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Ionic crosslinking of SO_3H-group-containing graft chains helps to capture lysozyme in a permeation mode

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Abstract

SO_3H-group-containing polymer chains were grafted onto a porous hollow-fiber membrane uniformly across the thickness of the membrane (about 0.9 mm) by radiation-induced graft polymerization of glycidyl methacrylate and subsequent reaction with sodium sulfite. The graft chains extended from the pore surface due to their mutual electrostatic repulsion, and were crosslinked with bivalent ions such as Mg^{2+} and Ca^{2+}, resulting in an increase in permeation rate of the liquid through the pores. Lysozyme was bound in multilayers to the SO_3H-group-containing graft chains, which had been previously crosslinked with Mg ions, and the permeation rate was increased with the progression of lysozyme binding based on the ion-exchange interaction with Mg ions.

Keywords: Membranes; Hollow-fiber membranes; Stationary phases, LC; Lysozyme; Proteins

1. Introduction

Both membrane chromatography [1–16] and perfusion chromatography [17–19] are based on similar principles that convective flow of a protein solution through the pores of a porous membrane and a porous bead, respectively, can minimize the diffusional mass-transfer path to the ligands immobilized on the pore surface. The membranes and beads have pore diameters ranging from 0.1 to 1 μm. Ligands for specific and selective adsorption of proteins, namely, affinity ligands [1,3,4,11,16,18], ion-exchange groups [2,5–8,11–15,17–19], and hydrophobic ligands [2,5,9,18], have so far been immobilized on the pore surfaces of membranes and beads.

We have attached epoxy-group-containing polymer chains onto a porous hollow-fiber membrane uniformly across the thickness of the membrane by radiation-induced graft polymerization [20], and subsequently converted the produced epoxy group into various ligands for highly efficient recovery of proteins. The ion-exchange-group-containing polymer chains extend from the pore surface toward the pore interior due to their mutual electrostatic repulsion. The expanding polymer chains capture proteins in multilayers, reducing the pore diameter [8]. For example, a higher density of sulfonic acid groups in the polymer chains results in a lower permeation rate of pure water.

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Tsuneda et al. [6] prepared a sulfonic-acid-type cation-exchange porous membrane for binding lysozyme; however, the permeability, i.e., the flux, which is defined by dividing the flow-rate by the inside surface area, of the resultant membrane was 77% of that of the original membrane at a molar conversion of the epoxy group to the sulfonic acid group of 35%. Higher protein adsorptivity and lower liquid permeability had a trade-off relationship. In this study, we proposed the ionic crosslinking of the graft chains to recover the flux while maintaining protein adsorptivity. This enabled a high throughput in protein recovery.

Intercrosslinking or intracrosslinking with bivalent cations such as Mg^{2+} and Ca^{2+}, i.e., ionic crosslinking, is effective in restricting the swelling of the graft chains. Latex membranes consisting of acrylonitrile–butyl acrylate–acrylic acid were ionically crosslinked with multivalent metal ions to improve the permeability and selectivity in the dehydration of an ethanol–water mixture by pervaporization using the membranes [21]. The polymer chains containing amidoxime groups as chelate-forming groups, grafted onto a porous membrane, were ionically crosslinked by permeating seawater through the pores to attain a much higher permeation rate, due to the fact that multivalent ions contained in seawater work well as crosslinkers [22].

In this study, the recovery of egg-white lysozyme at a high capacity was examined using cation-exchange porous membranes of a hollow-fiber form. Egg white originally contains Mg^{2+} and Ca^{2+} at a concentration of 0.005 and 0.015 *M*, respectively, which will help to ionically crosslink the cation-exchange-group-containing graft chains. The objective of our study was threefold: (1) to prepare SO_3H-type porous membranes, (2) to verify the increase in pore size by ionic crosslinking, and (3) to evaluate the multilayer binding of lysozyme to the graft chains.

2. Experimental

2.1. Materials

A porous hollow-fiber membrane (Asahi Chemical Industry) was used as the trunk polymer for grafting. This membrane, made of polyethylene (PE), had inner and outer diameters of 1.9 and 3.2 mm, respectively, with a pore diameter of 0.5 μm and a porosity of 70%. Technical-grade glycidyl methacrylate (GMA) ($CH_2=CCH_3COOCH_2CHOCH_2$) was purchased from Tokyo Chemical and used without further purification. Egg-white lysozyme (M_r 14 000; isoelectric point, p*I* 10.7) was obtained from Sigma. Lysozyme was dissolved in a 0.05 *M* $NaHCO_3$–NaOH buffer (pH 9.0). Sodium sulfite was purchased from Wako. Other reagents were of analytical grade or higher.

2.2. Preparation of graft chain containing sulfonic acid group

The preparation scheme for the sulfonic-acid (SO_3H)-group-containing hollow-fiber membrane is shown in Fig. 1. This scheme consists of the following four steps [6]. (1) Electron beam irradiation: the trunk polymer was irradiated with an electron beam in a nitrogen atmosphere at ambient temperature with a total dose of 200 kGy. (2) Grafting of GMA: the irradiated membrane was immersed in a 10% (v/v) GMA–methanol solution at 313 K for 15 min. (3) Introduction of the SO_3H group: the GMA-grafted membrane was immersed in a mixture of sodium sulfite–isopropanol–water (10:15:75, w/w). (4) Blocking of the remaining epoxy group: the membrane was immersed in 0.5 *M* H_2SO_4 at 353 K for 2 h to reduce nonselective adsorption [23].

The degree of GMA grafting, defined as:

$$dg\,(\%) = 100[(W_1 - W_0)/W_0] \quad (1)$$

where W_0 and W_1, are the masses of the original and GMA-grafted membranes, respectively, was set at 220%.

The molar conversion of the epoxy group to the SO_3H group was calculated to be 28%. The membrane containing both SO_3H and diol groups was referred to as SS-Diol fiber. For comparison, Diol fiber, i.e., the fiber exclusively containing the diol group, was obtainable by reacting the GMA-grafted membrane with water in 0.5 *M* H_2SO_4 [23,24].

The inner and outer diameters and the length of the hollow-fiber membrane in a wet state were

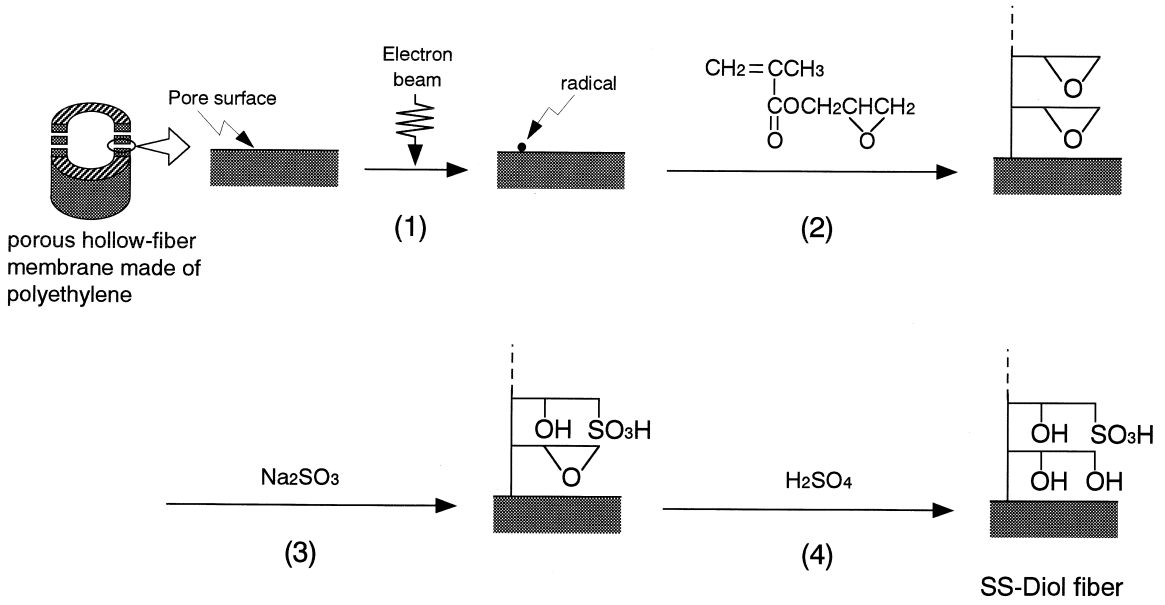


Fig. 1. Preparation scheme for graft chains containing sulfonic acid groups.

measured with a scale. After the membrane had been dried under reduced pressure, the specific surface area was determined using Quantasorb (Yuasa Ionics) according to the nitrogen adsorption method.

2.3. Permeation of Mg-ion-containing solution

The permeation flux was determined in the dead-end mode, as shown in Fig. 2. An SS-Diol fiber, approximately 5 cm long, was positioned in a U-configuration and the liquid was forced to permeate outward through the pores at a constant permeation pressure of 0.06 MPa. Five liquids were tested: pure water, 0.005 *M* NaCl, 0.005 *M* KCl, 0.005 *M* $CaCl_2$, and 0.005 *M* $MgCl_2$. The permeation rate of the effluent penetrating the outside surface of the hollow-fiber was determined. The permeation flux of the liquid was calculated by dividing the permeation rate by the inside surface area. For comparison, the permeation flux of the original and Diol fibers was also determined.

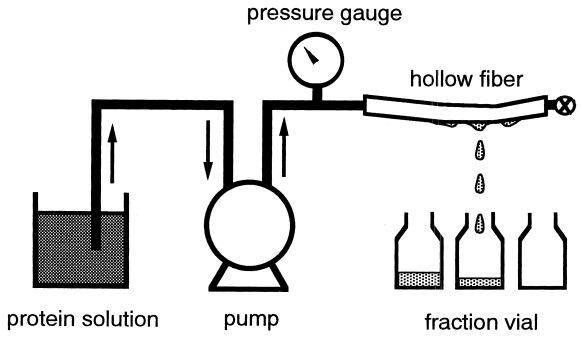


Fig. 2. Experimental apparatus for determination of permeation flux and amount of protein adsorbed.

To determine the concentration profile of Mg ions bound across the thickness of the membrane, after saturation with Mg ions, the intensity of the X-ray characteristic of sulfur and magnesium was determined using an X-ray microanalyzer (XMA).

2.4. Permeation of lysozyme solution

Lysozyme was adsorbed during permeation of the lysozyme solution and subsequently eluted by permeating an eluent through the SS-Diol fiber in the dead-end mode. The sequence of permeation was as follows: (1) a 0.05 *M* $NaHCO_3$–NaOH buffer (pH 9.0) containing 0.005 *M* $MgCl_2$ to ionically crosslink the graft chains, (2) pure water, (3) 0.5 g/l lysozyme buffer solution, (4) the 0.05 *M* $NaHCO_3$–NaOH buffer, and (5) the buffer containing 0.5 *M* NaCl.

The permeation pressure was maintained at 0.06 MPa. The effluent penetrating the outside surface of the hollowfiber was continuously collected in fraction vials. The concentration of lysozyme in each vial was determined by UV absorbance measurement at 280 nm. Magnesium concentration was determined by chelate titration with EDTA. Also, the change in the permeation flux was measured. Elution percentage was defined as follows:

$$\text{Elution percentage (\%)} = 100\ (\text{amount eluted})/[(\text{amount adsorbed}) - (\text{amount washed})] \quad (2)$$

All experiments were performed at ambient temperature.

3. Results and discussion

3.1. Properties of porous membrane

The properties of a cation-exchange porous hollow-fiber membrane, i.e., SS-Diol fiber, are summarized in Table 1. The hollow-fiber swelled on graft polymerization of GMA and subsequent sulfonation with sodium sulfite. This is mainly due to invasion of the polymer chains into the PE matrix [20]. The resultant density of the sulfonic acid group (-SO_3H) of 1.2 mmol per g of the H-type product was comparable to that of conventional cation-exchange beads. A uniform profile of sulfur across the SS-Diol fiber was demonstrated from XMA measurements, as shown in Fig. 3. This indicates that both GMA grafting and subsequent sulfonation proceeded uniformly across the thickness of the membrane.

Table 1
Properties of SO_3H-group-containing porous membrane of a hollow-fiber form (SS-Diol fiber)

Size (mm),	inner diameter	2.7
	outer diameter	4.5
SO_3H group density (mmol/g[a])		1.2
Specific surface area (m^2/g)		3.7
Pure water flux[b] (m/h)		0.10

[a] Grams of dry state of SS-Diol fiber.
[b] $\Delta P = 0.06$ MPa.

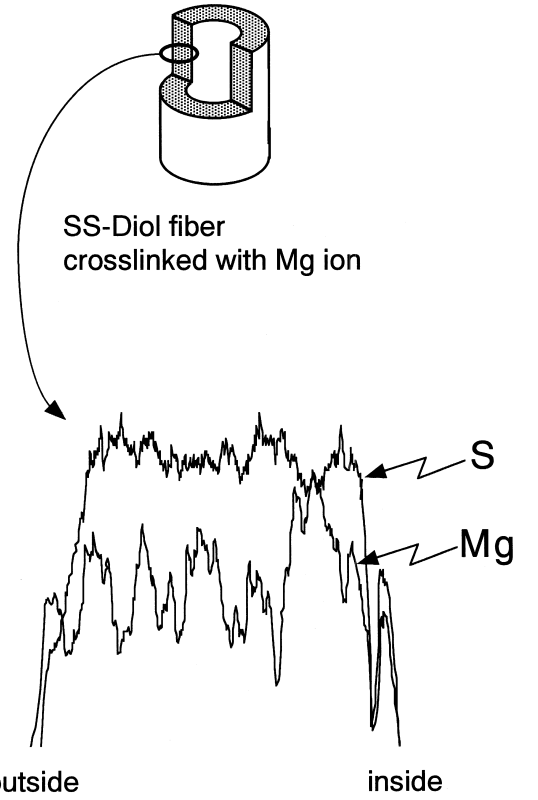
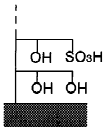
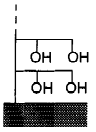


Fig. 3. Distribution of SO_3H groups and adsorbed Mg ions across the thickness of the membrane.

3.2. Ionic crosslinking of graft chains

Flux, i.e., the permeation rate of liquid per inside surface area of the hollow-fiber, is compared in Table 2 among SS-Diol and Diol fibers and the original hollow-fiber (PE fiber). The flux of the PE and Diol fibers was almost constant irrespective of the liquid permeated. The flux of the Diol fiber was about 65% that of the PE fiber because of pore volume reduction and thickness increase mainly due to graft polymerization. In contrast, the SS-Diol fiber exhibited a distinct difference in flux on permeation with water, monovalent cation (Na^+ and K^+) solution, and bivalent cation (Mg^{2+} and Ca^{2+}) solution. This difference can be explained by the action of bivalent cations crosslinking the SO_3H-group-containing polymer chain grafted onto the pore surface of the SS-Diol fiber, and thus restricting the exten-

Table 2
Flux of various liquids for hollow-fiber membranes (298 K, 0.06 MPa)

liquid	SS-Diol fiber	Diol fiber	PE fiber
			
water	0.10	1.9	3.1
0.005M NaCl	0.19	2.0	3.1
0.005M KCl	0.16	2.0	3.1
0.005M $CaCl_2$	1.5	2.0	3.1
0.005M $MgCl_2$	1.2	2.0	3.1

sion of the graft chains due to their mutual electrostatic repulsion.

The breakthrough curve of the buffer containing 0.005 *M* $MgCl_2$ solution (pH 9.0) for the SS-Diol fiber is shown in Fig. 4. The number of moles of Mg ions bound to the graft chains, and the degree of ionic crosslinking were calculated as follows:

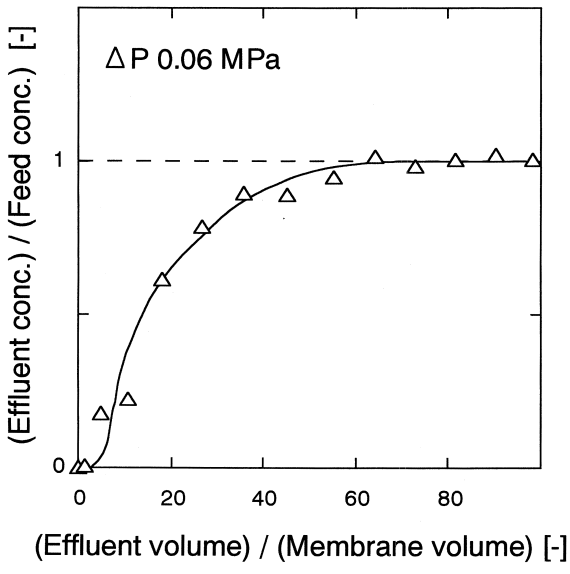


Fig. 4. Concentration change during permeation of the buffer containing 0.005 *M* $MgCl_2$ solution through the pores.

$$\text{Mol of } Mg^{2+} \text{ bound (mol)} = \int_0^{V_e} (C_0 - C)\, dV \quad (3)$$

Degree of ionic crosslinking (%)

$$= 100(\text{mol of } Mg^{2+} \text{ bound})/[(\text{moles of } SO_3H \text{ group})/2] \quad (4)$$

where *C*, *V*, and V_e are the concentration in the effluent, the effluent volume, and the effluent volume when *C* reached C_0, respectively. The degree of ionic crosslinking was 54%. A uniform profile of Mg^{2+} bound throughout the SS-Diol fiber is shown in Fig. 3.

3.3. Lysozyme recovery in a permeation mode

The breakthrough and elution curves of Mg^{2+} and lysozyme for the SS-Diol fiber are shown in Fig. 5. The abscissa is the dimensionless effluent volume (DEV) defined as the ratio of the effluent volume to the fiber volume excluding the lumen part, and the ordinate is the Mg^{2+} or lysozyme concentration ratio of the effluent to the feed. After ionic crosslinking, all lysozyme was bound to the SS-Diol fiber up to DEV=250, and an equilibrium was attained at DEV values greater than 1000. Meanwhile, Mg^{2+} as a crosslinker was quantitatively eluted by being replaced by lysozyme. The amount of lysozyme ad-

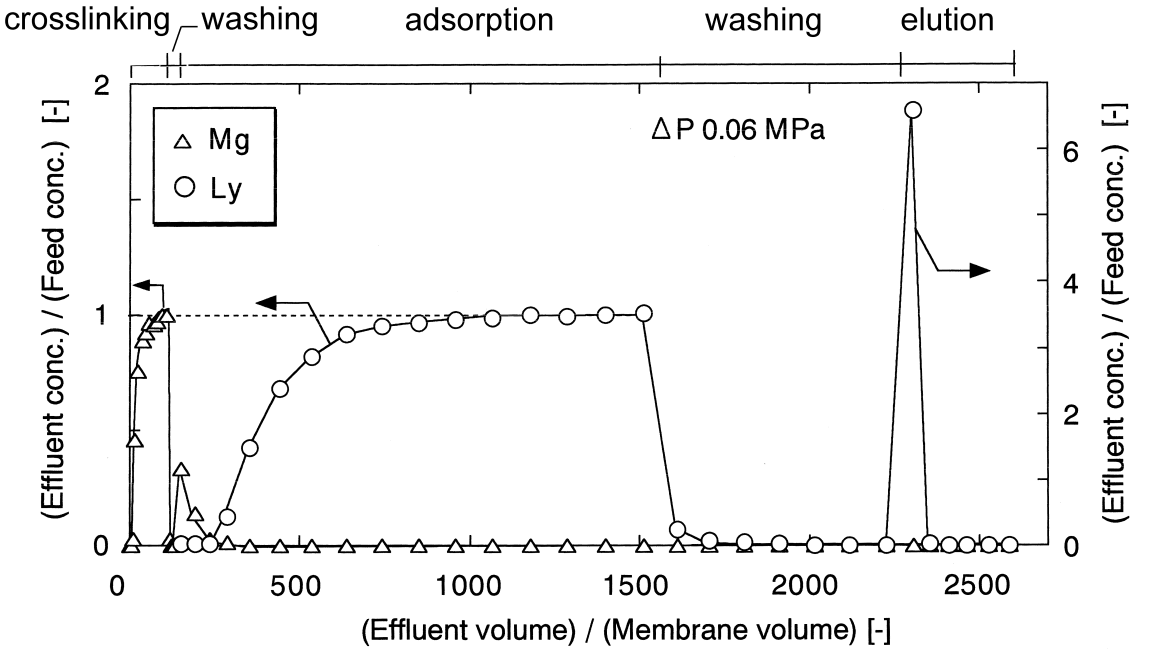


Fig. 5. Breakthrough and elution curves of Mg^{2+} and lysozyme for SO_3H-group-containing porous membrane (SS-Diol fiber).

sorbed was calculated as 0.42 g per g of the SS-Diol fiber. This value was equivalent to the degree of multilayer binding of lysozyme of 38 by dividing the amount of lysozyme bound by the theoretical amount of monolayer binding to the pore surface, i.e., 0.011 g/g [6]. The multilayer binding was due to the protein being bound to the SO_3H-group-containing graft chains, the extension of which had been restricted by ionic crosslinking with Mg^{2+}.

Lysozyme adsorbed via ion-exchange interaction with Mg^{2+} in the buffer solution (pH 9.0) was eluted by permeating a buffer containing 0.5 *M* NaCl. During three cycles of a series of adsorption, washing, and elution procedures, the amount of adsorbed lysozyme remained constant at 0.42 g/g with a 100% elution percentage defined by Eq. (2). No deterioration of the adsorption capacity of the SS-Diol fiber for lysozyme was observed. This is indicative of negligible nonselective adsorption of lysozyme onto the SS-Diol fiber because the coexistence of the diol and SO_3H groups conferred hydrophilicity to the pore surface [23,24].

The flux change accompanying a series of adsorption, washing, and elution under a constant permeation pressure of 0.06 MPa is shown in Fig. 6. As lysozyme adsorption proceeded, the flux increased, and reached a constant value of 1.7 m/h at adsorption equilibrium. The flux of the SS-Diol fiber remained constant throughout the washing step with buffer, and was reduced to a value of 1.5 m/h by permeating the eluent. The entrapment of the graft chains by lysozyme replacing Mg^{2+} was found to improve the permeability of the SS-Diol fiber.

4. Conclusion

Ionizable polymer chains grafted onto a porous hollow-fiber membrane are applicable to protein recovery based on electrostatic interaction. However, increasing the density of the ionizable group, e.g., sulfonic acid group, will enhance the extension of the graft chain from the pore surface toward the pore interior, resulting in the lowering of permeability. Ionic crosslinking of the graft chains by bivalent cations, e.g., Mg^{2+}, led to recovery of the permeability to overcome the trade-off between higher protein capacity and lower liquid permeability. The SO_3H-group-containing hollow-fiber membrane prepared here exhibited an equilibrium protein capacity

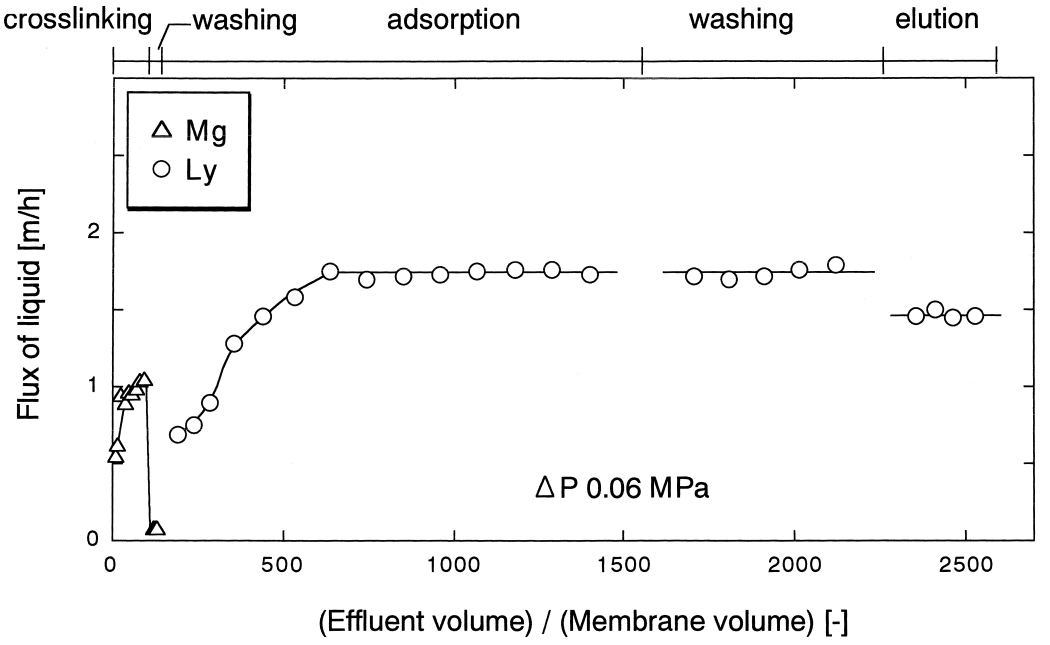


Fig. 6. Flux changes during adsorption, washing, and elution of lysozyme.

of 0.42 g per g of fiber, and an elution percentage of 100%. Since egg white originally contains Mg and Ca at a concentration of 0.005 and 0.015 *M*, respectively, ionic crosslinking by permeating egg white is applicable to purification of egg-white proteins.

In this study, we dealt with lysozyme dissolved in a buffer solution as a model solution of actual egg white. The egg white exhibits a viscosity of about four-fold that of the model solution. Therefore, in processing the egg-white protein using the modified porous hollow-fiber membrane, the operation in the crossflow mode is necessary so as not to reduce the permeability.

5. Symbols

C	Concentration of protein in the effluent (g/l)
C_0	Concentration of protein in the feed (g/l)
DEV	Dimensionless effluent volume
dg	Degree of grafting (%)
V	Effluent volume (l)
V_e	Effluent volume where C reaches C_0 (l)
W_0	Mass of the trunk polymer (g)
W_1	Mass of the GMA-grafted hollow-fiber (g)

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