Separation of Proteins by Polymeric Adsorbents Containing Azobenzene Moiety as a Ligand

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Synopsis

Polymeric adsorbents containing an azobenzene moiety as a ligand were prepared, and the photoinduced adsorption behavior of proteins to the adsorbent was studied. This method regulates by light the adsorption/desorption of proteins in hydrophobic chromatography. In the dark, the amount of protein adsorbed onto the adsorbent increased with increasing hydrophobicity of either adsorbent or protein. On irradiation with UV light, the amount of protein adsorbed decreased. Such a photoinduced change of the adsorption of protein was considered to be caused by the change of the hydrophobic interaction between the adsorbent and the protein due to the photoisomerization of the azobenzene moiety accompanying the polarity change of the adsorbent. It is also found that the desorption of protein was dependent on the balance of the hydrophobicity between the adsorbents and proteins. When column chromatography was carried out, the proteins were adsorbed in the dark and could be eluted after photoirradiation, with water as the single solvent. Furthermore, mixture of proteins could be separated by using a hydrophobic gradient column which was constituted by two polymeric adsorbents having different hydrophobicity.

INTRODUCTION

Hydrophobic interaction is a common phenomenon in biological systems.[1] In recent years, the hydrophobic interaction has been utilized for separation of biomaterials.[2] By introducing substituents of varying hydrophobicity into hydrophilic carriers, swelled in aqueous solutions, adsorbents with a highly varied degree of affinity for given biomaterials can be obtained. Er-el et al.[3] and Hofstee[4] studied purification of proteins using polymeric adsorbents containing alkyl or aryl groups as ligands. In this process, the proteins to be purified are adsorbed through hydrophobic interaction and then eluted with an eluent. If the adsorption and desorption steps in the hydrophobic chromatography can be controlled by external physical signals, such as light, heat, etc., a new technique which eliminates the use of eluents becomes available.

Azo dyes, a group of photochromic compounds, are known to undergo reversible isomerization when irradiated with light and interrupted, resulting in a change of solubility in water.[5] In our previous article, we reported that the polarity of the surface of an azoaromatic polymer film can be reversibly regulated by irradiation of light.[6] We have also reported on the column chromatography of antibiotics[7] and proteins,[8] which was carried out using photoresponsive polymeric adsorbents. These were prepared by coating azoaromatic polymers onto insoluble carriers. It was demonstrated that the antibiotics and the proteins can be desorbed by irradiation and then eluted with water.

In this article, in order to regulate the hydrophobic chromatography for proteins by light, polymeric hydrophilic adsorbents containing an azobenzene moiety

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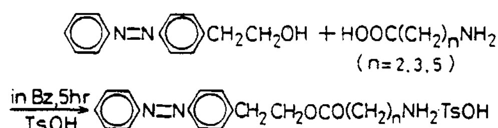

Fig. 1. Synthesis of azobenzene derivatives.

as a ligand were prepared and the photoinduced adsorption–desorption behavior of proteins was investigated. The separation of protein mixtures was also investigated using a gradient column which was constituted by two polymeric hydrophilic adsorbents having different hydrophobicity.

EXPERIMENTAL

Materials

p-Phenylazophenethyl alcohol was synthesized by the method of Ueno et al.[9] and recrystallized from hexane. Benzene, dimethylsulfoxide (DMSO), and acryloyl chloride were purified by the usual methods. Acrylamide (AAm) was recrystallized twice from benzene. Lysozyme (Seikagaku Kogyo Co., Ltd.), chymotripsin A_4 (Boehringer Mannheim GmbH), and chymotripsinogen A (Miles Lab. Ltd.) were used without further purification. Other reagents were extra pure grade commercial materials. *p*-Nitrophenyl acrylate (PNPA) was synthesized by adding dropwise acryloyl chloride to an aqueous solution of *p*-nitrophenol and potassium hydroxide at 0°C according to the method of Gaetjens and Morawetz.[10] The product was recrystallized from ethanol (mp 65.5°C).[10]

Syntheses of Azobenzene Derivatives with Primary Amino Group

The azobenzene derivatives with primary amino group were synthesized (Fig. 1) by condensation between ω-amino acid and *p*-phenylazophenethyl alcohol in benzene with *p*-toluenesulfonic acid (TsOH) as a catalyst for 5 h using the Dean–Stark apparatus.[11] The benzene was evaporated under reduced pressure, and the product was recrystallized from ethanol. The results of the syntheses of azobenzene derivatives are shown in Table I.

TABLE I
Syntheses of Azobenzene Derivatives with Primary Amino Group

Code		Elementary analysis (%) C	H	N	mp (°C)
PA2	calcd	61.4	5.80	8.95	185–187
	found	60.8	5.60	8.92	
PA3	calcd	62.1	6.04	8.69	159–161
	found	62.1	5.84	8.28	
PA5	calcd	63.4	6.50	8.22	164–166
	found	62.4	6.58	8.17	

Syntheses of Polymeric Adsorbents Containing Azobenzene Moiety

The DMSO solution containing the desired amounts (Table II) of PNPA, AAm, *N,N'*-methylenebisacrylamide(bis), and 2,2'-azobisisobutyronitrile (AIBN) was taken into a polymerization tube. The tube was evacuated and sealed under vacuum by conventional methods. The copolymerization was conducted at 60°C for 12 h under shaking, and the PNPA–AAm–bis copolymer gel formed was milled and suspended into methanol to exclude the unreacted monomers. The copolymer gel was filtered off and dried *in vacuo*, and divided into two portions, 100–200 mesh and 200–400 mesh, using wire gauge sieves. PNPA content in the copolymer gel was determined by the absorption coefficient at 404 nm of *p*-nitrophenol, which was liberated from the copolymer gel by 0.1*N* NaOH aqueous solution. Table II shows the results of PNPA–AAm–bis copolymerization. Introduction of the azobenzene derivatives with primary amino group into the PNPA–AAm–bis copolymer gel was done under the reactive group ratio $[NH_2]/[PNPA] = 2.0$ in DMSO at 60°C for 24 h. The product of the reaction was precipitated into methanol, filtered off, and dried *in vacuo*. The polymeric adsorbent containing PA2 as an azobenzene moiety is abbreviated as APA2, and also PA3 as APA3 and PA5 as APA5. The equilibrium hydration of these polymeric adsorbents were about 10 cm^3/g adsorbent both in the dark and after UV irradiation.

Measurement of Adsorption of Proteins

(a) Batch method: 0.05 g portions of the polymeric adsorbents (content of azobenzene moiety, 96.8 μmol/g adsorbent; 200–400 mesh) were transferred to test tubes to which 5 mL of 0.1 wt % aqueous solutions of proteins were added. The test tubes were shaken in a thermostated bath at 25°C for 24 h. The concentration of free protein was determined spectrophotometrically. Photoirradiation was carried out with a 500-W ultrahigh pressure mercury lamp (Ushio Denki, Model UI-501C), and the wavelength was selected with a filter ($\lambda = 350 \pm 50$ nm). Following the attainment of equilibrium adsorption in the dark, light irradiation was carried out for 1 h.

(b) Column method: The polymeric adsorbents (content of azobenzene moiety, 181 μmol/g adsorbent; 100–200 mesh) were packed into a glass column (7 cm × 0.5 cm, I.D.). After 200 μL of protein solutions with the concentration of 0.3 g/mL were applied on the column, fractions of 250 μL were collected, and their absorbance monitored. No effusion of protein from the column in the dark being found, photoirradiation was carried out. In order to obtain high irradiation efficiency, a reflective mirror was used.

TABLE II
Copolymerization of PNPA–AAm–Bis[a]

PNPA mole fraction in monomers	Bis mole fraction in monomers	[Monomer] (mol/L)	[AIBN] (mmol/L)	PNPA composition in copolymer (μmol/ g polymer)
0.0186	0.198	2.39	24.3	96.8
0.0204	0.118	2.85	24.3	181
0.0490	0	1.00	5.00	765

[a] Copolymerization was carried out in DMSO at 60°C for 12h.

Hydrophobic gradient column was constituted by connecting the two columns packed with adsorbents of different lengths of hydrocarbon chain (APA2 and APA5). The upper column was packed with adsorbent having a shorter length of hydrocarbon chain, APA2. The protein mixture was applied on the column, and the proteins were adsorbed in the dark. Then the connected columns were separated, and photoirradiation was carried out on each column. Fractions eluted from these columns were analyzed by GPC, and the amounts of protein in each fraction were determined.

Other Measurements

Fluorescence spectra were obtained with the aid of a recording spectrofluorophotometer (Hitachi, MDF-4). 1-Anilino-8-naphthalenesulfonate (ANS) was used as a hydrophobic probe. CD spectra were recorded on a JASCO J-500A spectropolarimeter with cell thickness of 1 cm (250–320 nm) and 0.1 cm (190–250 nm). GPC was run on Waters Protein Column I-125 at a flow rate of 1.2 mL/min.

RESULTS AND DISCUSSION

Preparation of Polymeric Adsorbents Containing Azobenzene Moiety as a Ligand and Their Photoresponsive Behavior

Esterification of a terminal carboxy group with *p*-nitrophenol is generally used as an activation method on the syntheses of polypeptides. By the condensation of a *p*-nitrophenyl ester with a primary amine, an amide linkage is formed and *p*-nitrophenol is liberted. Dattagupta and Buenemann[12] had given attention to this reaction, and prepared a new type of polymeric carrier with *p*-nitrophenyl ester moieties which can immobilize bioactive materials having primary amino groups.

When copolymerization of PNPA, AAm, and bis was carried out, light yellow gels were formed within 6 h. *p*-Nitrophenol was liberated from these gels by hydrolysis with 0.1*N* NaOH aqueous solution. The coupling reaction between azobenzene derivatives with primary amino group and the PNPA–AAm–bis copolymer gels proceeded quantitatively under the conditions described above, according to the measurement of the absorbance change of reactants. The uncrosslinked PNPA–AAm copolymer was soluble in water in the region of lower PNPA composition (<15 mol %). The copolymer, into which PA2 was introduced, i.e., uncrosslinked APA2, was also soluble in water.

Figure 2 shows the absorption spectra of aqueous solution of the water-soluble copolymer containing PA2 on UV irradiation. When UV irradiation was carried out, absorbance at 320 nm based on the π–π^* transition of *trans*-azobenzene moiety decreased and that at 430 nm based on the n–π^* transition of the *cis* one increased. Photostationary state was reached in a few minutes. Isosbestic points were recognized at 238, 273 and 388 nm. Therefore, it is considered that the change of absorption spectra was due to the trans to cis photoisomerization of azobenzene moieties. Assuming that the absorption of cis form in the wavelength region of 330–340 nm is negligible compared to that of the trans form, the percent of cis form was 54.5 at the photostationary state. The absorption spectra

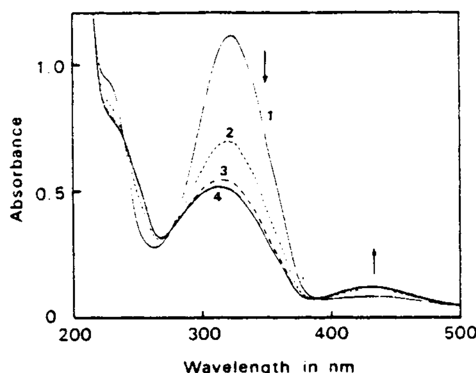

Fig. 2. Absorption spectra of PA2 bound to uncrosslinked PNPA–AAm copolymer in aqueous solution in the dark and under UV irradiation; UV irradiation time (min): (1) 0; (2) 0.5; (3) 1; (4) 2 (photostationary state). The arrows represent the direction of absorbance change.

returned to their original state, when the aqueous solution of the copolymer was allowed to stand in the dark or irradiation with visible light ($\lambda > 470$ nm). From these results, the azobenzene moieties introduced into the PNPA–AAm copolymer as side chains were isomerized reversibly by photoirradiation in water. Accordingly, it is considered that the azobenzene moieties attached to crosslinked polymeric adsorbents, APA2, APA3, and APA5, are also isomerized by photoirradiation.

Photoinduced Adsorption Behavior of Proteins on the Polymeric Adsorbents

Table III shows the amounts of proteins adsorbed on the photoresponsive polymeric adsorbents with azobenzene moieties before photoirradiation and after photoirradiation. These values were measured by the batch method. It is found that the longer the length of the alkyl chain of the azobenzene moieties is, the more protein is absorbed. When crosslinked polyacrylamide, which is generally said to show no hydrophobic adsorption, was used in this experiment, no adsorption of proteins could be obtained.

Hofstee investigated the adsorption behavior of proteins toward adsorbents having different lengths of alkyl chain as a ligand and concluded that the adsorption amount of proteins was increased with increasing the hydrophobicity of the adsorbents due to the elongation of the alkyl chain length.[4]

TABLE III
Adsorption Amount of Proteins onto Polymeric Adsorbent Containing Azobenzene Moiety at 25°C

	Lysozyme			Chymotripsin			Chymotripsinogen		
Adsorbent	Dark (%)	Light (%)	$(D\text{-}L)/D$[a]	Dark (%)	Light (%)	$(D\text{-}L)/D$[a]	Dark (%)	Light (%)	$(D\text{-}L)/D$[a]
APA2	44.7	26.3	0.412	33.3	28.7	0.138	29.9	25.8	0.137
APA3	57.9	47.4	0.181	60.0	33.3	0.445	35.1	27.1	0.228
APA5	80.5	80.0	·0.006	73.3	74.0	−0.010	66.8	35.8	0.467

[a] D = adsorption amount of proteins in the dark; L = adsorption amount of proteins under light irradiation; $(D\text{-}L)/D$ = adsorption–desorption efficiency of proteins by light irradiation.

Comparing the adsorption amount of every protein toward the same adsorbent, the adsorption amount increased in the following order: chymotripsinogen, chymotripsin, and lysozyme.

A fluorometric measurement was carried out on the aqueous solutions of these proteins in the presence of ANS for the purpose of the estimation of hydrophobicity of these proteins. The ANS molecule, a fluorescent probe for hydrophobicity, is formed preferentially to the hydrophobic region of proteins, resulting a shift of the emission maximum toward a shorter wavelength depending on the hydrophobicity around the binding site.[13] The maximum wavelength of emission for aqueous solutions of chymotripsinogen, chymotripsin, and lysozyme were 488, 477, and 458 nm, respectively. This result shows that highly hydrophobic region exists in the molecule of chymotripsinogen, chymotripsin, and lysozyme in the sequence to be more hydrophobic. That is to say, the adsorption amount of proteins was found to be in accord with the order of their hydrophobicity. These results suggest that the adsorption of the proteins to the adsorbents having azobenzene moieties is mainly attributed to the hydrophobic interaction between both of them.

A decrease was obtained for the adsorption amount on irradiation of light compared with that in darkness. When azobenzene is isomerized from trans form to cis form by UV irradiation, its hydrophobicity also changes, since the dipole moment across the azo bond is induced. Therefore, it seems that a decrease in the adsorption amount of proteins is induced by a decrement in the interaction force between proteins and adsorbents.

The value of $(D\text{-}L)/D$ indicates the adsorption–desorption efficiency of proteins, and the larger this value is, the higher efficiency is. It is found that the value in the case of lysozyme was the largest for the APA2, and in the case of chymotripsinogen for the APA5. The use of the APA3 produced the highest adsorption–desorption efficiency for chymotripsin. However, when the APA2, whose hydrophobicity is less than that of the APA3, was used for chymotripsin, the efficiency was lowered by a little adsorption amount in the dark. On the other hand, since chymotripsin adsorbed too strong to the APA5, having the highest hydrophobicity in the three, the change of the adsorption amount could not be observed even when the hydrophobicity of the azobenzene moiety was changed by photoirradiation; namely, the efficiency was low. It is expected from these results that selective adsorption–desorption is possible by selection of the adsorbent having the most suitable azobenzene moieties on the basis of the consideration of the balance of the hydrophobicity between the adsorbents and proteins.

Column Chromatography of Proteins on the Polymeric Adsorbents Containing Azobenzene Moieties

Figure 3 shows the profile of the chromatography of lysozyme on the column packed with the APA2 in comparison with crosslinked polyacrylamide. In the case of polyacrylamide, the effusion of lysozyme was detected around the column volume, i.e., fraction number at 5. The effusion of lysozyme was not detected; when the APA2 was used in the dark, however, the elution of lysozyme could appear in response to photoirradiation. By considering these results together with the results shown in Table III, the following consideration is produced.

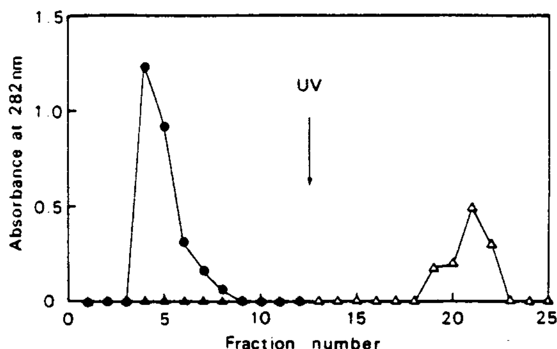

Fig. 3. Chromatography of lysozyme on crosslinked polyacrylamide and polymeric absorbent containing azobenzene moiety, APA2 at 25°C. Each 250 μL of fraction was collected at rate of 4 mL/h, and the absorbance at 282 nm was monitored. After sample solution application, each column was washed with water to effuse unbound protein in the dark and then UV irradiation to the column was carried out as indicated. (●) On crosslinked polyacrylamide; (▲,△) on APA2: closed plot, in the dark; open plot, under UV irradiation.

Lysozyme was not adsorbed owing to no occurrence of the appreciable interaction between lysozyme and the polyacrylamide, and it effused around the column volume, while, since lysozyme was adsorbed to the APA2 in the dark, the effusion of it from the column was not detected. The interaction between the adsorbent and lysozyme was weakened by photoinduced polarity change of azobenzene moiety. Consequently, lysozyme was desorbed from the adsorbent and eluted from the column. From these considerations, it is concluded that the adsorption–desorption steps of proteins are photocontrolled in the system with water as the only solvent by utilizing the polymeric adsorbents containing azobenzene moieties.

In order to examine the denaturation of lysozyme eluted from the column by photoirradiation, CD spectra were measured for the eluate containing lysozyme. The CD spectra were shown in Figure 4 compared with those of the aqueous solution of native lysozyme. It is found that the CD spectra of lysozyme eluted by photoirradiation quite agreed with those of native lysozyme in the range of 190–250 nm [Fig. 4(A)], in which reflects the conformation of polypeptide backbone, and 250–320 nm [Fig. 4(B)], in which reflects the orientation of the

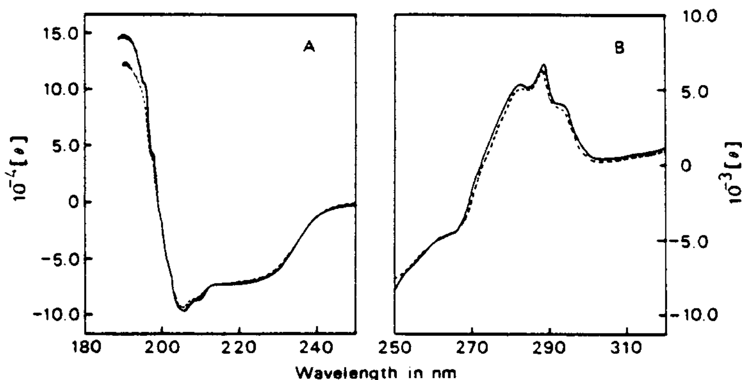

Fig. 4. The CD spectra of lysozyme: (---) in water: (—) in elution from the column packed with APA2 by photoirradiation.

residues of polypeptide side chains. These results suggest that the denaturation of lysozyme is not induced in the adsorption–desorption steps by photoirradiation.

Figure 5 shows the chromatography of chymotripsin on the photoresponsive polymeric adsorbents having different alkyl chain lengths. When the APA2 and APA3 were used as packing materials, effusion of chymotripsin was detected in the dark. However, the effusion was not detected at all in case of the APA5. When the columns were irradiated with light, the elution of chymotripsin appear for the APA2 and APA3, but not for the APA5. From the comparison of the amount of chymotripsin effused in the dark or eluted by light irradiation about the APA2 and APA3, it is clear that larger amount of effusion was obtained for the APA2 than that for the APA3 in the dark; however, the larger amount of elution was obtained for the APA3 than that for the APA2 under photoirradiation. It is considered from these results that chymotripsin was effused from the column packed with the APA2 and APA3 due to weak interaction between the adsorbents and the protein, whereas the protein was held in the column packed with the APA5 due to strong interaction. Thus the use of the APA2 or APA3 makes it possible to separate chymotripsin by photoirradiation, and, furthermore, it is evident that the use of the APA3 was most suitable for the separation by considering the efficiency of the photoinduced desorption. This finding agrees with the result obtained by the batch method. Consequently, selective and effective adsorption–desorption of the adsorbates by photoirradiation in hydrophobic chromatography becomes feasible when the adsorbents containing azobenzene moieties with suitable hydrophobicity are used.

Selective Desorption of Protein from Mixture Using Hydrophobic Gradient Column

Hofstee suggested that the selective separation of proteins from their mixture is achieved on the basis of the difference in the hydrophobicity of proteins by hydrophobic gradient column, which is made up by connecting the columns packed with the adsorbents having various hydrophobic groups in the order decreasing hydrophobicity.[14] Therefore, the hydrophobic gradient column was constituted by the APA2 and APA5 as packing materials, and the selective separation of lysozyme and chymotripsinogen was examined.

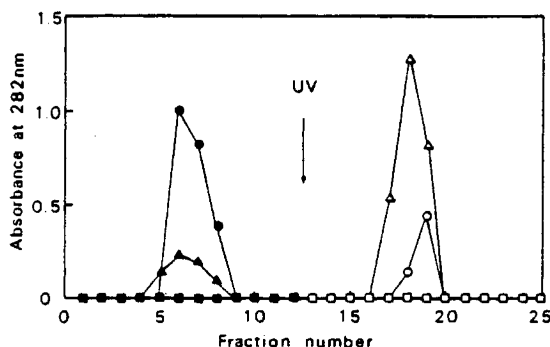

Fig. 5. Chromatography of chymotripsin on polymeric adsorbent containing various azobenzene moieties. Condition was the same as that in Figure 2. (●,○) On APA2; (▲,△) on APA3; (■,□) on APA5: closed plot, in the dark; open plot, under UV irradiation.

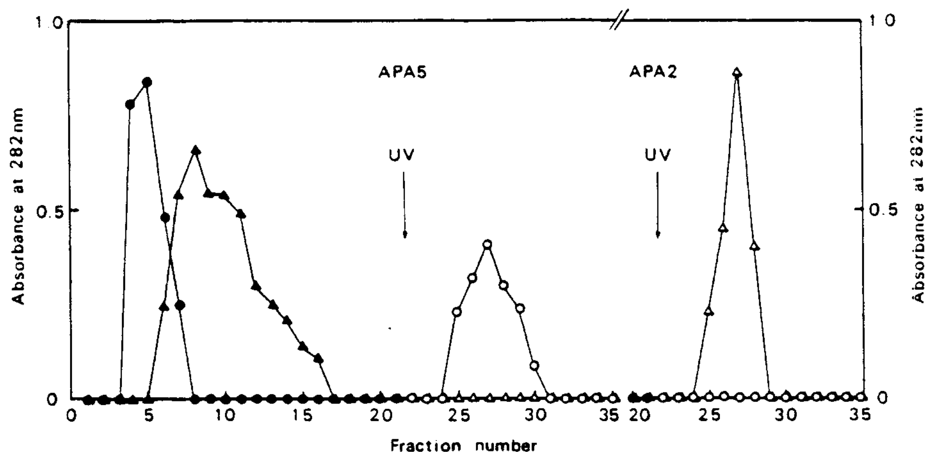

Fig. 6. Distribution of a mixture of lysozyme and chymotripsinogen on connected columns of APA2 and APA5. Condition was the same as that in Figure 2. After unbound proteins effused in the dark, the connected columns were separated and UV irradiation was carried out to each column as indicated. (●,○) Chymotripsinogen; (▲,△) lysozyme: closed plot, in the dark; open plot, under UV irradiation.

Figure 6 shows the result of the chromatography. After injection of the solution mixture of lysozyme and chymotripsinogen into the connected column, the effusion of proteins was observed in the dark. When the effused solution was analyzed by GPC, the first peak was ascribed to the effusion of chymotripsinogen and the second peak was ascribed to that of lysozyme. The region, in which both of the proteins were mixed, was also found. These results can be explained that molecular sieving effect contributed to the effusion of proteins in the dark. After the effusion of the proteins could not be detected in the dark, the connected columns were separated into two parts packed with the APA2 and APA5, and photoirradiation was carried out for each part of the separated columns. As the result of this performance, the elution of the proteins appeared on both of the columns. Analysis of the contents in the eluated solution indicated that lysozyme was selectively desorbed from the APA2 packed column and chymotripsinogen was desorbed from the APA5 packed one. From these results, it was proved that more hydrophobic lysozyme was suitably adsorbed to less hydrophobic APA2, and less hydrophobic chymotripsinogen was suitably adsorbed to more hydrophobic APA5. The proteins adsorbed were considered to be desorbed in response to the photoisomerization of azobenzene moiety and to be eluted from the columns. That is, when two kinds of proteins, of different hydrophobicity, are adsorbed and separated after, it is considered that the hydrophobic gradient column is useful and the proteins can be adsorbed by the minimum interaction as the result of contact with the adsorbents in the sequence increasing hydrophobicity. Such minimum interaction seems to suppress the denaturation of proteins while proteins are adsorbed in the column and makes it possible effectively to desorb the proteins adsorbed by the slight polarity change of the azobenzene ligand.

These results mentioned above lead to conclusion that photoinduced adsorption–desorption step can be realized in the system in which water is the only solvent by use of the adsorbents containing azobenzene moieties as hydrophobic binding sites and additionally that the hydrophobic gradient column enables proteins to be separated selectively.

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