Journal of Chromatography, 371 (1986) 29–35
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 942

MILD INTERACTION OF PROTEINS WITH BUTYL AND HYDROXYL GROUPS ON THE SURFACE OF POLYMER GELS TSK HW-65 AND BUTYL TOYOPEARL 650-M

H. IMAI*, G. TAMAI and S. SAKURA
Faculty of Pharmaceutical Sciences, Fukuyama University, Fukuyama City, 729-02 (Japan)

SUMMARY

Interaction of bovine serum albumin (BSA) with butyl and hydroxyl groups on adsorbent gel surfaces was investigated by using TSK HW-65 and Butyl Toyopearl 650-M gels. It was found that BSA was adsorbed on the gel not only from highly concentrated ammonium sulphate but also from dilute perchloric acid, trichloroacetic acid, etc. Some eluent modifiers, such as organic solvents (30% aqueous methanol or acetonitrile), salt solutions (0.18–0.2 *M* phosphate) and hydrogen-bond breaking reagents (3–7 *M* urea, 10 *M* ethylene glycol, 0.1% triethylamine) were found to be effective in facilitating the elution of trapped BSA from the gel. The conformational change of BSA in these solutions was slight except for urea, and it was reversibly recovered after removal of the modifier from the aqueous solutions, except for the hydrogen-bond breaking reagents.

INTRODUCTION

It has been pointed out that Toyopearl HW-65, one of the widely used gel-filtration matrices, can adsorb proteins at high concentrations of ammonium sulphate[1]. The mechanism of adsorption was assumed to be hydrogen bonding, made possible by a slight denaturation of proteins in solutions with high ionic strength. Recently, Butyl Toyopearl (BT) 650, a butyl derivative of HW-65, was synthesized, and characteristics of its hydrophobic interaction with proteins have been reported[2]. These gels attracted attention as chromatographic packings for protein separations[2,3]; moreover, BT 650 proved to be useful as an on-line deproteinization matrix in a pre-column for HPLC analysis of hydrophilic components in serum[4,5]. But, still, their fundamental properties have been poorly studied.

This paper presents our new findings on the properties of BT 650-M, a method for trapping proteins on BT 650-M and for releasing them, and some information from circular dichroism on the conformational changes in bovine serum albumin (BSA) molecules caused by adsorption and desorption.

0021-9673/86/$03.50

EXPERIMENTAL

Apparatus

An HPLC system, Model CCPD (Toyo Soda Kogyo, Tokyo, Japan) was used with a UV detector, Model UV-8000. Some (batchwise) experiments were carried out with a disposable syringe used as an open column. A Model J-20C spectropolarimeter (Japan Spectroscopic, Tokyo, Japan) with a Model J-DP2 data processor was used to measure the circular dichroism of BSA.

Adsorbents

TSK gels, HW-65, Phenyl 5-PW, and BT 650-M were kindly provided by Toyo Soda Kogyo. Toyopearl HW-65 has hydroxyl groups on its surface, and it is used for gel filtration with an exclusion limit of $5 \cdot 10^6$ daltons for proteins. Phenyl 5-PW and BT 650-M are used for hydrophobic interaction chromatography, the functional groups being phenyl groups and the base resin TSK gel G5000PW for the former, and butyl groups and HW-65, respectively, for the latter.

Chemicals

Fat-free BSA was obtained from Wako Pure Chemicals (Osaka, Japan). All other reagents were of analytical-reagent grade.

RESULTS AND DISCUSSION

Properties of BT 650-M

The particle size of BT 650-M was 44–88 μm with wide pores (exclusion limit of base resin, $5 \cdot 10^6$ daltons for protein). The ligand (butyl group) concentration was 850 μmol/ml of resin, and adsorbed about 35 mg BSA/ml of wet gel from an ammonium sulphate solution of 40% saturation. The resin was fragile, and easily collapsed at pressure higher than 30 kg/cm^2.

Trapping proteins on BT 650-M

It has been reported[2,3] that adsorption of proteins on HW–65 or BT 650-M is caused by salting out of proteins from solutions of high ionic strength. Ammonium sulphate solutions with concentrations as high as 5–50% of saturation are known to be effective for adsorption. We found that some denaturating reagents, such as perchloric acid (PCA), trichloroacetic acid (TCA), trifluoroacetic acid (TFA), tungstic acid, and molybdic acid are also effective for this purpose at concentrations lower than that needed to precipitate proteins. The results are summarized in Table I, and the adsorption curve is shown in Figs. 1 and 2. The adsorption curves were sigmoidal in all cases. It is noteworthy that even HW-65, which has no butyl groups, can adsorb BSA at high concentrations of ammonium sulphate, as shown in Fig. 1, even though the effect was smaller than in the case of BT 650-M. The mechanism of adsorption could presumably be hydrogen bonding. The discrepancy between the curves for BT 650-M and HW-65 may be due to butyl groups, which exhibit hydrophobic interaction toward protein molecules. As shown in Fig. 2, the effect of PCA was more pronounced than that of the other denaturation reagents investigated. When the quantity adsorbed in this case was replotted against the pH of the PCA solution, a

TABLE I

MINIMUM CONCENTRATION OF DENATURATING REAGENTS REQUIRED TO PRECIPITATE OR TRAP BSA ON BT 650-M

Reagent	*Concentration (%)*	
	Precipitation	*Trapping*
$(NH_4)_2SO_4$	75% of sat.	5–50% of sat.
TFA	6–10	0.05–1.0
TCA	1.5–5	0.05–0.75
PCA	1–2	0.02–0.5*

* pH 2.3–1.3.

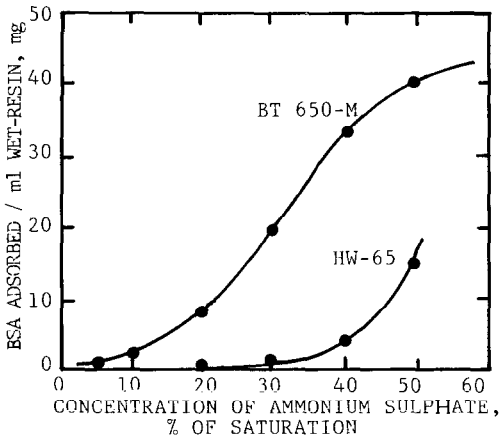

Fig. 1. Adsorption of BSA on BT 650-M and HW-65 from ammonium sulphate solutions.

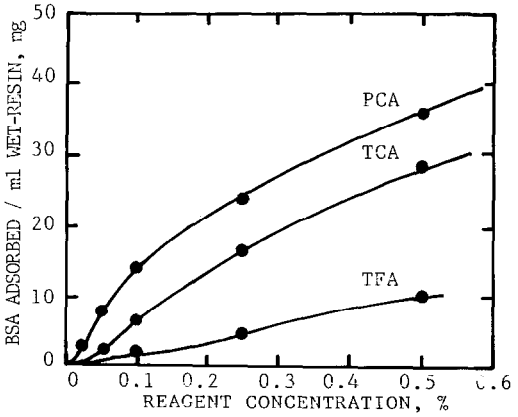

Fig. 2. Adsorption of BSA on BT 650-M from PCA, TCA and TFA solutions.

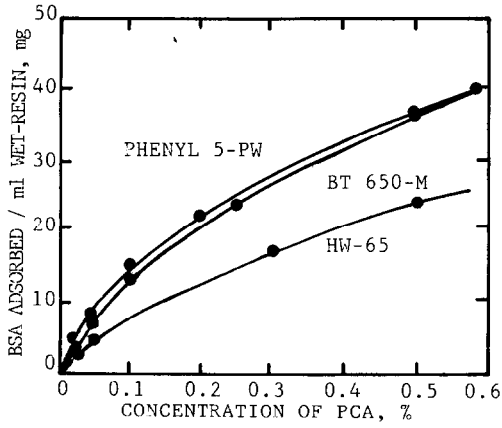

Fig. 3. Adsorption of BSA on Phenyl 5-PW, BT 650-M and HW-65 from PCA solutions.

linear relationship was obtained in the pH range from 1.5 to 2.3. In Fig. 3, the effects of hydroxyl, butyl, and phenyl groups are compared for 0.5% PCA solution. It is seen that the amount adsorbed on HW-65, which has only hydroxyl groups on the surface, was small, and that the effect of phenyl groups was almost the same as that of butyl groups. The adsorptivity of HW-65 was greater for the very dilute PCA solution than for the ammonium sulphate solution, as shown in Fig. 1. Hydrophobic interaction may exert its influence in the case of HW-65, as pointed out by Shin *et al.*[2].

Releasing proteins from BT 650-M

The elution profile of BSA, trapped on BT 650-M, was investigated with eluents containing various modifiers. When *ca.* 6 mg BSA was adsorbed on 1 ml of wet gel and desorbed batchwise by water, the recovery of BSA was almost quantitative. But the equilibration rate was rather slow, so that the chromatogram showed a broad peak, as shown in Fig. 4. When we used modifiers to facilitate the desorption

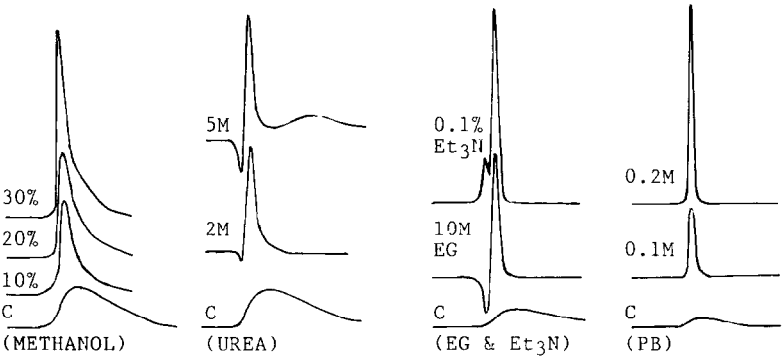

Fig. 4. Chromatograms of adsorbed BSA with various modifier solutions. Flow-rate, 1 ml/min; detection, 280 nm. C = control (water); EG = ethylene glycol; Et_3N = triethylamine; PB = phosphate buffer (pH 7.0).

TABLE II

RELATIVE PEAK HEIGHT, *h*, IN ELUTION WITH VARIOUS MODIFIER SOLUTIONS

Eluent	*h**
Water	1
5 *M* Urea	2.5
30% Methanol	4.0
10 *M* Ethylene glycol	7.0
0.1% Triethylamine	11.0
0.2 *M* Phosphate buffer	15.0

* Height relative to water.

the peak became sharper. The increase in solubility caused by organic solvents (30% aq. methanol or acetonitrile), hydrogen-bond breaking by 5–7 *M* urea, 10 *M* ethylene glycol, and 0.1% triethylamine solution, and salting in by phosphate solution are compared in Table II. The effect of salting in was the most remarkable among the modifiers used. The effect was maximal at a concentration of 0.18–0.2 *M* as shown in Fig. 5. At concentrations higher than 0.2 *M*, the effect of salting out seemed to overcome the effect of salting in, so that the peak height was decreased at higher concentrations.

As reported previously[5], rabbit serum was injected into a BT 650-M column in the mobile phase of 0.4% PCA solution, and the proteins eluted were determined by the Coomassie Brilliant Blue method. The recovery of proteins by elution with water was very poor. Even if the solubility was increased by using organic solvents or salting in effects, recoveries were only *ca.* 85–95%. Hydrogen-bond breaking was necessary to attain quantitative recovery.

The bond energy of hydrophobic interaction and hydrogen bonding are estimated to be 3–4 kcal/mol and 7–8 kcal/mol, respectively. In either case the interaction is mild, and proteins can easily be desorbed from the gel. The multidentate binding

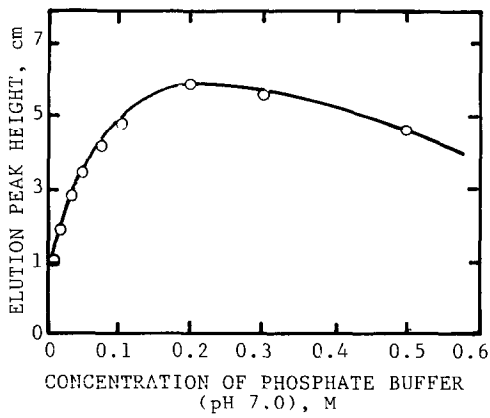

Fig. 5. Effect of salting in on elution of BSA, adsorbed on BT 650-M.

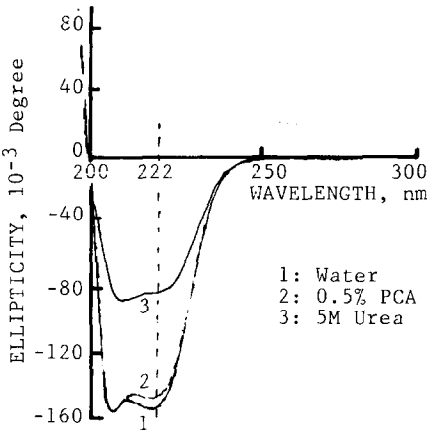

Fig. 6. CD spectra of BSA. Curve 1: control, 10 μM BSA in water; curve 2: 10 μM BSA in 0.5% PCA solution; curve 3: 10 μM BSA in 5 M urea solution.

of a protein molecule to BT 650-M by both hydrophobic interaction and hydrogen-bonding may be possible. If so, elution will be more difficult.

It is concluded that retention of protein molecules is enhanced from solutions containing PCA, TCA, TFA, or ammonium sulphate, and that the elution is facilitated by the presence of salts, organic solvents, or hydrogen-bond breaking reagents in aqueous solutions.

Conformational change of BSA by adsorption and desorption treatments

Circular dichroism was measured, and the decrease in the α-helix of BSA was estimated from the mean residue ellipticity at 222 nm (Fig. 6). Table III shows the results obtained with media containing various modifiers. As seen in Table III, PCA, ammonium sulphate, 20% acetonitrile, and 10 M ethylene glycol showed decrements in the α-helix of less than 15%. The conformation was reversibly recovered by removal of PCA and ammonium sulphate from the aqueous solution, even after the adsorption and desorption treatments. The effect of urea was rather deleterious; the

TABLE III

MEAN RESIDUE ELLIPTICITY OF BSA AND α-HELIX DECREASE IN VARIOUS MODIFIER SOLUTIONS

Medium	$-[\theta]_{222} \times 10^{-3}$ *(deg cm² dmole⁻¹)*	*α-Helix (%)*	*Decrement of α-helix (%)*
Water	17.3	50	
0.05–0.5% PCA	14.8	42	14
50% of sat. $(NH_4)_2SO_4$	14.4	41	14
20% Acetonitrile	16.6	47	3.6
10 M Ethylene glycol	15.9	45	8.2
0.1% Triethylamine	15.5	43	12.2
5 M Urea	9.6	27	43

original conformation of BSA could not be recovered by removal of this hydrogen-bond breaking reagent. The reversible recovery of proteins in their native conformation is important in separation processes involving, for example, enzymes and globulin. Investigation of this problem is now in progress in our laboratory.

CONCLUSIONS

Proteins were shown to be adsorbed on gels having functional hydroxyl (HW-65), butyl (BT 650-M), and phenyl (Phenyl 5-PW) groups on the surface, not only in highly concentrated solutions of ammonium sulphate but also in dilute solutions of PCA, TCA, etc. The elution was carried out effectively with a mobile phase containing organic solvents, salts, or hydrogen-bond breaking reagents. The conformational changes in the BSA molecule produced by this treatment were slight, except in the case of urea.

REFERENCES

1 N. Sakihama, H. Ohmori, N. Sugimoto, Y. Yamazaki, R. Oshino and M. Shin, *J. Biochem.*, 93 (1983) 129.
2 M. Shin, N. Sakihama, R. Oshino and H. Sasaki, *Anal. Biochem.*, 138 (1984) 259.
3 A. V. Galkin, I. E. Kovaleva and S. L. Kalnov, *Anal. Biochem.*, 142 (1984) 252.
4 G. Tamai, H. Yoshida and H. Imai, *Bunseki Kagaku*, 35 (1986) 335.
5 G. Tamai, H. Imai and H. Yoshida, *Chromatographia*, in press.