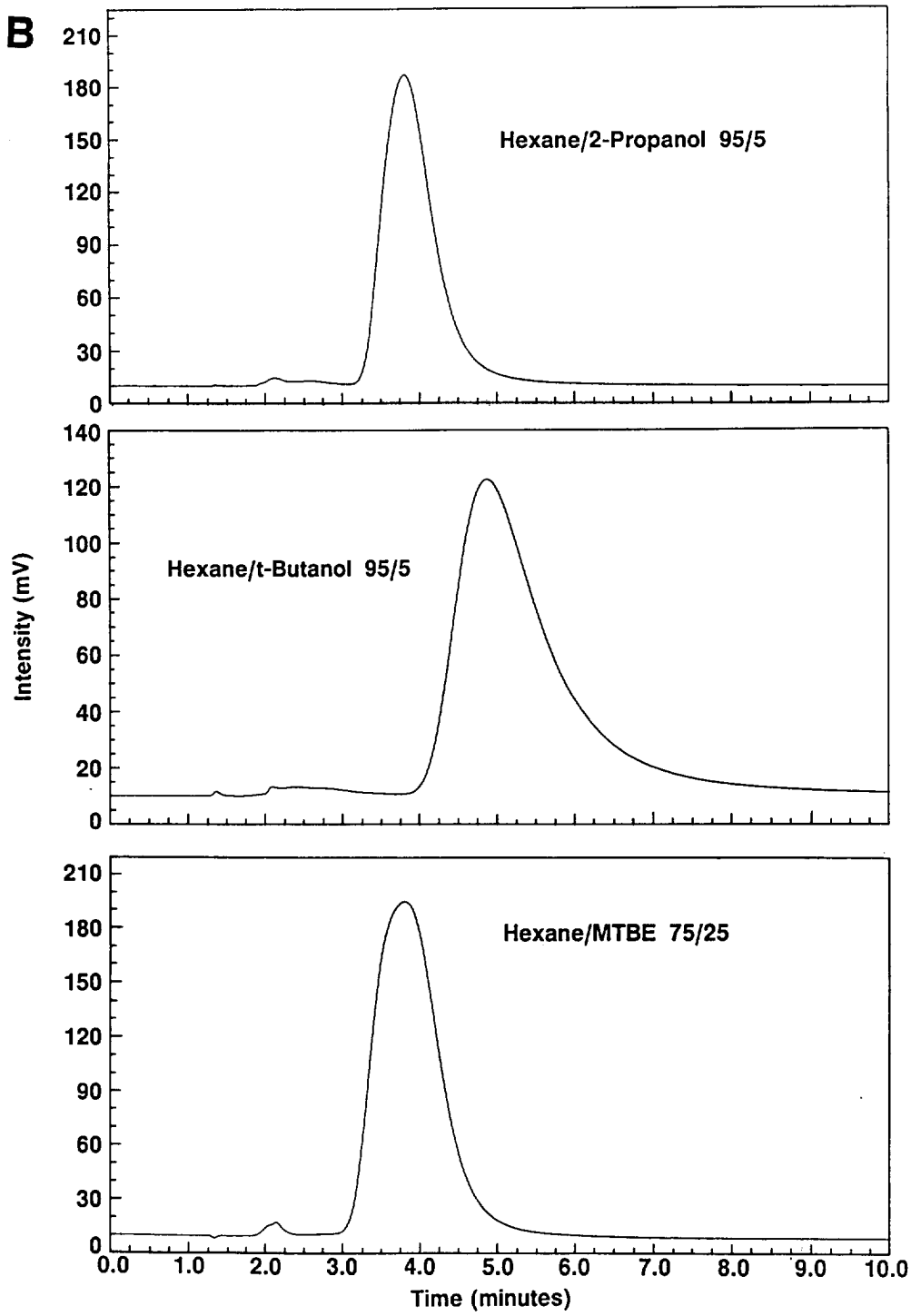

Fig. 10. (*continued*)

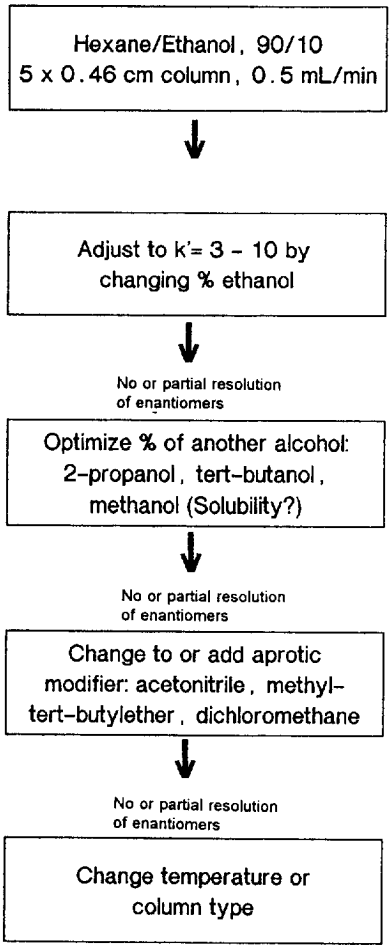

Fig. 11. Suggested strategy for optimizing separations on Chiralcel OD.

action. The Chiracel OD column appears stable to relatively high concentrations of certain aprotic solvent modifiers. Use of short Chiralcel OD columns with aprotic solvents expedites method development and is a cost-effective alternative to conventional approaches.

Acknowledgement

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Preparation of a hydrophobic porous membrane containing phenyl groups and its protein adsorption performance

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Abstract

A novel porous membrane was prepared by radiation-induced graft polymerization of an epoxy-group-containing vinyl monomer, glycidyl methacrylate, and subsequent addition of phenol and water. A phenyl group was appended to the polymer chain grafted on to a porous polyethylene hollow-fibre membrane. The presence of a diol group together with the phenyl group was required to reduce non-selective adsorption of the protein. A bovine serum albumin (BSA) phosphate buffer solution containing 2 *M* ammonium sulfate was forced to permeate through the resultant hydrophobic porous membrane, 0.36 mm thick with a phenyl-group density of 1.3 mmol/g. The breakthrough curves of BSA overlapped independent of the residence time in the range 12–1.2 s because of negligible diffusional mass-transfer resistance. A lower phenyl-group density resulted in a higher recovery of BSA after a series of adsorption and elution steps.

1. Introduction

Hydrophobic interaction chromatography (HIC) is recognized as a powerful technique for protein purification, along with affinity and ion-exchange chromatography [1]. HIC initially involved the use of charged beads, i.e., beads containing both a hydrophobic ligand and a charged group [2–6]. Later, uncharged beads were prepared to improve the performance of HIC [7–9].

To overcome the processing rate limitation caused by protein diffusion into the bead interior in conventional bead-packed column chromatography, the use of porous membranes [10–12], non-porous beads [13] and a continuous bed [14,15] has been suggested. Of these, a porous membrane, which has on its surface affinity ligands such as protein A [10,16], dye [17] and immobilized metal [18], and ion-exchange groups such as diethylamino [19,20] and sulfonic acid groups [21], is promising because of its high binding capacity for proteins and suitability for scale-up.

Tennikova and Svec [22] prepared a flat-sheet membrane consisting of a cross-linked terpolymer of glycidyl methacrylate–octyl methacrylate–ethylene dimethacrylate, in which the octyl group was directly bound to the matrix without a spacer and functioned as a hydro-

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phobic ligand. The spacing between the matrix and ligand is effective in improving accessibility of the protein to the ligand [23]. For example, HIC which utilized beads containing a phenyl group as the hydrophobic ligand with a 2-hydroxypropane group as a spacer exhibited a higher resolution than that without a spacer [24].

Radiation-induced graft polymerization (RIGP) is a powerful modification technique in that a flexible polymer chain can be grafted on to various forms of trunk polymers. We have prepared functionalized porous membranes containing affinity ligands [18] and ion-exchange groups [21,25] by RIGP of glycidyl methacrylate and subsequent ring-opening reactions of the epoxy group produced. The functional moiety was immobilized on the graft chain of submicrometre length. The flexible graft chain may provide a favourable binding space for the protein because it can also function as a spacer.

The objective of this study was twofold: (1) to prepare an uncharged porous membrane for HIC by RIGP and (2) to evaluate the adsorption and elution characteristics of the protein during permeation through the resultant membrane. We adopted a phenyl group and a porous polyethylene hollow-fibre membrane as hydrophobic group and feasible matrix, respectively.

2. Experimental

2.1. Materials

A porous membrane of hollow-fibre form was used as a trunk polymer for grafting. This membrane, which was made of polyethylene (PE), had inner and outer diameters of 0.07 and 0.12 cm, respectively, with a nominal pore diameter of 0.2 μm and a porosity of 0.67. Technical-grade glycidyl methacrylate (GMA) ($CH_2 = CCH_3COOCH_2CHOCH_2$) was purchased from Tokyo Kasei and used without further purification. Bovine serum albumin (BSA) was obtained from Sigma. Phosphate buffer (0.07 *M*, pH 7.4) was prepared by dissolving phosphate buffer powder (Wako) in deionized water. BSA was dissolved in phosphate buffer solution containing 2 *M* ammonium sulfate. Other reagents were of analytical-reagent grade or higher.

2.2. Introduction of phenyl group on to porous membrane

Ring opening of a poly-GMA chain was adopted to introduce a ligand on to a porous membrane [26,27]. Fig. 1 shows a schematic diagram of the procedure used to append a hydrophobic phenyl group to a PE membrane. This procedure consists of the following three steps. (1) Grafting of GMA on to the original membrane: the PE membrane irradiated with an electron beam is immersed in 10% (v/v) GMA–methanol solution solution at 313 K for 12 min [28]. (2) Introduction of the phenyl group for enhancement of HIC: the GMA-grafted membrane is immersed in 9.4% (w/w) aqueous phenol solution, the pH of which is adjusted to 9, at 353 K for 1–24 h. (3) Blocking of the

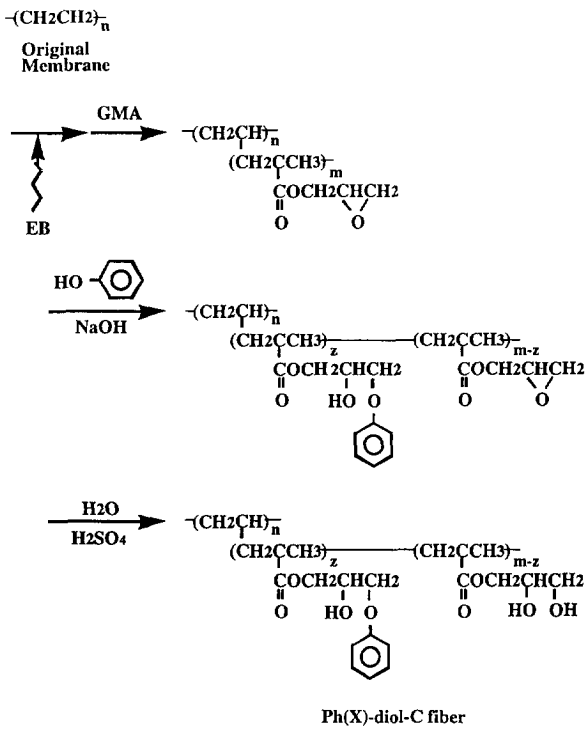

Fig. 1. Schematic diagram of procedure used to append phenyl and hydroxyl groups to polyethylene porous membrane.

remaining epoxy group for reduction of non-selective adsorption: the membrane is immersed in 0.5 *M* H_2SO_4 at 353 K for 2 h [25,29].

The degree of GMA grafting, defined as

$$d_g = 100[(W_1 - W_0)/W_0] \quad (1)$$

where W_0 and W_1 are the masses of the original and GMA-grafted membranes, respectively, was set to 150%.

The molar conversion of the epoxy group to the phenyl group, X, and phenyl-group density were calculated from the mass gain as follows [26,27]:

$$X = 100[142(W_2 - W_0)/(W_1 - W_0) - (142 + 18)]/(94 - 18) \quad (2)$$

phenyl-group density =

$$[(W_1 - W_0)/142](X/100)/W_2 \quad (3)$$

where W_2 is the mass of the resulting membrane and 142, 94 and 18 are the molecular masses of GMA, phenol and water, respectively. The membrane containing both phenyl and diol groups is referred to as a Ph(X)-diol-C fiber, where Ph, (X) and C denote phenyl group, conversion and capillary, respectively. For comparison, a Ph(0)-diol-C fibre, i.e., exclusively containing the diol group, was obtainable by reacting the GMA-grafted membrane with water.

2.3. Properties of the Ph-diol-C fibre

The inner and outer diameters and length of the hollow-fibre membrane in a wet state were measured with a scale. The porosity of the membrane was determined from measurement of its water content. 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.

Determination of the permeation flux in a dead-end mode has been described elsewhere [26,27]. Briefly, a Ph(X)-diol-C fibre about 15 cm long was positioned in a U-configuration and the liquid was forced to permeate outwards through the hollow fibre at a constant permeation pressure of 0.1 MPa. Four kinds of liquids were permeated: pure water, phosphate buffer (0.07 *M*, pH 7.4) and phosphate buffers containing 1 *M* and 2 *M* ammonium sulfate. The permeation rate of the effluent penetrating the outside surface of the hollow fibre was determined. The permeation flux of the liquid through the hollow fibre was calculated by dividing the permeation rate by the inside surface area. For comparison, the permeation flux of the original hollow-fibre membrane was also determined. The relative viscosity of the liquid to water was determined with an Ostwald viscometer at 298 K.

2.4. Adsorption and elution of BSA during permeation through the Ph-diol-C fibre

The protein was adsorbed during permeation of the protein solution and subsequently eluted by permeation of an eluent through the Ph-diol-C fibre in a dead-end mode, using a procedure similar to that described above. First, a 0.2 mg/ml BSA phosphate buffer solution containing 2 *M* $(NH_4)_2SO_4$ was forced to permeate through the hollow fibre until adsorption equilibrium was attained. Second, phosphate buffer containing 2 *M* $(NH_4)_2SO_4$ was permeated to wash the pores. Third, the adsorbed BSA was eluted by permeating $(NH_4)_2SO_4$-free phosphate buffer. The permeation pressure ranged from 0.01 to 0.1 MPa. All the experiments were performed at 298 K.

3. Results and discussion

3.1. Introduction of phenyl and hydroxyl groups on to porous membrane

The epoxy groups of poly(glycidyl methacrylate) (GMA) grafted on to hollow fibres were converted into phenyl groups by the addition of phenol, and the conversion is shown in Fig. 2 as a function of reaction time. A conversion of 0–44% corresponded to a phenyl (Ph)-group density of 0–1.6 mmol/g. The phenyl-group density of this Ph-diol-C fibre was compared with

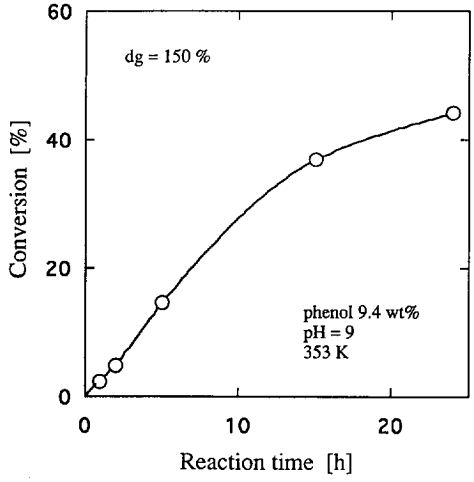

Fig. 2. Conversion of epoxy groups into phenyl groups as a function of reaction time.

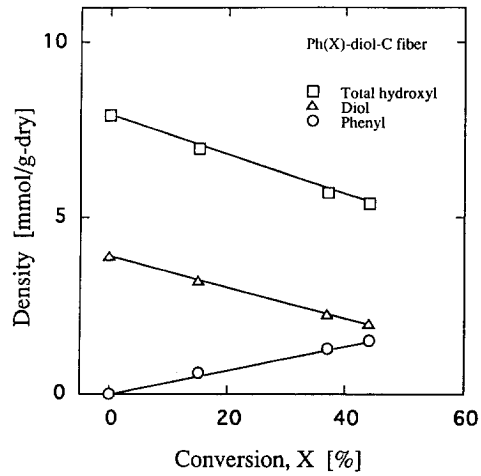

Fig. 3. Densities of phenyl, diol and total hydroxyl groups of the Ph(X)-diol-C fibre.

that of commercially available HIC gel beads: 0.1 mmol/ml of TSK gel Phenyl-5PW (Tosoh) [30] and 0.4 mmol/g of phenyl Sepharose FF (Pharmacia Biotech) [31] were equivalent to the phenyl-group densities of the Ph(5)-diol-C and Ph(10)-diol-C fibres, respectively.

The residual epoxy group was quantitatively converted into two adjacent hydroxyl (diol) groups via reaction with water. The density balance of the functional moieties on the graft chain, the units of which are represented mmol/g of the dry membrane, is shown in Fig. 3 as a function of the conversion. The total hydroxyl-group density was calculated as the sum of the density of monool groups produced by ring opening of the expoxy group with phenol and twice the density of diol groups produced by that of the residual epoxy groups with water. Kim et al. [29] reported that the critical density of the hydroxyl groups on the graft chain required for minimizing non-selective, i.e., irreversible, adsorption of the protein on to a porous polyethylene membrane was 14 mmol/g of original polyethylene membrane. This critical density corresponded to a total hydroxyl-group density of 4.3 mmol/g of Ph(67)-diol-C fibre. Therefore, the Ph-diol-C fibres below a conversion of 67% satisfy the requirement for hydrophilization, and will reduce non-selective adsorption of the protein.

3.2. Permeability of the Ph-diol-C fibre

Four kinds of liquids were permeated through the Ph-diol-C fibre with various conversions at a constant permeation pressure of 0.1 MPa: pure water, phosphate buffer and phosphate buffers containing 1 M and 2 M $(NH_4)_2SO_4$. Fig. 4 shows the permeation flux of the liquid as a function of the conversion. The permeation flux of each liquid of the Ph-diol-C fibre was independent of the conversion, i.e., phenyl-group density, and constant at 90% of the original hollow fibre. In contrast, the permeation flux of an ion-exchange porous hollow-fibre membrane is reported to decrease with increasing ion-exchange group density because mutual electrostatic repulsion of the graft chains induced stretching of the graft chains from the pore surface toward the pore interior [32,33]. The stability of the conformation of the polymer chains grafted on to the pore surface of the Ph-diol-C fibre towards the liquid is due to electrostatic neutrality of the graft chain, which is favourable for practical applications.

The permeation flux ratios of the phosphate

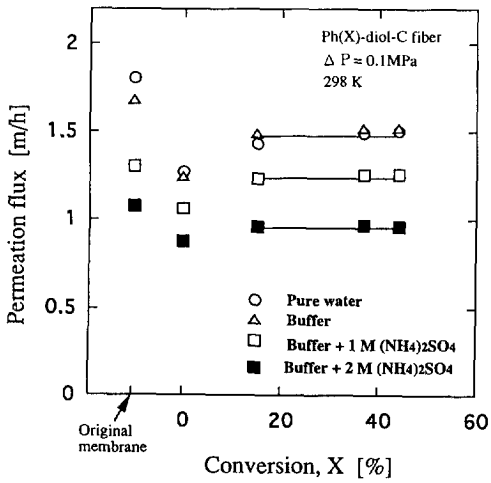

Fig. 4. Permeation flux of the Ph(X)-diol-C fibre as a function of conversion.

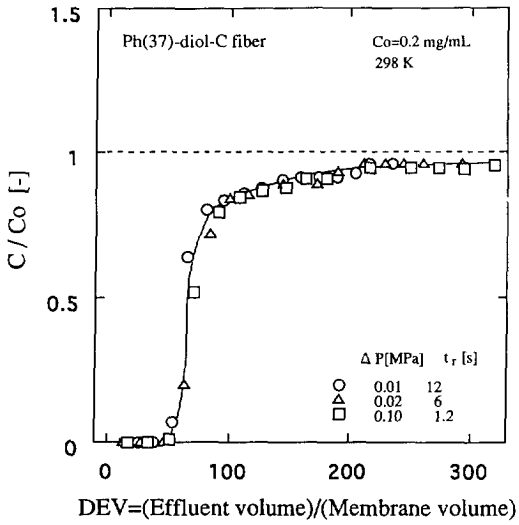

Fig. 5. Breakthrough curve of BSA across the Ph(37)-diol-C fibre with various permeation pressures.

buffer and phosphate buffers containing 1 M and 2 M $(NH_4)_2SO_4$ to water, 1.0, 0.84 and 0.66, respectively, were well correlated with the reciprocals of the relative viscosity, 0.97, 0.86 and 0.71, respectively. Therefore, the decrease in the permeation flux with increasing salt concentration could be explained by the increase in liquid viscosity.

3.3. Adsorption characteristics of BSA during permeation

A 0.2 mg/ml bovine serum albumin (BSA) buffer solution was fed to the inside surface of the pH(37)-diol-C fibre at a constant permeation pressure ranging from 0.01 to 0.1 MPa, i.e., a residence time ranging from 12 to 1.2 s. The residence time, t_r, was calculated as follows:

$$t_r = \varepsilon[\pi(d_o^2 - d_i^2)L/4](\text{permeation rate}) \quad (4)$$

where ε, d_i, d_o and L are the porosity, inner and outer diameters and length of the Ph-diol-C fibre in a wet state, 0.54, 0.073, 0.145 and 14.5 cm, respectively. The permeation rate of the BSA buffer solution for each permeation pressure remained constant during adsorption of the protein, e.g., 0.0083 ml/s at 0.01 MPa and 0.083 ml/s at 0.1 MPa. The time course of the protein concentration of the effluent, i.e., the breakthrough (BT) curve, is shown in Fig. 5 as a function of the dimensionless effluent volume (DEV), defined as effluent volume divided by the membrane volume. The BT curves overlapped independent of the permeation pressure, i.e., residence time. This indicates a negligible diffusional mass-transfer resistance of BSA to the phenyl group. Thus, a faster HIC of the protein can be realized using the Ph-diol-C fibre because the residence time of the protein solution across the membrane is much shorter (about $1/10^3$) than that across the conventional bead-packed column [10]. The advantage of using the porous membrane instead of beads has been demonstrated for HIC and also for pseudo-affinity [10,16–18,26,27] and ion-exchange [19–21,25] chromatography.

The amount of BSA adsorbed in equilibrium with the feed concentration, q_0, can be calculated by the following integration of the BT curve:

$$q_0 = \int_0^{V_s} (C_0 - C)\, dV/W_2 \quad (5)$$

where C_0(=0.2 mg/ml) and C are the concen-

trations of BSA in the feed and effluent, respectively, V is the effluent volume and V_s is the effluent volume when C reaches C_0. The q_0 values were compared with the theoretical saturation capacity, q_t, as shown in Fig. 6. The q_t of BSA on the Ph(X)-diol-C fibre can be calculated by assuming that BSA molecules cover the pore surface as a monolayer with end-on and side-on orientations, where the cross-sectional area, a, occupied by a BSA molecule is $1.4 \cdot 10^{-17}$ and $4.9 \cdot 10^{-17}$ m^2, respectively [34]:

$$q_t = a_v M_r / (a N_A) \quad (6)$$

where a_v, M_r and N_A are the specific surface area of the Ph(X)-diol-C fibre, molecular mass of BSA (66 300) and Avogadro's number, respectively. The a_v value was determined to be $6.7 \cdot 10^3$ m^2/kg irrespective of the conversion.

The amount of BSA adsorbed, i.e., the BSA binding capacity, was almost constant between two extreme orientations independent of the conversion, i.e., phenyl-group density.

The hollow-fibre membrane containing exclusively the diol group, i.e., the Ph(0)-diol-C fibre, had about 50% of the BSA binding capacity of the Ph(15)-diol-C fibre. The difference in BSA binding capacity between the Ph(0)-diol-C and Ph(15)-diol-C fibres is explained by the contribution of the phenyl group on the graft chain as a hydrophobic ligand. Poly(2-hydroxyethyl methacrylate) beads, which are analogues of the Ph(0)-diol-C fibre in terms of chemical structure, have been used as an HIC adsorbent [24]; the Ph(0)-diol-C fibre also exhibited hydrophobic interaction with the protein in the phosphate buffer containing 2 M $(NH_4)_2SO_4$.

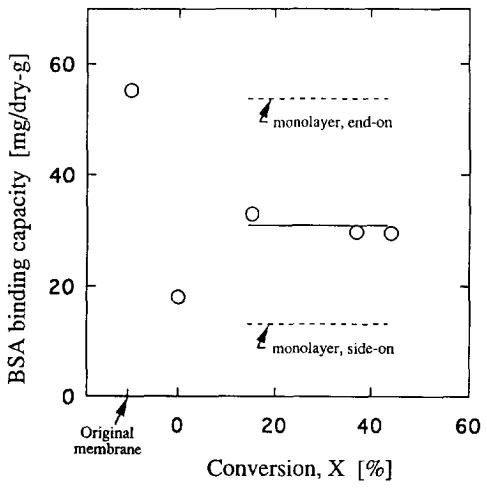

Fig. 6. BSA binding capacity of the Ph(X)-diol-C fibre.

3.4. Elution and recovery

A series of procedures for protein processing using the Ph-diol-C fibre in the conversion range 15–44% includes adsorption, washing and elution. Examples of breakthrough and elution curves of BSA are shown in Fig. 7, where the BSA solution and eluent were forced to permeate through the Ph(37)-diol-C fibre at a permeation pressure of 0.02 MPa. The recovery of the protein during permeation was defined as

$$\text{recovery}\ (\%) = 100\ [(\text{amount eluted})/(\text{amount adsorbed} - \text{amount washed})] \quad (7)$$

The recovery of BSA is shown in Fig. 8 as a function of the conversion. A higher density of the phenyl groups on the graft chain resulted in a lower recovery of BSA. This implies that BSA bound more strongly to the Ph-diol-C fibre which

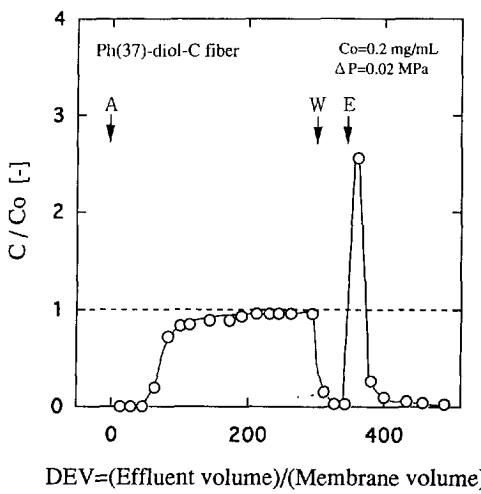

Fig. 7. Breakthrough and elution curves of BSA of the Ph(37)-diol-C fibre. A = adsorption; W = washing; E = elution.