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EXPRESSION OF POTATO DAHP SYNTHASE IN *ESCHERICHIA COLI*

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Abstract: Expression of a potato (*Solanum tuberosum* L.) cDNA encoding 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase, the first enzyme of the shikimate pathway, complemented an *Escherichia coli* mutant devoid of the enzyme. While the potato cDNA encodes a protein with an amino terminal putative transit sequence for chloroplast import, immunocytochemistry localized the *E. coli* synthesized potato enzyme to the bacterial cytosol.

Biosynthesis of the aromatic amino acids proceeds *via* the shikimate pathway. The first committed step in the shikimate pathway is the condensation of P-enolpyruvate and erythrose 4-phosphate to form 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP). This reaction is catalyzed by the enzyme DAHP synthase (EC 4.1.2.15) (1,2). DAHP synthase, only found in microorganisms and plants, regulates carbon flow through the shikimate pathway (3). Three unlinked *Escherichia coli* genes, *aroF*, *aroG*, and *aroH*, encode three DAHP synthase isoenzymes that are repressed and feedback inhibited by the three aromatic amino acids.

Multiple isoenzymes of DAHP synthase have also been described in higher plants (4-6). Potato contains DAHP synthase isoenzymes that have been characterized through their different metal requirements for enzyme activity (7). The Mn-isoenzyme is activated by Mn^{2+}, Mg^{2+}, and tryptophan, requires a reducing agent for activity, and has been purified to electrophoretic homogeneity from potato tuber (8). The Co-isoenzyme is activated by any divalent metal ion and has been partially purified from potato tubers (7). The two isoenzymes are immunologically distinct (9), and polyclonal monospecific antibodies against any of the *E. coli* isoenzymes do not crossreact with either of the two potato activities.

A cDNA encoding potato DAHP synthase has been isolated using antibodies to the pure tuber Mn-isoenzyme (10). The amino terminus of the cDNA-encoded DAHP synthase is rich in Ser, Thr, Ala, and Val residues and has a net positive charge, characteristics of a transit sequence for chloroplast import (11). The cDNA-encoded potato DAHP synthase has only about 22% amino acid sequence identity with the

homologous enzymes from *E. coli*. In order to show that the cloned cDNA in fact encodes a polypeptide with DAHP synthase enzyme activity and as a prerequisite for future work on site-directed mutagenesis, we report here the expression of the potato cDNA in *E. coli*, the complementation of an *E. coli* mutant void of DAHP synthase by the potato cDNA, and the partial characterization of the potato protein synthesized by *E. coli*.

Materials and Methods

Reagents and Antibodies - Nitroblue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, amino acids, sulfopropyl Sephadex, and triethanolamine buffer were from Sigma. Restriction endonucleases and T4 DNA ligase were from either New England Biolabs or Bethesda Research Laboratories. IPTG and agarose were from Bethesda Research Laboratories. Oligonucleotides were from the Purdue Laboratory for Macromolecular Structure. ^{35}S-dATP was from Amersham. Rabbit anti-potato DAHP synthase was described previously (12,13). Rabbit anti-ß-galactosidase was a gift of Dr. R. L. Somerville. Chicken anti-ribose binding protein and alkaline phosphatase conjugated rabbit anti-chicken antibodies were a gift of Dr. M. A. Hermodson.

Bacterial Strains and Plasmids - All strains are *E. coli* K12 derivatives. Strain JM109 is *recA1, endA1, gyrA96, thi, hsdR17* (r_K^-, m_K^+)· *supE44, relA1,* λ⁻, Δ(*lac-proAB*),[F', *traD36, proA*$^+$*B*$^+$, lacI^{q}ZΔM15 (14). Strain HE628 is Δ*(aroF tyrA)*, Δ*aroG* (15). Phage M13mp18 (14) and plasmid pKK233-2 (16) were from Pharmacia. Plasmid pLW3-210 is derived from pKK233-2 as described below. Bacteria were grown in either Luria broth (17) or Vogel-Bonner minimal medium (18) supplemented with ampicillin (50 μg/ml), amino acids (40 μg/ml), thiamine, niacin, biotin (1 μg/ml each), and IPTG (1 mM) where indicated. Solid medium, supplemented as above, was either nutrient agar or Vogel-Bonner minimal to which agar (15 g/l) was added.

Nucleic Acid Manipulations - Agarose gel electrophoresis, ligations, plasmid miniscreens, and transformations were performed as described (17). Site-directed mutagenesis was performed in strain JM109 with an Amersham kit according to (19). Dideoxy sequencing (20) was performed with kits from either New England Biolabs or United States Biochemical, according to the manufacturers' protocols.

Immunoblots - *E. coli* cells were grown in Luria broth containing 50 μl/ml ampicillin, harvested, boiled 10 min in SDS sample buffer (21), and the insoluble material was removed by centrifugation. The supernatant was then loaded onto SDS polyacrylamide gels. Alternatively, protein samples were precipitated with 20% trichloroacetic acid, ether washed, and dissolved by boiling for 10 min in SDS sample buffer. Proteins were detected either by silver stain (22), or with antibodies. For immunoblots, proteins from 2 x 10^8 cells were transferred to nitrocellulose (23,24) and detected with either anti-potato DAHP synthase, anti-ß-galactosidase, or anti-ribose binding protein followed by the appropriate phosphatase conjugated secondary antibodies.

Purification of DAHP Synthases - Potato tuber DAHP synthase was purified as described previously (8). Potato DAHP synthase made in *E. coli* was purified by a modification of this method. Strain HE628 bearing plasmid pLW3-210 was grown at 37°C in Luria broth supplemented with ampicillin. All further steps were performed at 4°C. The cells were harvested by centrifugation at 5000xg for 10 min, and resuspended in 50 mM potassium phosphate, pH 7.0, containing 1 mM P-enolpyruvate, 1.5 mM tryptophan, 50 mM ß-mercaptoethanol, and 0.1 mM each of $MnCl_2$, $MgCl_2$, and phenylmethylsulfonyl

fluoride (buffer A). The cells were disrupted using a French pressure cell, and 1/10 volume of 2% protamine sulfate in buffer A was added. The extract was clarified by centrifugation at 7000xg for 15 min.

The extract was applied to a phosphocellulose column equilibrated with buffer A, and DAHP synthase was eluted with a linear gradient of 50 to 250 mM potassium phosphate, pH 7.0, containing buffer A supplements. The fractions containing DAHP synthase activity were pooled and the protein precipitated by the addition of $(NH_4)_2SO_4$ to 80% saturation. The precipitate was resuspended in 5 mM potassium phosphate, pH 7.0, containing buffer A supplements, and desalted by passage over a 3 ml Sephadex G10 column. The protein was applied to a sulfopropyl Sephadex column equilibrated with 5 mM potassium phosphate, pH 7.0, containing buffer A supplements. The column was washed with the same buffer, and DAHP synthase was eluted by addition of 0.5 M potassium phosphate, pH 7.0, containing buffer A supplements. Alternatively, the $(NH_4)_2SO_4$ precipitate was resuspended in 25 mM potassium phosphate, pH 7.0, containing buffer A supplements, desalted, and applied to a sulfopropyl Sephadex column equilibrated with the same buffer and eluted with a linear gradient of 25 to 200 mM potassium phosphate, pH 7.0, containing buffer A supplements except phenylmethylsulfonyl fluoride.

DAHP Synthase Assay and Protein Determination - DAHP synthase was assayed by measuring the absorbance at 549 nm of the periodate degradation product of DAHP complexed with thiobarbiturate (25). To assay the Mn-isoenzyme (assay I), the reaction mixture contained 50 mM 1,3-bis[tris(hydroxymethyl)methylamino]propane, pH 7.0, with 1.5 mM tryptophan, 3 mM $MnCl_2$, 3 mM $MgCl_2$, 0.75 μmoles of P-enolpyruvate, and 0.3 μmoles of erythrose 4-phosphate in a total volume of 0.1 ml (8). To assay the Co-isoenzyme (assay II), the reaction mixture contained 25 mM potassium N-[2-hydroxyethyl]piperazine-N'-[3-propanesulfonate], pH 8.6, with 10 mM $MgCl_2$, 0.75 μmoles of P-enolpyruvate and 0.3 μmoles of erythrose 4-phosphate in a total volume of 0.1 ml (7). The assay for bacterial DAHP synthase (assay III) was as described (25). In all cases, the reaction was initiated by the addition of 50 μl of appropriately diluted enzyme, and the reaction mixtures were incubated for 10 min at 37°C. The reactions were stopped by the addition of 10% trichloroacetic acid. The unit of activity is defined as the amount of protein catalyzing the appearance of 1 μmole of DAHP per min. Protein was determined by the method of Bradford (26), with lysozyme as the standard.

Cross-linking - Cross-linking of enzyme subunits was performed using a kit (Anatrace) of diimidates with varying numbers of carbon atoms between their functional groups. For cross-linking of potato DAHP synthase made in *E. coli*, 40 μg of partially purified enzyme at approximately 1 mg/ml was added to an equal volume of 4 mg/ml diimidate in 0.2 M triethanolamine buffer, pH 8.0, and incubated at 30°C for 3 hours. SDS and ß-mercaptoethanol were added to a final concentration of 350 mM each and incubation was continued for 2 hours at 37°C (27,28). For cross-linking of potato DAHP synthase from tubers, 20 μg of partially purified enzyme at 0.3 mg/ml were added to an equal volume of 4 mg/ml diimidate and treated as above. The proteins were then subjected to electrophoresis on 6% polyacrylamide gels, and identified by immunoblotting.

Results

Subcloning of a Potato cDNA Encoding DAHP Synthase into the Expression Vector pKK233-2 - The plasmid pKK233-2 was chosen as a vector for expression of a potato cDNA encoding DAHP synthase (10). The vector contains the *trc* promoter with 17 bp between the *trp* -35 and the *lac*UV5 -10 region, the *lacZ*

ribosome binding site and ATG initiation codon, and transcriptional terminators *rrnB*T_1T_2. An *NcoI* restriction site spans the initiation codon. Open reading frames cloned into the *NcoI* site should be inducible with IPTG in *lacI*^{q} host strains. At the initiation codon for potato DAHP synthase, the cDNA sequence is CAATGG, a one bp mismatch from the *NcoI* restriction recognition sequence CCATGG. To change the existing sequence into an *NcoI* site, the 872 bp *EcoRI-KpnI* fragment containing the 5' end of the cDNA was cloned into M13mp18, and a new *NcoI* restriction site was created by site directed mutagenesis. The resulting fragment containing the 5' portion of the coding region and part of the M13mp18 polylinker was then cloned into NcoI/HindIII cut plasmid pKK233-2 as a NcoI/HindIII fragment. The 3' portion of the cDNA, deleted for all but 20 bp of the 3' untranslated region, was subcloned as a *KpnI-HindIII* fragment into the intermediate vector resulting in plasmid pLW3-210. This plasmid carries the entire coding region for the potato DAHP synthase under the control of the *trc* promoter. The junctions of the vector and the cDNA insert were verified by nucleotide sequence analysis.

Complementation of E. coli void of DAHP Synthase by Plasmid pLW3-210 - Strain HE628 contains deletions in *aroF tyrA* and *aroG* (15). Therefore, extracts of HE628 lack the tyrosine- and the phenylalanine-sensitive DAHP synthases and the first enzyme of the terminal pathway towards tyrosine, the chorismate mutase prephenate dehydrogenase. Thus, HE628 requires tyrosine for growth, but cannot grow on minimal media supplemented with tyrosine and tryptophan, due to repression of *aroH* and feedback inhibition of the tryptophan sensitive DAHP synthase by tryptophan. Strain HE628 was transformed with plasmid pLW3-210. Transformants were selected on nutrient agar containing ampicillin. The transformed cells grow on minimal medium containing tyrosine, tryptophan, and ampicillin, while HE628 transformed with the parental plasmid pKK233-2 does not grow on this medium. Therefore, the cDNA encoding a potato DAHP synthase complements the *aroF aroG* double deletion. HE628/pLW3-210 does grow more slowly on both nutrient agar with ampicillin, or minimal medium containing tyrosine, tryptophan, and ampicillin than HE628/pCG201 containing the bacterial *aroF* gene on a multicopy plasmid (29). The growth of HE628/pLW3-210 is further reduced through addition of IPTG to the medium.

Synthesis of Potato DAHP Synthase in Cells of Strain HE628/pLW3-210 - Cells of strain HE628 transformed with plasmids pLW3-210 or pKK233-2 were grown in Luria broth supplemented with ampicillin, and with or without 1 mM IPTG added at mid-log phase. Cells of HE628/pLW3-210 produced enzymatically active DAHP synthase (4.0 Eu/mg), but there is about 25% less enzyme activity (3.12 Eu/mg) in extracts of cells grown in media containing IPTG, a potential correlation to the reduced growth rate of HE628/pLW3-210 in media containing the inducer. Cell extracts of strain HE628/pKK233-2 contain negligible amounts of DAHP synthase enzyme activity (0.06 Eu/mg). HE628/pLW3-210 produces two polypeptides that cross-react with anti-potato DAHP synthase, while none of the proteins from HE628/pKK233-2 do (Fig. 1). Consistent with the enzyme assay, IPTG appears not to cause an increase in the amount of cross-reacting material. The two cross-reacting species of HE628/pLW3-210 have apparent MW of 59,000 and 50,000.

Localization of Potato DAHP Synthase in E. coli - Since the cDNA insert of pLW3-210 encodes a putative transit sequence and since the appearance of two immunoreactive polypeptides could suggest that this sequence may be removed in *E. coli*, we examined the intracellular location of the potato protein in *E. coli* by immunocytochemistry. Soluble cellular proteins were separated into periplasmic and cytoplasmic

fractions by chloroform shock (30). Whole cell, periplasmic, and cytoplasmic proteins were subjected to electrophoresis and immunoblotting. The blots were developed with anti-potato DAHP synthase, and as controls with anti-ß-galactosidase or anti-ribose binding proteins. Potato DAHP synthase and ß-galactosidase were detected in whole cells and in the cytoplasm but not in the periplasmic fraction, whereas ribose binding protein was detected in whole cells and in the periplasm but not in the cytoplasmic fraction. These results were confirmed by electron microscopic analysis of immunogold labelled thin sections of the bacterial cells (Fig. 2).

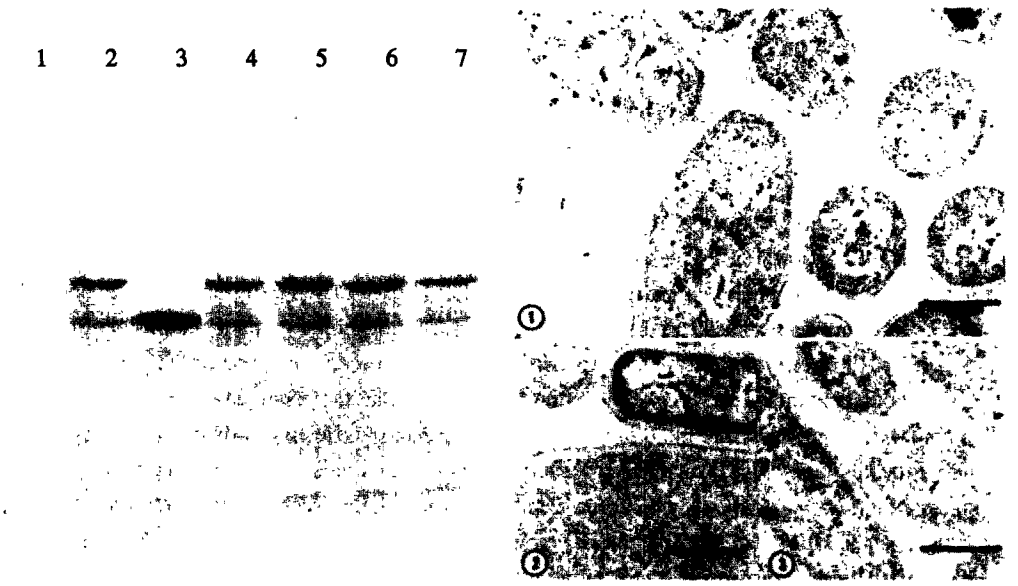

Fig. 1. (Left) Synthesis of potato DAHP synthase in *E.* coli. Proteins from extracts of HE628/pKK233-2 (lane 1), HE628/pLW3-210 (lanes 2 and 4-7), or tubers (lane 3) were subjected to electrophoresis and immunoblotting with anti-potato DAHP synthase. Lanes 4-7, cells grown in the presence of IPTG for 8, 6, 4, or 2 h, respectively.

Fig. 2. (Right) Immunocytochemical localization of potato DAHP synthase made in *E. coli* HE628/pLW3-210. *E. coli* cells of strain HE628/pLW3-210 (Figs. 2-1 and 2-2) or HE628/pKK233-2 (Fig 2-3) were fixed and thin sections labelled with anti-potato DAHP synthase (Figs. 2-1 and 2-3) or preimmune serum, followed by secondary immunogold antibodies; magnification x 57,000.

Molecular Properties of Potato DAHP Synthase Produced in E. coli - The potato DAHP synthase of *E. coli* HE628/pLW3-210 was purified. While this protein is retained on sulfopropyl Sephadex in 5 mM potassium phosphate, pH 7.0, the tuber enzyme is eluted from the matrix under these conditions (8). The HE628/pLW3-210 enzyme can be eluted from the matrix with a linear gradient of 25 to 200 mM potassium phosphate, pH 7.0. DAHP synthase purified through two chromatography steps is between 75 and 90% pure, as judged by a combination of silver stained and immunoblotted electropherograms. When the

protease inhibitor phenylmethylsulfonyl fluoride is omitted from buffers used in the purification procedure, only the 50 kD species is obtained; the "processing" of the larger species is apparently inhibited by the fluoride.

We examined the effects of aromatic amino acids and metal ions on enzyme activity of potato DAHP synthase produced by HE628/pLW3-210. The *E. coli* synthesized potato enzyme is only slightly activated by Mn^{2+} and tryptophan, much less than the enzyme from potato tuber. Also, a pronounced difference between the two enzymes is seen when the effect of Co^{2+} is tested: the *E. coli* synthesized potato enzyme is significantly inhibited, while the tuber enzyme is activated by this metal ion.

The quaternary structure of potato DAHP synthase synthesized by *E. coli* was examined by diimidate cross-linking. The enzyme subunits are best cross-linked when the distance between the functional group is at least five carbon atoms or about 8.5 Å. Dimers, trimers, and tetramers were found. Cross-linked enzyme retains about 75% of its activity. The tuber enzyme, crosslinked under the same conditions, exists predominantly as a dimer, in agreement with the MW of 110,000 estimated for the native tuber enzyme by gel filtration (8).

Discussion

A cDNA encoding a potato DAHP synthase has been expressed in *E. coli*. The cDNA complements an *E. coli* mutant void of DAHP synthase, despite the low amino acid sequence identity between bacterial DAHP synthases and the potato enzyme (10). *E. coli* strain HE628/pLW3-210, that produces potato DAHP synthase, grows very poorly in the presence of IPTG. Also, immunoblots of total cell protein from HE628/pLW3-210 or enzyme assays show no evidence for the expected induction of the potato enzyme by IPTG. The poor growth and the lack of induction may indicate that the potato enzyme is only tolerated in limited amounts. Since feedback inhibition is the major mechanism affecting carbon flow through the shikimate pathway in *E. coli* (3), and since the potato DAHP synthase is not feedback inhibited by aromatic amino acids, the potato enzyme may be detrimental to the general physiology of the bacterium. Interestingly, an *E. coli* strain that contains functional copies of all three genes encoding bacterial DAHP synthases and that was transformed with plasmid pLW3-210 produces no detectable potato DAHP synthase enzyme activity, and immunoblots of total protein reveal only small peptides that cross-react with anti-potato DAHP synthase. Presumably, the potato DAHP synthase is produced, then rapidly degraded, when its function is not required for growth.

Expression of the potato cDNA in *E. coli* results in a protein that contains a putative transit sequence for import into chloroplasts or other plastids. The potato DAHP synthase produced in *E. coli* appears as two species, one may correspond to the full-length product of the open reading frame, while the other is comparable in size to the purified DAHP synthase of potato tuber (8). The lower MW species could represent a product of the cDNA open reading frame without the plastid transit sequence.

The products encoded by the cDNA were localized in *E. coli* by cell fractionation and by electron microscopy of immunogold labelled thin sections. Both full-length and processed potato DAHP synthase are cytosolic proteins in *E. coli*, unlike acetolactate synthase from *Arabidopsis thaliana* that has recently been synthesized in this bacterium (31). The cDNA for acetolactate synthase also encodes a preenzyme, and the plastid transit sequence is apparently processed by *E. coli*; however, all of the enzyme activity was found in the periplasm. Presumably, the bacteria recognize this plastid transit sequence as an export signal,

while that is not the case for the DAHP synthase transit sequence. The amino terminus of DAHP synthase has a net positive charge, characteristic of plastid transit sequences, but positively charged residues inhibit protein transport across membranes in *E. coli* (32). Therefore, cleavage of the putative plastid transit sequence from the full-length potato DAHP synthase is probably due to nonspecific cytosolic proteases.

Since both long and processed forms of the cDNA product are present in *E. coli*, it is not clear if both are enzymatically active. Both forms are eluted together from both phosphocellulose and sulfopropyl matrices. Phosphocellulose is an affinity matrix for proteins that bind phosphorylated sugars (33), such as the substrates and the product of DAHP synthase. Thus, both forms of the enzyme may be active, since they both bind to phosphocellulose. A second explanation for the co-purification of the two forms could be the formation of hetero-oligomers. The net positive charge found in the amino terminus of the full-length cDNA product may be responsible for the retention of the enzyme by the sulfopropyl matrix. The enzyme that elutes as one species from this matrix contains both forms, again indicative of hetero-oligomers.

An examination of the quaternary structure of the enzyme produced by *E. coli* shows that at least a portion of the protein exists as a tetramer, in contrast to the DAHP synthase from potato tubers that is a dimer (8). Full-length and processed forms of potato DAHP synthase keep their nearly 1:1 ratio during cross-linking, another indication of hetero-oligomers.

The DAHP synthase encoded by the cloned cDNA cross-reacts with antibodies raised against the tuber enzyme, and both enzymes are activated by Mn^{2+}. However, there are subtle differences between these two DAHP synthases. Unlike the tuber enzyme, the enzyme synthesized in *E. coli* is retained by a sulfopropyl matrix at low ionic strength. The *E. coli* synthesized enzyme forms tetramers, while the tuber enzyme is a dimer. The effects of aromatic amino acids and metal ions are also slightly different for the two enzymes. These differences may be due to the presence of the plastid transit sequence in some subunits of the *E. coli* synthesized enzyme or to posttranslational modifications of the tuber enzyme that do not occur in *E. coli*. A third possibility is that the cDNA encoded enzyme is closely related to, but not identical with the tuber enzyme. A more detailed protein analysis is required to settle the question.

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References

1. Herrmann, K.M. *Amino Acids: Biosynthesis and Genetic Regulation*, Herrmann, K.M. Somerville, R.L. Eds; Addison Wesley, Reading, MA. **1983**, pp. 301-322.

2. Pittard, J. . *Escherichia coli* and *Salmonella typhimurium*: *cellular and molecular biology*, Neidhardt, F.C., Ingraham, J.L., Eds; American Society for Biology, Washington, D.C. **1987**, pp. 368-394.

3. Ogino, T.; Garner, C.C.; Markley, J.L.; Herrmann, K.M. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, 79:5828-5832.

4. Rothe, G.M.; Maurer, W.; Mielke, C. *Ber. Deutsch. Bot. Ges.* **1976**, 89:163-173.

5. Suzich, J.A.; Dean, J.F.D.; Herrmann, K.M. *Plant Physiol.* **1985**, 79:765-770.

6. Ganson, R.J.; d'Amato, T.A.; Jensen, R.A. *Plant Physiol.* **1986**, 82:203-210.

7. Morris, P.F.; Doong, R.L.; Jensen, R.A. *Plant Physiol.* **1989**, 89:10-14.
8. Pinto, J.E.B.P.; Suzich, J.A.; Herrmann, K.M. *Plant Physiol.* **1986**, 82:1040-1044.
9. Muday, G.K.; Herrmann, K.M. *Plant Physiol.* **1992**, 98:496-500.
10. Dyer, W.E.; Weaver, L.M.; Zhao, J.; Kuhn, D.; Weller, S.C.; Herrmann, K.M. *J. Biol. Chem.* **1990**, 265:1608-1614.
11. Keegstra, K.; Olsen, L.J. *Annu. Rev. Plant Physiol. Plant Molec. Biol.* **1989**, 40:471-501.
12. Pinto, J.E.B.P.; Dyer, W.E.; Weller, S.C.; Herrmann, K.M. *Plant Physiol.* **1988**, 87:891-893.
13. Herrmann, K.M.; Pinto, J.E.B.P.; Weaver, L.M.; Zhao, J. *FEBS-NATO Adv. Study Inst. Plant Mol. Biol.* 2, **1990**, pp. 729-735.
14. Yanisch-Perron, C.; Vieira, J.; Messing, J. *Gene* **1985**, 33:103-119.
15. Garner, C.C.; Herrmann, K.M. *J. Biol. Chem.* **1985**, 260:3820-3825.
16. Amann, E.; Brosius, J. *Gene* **1985**, 40:183-190.
17. Ausubel, F.M.; Brent, R.; Kingston, R.E.; Moore, D.D.; Siedman, J.G.; Smith, J.A.; Struhl, K. *Current Protocols in Molecular Biology*. **1987**, Green Publishing Associates and Wiley-Interscience, New York, NY.
18. Vogel, H.J.; Bonner, D.M. *J. Biol. Chem.* **1956**, 218:97-106.
19. Sayers, J.R.; Schmidt, W.; and Eckstein, F. *Nucleic Acids Res.* **1985**, 16:791-802.
20. Sanger, F.; Nicklen, S.; Coulson, A.R. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, 74:5463-5467.
21. Laemmli, U.K. *Nature*. **1970**, 227:680-685.
22. Wray, W.; Boulikas, R.; Wray, V.P.; Hancock, R. *Anal. Biochem.* **1981**, 118:197-203.
23. Burnette, W. N. *Anal. Biochem.* **1981**, 112:195-203.
24. Johnson, D.A.; Gautsch, J.W.; Sportsman, J.R.; Elder, J.H. *Gene Anal. Tech.* **1984**, 1:3-8.
25. Schoner, R.; Herrmann, K.M. *J. Biol. Chem.* **1976**, 251:5440-5447.
26. Bradford, M.M. *Anal. Biochem.* **1976**, 72:148-254.
27. Davies, G.E.; Stark, G.R. *Proc. Natl. Acad. Sci. U.S.A.* **1970**, 66:651-656.
28. Hadju, J.; Dombradi, V.; Bot, G.; Friedrich, P. *Biochemistry* **1979**, 18:4037-4041.
29. Shultz, J.; Hermodson, M.A.; Garner, C.C.; Herrmann, K.M. *J. Biol. Chem.* **1984**, 259: 9655-9661.
30. Ames, F.F.; Prody, C.; Kustu, S. *J. Bacteriol.* **1984**, 160:1181-1183.
31. Smith, J.K.; Schloss, J.V.; Mazur, B.J. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, 86:4179-4184.
32. Laws, J.K.; Dalbey, R.E. *EMBO J.* **1989**, 8:2095-2099.
33. Scopes, R.K. *Protein Purification - Principles and Practice*, Springer Verlag, New York, NY, **1987**, p. 127.