Preparative Nondenaturing Gel Electrophoresis to Purify NADP-Specific Glutamate Dehydrogenase From *Chlorella*

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Introduction

One of the research goals of this laboratory is to elucidate the pre- and posttranslational mechanisms that regulate both carbon and nitrogen metabolism in plants. *Chlorella sorokiniana,* a unicellular green alga, has been used extensively as a model system to study enzymes involved in higher plant metabolism. Research in this laboratory has shown that *Chlorella sorokiniana* possesses seven ammonium inducible, chloroplastic, NADP-specific glutamate dehydrogenase (NADP-GDH) isoenzymes that are regulated by both nitrogen and carbon metabolites (Prunkard et al. 1986). Biochemical, immunochemical, and physical characterization of purified GDH homohexamers revealed that the subunits (α, 55.5 kD; β, 53 kD) composing these holoenzymes are nearly identical; however, kinetic characterization of the enzymes showed them to have strikingly different K_m values, allosteric properties, and turnover rates (Bascomb and Schmidt 1987). Recent molecular genetic analysis indicates that minor modifications in the primary protein sequences may account for these observed properties. In order to further elucidate the regulation of these isoenzymes, it was necessary to purify to homogeneity a large quantity of active enzyme for polyclonal/monoclonal antibody production and additional protein analysis.

We report here the use of the Model 491 prep cell for the purification of native NADP-GDH protein by nondenaturing PAGE with <2% loss of enzyme activity.

Methods

Glutamate Dehydrogenase Isolation

Chlorella sorokiniana cells were cultured in 29 mM NH_4^+ medium as previously described (Baker and Schmidt 1963). Approximately 130 g fresh weight cells were harvested by centrifugation and washed two times in 0.01 M Tris-HCl (pH 8.5, 4°C). Pelleted cells were resuspended in an equal ratio of breakage buffer (w/v) and ruptured by two passages through a French pressure cell (American Inst. Co.) at 20,000 psi. The cell homogenate was centrifuged at 27,000 x g for 45 min and the supernatant was stored overnight at –20°C. Frozen supernatant was thawed and the resulting precipitate was removed by centrifugation at 27,000 x g. Initial purification of the NADP-GDH was accomplished using a modified procedure of Yeung et al. (1981), which employs sequential ion exchange and size exclusion chromatography to remove the bulk contaminating proteins from the cell lysate. Procedural modifications involved the addition of $NADP^+$, which functioned as a stabilizer, to a final concentration of 0.1 mM to the gel filtration buffer and all subsequent buffers. As a final modification, a preparative nondenaturing PAGE step using the Model 491 prep cell was substituted for an expensive NADP-affinity resin step.

Sample Preparation

Following ammonium sulfate precipitation and ion exchange chromatography, size exclusion column fractions, in 10 mM KPO_4, 2 mM dithiothreitol (DTT), 0.1 mM $NADP^+$ (pH 6.2), possessing NADP-GDH activity were pooled and concentrated via Diaflo filtration from 17.5 ml to 5 ml. The soluble enzyme was reduced by the addition of DTT to a final concentration of 10 mM and placed in 14,000 MW cutoff dialysis tubing. The concentrated sample was dialyzed at 4°C against 28.8 mM Tris, 192 mM glycine, 2 mM DTT (pH 8.4) for 30 min. The dialyzed enzyme preparation was clarified by centrifugation at 20,000 x g for 10 min and was combined with 3 ml of 40% sucrose and 1 ml of 0.02% Bromophenol Blue.

Preparative Nondenaturing Gel Electrophoresis

For preparative nondenaturing gel electrophoresis, a 3 cm high 7% acrylamide (28 acrylamide:0.735 bis-acrylamide, pH 8.8) resolving gel and a 2 cm high 2% acrylamide (1.6 acrylamide:0.4 bis-acrylamide, pH 6.6) stacking gel were cast in the 28 mm ID gel tube of the Model 491 prep cell. The resolving gel was polymerized in 374 mM Tris (pH 8.8) using 140 µg/ml ammonium persulfate and 1.12 µl/ml TEMED. The stacking gel was polymerized in 39 mM Tris (pH 6.6), 12.7% sucrose using 0.07% riboflavin-5-phosphate (Bio-Rad) and 0.365 µl/ml TEMED. Stacking gel polymerization was achieved by exposure to two 5 W fluorescent lights for 45 min. Both gels were cooled as per the Model 491 prep cell instruction manual during the polymerization process. All acrylamide stock solutions were pretreated with AG® 501-X8 mixed bed resin to remove any contaminating acrylic acid to prevent in vitro acylation of proteins during the electrophoresis process. The preparative gel was pre-electrophoresed for 10 min at 15 mA constant power in upper/lower gel electrophoresis buffer containing 28.8 mM Tris, 192 mM glycine (pH 8.4, 4°C). The elution buffer reservoir was filled with elution buffer comprised of 28.8 mM Tris, 192 mM glycine, 2 mM DTT, and 0.1 mM $NADP^+$ (pH 8.4, 4°C). The entire Model 491 prep cell was cooled to 4°C during the electrophoresis process by operating the unit in a 4°C coldroom. The protein sample, 68 mg total protein in 9 ml of loading buffer, was loaded on top of the stacking gel and electrophoresed for 20 min at 15 mA, and then for an additional 3.5 hr at a constant power of 30 mA using Bio-Rad's Model 3000/300 power supply.

Fraction Collection and Analysis

The elution buffer was pumped at a rate of 2 ml/min to a fraction collector and 6 ml fractions were collected. The first fraction after the Bromophenol Blue marker eluted, fraction 1, was collected after 2 hr of electrophoresis. A spectrophotometric assay was used to quantitate the deaminating activity of the NADP-GDH in each fraction. One unit of GDH activity was defined as the amount of enzyme activity required to reduce 1 µmol of $NADP^+$/min at 38.5°C. NADP-GDH activity was detected in fractions 35–68 (Figure 1). The multiple peaks of enzyme activity detected presumably correspond to the multiple isoenzymes of the NADP-GDH.

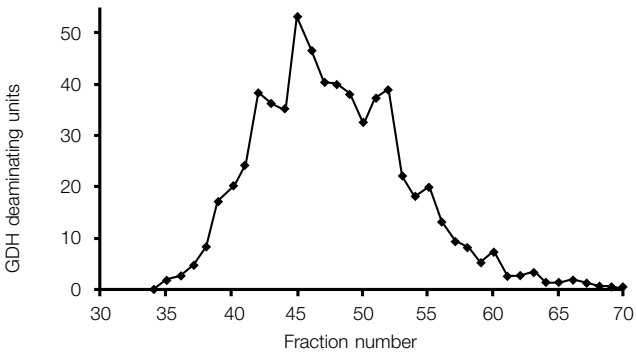

Fig. 1. NADP-GDH activity after preparative nondenaturing electrophoresis using the Model 491 prep cell. Fractions 35–68 contained NADP-GDH activity. Note multiple peaks, presumably corresponding to multiple GDH isoenzymes. Protein under the major peak was analyzed for purity by SDS-PAGE (Figure 2).

Analysis of Model 491 Prep Cell Purified NADP-GDH

NADP-GDH containing fractions were combined and the protein concentration was determined by the method of Bradford (1976) using the Bio-Rad protein assay kit with Bio-Rad's protein standard II as the standard. Fractions under the peaks were pooled, concentrated, and rinsed with 10 mM KPO_4 (pH 6.2), then resuspended in 10 mM KPO_4 (pH 6.2), 0.1 mM $NADP^+$ to a concentration of 1 mg/ml for further analysis and storage at –20°C. The purity of the protein was determined by Tris-Tricine SDS-PAGE (Schagger and von Jagow 1987) in a 10% polyacrylamide resolving, 3% stacking gel using the Mini-PROTEAN® II slab cell (Figure 2). Gels were stained using the Bio-Rad Silver Stain Plus™ kit.

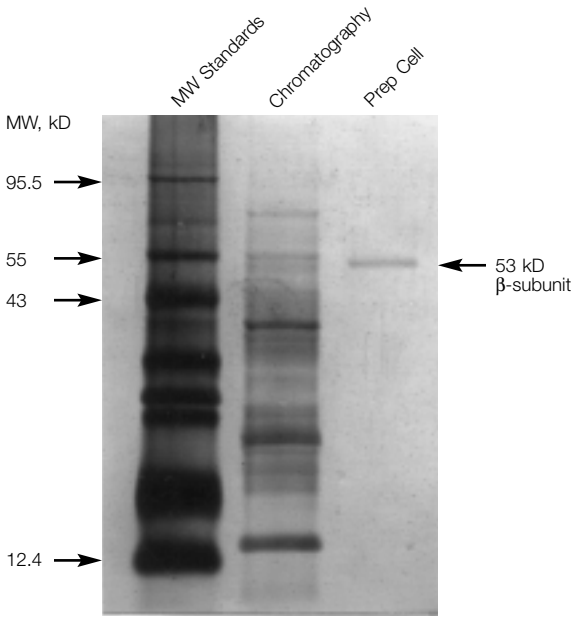

Fig. 2. Analytical SDS-PAGE of NADP-GDH purified by preparative nondenaturing gel electrophoresis using the Model 491 prep cell. Lane 1, MW standards; lane 2, partially purified sample prior to preparative native-PAGE; lane 3, prep cell purified NADP-GDH β-subunit from pooled fractions 40–45.

Table. Purification scheme for NADP-GDH β-subunit.

Step	Total Units[1]	Specific Activity[2]	% Recovery	Fold Purification[3]
Supernatant frozen/thawed cell homogenate	3,546	0.678	100	0
Ammonium sulfate fractionation (35–70%)	3,526	1.22	99	1.8
Ion exchange chromtography, pH 7.4	3,411	5.16	96	7.6
Ion exchange chromatography, pH 6.0	3,060	23.00	86	33.9
Size exclusion chromatography	3,011	30.00	85	44.2
Model 491 preparative native PAGE	2,938	254.00	83	375.0

[1] One unit is defined as the amount of enzyme necessary to reduce 1 mmol of $NADP^+$/min at 38.5°C

[2] Specific activity is defined as units/mg protein

[3] Fold purification is defined as the ratio of the specific activity of step to the specific activity of supernatant cell homogenate

Results

Chlorella sorokiniana NADP-specific GDH was purified an additional 8.5-fold for a final 375-fold purification using the Model 491 prep cell (see Table). Nondenaturing preparative electrophoresis of the partially purified NADP-GDH sample was performed under conditions determined to be optimal for analytical native slab gel electrophoresis. A total of 11.5 mg of extremely pure GDH protein was recovered with <2% loss of activity during the procedure. The resolution of the nondenaturing gel in the Model 491 prep cell was sufficient to separate multiple peaks of NADP-GDH activity corresponding to the multiple isoenzymes of the GDH isoenzymes. Analytical SDS-PAGE of the final purified product revealed a single 53 kD band corresponding to the NADP-GDH β-subunit and detected no contaminating proteins. The highly purified NADP-GDH protein was recovered in sufficient quantity in its native form to allow NH_2- and COOH-terminal sequencing, antigen affinity column production, and the production of high-titer polyclonal and monoclonal antibodies.

These results suggest that the Model 491 prep cell system is amenable to large-scale purification of partially purified proteins in their native, active form. The high resolution of this technique, evidenced by its ability to separate closely migrating isoenzymes, should allow separation of multiple isoenzymes for the purpose of kinetic, biochemical, and immunochemical analyses.

Acknowledgment

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