·—·—, immobilized tannin.

specificity for proteins as described in Table 7. Adsorption specificity for proteins of this adsorbent is the important characteristic. The adsorbents for proteins employed so far, such as alumina, charcoal, clay, glass, silica, starch, ion-exchange celluloses, and ion-exchange resins, are not specific for protein. On the other hand, the new adsorbent described in this paper is specific for protein, and this adsorbent can overcome the disadvantages of conventional adsorbents for protein.

This new adsorbent may be useful as a tool for the selective removal of contaminated proteins in solutions containing various organic and inorganic compounds. The possibilities in the applications of immobilized tannin to the recovery of proteins, purification of proteins, and immobilization of enzymes will be presented in another paper (19, 20).

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