# Preparation of Spinach Cold Acclimation Proteins for Gas Phase Sequencing, Oligonucleotide Derivation, and Monoclonal Antibody Production

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## Abstract

Previous work on the tolerance of spinach to freezing stress demonstrated a close correlation with the synthesis of several high molecular weight cold acclimation proteins (CAPs).[1] Preparative purification of three moderate abundance CAPs from spinach seedlings has now been accomplished using Rotofor® preparative isoelectric focusing. Rotofor cell purification of these proteins in a 5 hour run is estimated to be 17 to 22 fold. These proteins were then suitable for monoclonal antibody production and, after further purification, amino acid sequencing.

## Methods

### PROTEIN EXTRACTION

Protein was extracted from spinach hypocotyl tissues grown in the dark at 5 °C for 3 to 4 weeks. Frozen tissue was ground in a chilled mortar, then homogenized in a Polytron blender with 80% v/v distilled phenol buffered with 120 mM Tris-HCl (pH 6.8), 50 mM EDTA, 100 mM KCl, 2% v/v Triton® X-100, 5% v/v glycerol, and 2% v/v 2-mercaptoethanol. Phenol extraction irreversibly destroyed proteolytic activity, reduced salt concentration, and enriched the level of CAPs compared to alternative extraction methods. The extract was centrifuged and the aqueous phase was removed. The phenol phase was extracted three additional times with 50 ml of buffer. The final phenol phase was filtered through glass wool to remove cellular debris. Protein was precipitated from phenol with 5 volumes of -20 °C acetone with 1% v/v 2-mercaptoethanol for 2 hours at -20 °C and pelleted at 15,000 g for 10 minutes. The virtually salt-free protein pellet was vacuum dried, dissolved in 5 ml of protein solubilization buffer (PSB) (1 mM Tris-HCl, pH 8.0; 8 M urea; 8 mM CHAPS; 5% v/v glycerol) and centrifuged to remove undissolved material. The supernatant was saved and stored at -20 °C prior to purification in the Rotofor cell. Protein content was determined by the Bradford method.[2]

### RUNNING CONDITIONS

The following protocol was developed to substantially eliminate protein precipitation during focusing. Fifty ml of PSB with 2% v/v ampholytes (0.8%, pH 5–7; 0.8%, pH 4–6; 0.4%, pH 3–10) was prefocused in the Rotofor cell for 1 hour at 12 W constant power at 10 °C. The prefocus was carried out to avoid subjecting the proteins to rapid shifts in pH as the pH gradient was established. Ampholytes were added to the protein solution (usually 3.5–4.0 ml containing from 31 to 187 mg of protein) in the same percentages as above. The protein solution was loaded into the focusing chamber of the Rotofor cell through the port in the eleventh compartment from the anode following addition of ampholytes. This compartment was chosen since in this fraction the pH was closest to the pH of the protein solution. Focusing proceeded for 5 hours at 12 W constant power. Twenty fractions were collected and their pH values measured. Twenty-five µl aliquots from each fraction were loaded directly onto a 20 well, 0.75 mm, 7.5% acrylamide gel containing 1% SDS and run at 20 mA (or 4 mA overnight) at 10 °C. Proteins from cold-acclimated spinach were visualized with Coomassie® blue staining. The isoelectric points as well as the molecular weights of the CAPs were previously characterized by extensive 2-D analysis. Rotofor purification closely approximated analytical IEF separation in two dimensional gel electrophoresis, facilitating identification of Rotofor fractions containing CAPs. Rotofor fractions containing purified CAPs were separated by preparative SDS-PAGE and transferred to polyvinylidine difluoride transfer membrane (PVDF) by semi-dry blotting. Transferred CAPs were treated with cyanogen bromide (CNBr) because of blockage at the amino terminus. The CNBr generated fragments were then separated by SDS-PAGE and transferred to PVDF by semi-dry blotting for sequencing. Fragment sequences from the three CAPs permitted homology searches and oligonucleotide probe synthesis.

CAP antigen preparation for monoclonal antibody production was accomplished by precipitating the protein from the appropriate Rotofor fraction with acetone. Urea was removed with a methanol wash before resuspending the protein for injection into mice.

## Results

Initial attempts to fractionate spinach proteins with the Rotofor cell were unsuccessful due to considerable precipitation, which started in the end compartments and spread inward, at about 20 minutes into the run. This problem was substantially alleviated using urea, CHAPS, glycerol, and the prefocus of the pH gradient before loading the protein.

Rotofor fractionation closely approximated analytical IEF separation in two dimensional gel electrophoresis (see Figure 1). A pH and PAGE analysis of a representative run is shown in Figure 2, which demonstrates that from a single run of 104 mg protein all three CAPs are obtainable for further preparation for either sequencing or monoclonal antibody production.

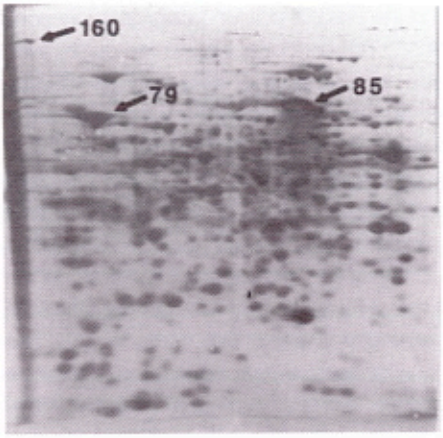


*Fig. 1. Phenol extracted and IEF-PAGE separated protein (200 µg) from cold acclimated-etiolated spinach (hypocotyl and cotyledon) electrophoretically blotted to PVDF membrane and stained with Coomassie blue. Ampholyte percentages were the same as used for Rotofor fractionations (see Methods). The acidic end of the isoelectric focusing gel is on the left and the basic end is on the right. Arrows point to cold acclimation proteins, and the numbers indicate the molecular weights (kD).*

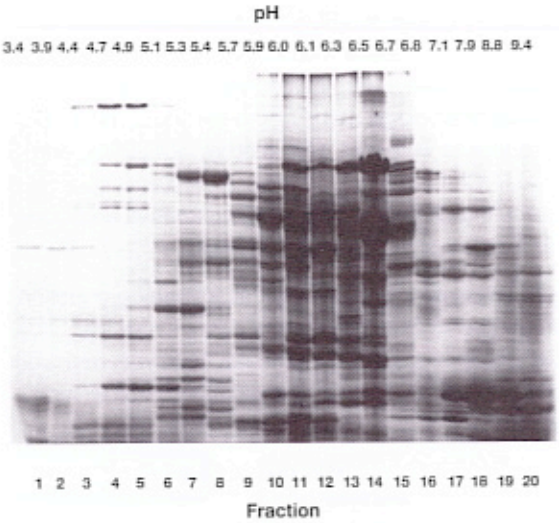


*Fig. 2. Rotofor compartment fractions 1–20 containing spinach proteins (104 mg) analyzed by pH and SDS-PAGE. The prefocus initial conditions were 900 V and 15 mA The end conditions after the 5 hour fractionation were 1,900 V and 6 mA. Compartments containing cold acclimation proteins: 3–6, CAP 160; 7 and 8, CAP 79; 14, CAP 85.*

Estimates by densitometry of SDS-PAGE gels indicated that the percentage of CAP proteins in the Rotofor fractions containing the highest levels of CAP were respectively, 8, 16, 13, for CAP 160, 85, and 79, an enrichment of 17–22 fold. Cleavage bands of CAP were successfully sequenced after elution from PVDF transfer membrane following SDS-PAGE.[3] One fragment of CAP 85 was highly suitable for deriving an oligonucleotide for screening the cold acclimated spinach cDNA library. A northern blot of non-cold acclimated and cold acclimated spinach leaf total RNA is being screened with the oligonucleotide (radiolabeled) to confirm the probe is derived from a protein with a cold induced mRNA. To date, sequencing of cleavage fragments from CAP 160 has revealed no convincing homologies with other proteins. Two CAP 79 fragments were also sequenced and a convincing homology to a known protein was found (to be published elsewhere).

Concentration and purification of substantial amounts of the three CAPs by Rotofor fractionation made it possible to inject enough antigen to raise high serum titers. Following the third boost, titers ranged from 1:3,200 to 1:51,000. With these high titers the likelihood of finding fusion cell lines with antibody to the CAPs was increased. Western blots of CAP antigens were used for screening cell fusion supernatants for CAP monoclonal antibody production (see Figure 3). Screening of fusion cell lines revealed four cell lines with strong reactivity to CAP 160, one of which will be cloned. The primary screening of CAP 79 and 85 fusion cell lines is now in progress.

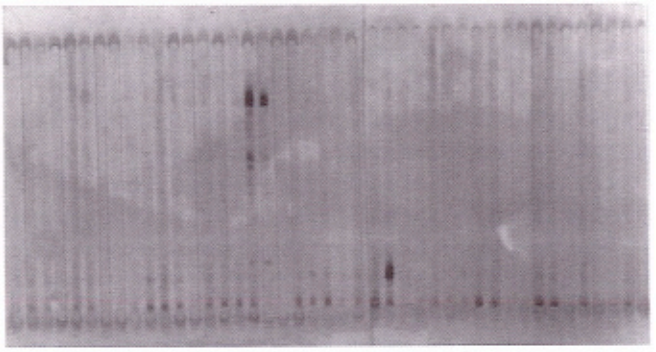

*Fig. 3. Western blot screening of fusion supernatants for CAP 160 monoclonal antibody production. Proteins from cold acclimated spinach hypocotyls and cotyledons (1.1 mg) separated by SDS-PAGE and electrophoretically blotted to PVDF. CAP 160 was estimated at 370 ng/cm of gel. After blocking with 1% BSA, western blots were placed in a miniblotter and 75 µl each of fusion serum was incubated for 2 hours on a rocking table. After washing, 75 µl of anti-mouse IgG (whole molecule) alkaline phosphatase conjugate (1:500 dilution) replaced each fusion serum (45 min on rocker table). Following washing, the blots were placed in developer (NBT/BCIP) for 10 min. Strips (not shown) from the western blots had been removed and stained in Coomassie blue before the remainder was blocked for screening. These stained strips confirmed effective electrophoretic blotting of CAP 160 and were aligned with the developed blots for identifying serums with antibody to CAP 160. The serums from two fusion cell lines, coincidentally incubated in adjacent slots, show strong antibody reaction to CAP 160. Another cell line shows strong antibody reaction to a lower molecular weight protein.*

## Conclusion

The demonstration that cold acclimation of spinach is inducible and coincides with the synthesis of special stress proteins has been substantiated by evidence from these experiments performed with the Rotofor cell. The capabilities of the Rotofor cell were well suited for obtaining large quantities of all the proteins of interest for microsequencing and monoclonal antibody production. After a single focusing, the cold acclimation proteins could easily be isolated by molecular weight with SDS-PAGE from other proteins of similar pI in the same Rotofor fraction.

## References


1 *Guy, C. L. and Haskell, D.*, Plant Physiol., ***84***, *872 (1987).*
2 *Bradford, M. M.*, Anal. Biochem., ***72***, *248 (1976).*
3 *Guy, C. L. and Haskell, D.*, Plant Physiol. Biochem., *in press.*

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