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RAPID METHOD FOR REMOVAL OF DETERGENTS AND SALTS FROM PROTEIN SOLUTIONS USING TOYOPEARL HW-40F

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SUMMARY

A simple and rapid technique is described for the removal of cholate, deoxycholate and Triton X-100 from protein solutions. The procedure involves a 15-min centrifugation of the sample on a Toyopearl HW-40F bed prepared in a microcentrifuge tube. The merit of this method is that desalting or exchange of buffer ions is possible simultaneously with the removal of detergents from protein solutions. The method is suitable for multiple assays with a high recovery of proteins and without dilution of the sample.

INTRODUCTION

Detergents such as Triton X-100, deoxycholate and cholate are commonly used in biochemical studies on membrane solubilization and the separation and purification of membrane proteins. In certain instances there is a need for desalting or exchange of buffer ions in small volumes of samples in addition to the removal of detergents from the sample. For the desalting of small volumes of samples, a gel centrifugation method using a mini-column of Sephadex G-25[1] or Bio-Gel P-2[2] has been developed and used for removal of [^{125}I]iodide following iodination of protein solution[1] and in the study of the degradation of ^{125}I-labelled plasminogen[2].

When we attempted the simultaneous removal of salts and detergents from protein solutions by using Sephadex G-25, efficient removal of Triton X-100 with large micellar sizes from the sample was not achieved, in contrast to approximately 90% removal of cholate and deoxycholate. Therefore, preliminary experiments were performed with Toyopearl HW-40F, which is a rigid polyvinyl resin for gel filtration with a slightly larger fractionation range than Sephadex G-25. With this material, complete removal of cholate and deoxycholate is possible and, in addition, this resin has a capability of adsorb detergents so that Triton X-100 could be completely removed from the sample.

In this paper, we describe a gel centrifugation method using a Toyopearl HW-40F bed prepared in a microcentrifuge tube. This method is capable of removing detergents from protein solutions simultaneously with desalting or exchange of the sample buffer. The procedure is simple and rapid and suitable for multiple assays of

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small volumes of samples with a high recovery of proteins and without dilution of the samples. The results are discussed in comparison with the rapid method for the removal of detergents from protein solutions using Bio-Beads SM-2[3].

EXPERIMENTAL

Materials

Toyopearl HW-40F (30–60 μm), a rigid polyvinyl gel with a fractionation range of 100–10,000 daltons, was obtained from Toyo Soda (Tokyo, Japan) and Triton X-100 from Kishida Chemicals (Osaka, Japan). Sodium deoxycholate and cholic acid were purchased from Sigma (St. Louis, MO, U.S.A.) and recrystallized from ethanol, and bovine serum albumin, myoglobin, cytochrome *c* and chymotrypsinogen also from Sigma. Submicrosomal membrane proteins were prepared from rat liver as described in a previous paper[4]. L-[4,5-^{3}H]Leucine and D-[U-^{14}C]glucose were purchased from the Radiochemical Centre (Amersham, U.K.).

Preparation of Toyopearl bed

A 1.5-ml conical polypropylene microcentrifuge tube was processed as described previously[3] and the pinhole at the bottom was filled with a small amount of siliconized glass-wool [the glass-wool was siliconized by soaking for 5 min with 2% (v/v) dimethyldichlorosilane in carbon tetrachloride, rinsing with water and baking for 20 min at 130°C]. The tube was filled with 0.5–1.5 ml of a thick slurry of Toyopearl HW-40F equilibrated in 20 m*M* Tris–HCl buffer (pH 8.6) containing 0.2 *M* potassium chloride, and then inserted into a 105 × 15 mm Pyrex glass tube. The combination was centrifuged for 15 min at 480 *g* (at the surface of the Toyopearl bed) to release extra buffer into the glass tube. Subsequent centrifugations should release no additional buffer into the glass tube. For buffer exchange, Toyopearl HW-40F should be equilibrated with the buffer to be exchanged. For removal of detergents, buffers such as glycine, acetate, borate and HEPES can be used within the pH range 6.0–9.5.

Standard procedure for desalting and/or removal of detergents

A 0.3-ml sample containing salt and/or deoxycholate or cholate at concentrations less than 10% or Triton X-100 at less than 1.2% was placed on the 1-ml Toyopearl bed in the microcentrifuge tube prepared as described above. After standing for 5 min with the septum cap open, the tube was capped and placed in the weighed glass tube and the combination was centrifuged for 15 min at 480 *g*. The amount of the solution released into the glass tube was determined from the gain in weight of the glass tube. Toyopearl HW-40F can be regenerated by washing it with three volumes of isopropanol for at least 30 min at room temperature.

Analysis

The concentrations of deoxycholate, cholate and phosphate were determined by the methods of Wollenweber *et al.*[5], Kottke *et al.*[6] and Fiske and Subbarow[7], respectively. Chloride was determined with a standard silver solution using potassium chromate as indicator. Triton X-100 and sodium azide were determined from the absorbance at 275 and 240 nm, respectively. When protein was present, the sample

TABLE I

REMOVAL OF SALTS AND LOW-MOLECULAR-WEIGHT COMPOUNDS

Each sample was placed on a 1-ml Toyopearl bed and allowed to stand for 5 min at 4°C. After centrifugation for 15 min at 480 *g*, the concentration of salt or compound in each eluate was determined. Media used: (a) water; (b) 20 m*M* Tris–HCl (pH 8.6) containing 0.2 *M* KCl; (c) 0.2 *M* sodium phosphate buffer (pH 7.6); (d) 0.2% bovine serum albumin containing 1% deoxycholate; (e) 25 m*M* Tris–192 m*M* glycine buffer (pH 8.4).

Compound	*Medium*		*Sample volume (ml)*		*Concentration (M)*		*Compound removed (%)*
	Sample	*Column*	*Initial*	*Final*	*Initial*	*Final*	
NaN_3	a	b	0.30	0.31	3.0	0.003	99.9
	a	b	0.35	0.36	3.0	0.065	97.8
	a	b	0.40	0.42	3.0	0.147	94.9
KCl	a	c	0.20	0.19	1.0	ND*	>99.9
	a	c	0.25	0.24	1.0	0.003	99.7
	a	c	0.30	0.29	1.0	0.026	97.5
[^{14}C]Glucose	b	b	0.30	0.30	0.05	0.0022	96
[^{3}H]Leucine	b	b	0.30	0.30	0.05	0.0041	92
Glycine	b	b	0.30	0.31	0.10	0.0099	90
Glutamic acid	b	b	0.25	0.25	0.10	0.011	89
	b	b	0.30	0.30	0.10	0.023	77
Na_2HPO_4	d	e	0.20	0.22	0.05	0.012	74
	d	e	0.25	0.26	0.05	0.015	69
	d	e	0.30	0.32	0.05	0.018	62

* ND = not detectable.

was processed as described previously before the determination of detergents[3]. The amount of protein was determined by the method of Lowry *et al.*[8]. Glycine and glutamic acid were analysed with a Hitachi KLA-3B amino acid analyser. Radioactivity was measured with an Aloka LSC-900 liquid scintillation spectrometer.

RESULTS

First, types and sample volumes of salts and low-molecular-weight compounds that are removable from the sample by gel centrifugation using a minicolumn of Toyopearl HW-40F were examined. Each sample was placed on a 1-ml Toyopearl bed prepared as described under Experimental and allowed to stand for 5 min. After centrifugation, the concentration of salt, amino acid or glucose in each eluate was determined. The results are shown in Table I. When a 0.3-ml sample was placed on a 1-ml Toyopearl bed and processed according to the standard procedure, over 96% of sodium azide, potassium chloride and glucose were removed from the sample. Leucine and glycine were removed to the extent of 90% or above, but glutamic acid and phsophate, which have negative charges, were less effectively removed. Thus, it was concluded that except for compounds with highly negative charges such as phosphate or adenosine triphosphate (data not shown), amino acids, salts and small sugar molecules could be easily and efficiently removed from 0.3 ml or less of sample by a single centrifugation using a 1-ml Toyopearl bed.

TABLE II

REMOVAL OF DETERGENTS

Samples were treated as in Table I. Media used: (a) 20 m*M* Tris–HCl buffer (pH 8.6) containing 0.025 *M* KCl; (b) 20 m*M* Tris–HCl buffer (pH 8.6) containing 0.2 *M* KCl; (c) 20 m*M* Tris–HCl buffer (pH 8.6) containing 1 *M* KCl.

Detergent	*Medium*		*Sample volume (ml)*		*Concentration (%)*		*Detergent removed (%)*
	Sample	*Column*	*Initial*	*Final*	*Initial*	*Final*	
Cholate	a	b	0.30	0.27	8.0	ND**	>99.9
	a	b	0.30	0.26	10	ND	>99.9
	c	b	1.0*	1.08	2.0	0.003	99.8
	c	b	1.2*	1.31	2.0	0.179	90.2
Deoxycholate	a	b	0.30	0.26	8.0	0.006	99.9
	a	b	0.30	0.26	10	0.01	99.9
	a	b	0.70	0.65	2.0	ND	>99.9
	a	b	0.80*	0.82	2.0	0.048	97.5
Triton X-100	b	b	0.35	0.37	1.0	0.009	99.1
	b	b	0.30	0.33	1.5	0.054	96.0
	b	b	0.40	0.40	1.0	0.047	95.3

* Experiments were performed using a 0.5-ml Toyopearl bed and the values obtained were doubled.
** ND = not detectable.

Removal of detergents was then examined. The sample volume applicable to the Toyopearl bed was examined by using various volumes of a sample containing 2% of cholate, 2% of deoxycholate or 1% of Triton X-100. As shown in Table II, over 99.9% of cholate and deoxycholate were removed from a sample of up to 0.7 ml on a 1-ml Toyopearl bed. For cholate, even the application of a 0.5-ml sample to a 0.5-ml Toyopearl bed resulted in 99.8% removal of cholate from the sample. Over 99% of Triton X-100 was removed from a sample of up to 0.35 ml on a 1-ml Toyopearl bed. These results suggest that highly efficient removal of detergents from a sample of above 0.3 ml on a 1-ml Toyopearl bed depends on the adsorption of detergents on the Toyopearl resin, as the removal of salts by gel filtration from a sample of above 0.3 ml was incomplete on a 1-ml Toyopearl bed. It should be noted that the removal of detergents showed a slightly higher efficiency at room temperature than at 4°C.

Next, experiments were carried out to determine the initial concentration of detergents removable from a 0.3-ml sample by a single Toyopearl centrifugation (Table II). The results revealed that over 99% of cholate or deoxycholate could be removed from a 0.3-ml sample placed on a 1-ml Toyopearl bed even when the detergent concentration was increased to 10%, and over 99% removal of Triton X-100 from a 0.3-ml sample was obtained at concentrations up to 1.2%. Removal of detergents from samples containing protein is shown in Table III, which indicate that the removal of detergents is not suppressed by the presence of proteins in the sample.

Fig. 1 shows the recovery of protein after Toyopearl centrifugation in the presence of detergent. The indicated amounts of bovine serum albumin, myoglobin, cytochrome c and submicrosomal proteins, prepared from rat liver microsomes as

TABLE III

REMOVAL OF DETERGENTS FROM PROTEIN SOLUTIONS

Each 0.3-ml sample containing 1.5 mg of protein per ml of 20 m*M* Tris–HCl (pH 8.6), detergent at the concentration indicated and 1 *M* (for cholate), 25 m*M* (for deoxycholate) or 0.2 *M* (for Triton X-100) KCl was placed on a 1-ml Toyopearl bed and allowed to stand for 5 min at 4°C. After centrifugation for 15 min at 480 *g*, the concentration of the detergent in each eluate was determined.

Detergent	*Protein*	*Recovered volume (ml)*	*Detergent concentration (%)*		*Detergent removed (%)*
			Initial	*Final*	
Cholate	BSA*	0.30	2.0	0.002	99.9
	R_1**	0.28	2.0	0.002	99.9
Deoxycholate	BSA	0.28	2.0	0.0004	>99.9
	R_1	0.27	2.0	0.001	>99.9
Triton X-100	BSA	0.29	1.0	0.004	99.6
	R_1	0.29	1.0	0.008	99.2

* Bovine serum albumin.

** Hydrophobic proteins prepared from rat liver microsomes as described under Experimental.

described under Experimental, were subjected to the Toyopearl treatment in the presence of cholate. The results showed that approximately 95% of bovine serum albumin was recovered in the eluate from the samples containing the protein at concentrations greater than 0.2 mg per 0.3 ml, whereas the recovery was slightly reduced for samples

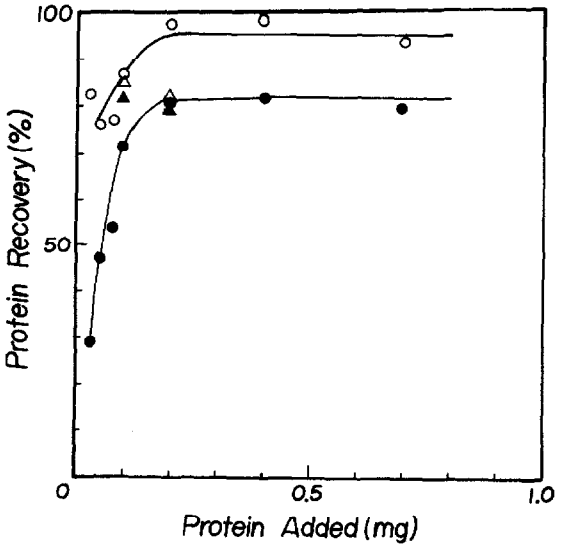

Fig. 1. Recovery of proteins after removal of detergents by the Toyopearl centrifugation. The indicated amounts of bovine serum albumin (○), myoglobin (△), cytochrome c (▲) and submicrosomal proteins (●) prepared as described under Experimental, dissolved in 0.3 ml of 20 m*M* Tris–HCl (pH 8.6), 0.2 *M* KCl and 2% cholate, were placed on a 1-ml Toyopearl bed and allowed to stand for 5 min at 4°C. After centrifugation for 15 min at 480 *g*, the amount of protein in each eluate was determined.

TABLE IV

RECOVERY OF PROTEIN UNDER LOW IONIC STRENGTH CONDITIONS

Each 0.3-ml sample containing 0.2 mg of protein in 20 m*M* Tris–HCl (pH 8.6) and 2% of cholate was placed on a 1-ml Toyopearl bed pre-equilibrated in 20 m*M* Tris–HCl (pH 8.6) and allowed to stand for 5 min at 4°C. After centrifugation for 15 min at 480 *g*, the amount of protein was determined.

Protein	*pI*	*Recovery of protein (%)*
Cytochrome *c*	10.6	0
Chymotrypsinogen	9.5	10
Myoglobin	7.0	92
Bovine serum albumin	5.0	93
R_1*		86

* Hydrophobic proteins prepared from rat liver microsomes as described under Experimental.

containing less than 0.1 mg per 0.3 ml of the protein. Myoglobin and cytochrome c showed approximately 80% recoveries at a concentration of 0.1 mg protein per 0.3 ml. Hydrophobic submicrosomal proteins also showed similar recoveries to the above at concentrations up to 0.2 mg per 0.3 ml, but showed a steep decline in recovery with decreasing protein concentration.

The Toyopearl bed used in the above experiments was prepared by using Toyopearl HW-40F pre-equilibrated in a buffer containing 0.2 *M* potassium chloride as described under Experimental. In the following experiment, the effects of lowering the ionic strength of the Toyopearl bed on the efficiency of detergent removal and the recovery of protein were examined. Each 0.3-ml sample containing 2% of cholate, 2% of deoxycholate or 1% of Triton X-100 in 20 m*M* Tris–HCl (pH 8.6) was placed on a 1-ml Toyopearl bed pre-equilibrated in 20 m*M* Tris–HCl (pH 8.6) and allowed to stand for 5 min at 4°C. After centrifugation for 15 min at 480 *g*, the concentration

TABLE V

SIMULTANEOUS REMOVAL OF DETERGENT AND SALT FROM PROTEIN SOLUTION

Experiment A: the indicated volumes of 0.20% bovine serum albumin dissolved in 200 m*M* sodium phosphate buffer (pH 7.6), 1 *M* KCl and 2% cholate were placed on a 1-ml Toyopearl bed pre-equilibrated in 200 m*M* sodium phosphate buffer (pH 7.6) and allowed to stand for 5 min at 4°C. After centrifugation for 15 min at 480 *g*, the amounts of protein, detergent and salt in each eluate were determined. Experiment B: the indicated volumes of 0.20% bovine serum albumin dissolved in 25 m*M* Tris–192 m*M* glycine buffer (pH 8.4) and 1% deoxycholate were treated as in Experiment A.

Experiment	*Sample volume (ml)*		*Protein recovered (%)*	*Cholate removed (%)*	*Deoxycholate removed (%)*	*KCl removed (%)*	*Glycine removed (%)*	*Phosphate recovered (mM)*
	Initial	*Final*						
A	0.20	0.20	90	>99.9		99.9		
	0.25	0.26	97	>99.9		>99.9		
	0.30	0.31	90	>99.9		>99.9		
B	0.20	0.18	93		>99.9		98	223
	0.25	0.21	86		>99.9		98	216
	0.30	0.26	94		>99.9		93	194

of detergent in each eluate was determined. The results showed that all three detergents were removed to the extent of 99.5% from the samples in the low ionic strength conditions. However, a different situation was found with respect to the recovery of proteins, as shown in Table IV. Cytochrome c and chymotrypsinogen, both of which have p*I* values higher than the pH of the buffer used, showed very low recoveries, whereas the other three proteins had over 86% recoveries.

Table V shows an example of the simultaneous removal of salt and detergent from protein solution. Experiment A demonstrates that simultaneous removal of potassium chloride and cholate from a protein solution is possible with the same efficiency as for the removal of potassium chloride or cholate from a solution containing only one of these compounds (Tables I and II). Experiment B indicates that the method is capable of exchanging buffer simultaneously with removal of detergent.

DISCUSSION

A rapid technique has been developed for the removal of cholate, deoxycholate and Triton X-100 from protein solution. In a previous paper[3], we reported a similar rapid method using Bio-Beads SM-2, suitable for removal of the three kinds of detergents from protein solutions. The advantage of the present method using Toyopearl HW-40F is the ability to desalt or to exchange buffer ions of the sample by the molecular sieve effect simultaneously with the removal of detergents from the sample. Moreover, the recovery of protein was higher, especially at low protein concentrations, than the method using Bio-Beads SM-2. According to preliminary experiments, Toyopearl HW-40F has the capability of adsorbing all of the detergents used here. Deoxycholate and Triton X-100 were more strongly adsorbed than cholate to Toyopearl HW-40F. The capacity of Toyopearl HW-40F to adsorb cholate and deoxycholate was much higher than that for Triton X-100. The reason for this difference in adsorption capacity is probably that the micellar species of cholate and deoxycholate are able to enter the gel matrix of Toyopearl HW-40F, whereas those of Triton X-100 are not.

It is known[9] that in gel chromatography using Toyopearl HW-55F, a protein having a p*I* value higher than the pH of the elution buffer is retarded compared with the elution volume expected on a molecular weight basis. This retarding effect was much improved by using an elution buffer containing 0.3 *M* sodium chloride[9]. These observations are consistent with the large decrease in the recovery of cytochrome c and chymotrypsinogen seen in the present study (Table IV), which was improved by using a Toyopearl bed pre-equilibrated in a buffer containing 0.2 *M* potassium chloride (Fig. 1). When a protein solution containing detergents is subjected to the Toyopearl centrifugation under low ionic strength conditions, the buffer used should have a pH value higher than the p*I* values of the protein species to be recovered from the sample.

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