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TITLE: THE PURIFICATION OF RECOMBINANT *ESCHERICHIA COLI* CP4 5-ENOLPYRUVAL-SHIKIMATE-3-PHOSPHATE SYNTHASE FOR EQUIVALENCE STUDIES

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CONTRIBUTORS:

ABSTRACT:

This report documents the purification process used to produce CP4 5-enolpyruval-shikimate-3-phosphate synthase (CP4 EPSPS) from recombinant *Escherichia coli* (*E. coli*)for use in safety assessment studies in support of the Monsanto Agricultural Group registration of transgenic plants. The enzyme CP4 EPSPS, introduced into the plants during the transformation process, gives the plant a tolerance to glyphosate. The protein was purified by a combination of cell extraction, ammonium sulfate precipitation, hydrophobic and anion exchange chromatography.

AUTHORS: R. A. Heeren, S. R. Padgette, and M. E. Gustafson
TITLE: THE PURIFICATION OF RECOMBINANT *ESCHERICHIA COLI* CP4 5-ENOLPYRUVAL-SHIKIMATE-3-PHOSPHATE SYNTHASE FOR EQUIVALENCE STUDIES

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INTRODUCTION

The Monsanto Agricultural Group's New Products Division is commercializing a number of transgenic plants expressing proteins which can impart resistance to selected herbicides, and certain fungal and insect pests. Registration of glyphosate tolerant soybeans with the FDA requires safety assessment of the expressed protein(s). Low expression levels in plants makes isolation of sufficient quantities from plants extremely difficult. CP4 5-enolpyruval-shikimate-3-phosphate synthase (CP4 EPSPS) was expressed in an *Escherichia coli* (*E. coli*) culture produced by recombinant DNA technology. This report documents the production of 2.2 grams of purified CP4 EPSPS used in the MAG safety assessment studies.

II. MATERIALS AND METHODS

1. BUFFER PREPARATION

All reagents used for this process were obtained, tested, and released in accordance with the Bioprocess Technology raw materials procurement procedures. Tris-HCl, Na_4EDTA, ammonium sulfate, and protamine sulfate were purchased from Sigma. The glycerol, hydrochloric acid, and sodium chloride were purchased from Mallinckrodt. The potassium chloride and sodium hydroxide were purchased from Fisher. The Phenyl-Sepharose Fast Flow and Q-Sepharose Fast Flow resins were purchased from Pharmacia. The DTT was purchased from Research Organics.

The buffers, and/or reagents, described in this report were used for the purification of CP4 EPSPS from recombinant *E. coli*. Unless otherwise referenced, all buffers used in GG Building were prepared and stored at 4-10°C, and used GG Building WFI (water for injection, Finn-Aqua System). The pH meter (Model PHM 84, Radiometer, located in GG3460), and the conductivity meter (Model CDM 83, Radiometer, located in GG3460) used for the buffer preparation in GG Building, were calibrated daily.

1) Sodium chloride cell wash solution (A): 15 liters of 0.9% NaCl solution was prepared as described in Isolation run # I92081.

2) Extraction buffer (B): 20 liters 100 mM Tris-Cl, 10% glycerol, 1 mM tetrasodium ethylene diamine tetraacetic acid (Na_4EDTA), 1 mM dithiothreitol (DTT), pH 7.5. The buffer was prepared as described in Isolation run # I92081.

3) Protamine sulfate stock solution (C): 10% (w/v) stock solution was prepared in water immediately prior to use.

4) Ammonium sulfate (D): 516 gram solid per liter of protamine sulfate treated supernatant. Approximately 3.3 kg used in this procedure.

5) Phenyl-Sepharose column equilibrating buffer (E): 50 liters of 100 mM Tris-Cl,10 % glycerol, 1 mM Na_4EDTA, 2 mM DTT, 1.0 M $(NH_4)_2SO_4$, pH 7.5. The pH was adjusted with NaOH.

6) Phenyl-Sepharose column elution buffer (F): 50 liters 100 mM Tris-Cl, 10 % glycerol, 1 mM Na_4EDTA, 2 mM DTT, pH 7.5. The pH was adjusted with NaOH. This buffer and the Phenyl-Sepharose column equilibrating buffer were combined proportionately, when necessary, to make the appropriate $(NH_4)_2SO_4$ concentration for each step of the Phenyl-Sepharose column.

7) Q-Sepharose equilibrating buffer (G): 200 liters of 10 mM Tris-Cl, 10 % glycerol, 2 mM DTT, pH 7.5. Adjust pH with NaOH.

8) Q-Sepharose eluting buffer (H): 50 liters of 10 mM Tris-Cl, 10 % glycerol, 2 mM DTT, 0.5 M KCl, pH 7.5. Adjust pH with NaOH. This buffer and the Q-Sepharose column equilibrating buffer were combined proportionately, when necessary, to make the appropriate KCl concentration for each step of the Q-Sepharose

9) Storage buffer (I): 50 liters of 50 mM Tris-Cl, 50 % glycerol, 2 mM DTT, 50 mM KCl, pH 7.5. Adjust pH with NaOH.

DTT, when required in a buffer, was added just prior to use of the buffer. There was little, if any, significant changes noticed in the pH or conductivity after DTT addition.

2. ASSAYS

CP4 EPSPS activity assays were done using a procedure outlined in MSL 11344 (1). In-process SDS-PAGE was performed by S. Becker of Bioprocess Technology and was done using the Laemmli method (2). Protein concentrations were done by M. Taylor (MAG) using the Bradford method (3).

3. PROCESSING PROCEDURES

The fermentation, homogenization, extraction, and ammonium sulfate precipitation steps of the purification are documented in fermentation batch record 307-92019 and isolation batch record I92081, and were performed in HH Building.

The Phenyl-Sepharose column chromatography and the Q-Sepharose chromatography were done in the GG3421 cold room. Fresh chromatography resin (Pharmacia) and new supplies (tubing, plasticware, etc.) were used whenever possible. Columns were cleaned with 0.1 N NaOH, rinsed with hot WFI, and were visually inspected for cleanliness prior to use. The column chromatography steps of the process were documented in Monsanto notebook 5,192,201.

FERMENTATION AND CELL RECOVERY

The recombinant *E. coli* fermentation (100L) was terminated 6 hours after induction by chilling the fermentor contents to 10°C. The cells were harvested by passing the fermentation broth through a Sharples centrifuge (Sharples 6" solid bowl centrifuge #147) operating at a flow rate of 500 ml/minute. The recovered cell paste was stored at -20°C in HH Building until further processing was required. The wet weight of the cell paste was 3.5 kg.

CELL HOMOGENIZATION AND SOLUBLE PROTEIN RECOVERY

The cell paste (approximately 1600 grams wet weight, or 50%), after overnight storage at 4-10°C, was resuspended in 0.9% NaCl at a concentration of 1 gram cell paste per 4 mls NaCl solution. The washed cells were recovered by passing the cell suspension through a Sharples centrifuge at a flow rate of 500 ml/minute. The washed cell pellet was resuspended in 6.2 liters of an extraction buffer that consisted of 100 mM Tris-Cl, 10% glycerol, 1 mM Na_4EDTA, 1 mM DTT, pH 7.5. Volume of the extraction buffer suspension was 7.3 liters. The cell suspension was disrupted by homogenization in a Gaulin 15M (#902) operated at 9000 PSI. The cell suspension was processed through the homogenizer with the output cycled back into the feed tank for the equivalent of two passes. The homogenate was centrifuged at 9,000 RPM for 60 minutes (Sorvall centrifuge; GS-3 rotor; 500 ml polypropylene bottles) to recover soluble proteins. The supernatant was decanted and held as a pool at 4-10°C until centrifugation was completed. The cell debris pellet was discarded.

PROTAMINE SULFATE PRECIPITATION

A 10% stock solution (100 mg/ml) of protamine sulfate was prepared immediately prior to use. The stock solution was added slowly to the extract supernatant so that the final protamine sulfate concentration was 0.2% (2 mg/ml). The protamine sulfate precipitation was allowed to react for two hours with gentle stirring at 4-10°C. The nucleic acid rich precipitate was removed by centrifugation at 9000 RPM for 60 minutes at 4-10°C (Sorvall centrifuge; GS-3 rotor; 500 ml polypropylene bottles). The supernatant was decanted and held as a pool at 4-10°C until centrifugation was completed. The volume of the pooled supernatant was 6.5 liters.

AMMONIUM SULFATE PRECIPITATION

Solid ammonium sulfate (516 g/liter- 80% final concentration) was added slowly to the protamine sulfate supernatant with moderate stirring from an overhead stirrer. The reaction was continued overnight with gentle stirring at 4-10°C. The precipitate was recovered by centrifugation at 9000 RPM for 30 minutes at 4-10°C (Sorvall centrifuge; GS-3 rotor; 500 ml polypropylene bottles). The pelleted precipitate was stored at -20°C in 24 approximately equal aliquots. The wet weight of the pelleted precipitate was approximately 760 grams total.

PHENYL-SEPHAROSE COLUMN CHROMATOGRAPHY

Approximately 290 grams of frozen ammonium sulfate precipitated protein containing CP4 EPSPS was dissolved in 1 liter of Phenyl-Sepharose column equilibrating buffer (see buffer E under Buffer Preparation). The precipitate was dissolved easily. The volume of the solution was approximately 1200 mls. The CP4 EPSPS solution was loaded onto a Phenyl-Sepharose Fast Flow column (Amicon G70 x 250 mm)that had previously been equilibrated in 10-20 column volumes of Phenyl-Sepharose column equilibrating buffer at a flow rate of 18 mls per minute. The absorbance at 280 nm (A280) was monitored and the AUFS setting was 2.0. The flow-through was collected as a pool and was retained until activity assays were done. The column was washed with Phenyl-Sepharose column equilibrating buffer after the protein was loaded onto the column. Fractions were collected (fraction volume was 45 ml). After fraction 17, the elution was initiated by washing the column with 100 mM Tris-Cl,10 % glycerol, 1 mM Na_4EDTA, 2 mM DTT, 0.45 M $(NH_4)_2SO_4$, pH 7.5, while continuing to collect fractions. The buffer containing 0.45 M $(NH_4)_2SO_4$ was prepared by mixing Phenyl-Sepharose column equilibrating buffer and the elution buffer (see buffer F under Buffer Preparation) in a 4.5 to 5.5 ratio, respectively. Column parameters remained the same. After fraction 57, the column was washed with 100 mM Tris-Cl, 10 % glycerol, 1 mM Na_4EDTA, 2 mM DTT, 0.20 M $(NH_4)_2SO_4$, pH 7.5, while continuing to collect fractions. The buffer containing 0.20 M

$(NH_4)_2SO_4$ was prepared by mixing Phenyl-Sepharose column equilibrating buffer and the elution buffer in a 2.0 to 8.0 ratio, respectively. Continued collecting fractions through fraction 100 using the 100 mM Tris-Cl,10 % glycerol, 1 mM Na_4EDTA, 2 mM DTT, 0.20 M $(NH_4)_2SO_4$ buffer. Fractions were pooled based upon the results from the activity assays. The pool volume was 850 ml (see Figure 2). The pool was dialyzed against two changes of Q-Sepharose column equilibrating buffer (see buffer H under Buffer Preparation) for 48 hours.

The column was washed with Phenyl-Sepharose column elution buffer for approximately 1 column volume. The flow-through was collected as a pool. The column was regenerated with Phenyl-Sepharose column equilibrating buffer in preparation for further use, if necessary.

A second run(5192227-5192230), identical to the first Phenyl-Sepharose run, was performed.

Q-SEPHAROSE COLUMN CHROMATOGRAPHY

The Phenyl-Sepharose dialysate was loaded onto a Q-Sepharose Fast Flow column (Amicon G70 x 250 mm)that had previously been equilibrated in 10-20 column volumes of Q-Sepharose column equilibrating buffer (see buffer H under Buffer Preparation) at a flow rate of 18 mls per minute. The A280 was monitored and the AUFS setting was 1.0. The flow through was collected as a pool and was retained until activity assays were done. The column was washed with Q-Sepharose column equilibrating buffer until the A280 returned to the baseline. The wash was collected as a pool. The elution of bound proteins was initiated by washing the column with 10 mM Tris-Cl, 10 % glycerol, 2 mM DTT, 50 mM KCl, pH 7.5. Fractions were collected (40 ml each). The Tris buffer containing 50 mM KCl was prepared by mixing Q-Sepharose column elution buffer and Q-Sepharose column equilibrating buffer (see buffers H and G under Buffer Preparation) in a ratio of 0.5 to 9.5, respectively. A total of 53 fractions (total volume of 2100 ml) were collected using the Tris buffer containing 50 mM KCl. At fraction 54 the column was eluted with 10 mM Tris-Cl, 10 % glycerol, 2 mM DTT, 100 mM KCl, pH 7.5. The Tris buffer containing 100 mM KCl was prepared by mixing Q-Sepharose column elution buffer and Q-Sepharose column equilibrating buffer (see buffers H and G under Buffer Preparation) in a ratio of 1.0 to 9.0, respectively. Fractions were collected through fraction 106, when, at this point, collected the remaining elution as a pool. The pool volume was 350 ml. Samples were submitted for activity assays. Fraction were pooled for final dialysis based upon results from the activity assays. The pooled volume was 650 ml (see Figure X for fractions pooled). The pool was dialyzed for 72 hours against 50 mM Tris-Cl, 50 % glycerol, 2 mM DTT, 50 mM KCl, pH 7.5 (see buffer I under Buffer Preparation). The lot number of this final Q-Sepharose dialysate was 5192220.

The second Phenyl Sepharose run, after dialysis, was loaded onto the Q-Sepharose column under the same conditions and results (5192232-5192235). Fractions from the second Q-Sepharose run were split into 2 pools based upon the results from activity assays and dialyzed for 72 hours against 50 mM Tris-Cl, 50 % glycerol, 2 mM DTT, 50 mM KCl, pH 7.5 (see buffer I under Buffer Preparation). The lot numbers were 5192235-1 and 5192235-2.

The three lots of purified CP4 EPSPS were pooled and transferred to MAG (see Monsanto notebook page 5192245). The final volume was 570 ml and the lot number was 5192245.

4. RESULTS AND DISCUSSION

Over 2.2 grams (Table 1), by Bradford assay, of high purity CP4 EPSPS (Figure 4) was purified and delivered to MAG during this campaign. The specific activity increased approximately four fold during the purification starting from 0.75 units/mg protein for the cell extract to 3.5 - 4.2 units/mg protein for the purified Q-Sepharose pools.

Figure 1 shows the process utilized to purify 2.2 grams of CP4 EPSPS. Typical A280 absorbance profiles for the Phenyl-Sepharose and Q-Sepharose chromatography steps are shown in Figures 2 and 3, respectively. Figure 4 shows an SDS-PAGE analysis of the three dialyzed Q-Sepharose pools that were combined to make the final pool of purified CP4 EPSPS transferred to MAG.

ACKNOWLEDGEMENTS

Mary Taylor for in process activity and protein assays.

Sandy Becker for electrophoresis support.

REFERENCES

1. Morgan, D.R., and Schilling, R.J. MSL-11344 (August 1991).

2. Laemmli, U. K., Nature 227: 680-685 (1970).

3. Bradford, M., Analytical Biochemistry 72: 248 (1976).

TABLE 1: Summary of data by lot number from purification of CP4 EPSPS showing specific activity (SA) and protein. Lots 5192220, 5192235-1, and 5192235-2 were combined (final lot no. 5192245) after dialysis into the storage buffer, and transferred to MAG. The specific activity of lot number 5192245 was 3.0 units/mg, the volume was 570 ml, and the protein concentration was 3.95 mg/ml.

SAMPLE	SA (U/mg)	PROTEIN* (mg/ml-	total g)
Cell Extract			
5192220	0.75	- - -	
5192235	0.75		
Am.SO4 Resuspension			
5192220	0.40	14.5	17.4
5192235	1.70	22.0	16.1
Phenyl-Seph. Pool			
5192220	1.13	11.6	9.90
5192235	1.80	8.70	10.4
Q-Seph. Pool			
5192220	3.50	2.10	1.40
5192235-1**	4.20	1.10	0.55
5192235-2**	3.40	1.09	0.30

* Bradford assay.

**Second Q-Sepharose run yielded two separate pools that were combined after further activity and gel analysis.

FIGURE 1: PURIFICATION SCHEME FOR CP4 EPSPS.

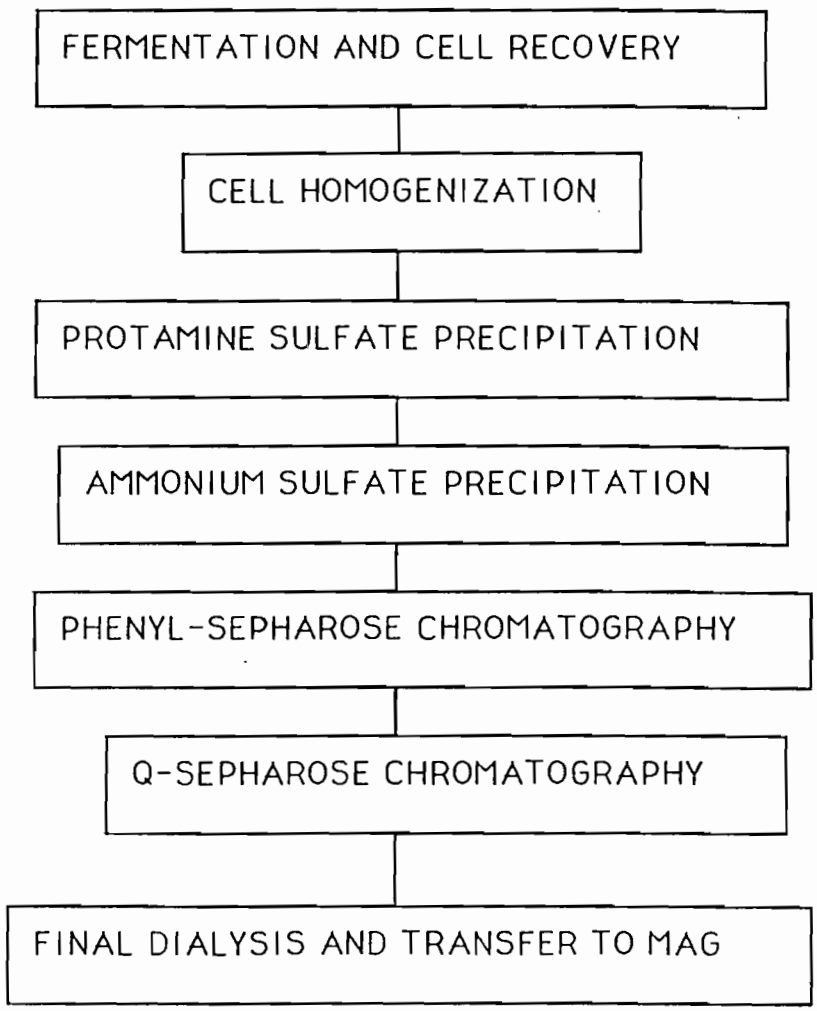


Figure 2. Schematic of a portion of the A280 profile from the Phenyl-Sepharose Run 1 column. Fractions 22-40 were pooled and dialyzed against Q-Sepharose equilibation buffer. No significant CP4 EPSPS activity was found in other A280 peaks.

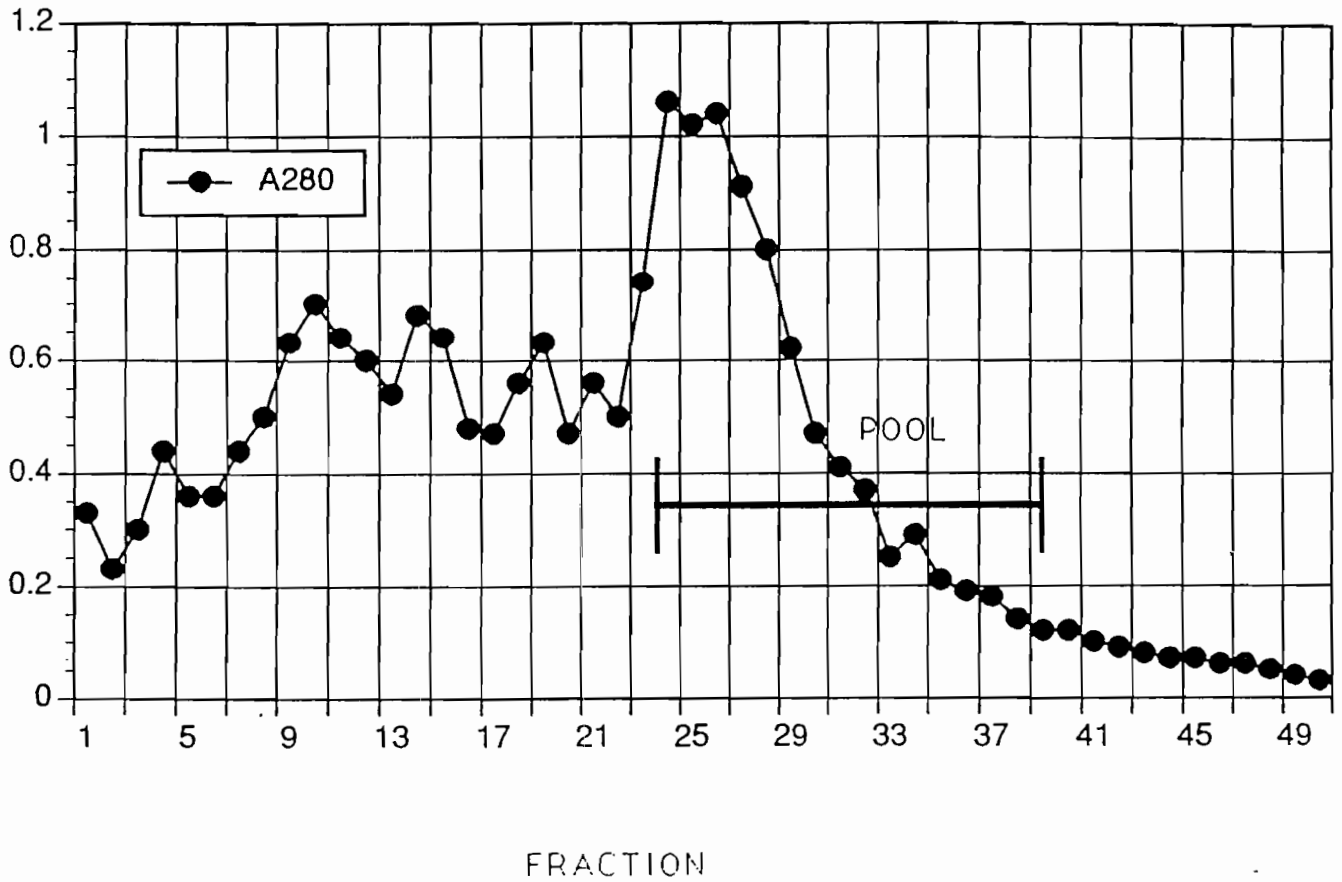
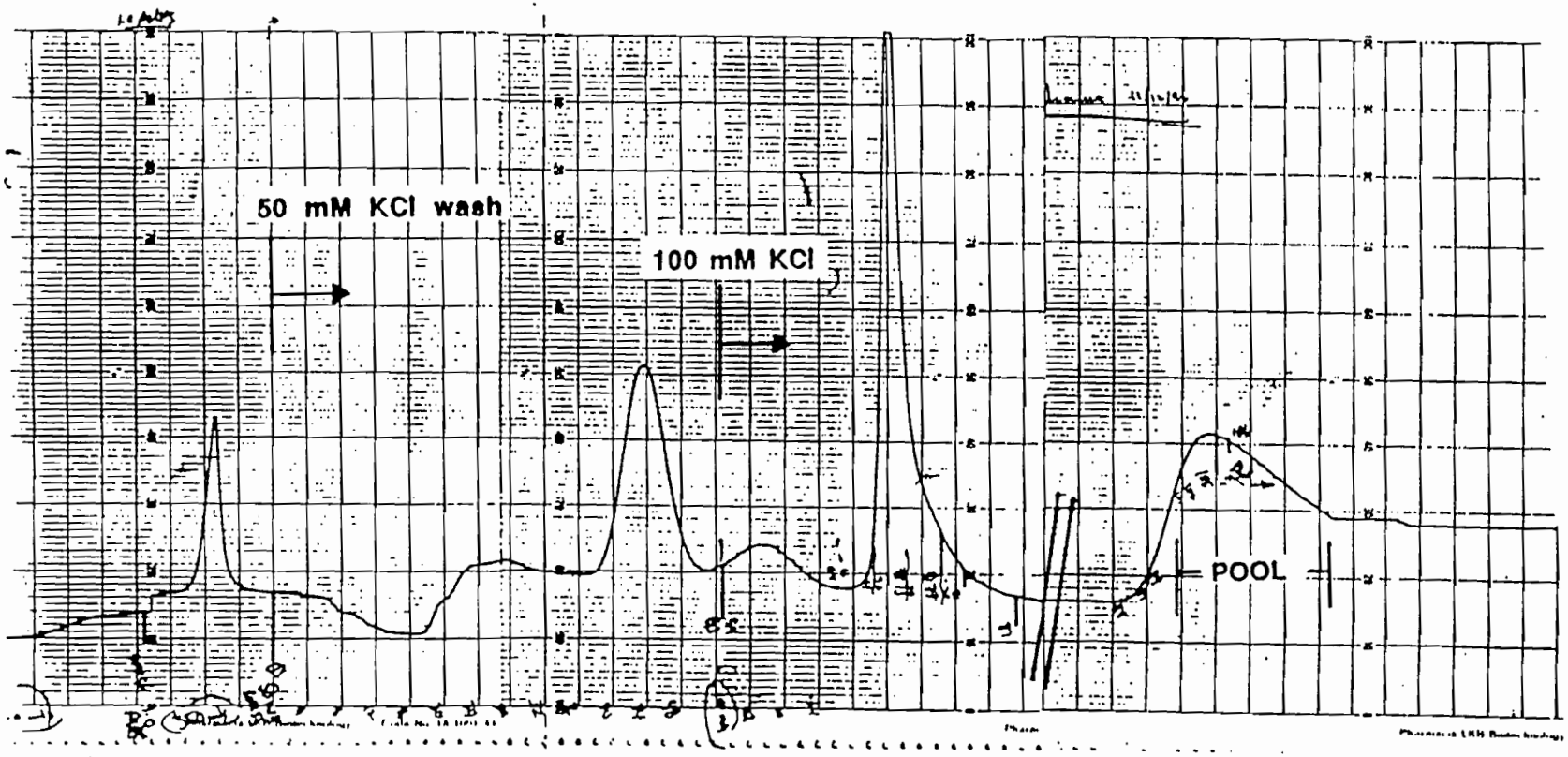


Figure 3: Chromatogram from Q-Sepharose Run 1 chromatography. Fractions 100-106, and the subsequent eluant pool after fraction 106, were found to have a significant CP4 EPSPS activity. These fractions were pooled and dialyzed into storage buffer.



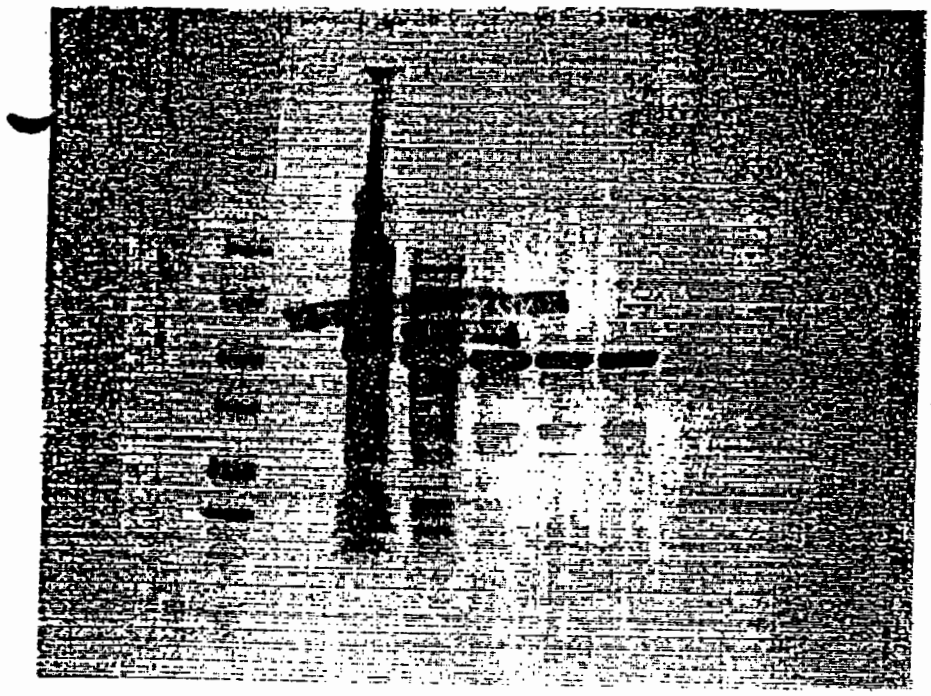


Figure 4: SDS-PAGE of the 3 Q-Sepharose pools (one pool from run1 and two pools from run2), post-storage buffer dialysis, that were transferred to MAG. Lanes D,E, and F were the Q-Sepharose pools. Lane B is the protein isolated from the ammonium sulfate precipitation, and lane C is the Phenyl-Sepharose column pool (see Figure 2). Lane A is a MW marker.

Figure 5: Transfer documentation of CP4 EPSPS lot number 5192245.

[redacted]

MONSANTO PLANT SCIENCES SAMPLE TRANSFER FORM

FROM: Robert A. Heeren

TO: Barbara Fay

The following should be completed by Shipper:

	Sample Type	Sample ID	Comments Protocol/Exp #	No. of Pkgs	Rcv'd ✓
1.	Ld # 5192245	Purified CP4 EPSPS			
2.					
3.					
4.					
5.					

Samples Shipped by: Robert A Heeren (SIGNATURE) Date and Time: 12/11/92 1600 Hours

Number of Shipping Containers: _____ Method of Shipment: _____

Shipping Conditions (✓): ___ Frozen on Dry Ice ___ Cool on Ice ___ Ambient Temp

X Other (Please specify): 4-10°C

The following should be completed by Recipient:

Samples Received and Checked by: [signature] (SIGNATURE) Date and Time: 12/11/92

Condition of samples upon receipt: chilled (4-10°C) liquid

Distribution of Copies:
WHITE COPY Include with shipment, recipient sends finalized version to Study Director
YELLOW COPY Include with shipment, recipient keeps for his re
PINK COPY Shipper keeps for his records

Figure 6: Transfer documentation of CP4 EPSPS lot number 5192245.

Monsanto
The Agricultural Group

Test/Reference Substance Sample Transfer Form
SOP Reference: GEN-POL-006 Page ___ of ___

New Products Division - Regulatory Sciences [redacted]

Study #: _____ Date of Transfer: 12/11/92

Experiment #: _____ Number of Samples being Transferred: 1

SIGNATURES:

[illegible] 12/11/92
PERSON PROVIDING SAMPLE DATE

[illegible] 12/11/92
PERSON RECIEVING SAMPLE DATE

SAMPLE #1

ID of Test Material: Purified CP4EPSPS (Synthase) [Protein, Source]
Lot #: 5192245 Purity: _____
Reference for Test Material Prep: NBP 5192245 [Monsanto Notebook Pg, MSL #, or Study & Exp
Physical State of Sample: Liquid (50% glycerol, 50mM Tris-Cl) [Powder, Solution, Buffer, Conc.]
Quantity: 3.96mg/ml — 570 ml's (50mM KCl, 2mM DTT) [Weight or Volume]

Required Storage Conditions: -20°C

Storage Location: _____

Test(s) Requested for this sample: _____
Misc; Notes: _____

SAMPLE #2

ID of Test Material: _____ [Protein, Source]
Lot #: _____ Purity: _____
Reference for Test Material Prep: _____ [Monsanto Notebook Pg, MSL #, or Study & Exp
Physical State of Sample: _____ [Powder, Solution, Buffer, Conc.]
Quantity: _____ [Weight or Volume]

Required Storage Conditions: _____

Storage Location: _____

Test(s) Requested for this sample: _____
Misc; Notes: _____

SAMPLE #3

ID of Test Material: _____ [Protein, Source]
Lot #: _____ Purity: _____
Reference for Test Material Prep: _____ [Monsanto Notebook Pg, MSL #, or Study & Exp
Physical State of Sample: _____ [Powder, Solution, Buffer, Conc.]
Quantity: _____ [Weight or Volume]

Required Storage Conditions: _____

Storage Location: _____