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A Rapid Method for the Purification of Analytical Grade Proteins from Plants Using Preparative SDS-PAGE and Preparative Isoelectric Focusing

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Introduction

BiPs (Immunoglobulin Heavy Chain Binding Proteins, also known as GRP78; Glucose Regulated Proteins) are ER-luminal proteins and members of the HSP70 family.[1] BiPs are associated with the transport and folding process of proteins involved in the secretory pathway. BiPs and many other HSP70 proteins can easily be partially purified by affinity chromatography with ATP-agarose. However, due to BiPs ability to bind proteins, we have found it associated with other contaminating proteins over a broad range of salt gradients when using ion-exchange chromatography, and over a broad molecular weight range when using size-exclusion chromatography. Here we report a rapid method for the further purification of affinity selected BiP suitable for amino acid sequencing and antibody production.

Methods

Sample Preparation

Native extracts from 3 week old spinach leaf tissue were taken through 40 and 80% ammonium sulfate (AS) precipitation. The resuspended 80% ammonium sulfate pellet was refractionated by an additional 1 M and 3 M ammonium sulfate precipitation. The resulting 3 M pellet was resuspended in 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 0.1 mM DTT (Buffer A) and then dialyzed overnight against two changes of buffer A plus 3 mM $MgCl_2$ (Buffer B). The dialyzed sample was centrifuged at 20,000 x g to remove insoluble material. The clarified sample was loaded onto an ATP-agarose affinity column (1.5 x 10 cm bed volume) equilibrated with buffer B at a flow rate of 0.5 ml/min. Sample loading, UV-detection, and fraction collection were all done using Bio-Rad's Econo System low pressure chromatography workstation. The affinity column was washed with 2 bed volumes of buffer B, followed by 10 ml of 1 mM GTP in buffer B. The column was developed with 10 ml of 3 mM ATP at a flow rate of 1 ml/min. Fractions eluted with 3 mM ATP were pooled and then concentrated using an Amicon Ultrafiltration Cell with a PM30 membrane (30,000 MW cutoff).

Preparative SDS-PAGE

Preparative SDS-PAGE was run according to the discontinuous buffer system of Laemmli (1970).[2] A sample was prepared by mixing 3.75 mg of concentrated affinity purified protein with 2x SDS sample buffer at a 1:1 ratio (v/v) and heating at 95 ˚C for 2 minutes. A total sample volume of 2.5 ml (1.5 mg protein/ml) was applied to a 7.5% polyacrylamide separating gel poured to 5.5 cm height and a 1 cm high stacking gel consisting of 4% polyacrylamide. The separating and stacking gels were polymerized in a 37 mm diameter Model 491 Prep Cell casting tube assembly.

Running Conditions

The upper and lower electrophoresis chambers and the elution reservoir of the Model 491 Prep Cell were filled with a 1:10 dilution of Bio-Rad's 10x Tris/Glycine/SDS buffer. The lower chamber buffer circulated through the cooling core of the Model 491 Prep Cell at 100 ml/min at room temperature. The Model 491 Prep Cell was run at a constant current of 40 mA for the first 3 hours and at 60 mA constant current for the remainder of the electrophoretic separation. The dye front eluted from the gel at approximately 5 hours of run time. Starting at the dye front, fractions of 2.5 ml were collected at an elution rate of 0.5 ml/min using Bio-Rad's Econo System low pressure chromatography workstation. Fractions were collected for 5 hours to give a total Model 491 Prep Cell electrophoresis run of 10 hours.

Sample Analysis

Fractions collected from the Model 491 Prep Cell were analyzed by SDS-PAGE using the Mini-Protean® II system. Starting with fraction 4, 60 µl of every other fraction was mixed with 40 µl of sample buffer (125 mM Tris-HCl (6.8), 30% glycerol, 4% β-mercaptoethanol, 4% SDS, and 0.001% bromophenol blue). After being electrophoresed on 7.5% polyacrylamide gels, separated proteins were electrophoretically blotted to PVDF membranes (5.5 x 8.5 cm) at a constant current of 2.0 mA/ cm^2 of gel surface for 30 minutes. Blots were either stained with 0.05% Coomassie Blue or probed with antibody developed to tobacco BiP fusion protein (TOBBLP4, gift from Dr. J. Denecke).[3]

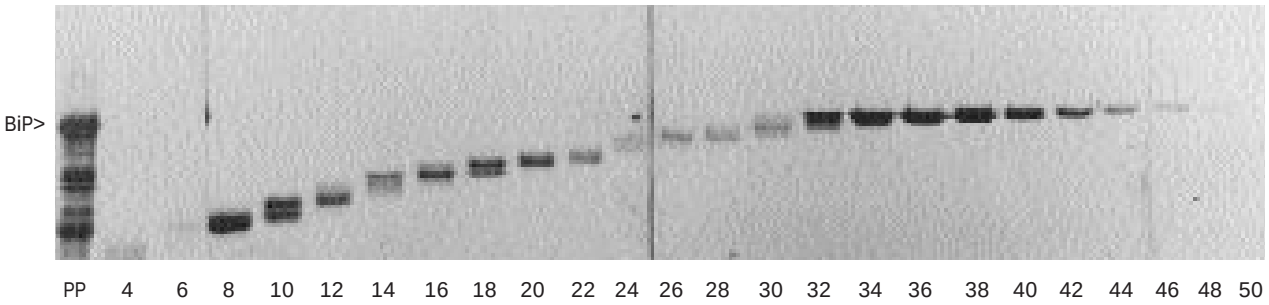

Fig. 1. Coomassie-stained Western blot of SDS-PAGE gel with fractions eluted from the Model 491 Prep Cell. The 79 kd BiP is marked. PP, 20 µg of sample eluted from ATP-agarose affinity column with 3 mM ATP. Each lane represents a 12.5 µl load volume of fraction indicated.

Preparative Isoelectric Focusing

Fractions 35, 36, and 37 from the Model 491 Prep Cell run were pooled and precipitated with 80% AS. The resulting 80% AS pellet was resuspended in 10 ml of dd H_2O and dialyzed overnight against the same. The dialyzed sample was then mixed with 40 ml of Rotofor® buffer (1% Bio-Lyte® 5/7 ampholyte, 1% urea, and 5% glycerol). The sample was focused on the Rotofor cell at a constant power of 10 W for 4 hours at 4 ˚C. Initial voltage was 1,250 and reached the 2,000 volts limit after 1 hour run time. Twenty fractions were collected from the Rotofor cell and each was lyophilized to a final volume of 500 µl. Lyophilized samples were analyzed by SDS-PAGE as previously described.

Results

The data in Figure 1 demonstrate the high degree of purification that can be accomplished in a single run on the Model 491 Prep Cell. Fraction 36 of the Model 491 Prep Cell run shows sufficient purity and quantity (approximately 100 µg total protein) suitable for direct amino acid sequence analysis. The data in Figure 2 demonstrate the advantage of using the Model 491 Prep Cell, compared to conventional chromatography techniques, as a quick means of obtaining protein suitable for sequence analysis. Those proteins (Figure 2) which were purified under native conditions, by conventional chromatography steps using an FPLC® system, required additional lab time and cost but resulted in lower protein yields due to the extra steps.

Trying to further resolve fractions 35–37 from the Model 491 Prep Cell run using preparative isoelectric focusing produced what appears to be a highly pure form of BiP in fraction 1 (Figure 2). However, this fraction had a pH of 3.05 which is approximately 2.2 pH units lower than that of BiP analysed on 2-D gels. We attribute this phenomenon to the probability that BiP still had SDS attached to it, thus causing it to migrate towards the cathode.

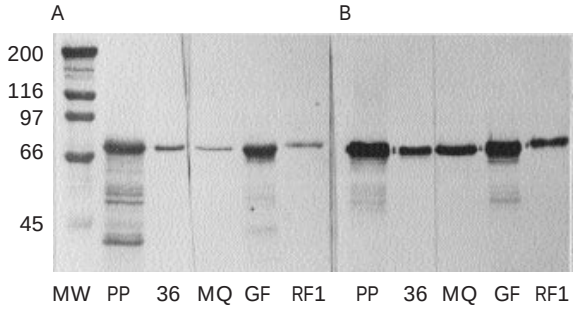

Fig. 2. Comparison of Model 491 Prep Cell purification of BiP under denatured conditions vs. purification of BiP by FPLC under native conditions. A.) Coomassie-stained Western blot of SDS-PAGE gel. B.) Western blot of SDS-PAGE gel probed with 1:7,000 dilution of antibody to tobacco BiP fusion protein. MW, Molecular weight markers; PP, 10 µg of sample eluted from ATP-agarose affinity column with 3 mM ATP; 36, 1 µg of fraction 36 eluted from Model 491 Prep Cell under denaturing conditions; RF1, 15 µl of lyophilyzed Rotofor fraction 1 from isoelectric focusing of Model 491 Prep Cell fractions 35-37; GF, 2.25 µg of affinity selected BiP after gel filtration under native conditions; MQ, 500 ng of gel filtered BiP run on Mono Q® (ion-exchange) column under native conditions.

Here we have reported a quick method for the purification of protein suitable for both amino acid sequence analysis and antibody production. However, for a higher degree of resolution, we would recommend altering the Model 491 Prep Cell conditions by either increasing the elution rate from the Model 491 Prep Cell, or by decreasing the sample volume collected per fraction, or both. The previous recommendation should also prevent unnecessary loss of protein from additional purification steps.

References

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2. Laemmli, U. K., *Nature,* **227,** 680–685 (1970).
3. Denecke, J., *Plant Cell,* **3,** 1025–1035 (1991).

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