Immobilization of Proteins on TOYOPEARL gels

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Various proteins have been immobilized on TOYOPEARL gels which are used for high performance gel filtration chromatography. The procedures used for immobilization include the activation via glutaraldehyde or cyanuric chloride, condensation through water-soluble carbodiimide, and diazo coupling. Measurement of the protein content in the enzyme-immobilized gels revealed that the amounts of adsorbed and chemically attached proteins differ each other appreciably according to the methods employed.

The effects of the molecular weight of protein and the pore size of the gel on the immobilization efficiency are investigated using the cyanuric chloride activation method; the results indicate that the amounts of immobilized proteins decrease in parallel with an increase in pore size of TOYOPEARL gels, while the number of immobilizable proteins decreases with an increase in their molecular weight.

1. INTRODUCTION

In recent years many kinds of immobilized proteins were prepared and applied to various fields[1,2]. However, proteins were rarely immobilized on the gels which are used for gel filtration chromatography and have a considerable strength. So in present paper TOYOPEARL gels for high performance gel filtration chromatography were used as immobilization supports, and the effects of the immobilization method and the pore size of the gel on amounts of proteins immobilized on the gel were studied.

2. MATERIALS AND METHODS

[1] Reagents

Thermoase (Daiwa Kasei K. K., 160×10^4 pu/g, from *Bacillus thermoproteolyticus*), Myoglobin (Sigma Type III, from horse heart), Thermolysin (Daiwa Kasei, 9810 pu/mg, from *Bacillus thermoprotelyticus*), Albumin (Nakarai Chemicals, Ltd., from bovine serum) and Catalase (Sigma, 2000-5000 Sigma units/mg protein, from bovine liver) were used without further purification. TOYOPEARL gels were obtained from Toyo Soda Kogyo Co., Ltd. and their grades were Hw40, Hw50 Hw60 and Hw75. Other reagents were purchased from Nakarai Chemicals Ltd. and Wako Pure Chemical Ind. Ltd., and Institute for Protein Research Foundation, and used without further purification.

[2] Measurement of amounts of proteins immobilized on the gel[3]

Immobilized enzymes on gels (dried weight of 10 to 100 mg) and 1.0 mℓ of 6N-HCl were placed in test tubes (16.5 × 165 mm), and the tubes were sealed under low pressure. And then they were incubated at 110°C for 24 hours. After the incubation the solution containing gels in the test tubes were filtrated by a glass filter (G4), and the filtrates were evaporated to dryness.

The dried samples were dissolved by the 2/3 M-citrate-sodium citrate buffer (pH 2.2), and amino acid contained in the hydrolyzed samples were analyzed by an automatic amino-acid analyzer (TOYO SODA HLC 805). Known amounts of proteins were hydrolyzed with 6N-HCl solution and the amounts of amino acids in hydrolyzed solution were analyzed and used as the calibration. In this paper we determined the amounts of proteins by the peak area of glutamic acid of the hydrolyzed solution because it was eluted early in the chromatogram and also it gave the excellent correlation between the height of the peak and the amount of protein (**Fig. 1**).

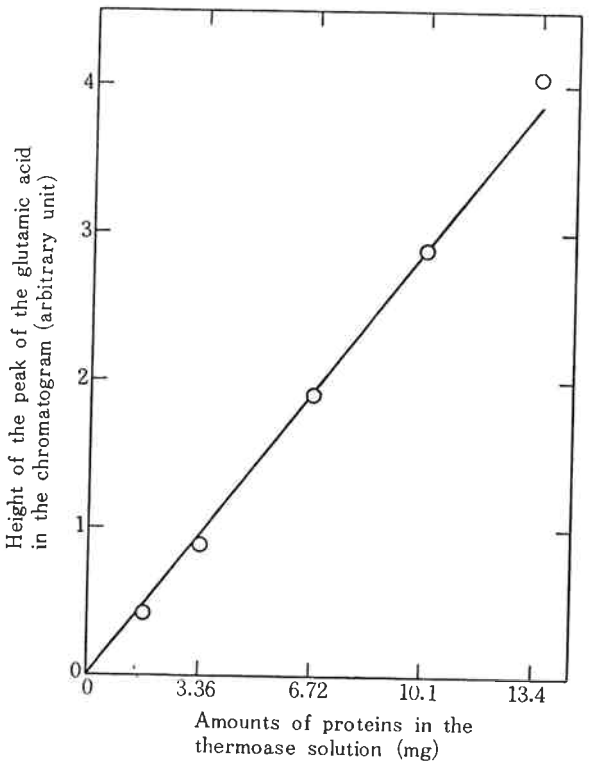

Fig. 1 Correlation between the height of the hplc peak of the glutamic acid and the amounts of the proteins in thermoase solution

〔3〕 Preparation of immobilized proteins

TOYOPEARL gel was washed on a glass filter (G4) with distilled water, and dried in vacuo at 60°C. Fifty grams of the dried gel, 200 mℓ of 1N-NaOH, and 11 mℓ of epichlorhydrin were placed in a 500 mℓ-separable flask and stirred for 3 hours at 30°C. And they were filtered by a glass filter (G4), and washed with cold distilled water. This gel was placed in a 500 mℓ-separable flask, and to it 63 mℓ of ethylenediamine and 35 mℓ of distilled water were added, then the mixture was stirred for 1.5 hours at 80°C. After the reaction the mixture was filtered by a glass filter (G4), the gel was washed with acetone several times to remove unreacted ethylenedimane, and was dried in vacuo at 60°C overnight. This gel was termed as TPL-EDA (**Fig. 2** (II))[4].

(1) Glutaraldehyde activating method[5]

1) Fifty grams of dried weight of (II) and 500 mℓ of 5% (v/v) glutaraldehyde solution were placed in a 1ℓ-beaker, and adjusted to pH 7.0 with 5N-NaOH. This mixture was stirred overnight at 20°C. And then the mixture was filtered by a glass filter (G4) and washed with

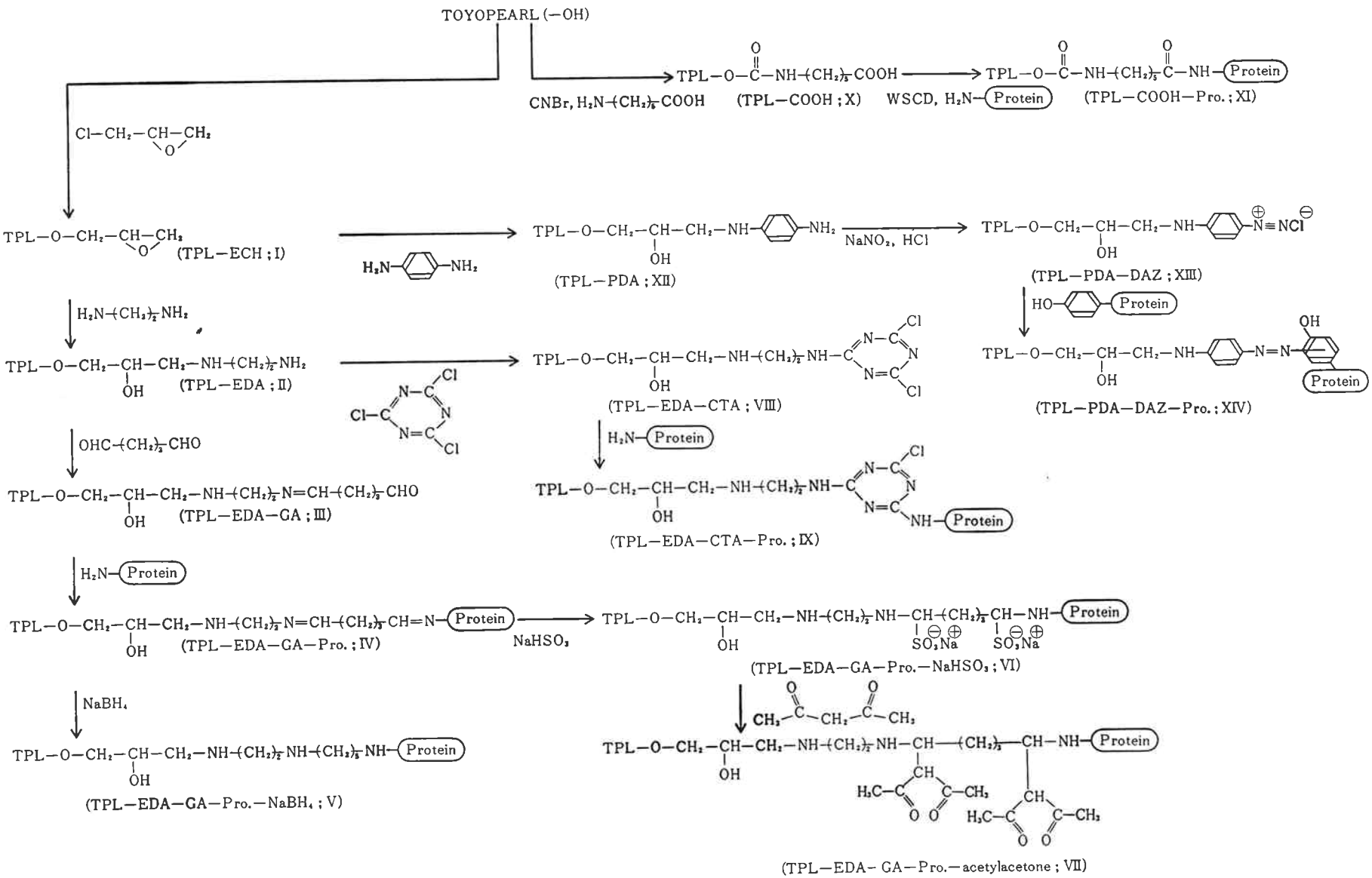

Fig. 2 Reaction scheme of immobilization methods *WSCD· water-soluble carbodiimide

distilled water, and then with acetone several times to remove unreacted glutaraldehyde. This gel was dried in vacuo at 60°C overnight, and was termed as TPL-EDA-GA (**Fig. 2** (III)).

Subsequently 13.3 grams of dried weight of (III) and 250 mℓ of 5% (w/v) thermoase solution (10 mM-$CaCl_2$, pH 8.0) were placed in a 500 mℓ-beaker, stirred at 4°C for 24 hours. The mixture was filtered and washed with 10 mM-$CaCl_2$ (pH 8.0) several times by a glass filter (G4). This gel was termed as TPL-EDA-GA-ThA (wet) (**Fig. 2** (IV)) and kept in a refregerator.

2) $NaBH_4$ reduction[5]

Ten grams of wet weight of (IV) and 100 mℓ of 10 mM-$CaCl_2$ (pH 8.0) were placed in a 500 mℓ-beaker, and to it one gram of $NaBH_4$ was added with stirring at 4°C. The mixture was stirred for 24 hours, and then filtered by a glass filter (G4) and washed with 10 mM-$CaCl_2$ (pH 8.0). In this manner the unsaturated C=N bonds of the gel were reduced by $NaBH_4$ (TPL-EDA-GA-ThA-$NaBH_4$, **Fig. 2** (V)).

3) $NaHSO_3$ addition[6,7]

Ten grams of wet weight of (V), 3.43 grams of $NaHSO_3$, 2.34 grams of NaCl and 40 mℓ of 10 mM-$CaCl_2$ (pH 5.7) were placed in a 200 mℓ-flask, and shaken at pH 5.7 and at 25°C for 5 hours. And then the mixture was filtered by a glass filter (G4) and washed with 10 mM-$CaCl_2$ (pH 8.0). In this manner the unsaturated C=N bonds of the gel were added by $NaHSO_3$ (TPL-EDA-GA-ThA-$NaHSO_3$, **Fig. 2** (VI)).

4) Acetylacetone subsititution[6,7]

Ten grams of weight of (VI), 15.6 mℓ of acetylacetone, 2.6 mℓ of piperidine and 100 mℓ of 10 mM-$CaCl_2$ (pH 8.0) were placed in a 500 mℓ-separable flask, stirred at pH 8.0 and at 20°C for 2 days. And then the mixture was filtered by a glass filter (G4) and washed with 10 mM-$CaCl_2$ (pH 8.0). In this manner we obtained TPL-EDA-GA-ThA-acetylacetone (wet) (**Fig. 2** (VII)).

(2) Cyanuric chloride activating method[5,8,9]

Ten grams of dried weight of (II), 132 mℓ of acetone and 21.8 grams cyanuric chloride were placed in a 500 mℓ-beaker, cooled to −10～−5°C in a dryice-methanol bath and stirred for 30 minutes. Subsequently 9.88 grams of $NaHCO_3$ in 58.8 mℓ of distilled water were added dropwise to the beaker, and then the mixture was stirred at 0°C. With the progress of the reaction, pH of the mixture decreased due to the generation of hydrochloric acid, so 5N-NaOH was added to keep pH at the constant value of 7.5. When the decrease of pH in the mixture was no more observed (after 2～3 hours), the mixture was filtered by a glass filter (G4) and washed with cooled acetone several times to remove unreacted cyanuric chloride. Further, the gel was dried in vacuo at 20°C overnight. In this manner we obtained TPL-EDA-CTA (**Fig. 2** (VIII)).

Subsequently 3.3 grams of dried weight of (VIII) and 50 mℓ of 5% thermoase solution (10 mM-$CaCl_2$, pH 8.0) were placed in a 200 mℓ-flask, shaken at pH 8.0 and at 4°C for 24 hours. The mixture was filtered by a glass filter (G4) and washed with 10 mM-$CaCl_2$ (pH 8.0). In this manner we obtained TPL-EDA-CTA-ThA (**Fig. 2** (IX)).

(3) Introduction of the carboxyl group on TOYOPEARL gel by CNBr-ε-amino-n-caproic acid, followed by condensation reaction with enzyme solution by water-soluble carbodiimide[5]

Ten grams of wet weight of TOYOPEARL gel and 100 mℓ of distilled water were placed in a 300 mℓ-beaker, stirred at pH 10 and at 20°C. Five grams of dried CNBr was added to the mixture by portions. With the progress of the reaction pH in the mixture decreased and temperature rose, and so 6N-NaOH and small ice cubes were added to keep pH at constant value of 10 and temperature at 20°C. When pH in the mixture became constant after 2～3 hours, 12 grams of ε-amino-n-capronic acid was added to the mixture. The mixture was stirred at pH 8.5～9.5 for 2 hours, filtered and then washed with distilled water until pH of the mixture reached the value of 7.

Subsequently 10 grams of wet weight of the gel and 50 mℓ of 5% thermoase solution (10 mM-$CaCl_2$, pH 6.0) were placed in a 200 mℓ-beaker, and 9.58 grams of water-soluble carbodiimide hydrochloride (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochoride) was added to this mixture with stirring at pH 6.0 and at 15°C. With the progress of the reaction pH in the mixture increased, and so pH was kept pH at the constant value of 6.0 by addition of 0.1N-HCl. After stirring for 2 hours at 15°C, the mixture was stirred at 4°C overnight. Then it was filtered by a glass filter (G4) and washed with 10 mM-$CaCl_2$ (pH 8.0). In this manner we obtained TPL-COOH-ThA (**Fig. 2** (XI)).

(4) Diazo coupling method[2,5]

One hundred grams of wet weight of (I), 231 grams of p-phenylenediamine and 400 mℓ of dioxane were placed in a 1ℓ-separable flask, and the mixture was stirred at 80°C for 6 hours. After the reaction the mixture was filtered and washed with acetone to remove unreacted p-phenylenediamine. The gel was dried in vacuo at 60°C overnight. In this manner we obtained TPL-PDA (**Fig. 2** (XII)).

Subsequently ten grams of dried weight of (XII) in 60 mℓ of 1N-HCl were added dropwise by 6.90 grams of $NaNO_2$ in 50 mℓ of distilled water with stirring at 0-3°C for 75 minutes. The mixture was filtered and washed with cooled acetone (−20°C). This freshly prepared gel and 150 mℓ of 5% thermoase solution (10 mM-$CaCl_2$, pH 6.4) were placed in a 500 mℓ-flask, shaken at pH 6.4 and at 4°C for 18 hours. Then the mixture was filtered by a glass filter (G4) and washed with 10 mM-$CaCl_2$ (pH 8.0). In this manner we obtained TPL-PDA-DAZ-ThA (**Fig. 2** (XIV)).

〔4〕 Measurement of amounts of proteins in immobilized protein gel during batchwise washing

Two grams of wet weight of thermoase immobilized by various methods and 20 mℓ of the substrate mixture[10] containing 0.5 mmoles of carbobenzoxy-L-aspartic acid, 1.0 mmoles of L-phenylalanine methyl ester monohydrochloride and 0.2 mmoles of $CaCl_2$ were placed in a 50 mℓ-flask, and shaken at pH 6.0 and at 40°C with the frequency of 100 strokes·min^{-1}. The mixture was filtered by a glass filter (G4) and washed with 10 mℓ of 10 mM-$CaCl_2$ (pH 6.0). Subsequently the recovered immobilized thermoase was mixed with 20 mℓ of the substrate mixture as described above, and was placed in a 50 mℓ-flask. The same procedure as described above was repeated. At arbitrary number of repeated run, about 300 mg of wet weight of immobilized thermoase was collected, the gel was dried in vacuo at 60°C overnight. The amounts of proteins in the dried immobilized thermoase gel were measured as follows. Amounts of proteins in thermoase solution was measured by Lowry method[10] with bovine serum albumin

as the standard. The thermoase solution containing known amounts of proteins was hydrolyzed with HCl, and amounts of glutamic acids in hydrolyzed solution were measured by an automatic amino acid-analyzer (TOYO SODA HLC 805). In this manner the calibration between protein content *vs.* amounts of glutamic acids in the protein solution was made (**Fig. 1**). And amounts of glutamic acids in dried immobilized themoase gel were measured as described above. So the amounts of proteins in dried immobilized thermoase could be determined as the equivalent amounts of bovine serum albumin.

[5] Effects of the size of the proteins and the pore size of the gels on the amounts of the proteins immobilized on the gels

TOYOPEARL gels[11] (Hw 40, Hw50, Hw60 and Hw75) were used as supports for immobilization. The gels were activated by the cyanuric chloride activating method as described before. Horse heart myoglobin (moleculer weight: 18, 800), thermolysin (34, 600), bovine serum albumin (67, 000) and bovine serum catalase (240, 000) were used as proteins for immobilization. Measurements of the amounts of various proteins in dried immobilized gels were done as the same procedure as before with respective proteins as standards.

3. RESULTS

From figure 3 it can be seen the followings. (i) The amounts of proteins in dried immobilized

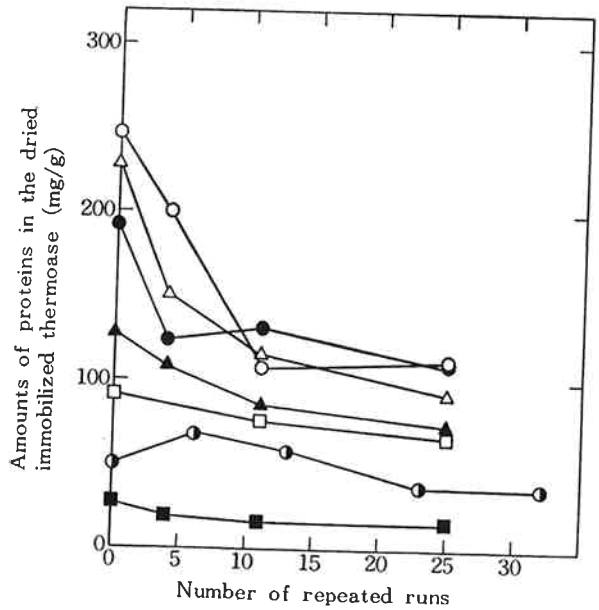

Fig. 3 Amounts of the proteins in the dried immobilized thermoase during repeated run
○, TPL-EDA-GA-ThA (IV)
△, TPL-EDA-GA-ThA-$NaBH_4$ (V)
●, TPL-EDA-GA-ThA-$NaHSO_3$ (VI)
▲, TPL-EDA-GA-ThA-acetylacetone (VII)
□, TPL-EDA-CTA-ThA (IX)
■, TPL-COOH-ThA (XI)
◑, TPL-PDA-DAZ-ThA (XIV)

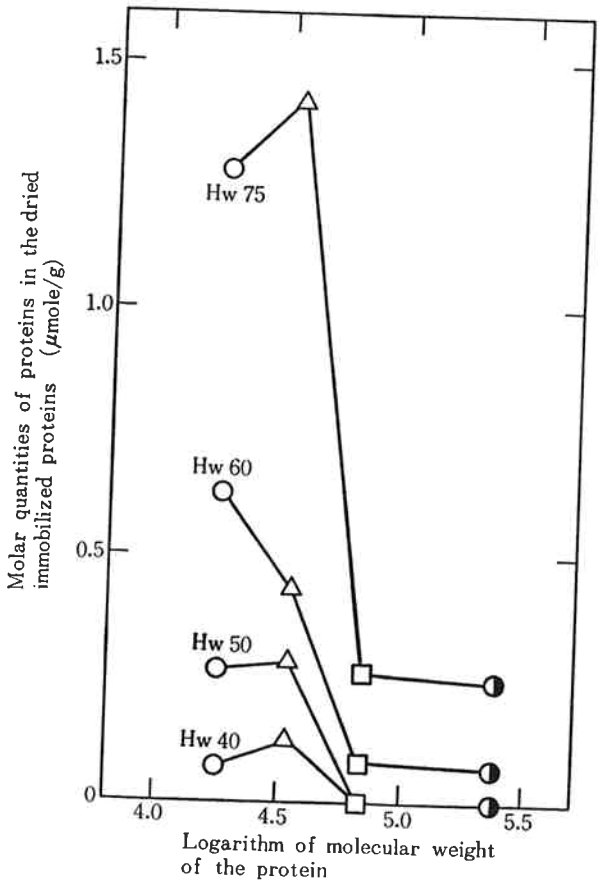

Fig. 4 Effects of the size of the proteins and the pore size of the gels on the amounts of the proteins immobilized on the gels
○, myoglobin △, albumin □, thermolysin
◑, catalase

thermoase by glutaraldehyde activating methods (2-[3]-(1)-1), 2), 3) and 4)) decreased until 11 times on repeated run, and didn't change very much in the subsequent repeated runs. (ii) In the cyanuric chloride activating method the amounts of proteins in dried immobilized thermoase didn't change very much by repeated runs. (iii) In the water-soluble carbodiimide condensation method and the diazo coupling method the amounts of proteins in dried immobilized thermoase were relatively small, but didn't change very much by repeated runs.

Next the effects of the size of the proteins and the pore size of the gels on the amounts of the immobilized proteins were studied in the cyanuric chloride activating method (**Fig. 4**). From figure 4 it can be seen the following. (i) The amounts of the immobilized proteins are increased with the pore size of gels. (ii) The numbers of the immobilized proteins are increased with the decrease of the size of the proteins.

4. DISCUSSION

TOYOPEARL gels for high performance gel filtration chromatography were used as supports for immobilization of proteins. In the present paper the differences of the amounts of the immobilized proteins and the tendency to adsorb the proteins on the supports were studied with respect to the various immobilization methods. It was found that the gels activated by glutaraldehyde (2-(3)-(1)-1), 2), 3) and 4)) have marked tendency to adsorb the proteins on the supports, because the amounts of the proteins in dried immobilized thermoase decreased considerably during repeated runs. The causes of adsorption on the supports are not clear yet, but it might be due to the unsaturated bonds (*e. i.*, Shiff base: $-CH=N-$) made by the activation of the gels. If the immobilization reaction was done in the solution of the high ionic strength, it might be possible to reduce the amounts of the adsorbed proteins. Further, since the immobilized proteins by the glutaraldehyde activating method have Shiff bases, the bond between the proteins and the supports might be broken in the presence of water. The amounts of the proteins in the dried immobilized proteins remained almost constant after 11 times of repeated runs, but additional repeated run might cause the decrease in the amounts of the proteins in the immobilized thermoase. The procedure of the reduction with $NaBH_4$, the addition of $NaHSO_3$ and the substitution by the acetylacetone had no effects on the decrease of the adsorbed proteins and the block of the cleavage of the bond between the proteins and the supports. But some effects might be able to be seen in the extremely long run.

The amounts of the proteins immobilized by the cyanuric chloride activating method were relatively high and had slightly adsorbed parts. However, in aqueous basic solution, the activated groups in supports might be inactivated by $OH^{\ominus}$.

With the water-soluble carbodiimide condensation method the amounts of the immobilized proteins were small. This may be because the activated groups in the supports are unstable. However, the amounts of the adsorbed proteins were also small, so if activation of the supports and immobilization of the proteins are done in appropriate conditions, larger amounts of the immobilized proteins might be resulted.

In the diazo coupling method the amounts of the proteins in the immobilized thermoase were relatively small. It is considered that the activated groups are inactivated in water. For example when (XII) is activated by cyanuric chloride the amounts of the immobilized proteins are approximately twice as much as the amounts of the proteins immobilized by the diazo coupling

method (unpublished data).

Next the proteins of various moleculer weight were immobilized on TOYOPEARL gels with various pore size by the cyanuric chloride activating method. It was found that greater amounts of the proteins were immobilized on the gel having larger pore size, and more numbers of the immobilized proteins were obtained with smaller size protein. These facts may be explained as follows: when the gels for gel filtration chromatography are used for immobilization of proteins the amounts of the proteins which penetrate into the inner sphere of the gel are controlled by the pore size of the gel and the size of the protein. Hence, one may expect to control the distribution of the proteins in the inner field of the gel. Further, it is thought that the model of the structure of the gel for gel filtration chromatography is that of providing a cone-shaped opening[12], but in the case of the supports for immobilization of the proteins the gel which provides a column-shaped opening seems to be more desirable because the column-shaped opening gel is more sensitive to the pore size of the gel or the size of the protein. So we might be able to control the amounts and the distributions of the proteins immobilized on the support by choosing the pore size and the shape of the support and the size of the protein.

References and a note

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