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Concentrating protein samples for sodium dodecyl sulphate–polyacrylamide gel electrophoresis and isoelectric focusing using protein-blotting membranes

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Abstract

The protein concentration in biological samples is a crucial limiting factor for a successful analysis by electrophoresis. Many techniques have been adopted to increase protein concentrations, however, they are often insufficient and require special equipment or poisonous chemicals. Herein, we report a simple and efficient technique for concentrating dilute protein samples by absorbing proteins onto protein-blotting membrane strips. In this technique, blotting membrane strips were incubated in dilute protein solutions to capture proteins. For either sodium dodecyl sulphate–polyacrylamide gel electrophoresis or isoelectric focusing, the protein-absorbed membrane strips were directly loaded to the sample wells which contained a strong protein elution buffer and electrophoresis was performed under standard conditions.

Keywords: Membranes; Sample preparation; Proteins

1. Introduction

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS–PAGE) and isoelectric focusing (IEF) are two of the most important techniques for analyzing and preparing proteins [1,2]. However, many biological samples are too dilute and have too high a salt content to be analyzed by these techniques. Many techniques have been developed to analyze such dilute samples, including more sensitive protein staining procedures, for instance, silver staining [3–5]. However, these techniques often use poisonous chemicals and are time-consuming. For most protein analyses, such as immunoblotting, protein sequencing, peptide mapping and amino acid analyzing, a certain concentration of protein is required.

A number of methods have been developed for concentrating protein samples for electrophoretic analysis. These include lysophilization, ammonium sulphate precipitation and dialysis against a high concentration of poly(ethylene glycol) (PEG). Nevertheless, when concentrated by any of these methods, the protein samples must be dialyzed to remove salts or low-molecular-mass PEG impurities which may interfere with electrophoresis. Each of these manipulation steps will result in the loss of proteins, especially when only a small amount of

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protein is available. Some researchers concentrated their samples by precipitation with trichloroacetic acid [6,7], acetone [8], or a chloroform–methanol–water system [9], all of which utilize toxic chemicals. Moreover, when the protein concentration is very low, the sample recovery is very difficult.

Reversed-phase HPLC has also been applied to concentrate and desalt peptide and protein samples [10–14]. This technique can generally give satisfactory results. However, HPLC set-up is expensive and may not be available to some laboratories. Furthermore, due to introduction of organic solvents into elution solutions, the HPLC-concentrated samples are not applicable to electrophoresis unless they are further processed, which complicates the procedure and reduces protein recoveries.

Protein-blotting membranes, such as polyvinylidene diflouride (PVDF) membrane, are widely used in research and diagnostic laboratories. They have high protein-binding capacity, and bound proteins can be reversibly eluted in strong detergent buffers. Based on these two properties, we have developed a protocol for concentrating protein samples for SDS–PAGE and IEF. It is a simple and efficient way to concentrate protein samples. It also can be used to concentrate protein samples with high salt contents.

2. Experimental

2.1. Chemicals

Bovine serum albumin (BSA), lysozyme and soybean trypsin inhibitor were obtained from Sigma (St. Louis, MO, USA); and goat IgG from Pierce (Rockford, IL, USA). PolyScreen PVDF Transfer Membrane was purchased from NEF Research Products (Boston, MA, USA). Ampholine was obtained from Bio-Rad (Hercules, CA, USA) and Bio-Lyte was from Pharmacia LKB Biotechnology (Uppsala, Sweden).

2.2. Processing membranes and absorbing proteins onto membrane strips

The PVDF membrane was cut into 2.0×0.8 cm strips. The strips were wetted in 100% methanol followed by a single rinse in doubly distilled water. The wet strips were put in protein solutions and rocked at 4°C to capture proteins for 6 to 48 h.

2.3. Gel preparation

All gels for both SDS–PAGE and IEF were cast and run with a Vertical Slab Gel Unit, Model SE-400 (Hoefer Scientific Instruments, San Francisco, CA, USA). The SDS–PAGE (10% polyacrylamide with 10 sample wells) was performed using a discontinuous buffer system [15]. The IEF gel was prepared as per the following procedure: a 20-ml solution consisting of 9.6 g urea (final concentration: 48%), 8.0 ml doubly distilled water, 3.2 ml of 28% acrylamide and 2% bisacrylamide, 0.5 ml Triton X-100, 0.6 ml Ampholine of pH range 3.5–5.0 and 0.4 ml Bio-Lyte of pH range 6.0–8.0 was made and degassed. Polymerization was initiated by the addition of 0.2 ml of 10% ammonium persulfide and 15 μl N,N,N′,N′-tetramethylethylenediamine (TEMED). The polymerizing solution was then poured into a glass plate sandwich. A 10-well comb was used. The filled sandwich was immersed in distilled water to allow a better polymerization.

2.4. Loading protein-absorbed membrane strips and sample well buffers

The protein-absorbed membrane strips were loaded into sample wells prefilled with a sample well buffer. The nonreducing sample well buffer for SDS–PAGE contained 125 m*M* Tris, 2% SDS and 48% urea, pH 6.8. The reducing sample well buffer consisted of the nonreducing well buffer and 2% 2-mercaptoethanol. The sample well buffer for IEF composed of 48% urea, 2.5% Triton X-100 and 5% Bio-Lyte of pH range 6.0–8.0. Three drops of 0.5% bromophenol blue was added to the reservoir buffer in the upper chamber as a tracking dye of SDS–PAGE after the reservoir buffer was applied.

2.5. Running conditions

The SDS–PAGE gel was run at 10 mA for the first 5 min, then at 2.0 mA until the tracking dye entered the stacking gel, and finally at 4.0 mA to finish

electrophoresis at room temperature. The electrode solutions for IEF were 0.1 M H_3PO_4 for the anode and 0.1 M NaOH for the cathode. The IEF gel was run at 2.0 W for 12 000 V h at room temperature.

2.6. Gel staining

After electrophoresis, the SDS–PAGE gel was stained with Coomassie blue [16]; the IEF gel was fixed in 20% trichloroacetic acid for 30 min, then washed in a destaining solution (50% methanol, 10% acetic acid) for 30 min, and stained with Coomassie blue.

2.7. Densitometric analysis and enrichment estimation

IS-1000 Digital Imaging System (Alpha Innotech, San Leandro, CA, USA) was used to analyze the amount of proteins on gels upon Coomassie blue staining. To all concentration standard lanes, proteins treated in a SDS–PAGE sample buffer were loaded. The sample buffer composed of 125 mM Tris, 4% SDS, 20% glycerol and 3% 2-mercaptoethanol (v/v), adjusted pH 6.8. The calibration curve was generated from band density values of the concentration standard lanes. The amount of protein captured by a PVDF membrane strip was calculated based on its band density value and the respective calibration curve. The enrichment was estimated according to the following formula: Enrichment=(μg protein captured by PVDF membrane)/(μg protein contained in 15 μl protein solution), since the loading capacity of one well was about 30 μl and an original protein solution required a treatment with the SDS–PAGE sample buffer by 1:1.

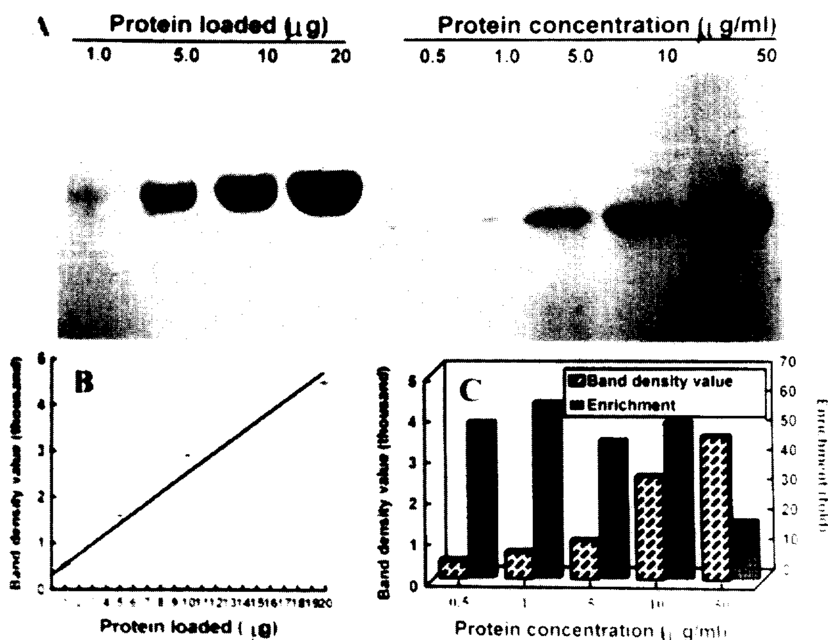

Fig. 1. Effect of protein concentrations on the enrichment. (A) Coomassie blue stained SDS–PAGE gel. The first four lanes (left) contained the SDS–PAGE sample buffer-treated BSA concentration standard. The rest of the lanes contained protein-absorbed PVDF membrane strips. BSA was diluted in 50 mM Tris–HCl buffer (pH, 7.5) at concentrations from 0.5 to 50 μg/ml. PVDF strips were incubated in the BSA solutions (each containing 50 μg BSA) for 24 h. The protein-absorbed strips were transferred into sample wells which were prefilled with the nonreducing SDS–PAGE sample well buffer, and electrophoresis was conducted. The gel was stained with Coomassie blue. (B) Densitometric analysis of the four standard lanes and resultant calibration curve. (C) Densitometric analysis of the membrane-captured BSA and estimated enrichment.

3. Results

3.1. Effect of protein concentrations on the enrichment

Blotting membranes are widely used as a carrier matrix for the analysis of proteins and nucleic acids. We employed PVDF membranes to capture proteins from dilute protein samples and found them to be effective. We used BSA at different concentrations as our model system. As shown in Fig. 1A, the captured BSA from dilute samples produced sharp bands and low backgrounds just as those resulted from SDS–PAGE sample buffer-treated BSA. Since the disulfide bonds of BSA were reduced by 2-mercaptoethanol contained in the SDS–PAGE sample buffer, BSA in the concentration standard lanes migrated slightly slower than those absorbed by PVDF membranes. All the treatments enriched the protein concentration about 45-fold except 50 μg/ml treatment, whose enrichment was about 20-fold (Fig. 1C).

3.2. Effect of incubation times on the enrichment

We tried to improve the enrichment by increasing incubation times. We found that in the first 24 h, the enrichment increased as the incubation proceeded, and than became steady. A 24-h incubation was sufficient for the 2 μg/ml treatment and necessary for the 20 μg/ml treatment to achieve the maximum enrichment (Figs. 2 and 3).

3.3. Effect of salt concentrations

For protein purification, salt concentrates are often utilized especially when ion-exchange chromatography is employed. As a high salt concentration is not permitted in both SDS–PAGE and IEF samples, it is desirable to eliminate salts in the sample. Though desalting procedures exist, they are often cumbersome. Salts can increase hydrophobic interactions. As expected, the present technique tolerated

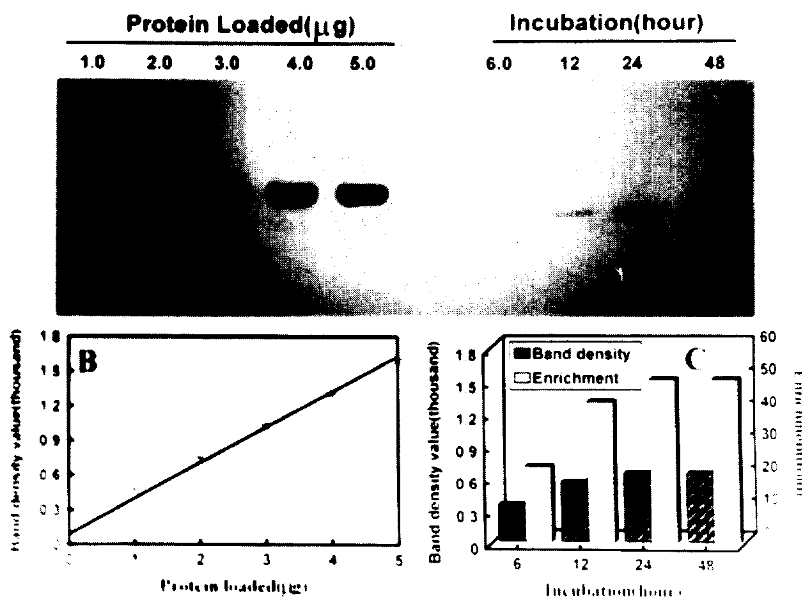

Fig. 2. Effect of incubation times on the enrichment of 2 μg/ml BSA treatment. (A) Coomassie blue stained SDS–PAGE gel. Five concentration standard lanes (left) were loaded with 1, 2, 3, 4 or 5 μg BSA in the SDS–PAGE sample buffer. Protein-absorbed PVDF membrane strips were applied into the four lanes on the right. Membrane strips were incubated in 200 ml of 2 μg/ml BSA solution for 6, 12, 24 and 48 h before placement into the sample wells which were prefilled with the nonreducing SDS–PAGE sample well buffer. (B) Densitometric analysis of the five standard lanes and resultant calibration curve. (C) Densitometric analysis of the membrane-captured BSA at different incubation times and estimated enrichment.

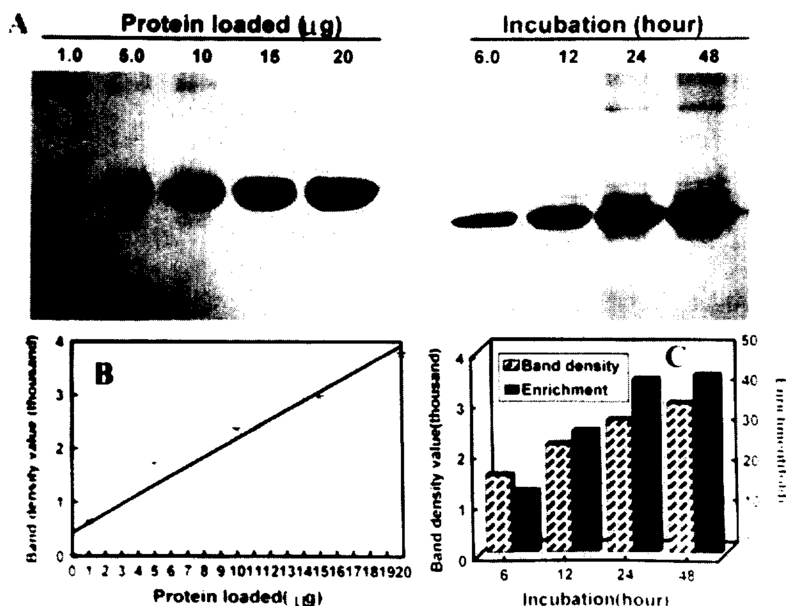

Fig. 3. Effect of incubation times on the enrichment of 20 μg/ml BSA treatment. (A) Coomassie blue stained SDS–PAGE gel. Five concentration standard lanes (left) were loaded with 1, 5, 10, 15 or 20 μg BSA in the SDS–PAGE sample buffer. Protein-absorbed PVDF membrane strips were placed into the four lanes on the right. The membrane strips were incubated in 20 ml of 20 μg/ml BSA solution for 6, 12, 24 and 48 h before being placed into the sample wells which were prefilled with the nonreducing SDS–PAGE sample well buffer. (B) Densitometric analysis of the five standard lanes and resultant calibration curve. (C) Densitometric analysis of the membrane-captured BSA and estimated enrichment.

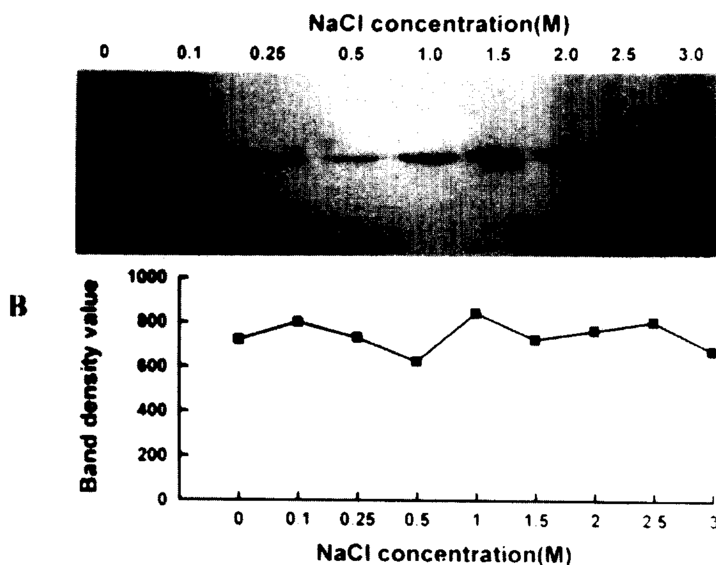

Fig. 4. Effect of salt concentrations on PVDF efficiency to capture protein. (A) Coomassie blue stained SDS–PAGE gel. BSA (5 μg/ml) was prepared in solutions (50 m*M* Tris–HCl buffer, pH 7.5) containing different NaCl concentrations (0 to 3.0 *M*). PVDF strips were incubated in the BSA–NaCl solutions for 24 h. The protein-absorbed strips were rinsed once in doubly distilled water, and placed in sample wells which were prefilled with the nonreducing SDS–PAGE sample well buffer. After electrophoresis the gel was stained with Coomassie blue. (B) Densitometric analysis of captured BSA by the membrane at different salt concentrations.

high salt concentrations (Fig. 4). Up to 3 *M* NaCl did not reduce protein quantities captured by the membranes.

3.4. Breakdown of disulfide bonds

For most purposes, SDS–PAGE analyses are conducted under reducing conditions. We tried to add 2-mercaptoethanol to dilute protein solutions to reduce disulfide bonds of proteins before they absorbed to the membrane strips. However, the reducing agent interfered with protein binding to the membrane (data not shown). We supplemented 2-mercaptoethanol to the sample well buffer. The result was satisfactory (Fig. 5). BSA absorbed by the membrane migrated at the same speed with that treated in the SDS–PAGE sample buffer when the sample well buffer contained 2-mercaptoethanol.

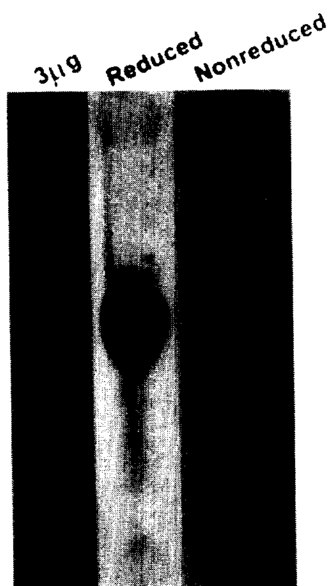

Fig. 5. Breakdown of protein disulfide bonds of PVDF membrane-absorbed protein. The SDS–PAGE gel was stained with Coomassie blue. The first lane (left) contained 3 µg BSA treated in the SDS–PAGE sample buffer. The middle and right lanes were protein-absorbed PVDF strips, which were incubated in 10 ml of 20 µg/ml BSA for 24 h. The sample well of the middle lane was prefilled with the reducing SDS–PAGE sample well buffer; and the right lane, with the nonreducing SDS–PAGE sample well buffer.

3.5. Concentrating protein samples for IEF

We have also examined the possible application of this technique to capture proteins for IEF. The focused bands shown in Fig. 6 demonstrate that the technique is applicable for IEF sample concentration.

3.6. Concentrating protein mixtures

Since the PVDF membrane can efficiently bind almost all known proteins, it is widely used to protein blotting even without consideration of the properties of the target proteins. As shown in Fig. 7, the technique efficiently concentrated different proteins, although the membrane showed different binding capacities for them. In the BSA and IgG mixture, approximately an equal amount of the two proteins (each about 10 µg) was captured, suggesting that these proteins have similar ability to win binding sites on the membrane. More soybean trypsin inhibitor was bound to the membrane than BSA, but the latter was greatly enriched. The amounts of both lysozyme and BSA captured from the mixture were similar to those bound in their individual solutions.

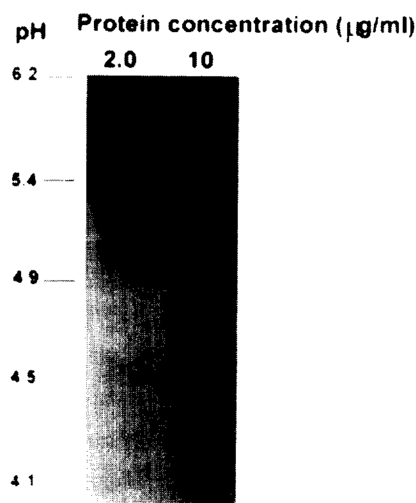

Fig. 6. Concentrating protein samples with the PVDF membrane for IEF. BSA was diluted in 50 m*M* Tris–HCl buffer (pH, 7.5) at 2.0 or 10 µg/ml. PVDF strips were incubated in the BSA solutions for 24 h. The protein-absorbed membrane strips were transferred into the sample wells of an IEF gel, which were prefilled with the IEF sample well buffer. After electric focusing, the gel was fixed, washed and then stained with Coomassie blue.

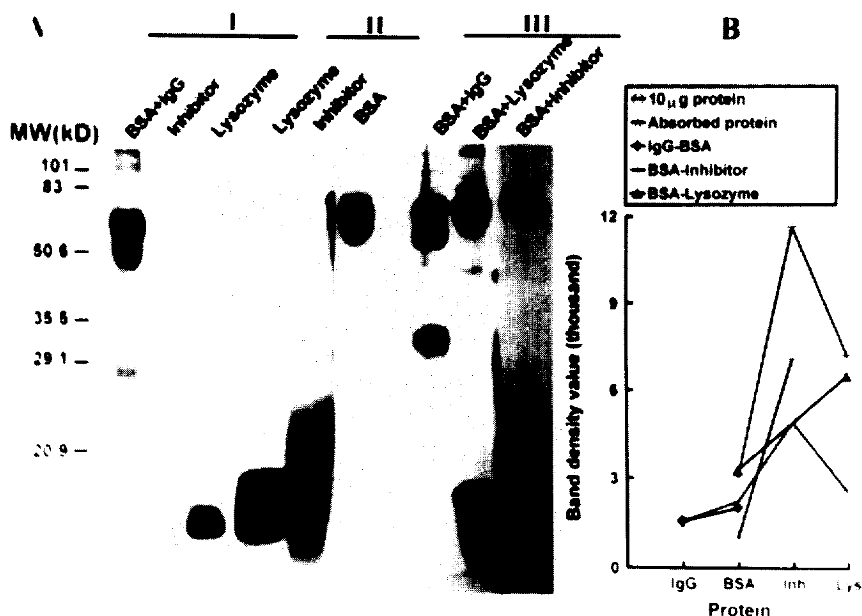

Fig. 7. Concentrating protein mixtures with the PVDF membrane. (A) Coomassie blue stained SDS–PAGE gel. (A-I) Protein concentration standards in the SDS–PAGE sample buffer were loaded. BSA+IgG lane, 20 μg protein (BSA and IgG each 10 μg) was loaded; inhibitor lane, 10 μg soybean trypsin inhibitor; lysozyme lane, 10 μg lysozyme. (A-II) Protein-absorbed membrane strips were applied. Membrane strips were incubated in 5.0 ml of 20 μg/ml lysozyme, soybean trypsin inhibitor or BSA for 24 h. (A-III) The three lanes were run for protein-absorbed membrane strips. Membrane strips were incubated in 5.0 ml of protein mixture of BSA and IgG, BSA and lysozyme, or BSA and soybean trypsin inhibitor (all the three mixtures contained 40 μg/ml protein; each protein 20 μg/ml). The reducing SDS–PAGE sample well buffer was placed in all (A-II) and (A-III) sample wells. (B): Densitometric analysis. The band density values of IgG were represented by IgG heavy chain bands. Soybean trypsin inhibitor and lysozyme were abbreviated Inh and Lys, respectively. The 10-μg protein curve was drawn from (A-I); and the absorbed protein curve, from (A-II). IgG–BSA, BSA–inhibitor and BSA–lysozyme curves were generated from the band density values of BSA+IgG lane, BSA+inhibitor lane and BSA+lysozyme lane of (A-III), respectively.

4. Discussion

Protein electrophoresis is widely employed in biomedical research. One of the major obstacles is that the concentrations of proteins in samples are too low to be detected. In this paper, we have described a simple, efficient technique to concentrate protein samples for SDS–PAGE and IEF utilizing blotting membranes. The adaptation of blotting membranes is a straightforward procedure and does not require any pretreatment of the sample.

This method relies on the successful elution of proteins from the blotting membrane. Both buffers described in this paper contain a strong protein detergent (SDS or Triton X-100) and urea which are widely used to dissolve and elute proteins. These buffers could efficiently remove proteins from the blotting membrane and resulted in sharp protein bands and low backgrounds on both SDS–PAGE and IEF gels. These chemicals must be removed from a protein sample before this technique is applied. When BSA was dissolved in a lysis buffer (50 m*M* Tris, 1% Triton X-100 and 0.1% SDS), protein bound to the blotting membrane was greatly reduced (data not shown). This technique is not satisfactory for concentration of proteins from solutions containing these agents.

The protein quantity captured by the membrane crucially depended on protein concentrations (Fig. 1). The enrichment achieved by this technique was about 45-fold for a protein concentration below 20 μg/ml (Figs. 1–3). For a protein concentration above 50 μg/ml, although more protein was captured, the enrichment decreased to 20-fold. The enrichment by elongating an incubation time was limited (Figs. 2 and 3). However, for the analysis of

an extremely low protein sample (below 1 μg/ml), the sample could be concentrated by lysophilization before the membrane is applied. Though lysophilization is a high recovery protocol for concentrating protein samples, it increases salt concentrations. Fortunately, salts did not prevent the membrane from capturing proteins as shown in Fig. 4.

When a reducing agent was added to the sample well buffer, it successfully disrupted disulfide bonds of proteins (Figs. 5 and 7). This expands the application of this technique.

Although different proteins in the solutions showed different binding abilities to the blotting membrane, they were greatly enriched by this technique (Fig. 7). Different proteins in a solution showed competitive or noncompetitive binding to the membrane. Two proteins in the same solution were bound equally, such as IgG and BSA; or one was selectively bound in greater amounts, such as soybean trypsin inhibitor and BSA. They showed competitive binding. Because binding sites on the blotting membrane are limited, the proteins in a solution are believed to compete for the binding sites. However, the different proteins in the same mixture also expressed noncompetitive binding, such as lysozyme and BSA. These two proteins did not interfere with each other's binding just as in each of the individual solutions. We expected the competitive binding between proteins but we do not know why noncompetitive binding occurred.

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