Characterization of Plant L-Isoaspartyl Methyltransferases That May Be Involved in Seed Survival: Purification, Cloning, and Sequence Analysis of the Wheat Germ Enzyme[†,‡]

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ABSTRACT: Protein carboxyl methyltransferases (EC 2.1.1.77) that catalyze the transfer of a methyl group from *S*-adenosylmethionine to L-isoaspartyl and D-aspartyl residues in a variety of peptides and proteins are widely, but not universally, distributed in nature. These enzymes can participate in the repair of damaged proteins by facilitating the conversion of abnormal L-isoaspartyl residues to normal L-aspartyl residues. In this work, we have identified L-isoaspartyl methyltransferase activity in a variety of higher plant species and a green alga. Interestingly, the highest levels of methyltransferase were located in seeds, where the problem of spontaneous protein degradation may become particularly severe upon aging. The wheat germ methyltransferase was purified as a monomeric 28 000-Da species by DEAE–cellulose chromatography, reverse ammonium sulfate gradient solubilization, and gel filtration chromatography. The purified enzyme recognized a variety of L-isoaspartyl-containing peptides, but did not recognize two D-aspartyl-containing peptides that are substrates for the mammalian enzyme. The partial amino acid sequence was utilized to design oligonucleotides to isolate a full-length cDNA clone, pMBM1. Its nucleotide sequence demonstrated an open reading frame encoding a polypeptide of 230 amino acid residues with a calculated molecular weight of 24 710. This sequence shares 31% identity with the L-isoaspartyl methyltransferase from *Escherichia coli* and 50% identity with the L-isoaspartyl/D-aspartyl methyltransferase from human erythrocytes. Such conservation in sequence is consistent with a fundamental role of this enzyme in the metabolism of spontaneously damaged polypeptides.

The recognition and repair or metabolism of spontaneously altered proteins may play an important role in limiting the aging process in cells and tissues. One enzyme that appears to play an important role in this process is the L-isoaspartate (D-aspartate) *O*-methyltransferase (EC 2.1.1.77) active on a variety of peptides and proteins. This cytosolic enzyme is present in many cells and catalyzes the transfer of a methyl group from *S*-adenosylmethionine to the α-carboxyl group of L-isoaspartyl residues (as well as the β-carboxyl group of D-aspartyl residues in humans), but not the β-carboxyl group of normal L-aspartyl residues (McFadden & Clarke, 1982; Murray & Clarke, 1984; Aswad, 1984; Clarke, 1985; Aswad & Johnson, 1987; Barten & O'Dea, 1990; Lowenson & Clarke, 1992). The substrates for this methyltransferase thus are not intrinsic residues of newly synthesized polypeptides, but arise from the spontaneous deamidation, isomerization, and racemization of L-asparaginyl and L-aspartyl residues (Geiger & Clarke, 1987; Stephenson & Clarke, 1989). The cumulative effect of these degradation reactions can inhibit the functional properties of such modified proteins (Johnson et al., 1987b; George-Nascimento et al., 1990). This methyltransferase can catalyze the first step in the conversion of L-isoaspartyl residues to normal L-aspartyl residues (McFadden & Clarke, 1987; Johnson et al., 1987a,b; Galletti et al., 1988; Lowenson & Clarke, 1991a), suggesting that repair of these damaged residues in vivo is possible. Recently, it has been shown that *Escherichia coli* mutants lacking the L-isoaspartyl methyltransferase survive poorly in stationary phase or under thermal and oxidative stresses (Li & Clarke, 1992b). This evidence suggests that the methyltransferase contributes to the long term survival of cells by preventing the accumulation of potentially inactive or labile proteins containing L-isoaspartyl residues (Li & Clarke, 1992b).

The similarity of this enzyme in Gram-negative bacteria and mammals (Fu et al., 1991; O'Conner & Clarke, 1985) is consistent with a fundamental role in cells. However, no methyltransferase activity has been found in several Gram-positive bacteria (Li & Clarke, 1992a) nor in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*,[1] suggesting that alternate pathways exist in some cells to metabolize proteins containing isomerized and racemized aspartyl residues. We were thus interested in asking whether representatives of the plant kingdom possess an L-isoaspartyl methyltransferase-mediated protein repair pathway or whether these organisms also utilize other strategies for dealing with spontaneous protein degradation. We were especially interested in reinvestigating the protein carboxyl methyltransferase activity reported earlier in wheat germ (Trivedi et al., 1982). The chemical nature of the groups modified by this enzyme was not identified, but its methyl acceptor activity on exogenous proteins had some similarities to that of the mammalian L-isoaspartyl/D-aspartyl methyltransferase. Preliminary studies by Johnson et al. (1991) indicate that wheat germ does, in fact, possess an activity capable of methylating at least one L-isoaspartyl-containing peptide.

In this work, we provide clear evidence that the L-isoaspartyl methyltransferase is present in a variety of plant species and

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[‡] The nucleotide sequence(s) reported in this paper has been submitted to the GenBank/EMBL Data Bank with accession number(s) L07941.

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[1] R. M. Kagan and S. Clarke, unpublished results.

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is found in the highest levels in seeds. In addition, we have purified the wheat germ L-isoaspartyl methyltransferase to homogeneity and characterized its interactions with L-isoaspartyl- and D-aspartyl-containing peptides and protein substrates. Finally, a comparison of the partial amino acid sequence of this enzyme with that of the deduced amino acid sequence of a cDNA clone suggests the presence of multiple similar genes and/or a high degree of genetic polymorphism for this enzyme in wheat.

EXPERIMENTAL PROCEDURES

Biological Materials. Fresh carrots, yellow corn, Romaine lettuce, green peas, white potatoes, spinach, cherry tomatoes, and alfalfa sprouts were purchased at a local distributor. Alfalfa seeds and raw wheat germ were from Rainbow Acres, Inc. (Los Angeles, CA), while soybean seeds were from Arrowhead Mills, Inc. (Hereford, TX). Winter wheat (*Triticum aestivum* cultivar Augusta) seeds were provided by Dr. Robert Forsberg of the University of Wisconsin (Madison, WI). Danver Half Long carrot seeds, Golden Jubilee corn seeds, Romaine lettuce seeds, sugar snap pea seeds, New Zealand spinach seeds, and Bonny Best tomato seeds were from the Chas. H. Lilly Co. (Portland, OR). A cytosolic fraction of *Chlamydomonas reinhardtii* (Wt strain 2137) was provided by Drs. Greg Howe and Sabeeha Merchant of the University of California at Los Angeles (Howe & Merchant, 1992).

Preparation of Plant Cytosol. Crude cytosol was extracted from the plant tissues by homogenization using a mortar and pestle. In a chilled mortar, liquid nitrogen was poured over plant tissue (typically, 20 g of fresh tissue or 5 g of seeds) until the tissue was completely frozen. To remove undesirable polyphenol oxidases potentially released from the tissue upon homogenization, 3 g of hydrated PVPP[2] (Loomis & Battaile, 1966) was thoroughly mixed with the frozen tissue before the tissue was ground with a pestle. Extraction buffer (20 mL of 100 mM sodium HEPES (pH 7.5), 10 mM 2-mercaptoethanol, 1 μM leupeptin, 1 mM phenylmethanesulfonyl fluoride, 10 mM sodium hydrosulfite, and 10 mM sodium metabisulfite at 4 °C) was added to the mortar, and the slurry was ground further. The resulting crude homogenate was pressed through four layers of cheesecloth and then centrifuged at 2200*g* for 30 min at 4 °C to remove the insoluble PVPP and undisrupted plant material. The resulting supernatant was centrifuged further at 172200*g* for 60 min at 4 °C and then filtered through two layers of Miracloth (Calbiochem) to remove the floating lipid layer. This fraction, identified as crude cytosol, was stored at −80 °C and utilized as the source of methyltransferase.

Methylation Assay. Methyltransferase activity was identified using a vapor-phase diffusion assay that quantitates the number of radiolabeled methyl groups transferred from *S*-adenosyl-L-[*methyl*-^{14}C]methionine to a peptide substrate by quantitating the release of [^{14}C]methanol resulting from the hydrolysis of base-labile methyl esters. In a total reaction volume of 40 μL, 12 μL of enzyme preparation was incubated with 10 μM *S*-adenosyl-L-[*methyl*-^{14}C]methionine (ICN Biomedicals, 50 mCi/mmol), 500 μM peptide substrate, and 0.33 M sodium HEPES, pH 7.5. Peptide substrates [VYP-(L-isoAsp)-HA, KASA-(L-isoAsp)-LAKY, AA-(L-isoAsp)-F-NH_2, VYG-(D-Asp)-PA, and KASA-(D-Asp)-LAKY] were synthesized by Dr. Janis Young at the UCLA Peptide Synthesis Facility and characterized as described previously (Lowenson & Clarke, 1991b, 1992). Alternatively, during the purification of the wheat germ methyltransferase (see below), samples were assayed in buffer containing a final concentration of 0.2 M sodium citrate, pH 6.0. In either case, incubations were performed at 25 °C for 60 min. Each reaction was then quenched with 40 μL of 0.2 M NaOH and 1% (w/v) SDS and vortexed, and a 60-μL aliquot was spotted onto a 1.5 × 8 cm pleated filter paper (Bio-Rad no. 165-090) and placed in the neck of a 20-mL scintillation vial containing 5 mL of Bio-Safe II (RPI, Mount Prospect, IL) counting fluor. The vials were capped, and [^{14}C]methanol was allowed to diffuse from the paper through the vapor phase to the fluor, while the nonvolatile ^{14}C radioactivity remained on the paper. After 2 h at room temperature, the paper was removed from the necks of the vials and the vials were counted.

Protein Determination. A modification of the Lowry procedure (Bailey, 1967) was used to determine the concentration of protein after precipitation with 1 mL of 10% (w/v) trichloroacetic acid.

Preparation of Wheat Germ Cytosol for Enzyme Purification. Raw wheat germ (150 g) was suspended in 750 mL of buffer (20 mM sodium borate (pH 9.3), 5 mM EDTA, 2.4 mM 2-mercaptoethanol, and 25 mM NaCl) and stirred for 30 min at 4 °C. The slurry was then squeezed through four layers of cheesecloth, and the resulting crude homogenate (585 mL) was centrifuged at 7000*g* for 60 min at 4 °C to remove membrane and cell debris. The supernatant (520 mL) was poured through two layers of Miracloth to filter the floating lipid layer.

Mono Q Anion Exchange Chromatography. Fractions containing L-isoaspartyl methyltransferase from several Sephacryl S-200 gel filtration columns were pooled and then dialyzed (Spectropor, cutoff 3500 Da) in buffer A (20 mM Tris–acetate (pH 7.0), 0.2 mM EDTA, 15 mM 2-mercaptoethanol, and 10% glycerol). Dialyzed methyltransferase (0.6 mg of protein) was fractionated on a Mono Q HR 5/5 anion exchange (Pharmacia) column (5 mm diameter × 50 mm resin height, 1 mL) previously equilibrated with buffer A. One-minute fractions were collected at a flow rate of 0.5 mL/min. The loaded column was washed isocratically with buffer A for 15 min followed by a linear gradient of 0 to 100% buffer B (20 mM Tris–acetate (pH 7.0), 0.2 mM EDTA, 15 mM 2-mercaptoethanol, 10% glycerol, and 1 M sodium acetate) over 60 min. The column effluent was monitored at 280 nm. Typically, methyltransferase activity was detected in fractions 43–44. Fractions containing active methyltransferase were pooled and used for enzymological studies.

Reverse-Phase HPLC. Homogeneous methyltransferase suitable for sequence analysis was obtained by reverse-phase HPLC of the enzyme purified through the Sephacryl S-200 step. Fractions were loaded onto a Vydac C-4 column (1 cm i.d. × 25 cm, 300-Å pore, 5-μm spherical silica support) equilibrated with 65% solvent B and eluted with a linear gradient of 65–80% solvent B over 45 min at 3.0 mL/min flow rate, where solvent A is 0.1% trifluoroacetic acid in water (w/v) and solvent B is 0.1% trifluoroacetic acid in 99% methanol/0.9% water (w/v/v). The column effluent was monitored at 280 nm as 1-min fractions were collected. Volatile reagents were removed from the fractions in a Savant Speedvac apparatus, and then these fractions were subjected

[2] Abbreviations: PVPP, polyvinyl–polypyrrolidone; SDS, sodium dodecyl sulfate; EDTA, ethylenedinitrilotetraacetic acid; HPLC, high-performance liquid chromatography; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PCR, polymerase chain reaction; dNTPs, deoxynucleotide triphophates; bp, base pair(s).

to SDS–polyacrylamide slab gel electrophoresis and silver staining (Jones, 1990). The methyltransferase eluted at about 40 min as a single polypeptide band with an apparent molecular mass of 28 000 Da.

Trypsin Digestion. Reverse-phase HPLC-purified methyltransferase was digested with trypsin as described by Gilbert et al. (1988). Methyltransferase (0.9–1.8 nmol) was incubated with freshly prepared 0.2 M ammonium bicarbonate (0.2 mL) (pH 7.82), and 1 μg of trypsin [L-1-(tosylamido)-2-phenylethyl chloromethyl ketone treated from bovine pancreas (Sigma type XIII)] in the ammonium bicarbonate buffer and mixed thoroughly. Digestion proceeded for 16 h at room temperature. The resulting peptides were separated by HPLC as described below.

Staphylococcus aureus V8 Protease Digestion. HPLC-purified methyltransferase (approximately 3 nmol) was incubated with freshly prepared 0.1 M ammonium bicarbonate (0.1 mL) (pH 7.73), and 0.6 nmol of *S. aureus* V8 protease (Sigma, type XVII, specific activity 500 units/mg) and mixed thoroughly. Digestion was carried out at 37 °C for 12 h. The resulting peptides were separated by HPLC as described below.

HPLC Peptide Mapping. Peptides were recovered by reverse-phase HPLC using a Vydac C-18 column (4.6 mm i.d. × 250 mm, 300-Å pore, 5-μm spherical silica support). The column was equilibrated with 100% solvent A and eluted with a linear gradient of 0–70% solvent B over 90 min at a 1.0 mL/min flow rate, where solvent A is 0.1% trifluoroacetic acid in water (w/v) and solvent B is 0.1% trifluoroacetic acid in 99% acetonitrile/0.9% water (w/v/v). The column effluent was monitored at both 214 and 280 nm to distinguish tryptophan-containing peptides from other peptides.

Amino Acid Sequence Analysis. Sequence analysis was performed by Dr. Audree Fowler at the UCLA Protein Microsequencing Facility with an Applied Biosystems Model 470A gas-phase sequencer with on-line HPLC detection (120 PTH analyzer).

Synthetic Oligonucleotide Probes. Oligonucleotide probes were synthesized using β-cyanoethyl *N,N*-diisopropylphosphoramidite chemistry in a Gene Assembler Plus DNA synthesizer (Pharmacia LKB Biotechnology). An oligonucleotide representing the T7 promoter of the pBluescript SK± phagemid, T7 [DMT-TAATACGACTCACTATAGGG], and three degenerate oligonucleotides, MB1 [TCTGG(G/A)AT(G/A)TG(C/T)TC(G/A)ATNCCCAT], MB3 containing an *Eco*RI linker [CTCGAATTCTA(C/T)(C/T)TNAA(G/A)CA(G/A)TA(C/T)GGNGT], and MB4 containing a *Hin*dIII linker [TCAAAGCTTTT(G/A)TC(T/G/A)ATNAC(C/T)TGNAG], were synthesized for use as probes and as primers in PCR amplification of a wheat cDNA library (described below). The primers were purified by size exclusion chromatography using Bio-Spin 6 columns (Bio-Rad).

PCR Amplification of a Wheat cDNA Library. An amplified λ-ZAP cDNA expression library (5×10^8 pfu/mL) prepared with poly(A)$^+$ RNA isolated from 48-h-etiolated wheat seedlings and tailed with an octanucleotide (GGAATTCC) containing an *Eco*RI site (Hatfield et al., 1990) was obtained from Dr. Richard Vierstra of the University of Wisconsin (Madison, WI). The degenerate primer MB3 and the pBluescript primer T7 were used to amplify a portion of the wheat L-isoaspartyl methyltransferase cDNA. The PCR reaction mixture contained 2.5 mM $MgCl_2$, 100 μg/mL bovine serum albumin, 200 μM dNTPs, 100 pmol of MB3 primer, 20 pmol of T7 primer, and 5 μL of λ phage lysate in reaction buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0), and 0.1% Triton X-100, Promega) in a total volume of 100 μL. The reaction tubes were heated to 98 °C for 5 min to rupture the phage and release the packaged DNA. The tubes were then cooled to 95 °C for 5 min prior to the addition of 2.5 units of *Taq* DNA polymerase (*Thermus aquaticus* DNA polymerase, Promega). Thirty-five amplification cycles were performed (denaturation at 95 °C for 1 min, primer annealing at 48 °C for 1 min, and primer extension at 72 °C for 2 min). PCR products were separated by gel electrophoresis and analyzed by Southern hybridization using the ^{32}P-end-labeled MB1 oligonucleotide as a probe (Sambrook et al., 1989). An 850-bp PCR product was identified and gel-purified using Magic PCR Preps DNA purification system (Promega). This fragment was then utilized as template DNA in a similar PCR reaction using degenerate primers MB3 and MB4. A 600-bp PCR product was gel-purified, and its sequence was determined by dideoxy chain-termination sequencing using the Δ*Taq* Cycle-Sequencing Kit (United States Biochemical; Sanger et al., 1977; Tabor & Richardson, 1989).

cDNA Cloning and Molecular Characterization of L-*Isoaspartyl Methyltransferase from Wheat.* The wheat cDNA library described above was screened using the radiolabeled 600-bp PCR product coding for a region of the wheat L-isoaspartyl methyltransferase cDNA. The library was propagated in *E. coli* BB4 cells and then plated in NZY (Becton Dickinson Microbiology Systems) top agar on 150-mm NZY plates (50000 pfu/plate). Plaques were transferred (Sambrook et al., 1989) in duplicate onto Immobilon-NC membranes (Millipore). The 600-bp PCR product was labeled to a specific activity of 2×10^9 cpm/μg with [α-^{32}P]dATP (ICN Biomedicals) using a Prime It II random priming kit (Stratagene). Membranes were prewashed in 0.75 M NaCl, 0.83 M sodium citrate, 0.5% (w/v) SDS, and 1 mM EDTA (pH 8.0) at 42 °C for 60 min and then prehybridized at 65 °C for 3 h in 0.75 M NaCl, 50 mM sodium phosphate (pH 7.4), 5 mM EDTA, 5× Denhardt's solution (Sambrook et al., 1989), 0.5% (w/v) SDS, and 100 μg/mL sonicated and denatured salmon sperm DNA. Hybridization was performed at 65 °C overnight by incubating membranes with denatured probe and fresh solution. Membranes were washed twice in 0.9 M NaCl, 1 M sodium citrate (pH 7.0), and 0.1% (w/v) SDS at room temperature for 15 min, with an additional 15-min wash at 65 °C in 0.3 M NaCl, 0.3 M sodium citrate (pH 7.0), and 0.1% (w/v) SDS. After air-drying, the membranes were autoradiographed by exposure to Hyperfilm-MP (Amersham) for 48 h. Screening produced one positive signal. The clone for this plaque was isolated by subsequent screenings and converted to a pBluescript phagemid using helper phage in *E. coli* XL-1 Blue cells (Short et al., 1988). *Eco*RI restriction endonuclease digestion of the pBluescript phagemid generated a 952-bp fragment, which was resolved by agarose gel electrophoresis. The 952-bp *Eco*RI fragment was isolated from the gel using the Magic PCR Prep DNA purification system (Promega) and ligated into the *Eco*RI site of pUC19 (Yanisch-Perron et al., 1985) to give the pMBM1 plasmid. This construct was then used to transform subcloning efficiency DH5α competent cells as described by the manufacturer (Bethesda Research Laboratories). Transformed cells selected by ampicillin resistance were harvested and utilized for plasmid preparation using Qiagen-tip 500 columns as directed by the manufacturer (QIAGEN). Plasmids were analyzed by restriction analysis and Southern hybridization using the radiolabeled 600-bp PCR product as a probe.

Sequencing of the DNA Insert of pMBM1. The DNA sequence of the 952-bp wheat cDNA clone insert was

Table I: Occurrence of L-Isoaspartyl Methyltransferase Activity in the Soluble Fraction of Plants

species	plant material	methyltransferase activity[a] (pmol/min/mg of protein) endogenous substrates	L-isoAsp peptide
green alga			
C. reinhardtii	vegetative cells[b]	0.15 ± 0.00	0.43 ± 0.01
monocots			
corn	fresh kernels	0.30 ± 0.04	1.46 ± 0.11
	dry kernels	0.71 ± 0.03	6.86 ± 0.42
wheat	embryos (germ)	0.33 ± 0.05	14.0 ± 0.14
	kernels	0.39 ± 0.01	4.36 ± 0.09
dicots			
alfalfa	seedlings	0.35 ± 0.01	0.47 ± 0.02
	seeds	0.34 ± 0.03	3.42 ± 0.25
carrot	roots	0.96 ± 0.07	2.64 ± 0.28
	seeds	0.44 ± 0.03	1.37 ± 0.04
lettuce	leaves	0.27 ± 0.00	0.29 ± 0.01
	seeds	0.14 ± 0.01	0.66 ± 0.01
pea	fresh seeds	0.24 ± 0.04	1.31 ± 0.05
	dry seeds	0.12 ± 0.00	1.79 ± 0.10
potato	roots	0.19 ± 0.01	1.04 ± 0.00
soybean	seeds	0.12 ± 0.00	0.69 ± 0.03
spinach	leaves	0.22 ± 0.01	1.10 ± 0.03
	seeds	2.16 ± 0.12	2.60 ± 0.05
tomato	fruit	2.90 ± 0.16	3.03 ± 0.17
	seeds	1.17 ± 0.02	8.07 ± 0.15

[a] Methylation assays were performed in triplicate as described in the Experimental Procedures. Endogenous substrates represent the methylatable polypeptides in the soluble fraction that give base-labile volatile radioactivity. The L-isoAsp peptide substrate was VYP-(L-isoAsp)-HA and was used at a concentration of 500 μM. Methyltransferase activity detected with the L-isoaspartyl peptide includes the endogenous methyltransferase activity. [b] *Chlamydomonas reinhardtii* vegetative cells were isolated as described by Howe and Merchant (1992).

determined by dideoxy chain-termination sequencing using the Sequenase Version 2.0 kit (United States Biochemical; Sanger et al., 1977; Tabor & Richardson, 1989) and [α-^{35}S]-dATP (New England Nuclear Research Products) as described by Fu et al. (1991). Oligonucleotides were designed using the sequence data obtained from the 600-bp PCR product.

RESULTS

Identification of L-*Isoaspartyl Methyltransferase in Plants.* Representatives from both classes of angiosperms as well as a green alga were surveyed for the presence of L-isoaspartyl methyltransferase. Crude cytosol was isolated from different types of plant material and then assayed for methyltransferase activity using the L-isoaspartyl-containing peptide, VYP-(isoAsp)-HA, which has been shown to be an excellent peptide substrate for the human erythrocyte methyltransferase (K_m = 0.29 μM; Lowenson & Clarke, 1991b). Endogenous cytosolic polypeptides are also potential methyl acceptors; therefore, parallel experiments were conducted in the presence and absence of the peptide substrate (Table I). Peptide-dependent L-isoaspartyl methyltransferase was found in the vegetative cells of the green alga *C. reinhardtii*, demonstrating its presence in at least one species in the Kingdom Protista. In the Kingdom Plantae, methyltransferase activity was detected in both classes of the angiosperms, the monocots and the dicots. The level of activity in different tissues varied considerably. Of the species assayed, the highest specific activity of the methyltransferase was found in wheat embryos. In contrast, almost no detectable L-isoaspartyl peptide-specific methyltransferase activity was found in the leaves of lettuce or the fruits of tomato. Significantly, we found high levels of methyltransferase activity in the seeds of all the plants assayed, including corn, alfalfa, lettuce, pea, spinach, and tomato, as well as in the roots of carrots and potatoes. The specific activity of the enzyme in plant seeds (0.66–14.0 pmol/min/mg) is comparable to the levels found in *E. coli* (1–2.5 pmol/min/mg; Fu et al., 1991) and human erythrocytes (1.9–9.4 pmol/min/mg; Ota et al., 1988; Gilbert et al., 1988).

Purification of L-*Isoaspartyl Methyltransferase from Wheat Germ.* Because of its high methyltransferase activity, we chose the wheat system to study the function of this enzyme in plants. Our purification strategy was based on the partial purification of the protein carboxyl methyltransferase reported by Trivedi et al. (1982). Methyltransferase was purified from a cytosolic fraction of raw wheat germ (Figure 1). This material was first fractionated by DEAE–cellulose chromatography at pH 9.3. Active fractions were then saturated to 80% with ammonium sulfate in the presence of a protein carrier, Celite 545, poured into a column, and fractionated by reverse ammonium sulfate gradient solubilization at room temperature. Active fractions containing approximately 26–31% saturated ammonium sulfate were further purified by Sephacryl S-200 gel filtration chromatography. Surprisingly, the L-isoaspartyl methyltransferase eluted in a highly purified state in a fraction nearly corresponding to the total volume of the column. In this step, the methyltransferase was not fractionated on the basis of its size. We suggest that the methyltransferase associated with the Sephacryl S-200 resin through hydrophobic interactions due to a solvent effect created by the relatively high concentration of ammonium sulfate in the fractions (Belew et al., 1978). In the absence of ammonium sulfate, the methyltransferase eluted from the Sephacryl S-200 column in a position consistent with a monomeric molecular weight (Figure 2), along with numerous contaminating polypeptides. Thus, we attribute our success in obtaining a highly purified enzyme preparation from the Sephacryl S-200 gel filtration column to this unusual absorption phenomenon. The native molecular mass of the wheat germ methyltransferase was determined to be 33 000 Da using the calculated distribution coefficients (K_d) for the protein standards from two independent gel filtration experiments (Figure 2, insert).

The overall purification of the L-isoaspartyl methyltransferase from wheat germ is summarized in Table II, and the typical polypeptide composition corresponding to each step in the purification is shown in Figure 3. The calculated molecular mass of the major polypeptide determined by SDS–polyacrylamide slab gel electrophoresis was 28 000 Da. We demonstrated that this polypeptide corresponds to the L-isoaspartyl methyltransferase by renaturing individual gel slices in the presence of Triton X-100 as described by Clarke (1981) (data not shown). The purity of this preparation was estimated at 86% from densitometry of the Coomassie-stained gel. The remaining minor polypeptide contaminants could be removed by an additional chromatography step. Dialyzed methyltransferase was loaded onto a Mono Q anion exchange column and eluted with a linear gradient of 0–1 M sodium acetate. Active methyltransferase eluted at approximately 0.5 M sodium acetate.

Characterization of L-*Isoaspartyl Methyltransferase from Wheat Germ.* Methyltransferase purified through the Mono Q step [12 500 pmol/min/mg at pH 7.5 with the VYP-(isoAsp)-HA peptide as the substrate] was used to study the specificity of the wheat germ enzyme. Like the *E. coli* and human erythrocyte methyltransferases, the wheat germ enzyme efficiently methylates L-isoaspartyl residues in synthetic peptides (Figure 4). However, the affinity of the wheat

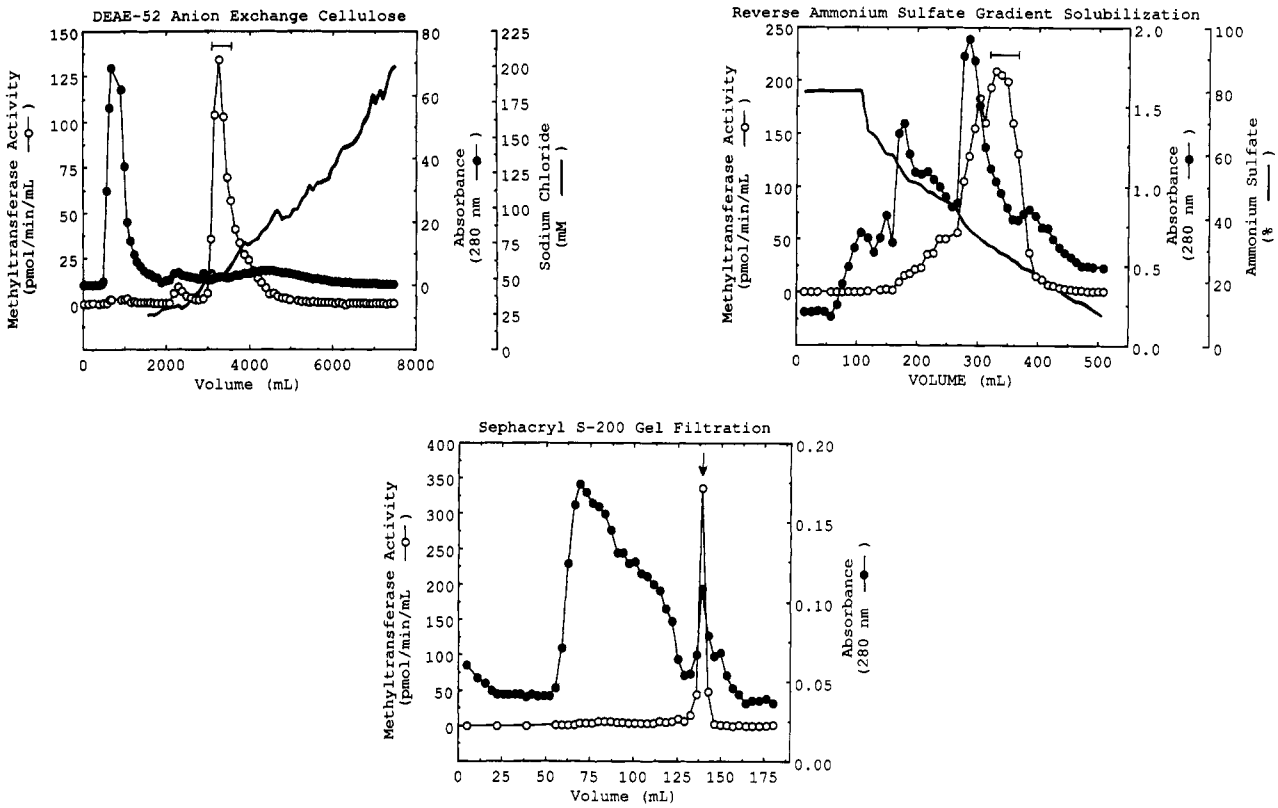

FIGURE 1: Purification of wheat germ L-isoaspartyl methyltransferase.Crude wheat germ cytosol (515 mL, 30 mg of protein/mL) was loaded onto a DE-52 (Whatman) column (9 cm diameter × 13 cm resin height, 827 mL) which was previously equilibrated at 4 °C with buffer (20 mM sodium borate (pH 9.3), 5 mM EDTA, 2.4 mM 2-mercaptoethanol, and 25 mM NaCl). Two-minute fractions were collected at an average flow rate of 8–10 mL/min. The loaded column was washed isocratically with 1 L of buffer followed by a 6-L gradient of 25–200 mM NaCl in the above buffer. The protein profile and the NaCl gradient were monitored by measuring absorbance at 280 nm and conductivity, respectively, in the corresponding fractions. Every fifth fraction was assayed for L-isoaspartyl methyltransferase using VYP-(L-isoAsp)-HA as the peptide substrate. One peak of methyltransferase activity was pooled (fractions 80–110, 600 mL, see brackets) and further purified by reverse ammonium sulfate gradient solubilization as described by King (1972). The pH of the DE-52 pooled material was adjusted to 8.38 with 20 mL of 1 M Tris-HCl (pH 7.97). Then 15.62 g of Celite 545 (Baker Analyzed Reagent, 11 g of Celite/1 g of protein) was added to this material (approximately 1.42 g of protein based on absorbance at 280 nm) to act as a protein carrier. Solid ammonium sulfate (338.28 g) was added with stirring to 80% saturation (56.1 g of ammonium sulfate/100 mL initial volume) in a 30-min period at room temperature, and then stirring was continued for an additional 45 min. This Celite mixture containing precipitated cytosolic proteins was poured into a 3 cm diameter × 19 cm column and packed with the aid of a peristaltic pump at room temperature. The column was washed isocratically with 150 mL (approximately two column vol) of 80% saturated ammonium sulfate solution containing 0.05 M Tris-HCl (pH 7.97). Then the column was eluted with a 550-mL linear gradient decreasing from 80 to 0% saturation in ammonium sulfate. The flow rate of the gradient was approximately 0.6 mL/min, and 7.5-min fractions were collected. The percent of ammonium sulfate and protein in the corresponding fractions was determined by conductance and absorbance at 280 nm, respectively. Every second fraction was assayed for L-isoaspartyl methyltransferase using VYP-(L-isoAsp)-HA as the peptide substrate. Fractions (65–74), containing the highest specific activity of the methyltransferase, were pooled (95 mL, see brackets) and subsequently purified on a Sephacryl S-200 (Sigma) gel filtration column (2 cm diameter × 77.5 cm resin high, 243 mL). Buffer containing 20 mM Tris–acetate (pH 7.0), 0.2 mM EDTA, 15 mM 2-mercaptoethanol, and 10 mM NaCl was used to equilibrate and run the column at 4 °C. The flow rate of the column was maintained at 0.12 mL/min and 30-min fractions were collected. Every second fraction was assayed for L-isoaspartyl methyltransferase using VYP-(L-isoAsp)-HA as the peptide substrate. Absorbance at 280 nm was measured to determine the protein concentration of these fractions. Purified wheat germ L-isoaspartyl methyltransferase consistently eluted in one or two fractions, roughly corresponding to a fraction volume of 134–139 mL (see arrow).

germ methyltransferase for L-isoaspartyl peptides is lower than that of the human erythrocyte methyltransferase. We found that the K_m value of the wheat germ methyltransferase for the peptide VYP-(isoAsp)-HA is 51.7 μM, compared to 0.29 μM for the human erythrocyte methyltransferase (Lowenson & Clarke, 1991b). Similarly, the K_m value of the wheat germ enzyme for the peptide KASA-(isoAsp)-LAKY is 12.7 μM, compared to 0.52 μM for the human erythrocyte enzyme (Lowenson & Clarke, 1991b). Interestingly, the corresponding D-aspartyl peptides were not found to be substrates for the wheat germ methyltransferase (Figure 4) at concentrations up to 16 mM. These same substrates were methylated by the human erythrocyte enzyme, with K_m values of 2.7 mM for the peptide KASA-(D-Asp)-LAKY and 3.0 mM for the peptide VYP-(D-Asp)-HA (Lowenson & Clarke, 1992). The *E. coli* methyltransferase is also unable to catalyze the methylation of D-aspartyl-containing peptides under similar conditions (Fu et al., 1991). In contrast to the situation for the mammalian enzyme where ovalbumin is a methyl acceptor with Michaelis–Menten kinetics (K_m = 35 μM, and the V_{max} value is comparable to that obtained with L-isoaspartyl containing peptides; Lowenson & Clarke, 1991b), saturation was not observed with ovalbumin as a substrate for the wheat germ enzyme. At an ovalbumin concentration of 1.5 mM, the velocity was only 11% of that of an L-isoaspartyl-containing peptide. A similar situation was seen with γ-globulin where the velocity at 40 mg/mL was less than 5% of that of the peptide substrate. These latter results are similar to those observed with the partially purified enzyme of Trivedi et al. (1982) and suggest that there are differences in the abilities of the mammalian and wheat enzymes to recognize particular protein substrates. We speculate that the preferred substrates for the wheat germ methyltransferase will be those proteins found in the seed.

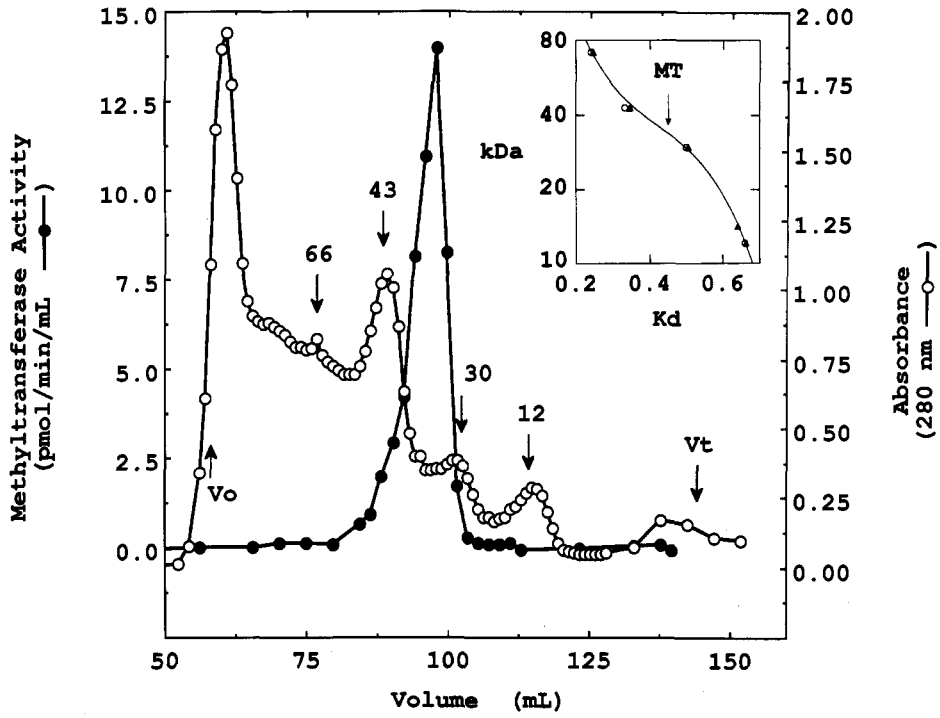

FIGURE 2: Native molecular weight determination of wheat germ L-isoaspartyl methyltransferase by gel filtration chromatography. A sample containing L-isoaspartyl methyltransferase (3 mL, 10.44 mg of protein/mL) purified by DEAE–cellulose chromatography was combined with molecular weight standards and separated on a Sephacryl S-200 column as described in the caption for Figure 1, except that 10 mM NaCl was deleted from the buffer and 10% glycerol was added. The column was calibrated using the following standards: 1.03 mg of blue dextran, 2000 kDa (V_0); 18 μg of malic dehydrogenase, 66 kDa (66); 10 mg of ovalbumin, 43 kDa (43); 0.93 mg of carbonic anhydrase, 29.5 kDa (30); 1.06 mg of cytochrome *c*, 12 kDa (12); and 0.9 pmol of L-[*methyl*-^{3}H]methionine (V_t, ICN Biomedicals, 77 Ci/mmol). Fractions were assayed for L-isoaspartyl methyltransferase using the peptide AA-(L-isoAsp)-F-NH_2 as described in the Experimental Procedures, except that reactions were incubated at 37 °C. Malic dehydrogenase and carbonic anhydrase enzyme assays were performed as described by Worthington (1988). Distribution coefficients (K_d) for the standards were calculated from two independent gel filtration experiments (see insert).

Table II: Purification of L-Isoaspartyl Carboxyl Methyltransferase from Wheat Germ Cytosol

sample	volume (mL)	total protein (mg)	total activity[a] (pmol/min)	% recovery	specific activity (pmol/min/mg)	purification
crude homogenate	585	19012	69205	100	3.64	1.0
crude cytosol (7000*g*)	520	15600	57616	83.3	3.69	1.0
DEAE–cellulose	600	600	52020	75.2	86.7	23.8
reverse ammonium sulfate gradient solubilization	45.3	24.9	10140	14.7	436.4	119.9
Sephacryl S-200	15.0	1.04	5025	7.3	4855.1	1333

[a] Measured at pH 6.0 using 500 μM VYP-(isoAsp)-HA as a methyl acceptor.

Since methyltransferase activity was detected in the absence of exogenous substrates in the wheat germ cytosol (Table I), it was evident that methyl-accepting substrates exist for the methyltransferase in vivo. Analysis of in vitro methylated endogenous polypeptides by SDS–polyacrylamide gel electrophoresis showed that a variety of polypeptides possessed base-labile methyl esters (Figure 5; cf. Trivedi et al., 1982). To verify that the methyltransferase activity detected in the crude cytosol is characteristic of the L-isoaspartyl methyltransferase, inhibitors of the L-isoaspartyl methyltransferase were used in the methylation assay. Incubation of a competitive peptide substrate, VYP-(L-isoAsp)-HA, and an inhibitor of the enzyme, *S*-adenosylhomocysteine, with wheat germ cytosol in independent methylation assays drastically reduced the methyl esterification of endogenous polypeptides (Figure 5), indicating that the endogenous methyltransferase activity is characteristic of the L-isoaspartyl methyltransferase.

We found that the pH optimum was about 7.5 using VYP-(isoAsp)-HA as the methyl-accepting peptide for the purified wheat germ methyltransferase (Figure 6). This value is more alkaline than the optimum previously measured for the methyltransferases from *E. coli* (pH 5.5; Clarke et al., 1980) and from rat and human erythrocytes (pH 6–7; Kim, 1974; cf. Trivedi et al., 1982). This pH optimum was somewhat dependent on the L-isoaspartyl peptide substrates used in the reactions. For example, the pH optimum for the purified wheat germ methyltransferase was 7.0 for the peptide VYP-(isoAsp)-CA and 8.0 for the peptide KASA-(isoAsp)-LAKY (data not shown). The pH optima of the methyltransferases from rat and human erythrocytes were also substrate dependent (Kim, 1974).

Amino Acid Sequence Determination by Tryptic and Staphylococcus aureus V8 Protease Mapping. Homogeneous methyltransferase suitable for amino acid sequence analysis was obtained by reverse-phase HPLC analysis as described in the Experimental Procedures. This material was digested with trypsin and *Staphylococcus aureus* V8 protease, and the resulting peptides were recovered by reverse-phase HPLC using a Vydac C-18 column. N-Terminal Edman sequencing was then performed on these peptides. The partial peptide sequence data obtained were used to generate oligonucleotide

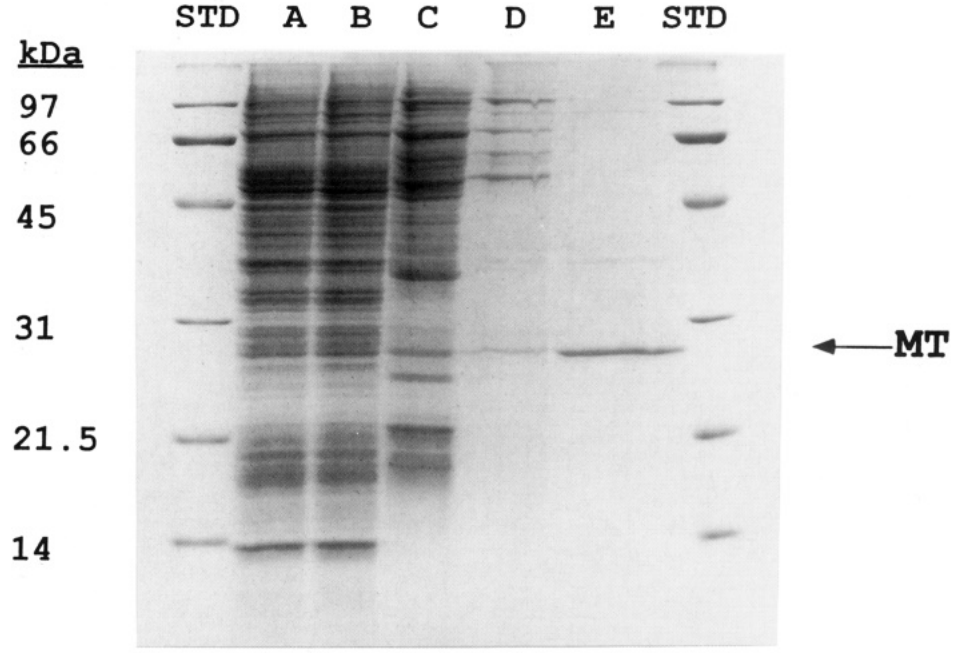

FIGURE 3: Polypeptide analysis of L-isoaspartyl methyltransferase from wheat germ. Active fractions containing methyltransferase from each purification step were analyzed by SDS–polyacrylamide slab gel electrophoresis using the buffer system described by Laemmli (1970). Protein fractions were mixed in a ratio of 2:1 (v/v) with sample buffer [180 mM Tris-HCl (pH 6.8), 6.0% (w/v) SDS, 2.1 M 2-mercaptoethanol, 35.5% (v/v) glycerol, and 0.004% (w/v) bromophenol blue] and boiled for 3 min. These fractions were electrophoresed in a 12.5% (w/v) acrylamide/0.43% (w/v) *N,N*-methylenebisacrylamide separating gel. Gels were stained in Coomassie brilliant blue. The molecular mass standards (Bio-Rad) included phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14 kDa). The samples analyzed were crude wheat germ homogenate (lane A), filtered crude cytosol (lane B), fractions 80–100 from the DEAE–cellulose column (lane C), fractions 65–74 from the reverse ammonium sulfate gradient solubilization step (lane D), and fraction 39 from the Sephacryl S-200 column (lane E). The position of the methyltransferase polypeptide (MT) is indicated at the right with an arrow.

probes and to confirm the presence of polymorphisms and/or multiple genes (see below).

Isolation of a cDNA Clone for L-*Isoaspartyl Methyltransferase From Wheat.* Degenerate oligonucleotides were synthesized on the basis of the partial amino acid sequence data (see above) and then used to amplify a region of the *pcm* cDNA from a wheat cDNA library constructed with poly-$(A)^+$ RNA isolated from 48-h-etiolated wheat seedlings (Hatfield et al., 1990). An 850-bp PCR product was amplified using a 384-fold degenerate oligonucleotide, MB3, representing the nucleic acid sequence at the 5′–region of the *pcm* cDNA (corresponding to the peptide YLKQYGV) and a primer encoding the T7 promoter of the pBluescript vector. The identity of the 850-bp PCR product was verified by Southern hybridization (data not shown) using a 64-fold degenerate oligonucleotide, MB1, representing the nucleic acid sequence in the middle region of the *pcm* cDNA (corresponding to the peptide GIEHIPE). In order to obtain a PCR product containing the *pcm* cDNA without the pBluescript vector sequence, the 850-bp PCR product was used as a template in a PCR reaction with the MB3 primer and a 288-fold degenerate oligonucleotide, MB4, representing the nucleic acid sequence at the 3′–region of the *pcm* cDNA (corresponding to the peptide LQVIDK). PCR amplification produced a 600-bp fragment containing only the *pcm* cDNA sequence, as determined by PCR dideoxy chain-termination sequencing. Screening of the wheat cDNA library with this 600-bp product resulted in the isolation of one positive plaque which was rescued to a pBluescript phagemid in *E. coli* XL-1 Blue cells. The 952-bp cDNA insert was subcloned into pUC19 to give the pMBM1 plasmid, which was then used to transform *E. coli* DH5α cells.

DNA Sequence of the Gene Encoding L-*Isoaspartyl Methyltransferase from Wheat.* We determined the DNA sequence of the 952-bp cDNA insert in the plasmid pMBM1 using the sequencing strategy shown in Figure 7. The DNA sequence of the *pcm* cDNA and its deduced amino acid sequence are shown in Figure 8. The calculated molecular weight of the 230 amino acid polypeptide deduced for the

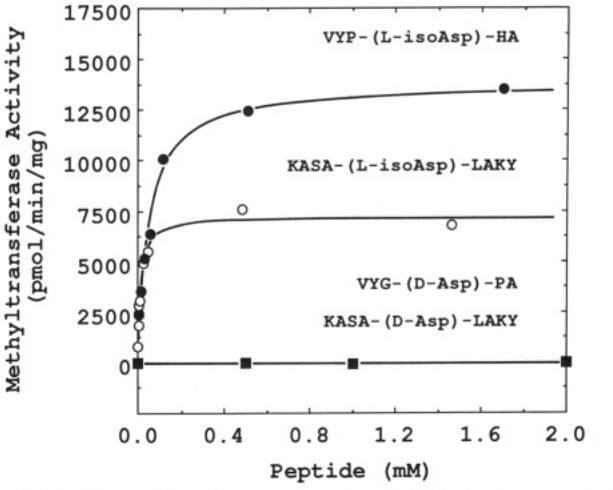

FIGURE 4: Recognition of L-isoaspartyl peptides, but not D-aspartyl peptides, by wheat germ methyltransferase. Wheat germ methyltransferase purified by Mono Q anion exchange chromatography as described under Experimental Procedures was used to determine the methylation kinetics with synthetic peptides, including VYP-(L-isoAsp)-HA, KASA-(L-isoAsp)-LAKY, VYG-(D-Asp)-PA, and KASA-(D-Asp)-LAKY. The initial velocity of the methylation reaction at different substrate concentrations was analyzed by nonlinear regression using the ENZFITTER program (Elsevier Biosoft). Lines were drawn to represent the results of this analysis. The K_m and the V_{max} values for the peptide VYP-(L-isoAsp)-HA are 51.7 μM and 13 800 pmol/min/mg of protein, respectively. For the peptide KASA-(L-isoAsp)-LAKY, the K_m is 12.7 μM and the V_{max} is 7250 pmol/min/mg of protein.

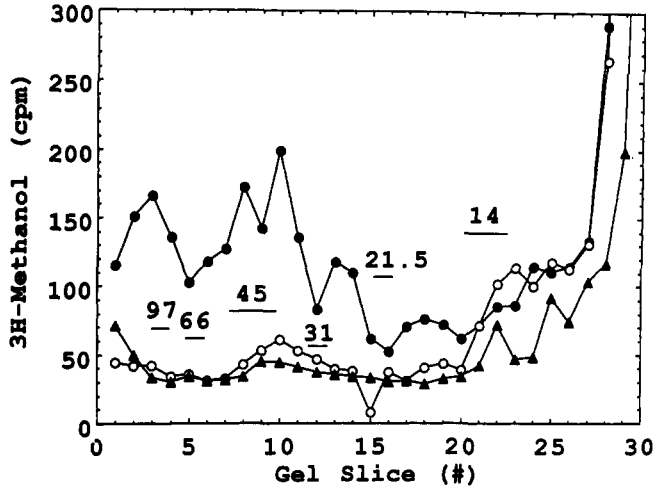

FIGURE 5: Identification of endogenous methyl-accepting polypeptides for wheat germ methyltransferase. Wheat germ cytosol (90 μg of protein) was incubated with *S*-adenosyl-L-[*methyl*-^{3}H]methionine (15–20 μM final concentration; New England Nuclear Research Products, 11 Ci/mmol) and 0.2 N sodium citrate (pH 6.0) at 25 °C for 60 min (●) in a final volume of 12 μL. In two additional incubations, the sodium citrate buffer was replaced with either the VYP-(L-isoAsp)-HA peptide (2.5 mM final concentration) in 0.2 N sodium citrate (pH 6.0) (○) or *S*-adenosylhomocysteine (100 μM final concentration) in 0.2 N sodium citrate (pH 6.0) (▲). Following incubation, the cytosolic polypeptides were mixed with 24 μL of sample buffer (75 mM sodium phosphate (pH 2.4), 8% SDS, 24% glycerol, 0.009% Pyronin Y, and 3.6 mM 2-mercaptoethanol), boiled for 3 min, and separated along with low molecular weight standards (Bio-Rad) in a 10% (w/v) acrylamide gel by SDS–polyacrylamide slab gel electrophoresis, at pH 2.4 (Fairbanks & Avruch, 1972). The gel was stained in Coomassie brilliant blue. After the gel was dried for 40 min at 60 °C, the lanes on the dried gel were cut into 2-mm slices and mixed with 100 μL of 0.2 N sodium hydroxide in a microcentrifuge tube. The tube was placed in a scintillation vial containing 5 mL of Safety-Solve (Research Products International) counting fluor and capped. After incubation at 37 °C for approximately 3 days, the vials were counted for [^{3}H]methanol. By quantitating the release of volatile [^{3}H]methanol, the incorporation of base-labile methyl esters into the endogenous polypeptides by the methyltransferase was measured. Gel slices 1 and 29 represent the top and bottom of the original gel, respectively. The locations of the molecular mass standards in Figure 3 are indicated by the bars.

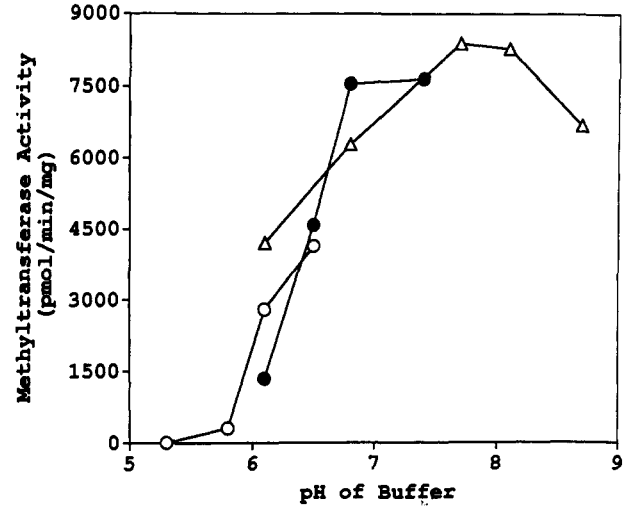

FIGURE 6: pH optimum of L-isoaspartyl methyltransferase from wheat germ. The activity of the wheat germ methyltransferase purified through the Mono Q step was measured at various pH values with the peptide VYP-(isoAsp)-HA as the substrate. Reaction mixtures were buffered with either sodium citrate (○), sodium phosphate (●), or sodium HEPES (△), each at a final concentration of 0.33 M.

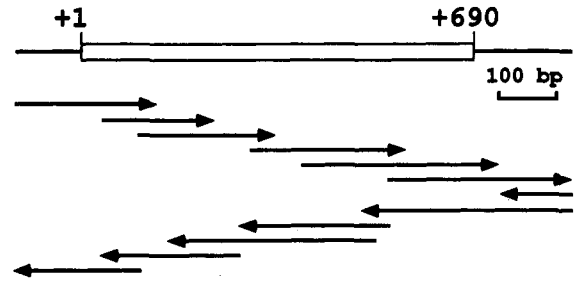

FIGURE 7: DNA sequencing strategy. Both strands of the pMBM1 clone containing the wheat *pcm* cDNA insert (a 952-bp *Eco*RI fragment) were sequenced by dideoxy chain-termination sequencing as described in the Experimental Procedures. Oligonucleotides were synthesized using the sequence of a PCR product containing 600 bp of the wheat *pcm* cDNA (see Experimental Procedures) and then used as primers to sequence the 952-bp fragment as shown in the sequencing strategy in this figure.

690-bp open reading frame is 24 710. In contrast, purified methyltransferase migrated as a 28 000-Da polypeptide as determined by SDS–polyacrylamide gel electrophoresis (Figure 3).

Comparison of Sequenced Peptide Fragments of L-*Isoaspartyl Methyltransferase from Wheat Germ and Its Predicted Amino Acid Sequence from pMBM1.* Interestingly, we found discrepancies at 12 sites between the predicted amino acid sequence of the wheat cDNA and the sequence of the peptide fragments of the wheat germ L-isoaspartyl methyltransferase (Figure 9). In six of these positions, the experimentally determined amino acid sequence data clearly show the presence of an amino acid not encoded by the cDNA. At the other six positions, residues in addition to the encoded residue were identified by Edman sequencing. These results are consistent with the hexaploid nature of this species of wheat, where the three diploid genomes (AABBDD) can contain alleles with variant sequences, leading to the production of variant gene products (Peumans et al., 1982; Wright & Raikhel, 1989). Most of the amino acid changes are located outside of the three highly conserved regions shared among methyltransferases. It is interesting to speculate that these amino acid differences can result in enzymes with slightly different methyl acceptor specificities, which would give the cell the ability to recognize and potentially repair a wider range of damaged proteins. Polymorphisms in the human *pcm* gene have also been identified (Ingrosso et al., 1989; MacLaren et al., 1992).

Comparison of L-*Isoaspartyl Methyltransferases from Wheat, Humans, and E. coli.* The amino acid sequence of the wheat L-isoaspartyl methyltransferase displays remarkable identity with both the L-isoaspartyl/D-aspartyl methyltransferase from human erythrocytes (Ingrosso et al., 1989) and the L-isoaspartyl methyltransferase from *E. coli* (Fu et al., 1991). On the basis of the 230 residues in the wheat polypeptide, the *E. coli* and wheat methyltransferases are identical at 72 residues (31.3%). An even more striking resemblance is seen between the human and wheat methyltransferases, which are identical at 114 residues out of 230 (49.6%). In this analysis, alignment was maximized by the insertion of two gaps in the wheat sequence, six gaps in the *E. coli* sequence, and two gaps in the human sequence (Figure 10). It is not surprising that the regions where all three methyltransferases are most similar are in regions that have been designated as highly conserved sequence motifs among different families of methyltransferases (Ingrosso et al., 1989). These motifs (regions I, II, and III; see Figure 10) are thought to play a role in the binding of the substrate, *S*-adenosylmethionine. Interestingly, with the addition of the deduced amino acid sequence for the wheat methyltransferase, we have been able to identify sequences outside of regions I, II, and III

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  1                  CG TCC CGC TTC CTG TTG CCC TCC ACG GCC GCC   32
 33   CGC CGC CGG TTC CTC CAC CAC CTC CTC GCC GCG CCC GCG CCC   74
 75   CCG AGG CCG CCG CAG CTT CGG CGC TGC TCC CCG TAC CAC TGG   116

117   ATG GCG CAA TTT TGG GCT GAA GGA TCA CTG GAG AAG AAC AAC   158
  1    M   A   Q   F   W   A   E   G   S   L   E   K   N   N    14

159   GCT CTG GTT GAA TAC CTG AAA CAG TAT GGT GTT GTT CGA ACC   200
 15    A   L   V   E   Y   L   K   Q   Y   G   V   V   R   T    28

201   GAT AAA GTG GCA GAA GTT ATG GAA ACT ATC GAC CGA GCC TTA   242
 29    D   K   V   A   E   V   M   E   T   I   D   R   A   L    42

243   TTT GTA CCG GAG GGC TTT ACC CCT TAC ACC GAC AGT CCT ATG   284
 43    F   V   P   E   G   F   T   P   Y   T   D   S   P   M    56

285   CCT ATT GGT TAC AAT GCA ACA ATA TCT GCT CCT CAC ATG CAC   326
 57    P   I   G   Y   N   A   T   I   S   A   P   H   M   H    70

327   GCA ACC TGC TTA GAA CTG TTG AAG GAT TAT TTA CAG CCA GGC   368
 71    A   T   C   L   E   L   L   K   D   Y   L   Q   P   G    84

369   ATG CAT GCT CTG GAC GTT GGA TCA GGC AGT GGT TAC TTG ACT   410
 85    M   H   A   L   D   V   G   S   G   S   G   Y   L   T    98

411   GCT TGC TTT GCA ATG ATG GTC GGA CCA GAA GGT CGC GCA GTG   452
 99    A   C   F   A   M   M   V   G   P   E   G   R   A   V   112

453   GGG ATT GAA CAC ATT CCT GAA CTC GTT GTT GCT TCT ACT GAA   494
113    G   I   E   H   I   P   E   L   V   V   A   S   T   E   126

495   AAT GTC GAA CGG AGT GCT GCA GCA GCA CTA ATG AAG GAT GGT   536
127    N   V   E   R   S   A   A   A   A   L   M   K   D   G   140

537   TCA CTT TCT TTT CAT GTT TCA GAT GGA AGG CTT GGC TGG CCG   578
141    S   L   S   F   H   V   S   D   G   R   L   G   W   P   154

579   GAT GCG GCG CCA TAC GAT GCT ATT CAT GTG GGC GCA GCG GCA   620
155    D   A   A   P   Y   D   A   I   H   V   G   A   A   A   168

621   CCT GAG ATC CCT CGG CCA CTG CTG GAG CAG CTG AAG CCT GGC   662
169    P   E   I   P   R   P   L   L   E   Q   L   K   P   G   182

663   GGG CGG ATG GTC ATA CCC GTT GGC ACA TAC TCT CAG GAC CTG   704
183    G   R   M   V   I   P   V   G   T   Y   S   Q   D   L   196

705   CAG GTG ATT GAC AAG AGC GCC GAC GGA TCC ACC AGC GTC CGC   746
197    Q   V   I   D   K   S   A   D   G   S   T   S   V   R   210

747   AAC GAT GCC TCT GTT CGC TAC GTC CCT CTG ACC AGC CGC TCT   788
211    N   D   A   S   V   R   Y   V   P   L   T   S   R   S   224

789   GCT CAG CTG CAG GAC AGC TGA GAT CCT TCG CTC TGG ATC TGG   830
225    A   Q   L   Q   D   S                                   230

831   AAA TGT GTG TGT ATA TAT GTG AGT GCC GAT GAT CTT TGT CTA   872
873   CCA ATG TGG CGT CTG ATG TTT TAG ATG GTT TGG TTT TGT ATA   914
915   ATG CTT ACT GCT GGT TGA TGT TGC TTA AAA                   944
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FIGURE 8: Nucleotide sequence of wheat *pcm* cDNA from pMBM1 and its predicted amino acid sequence. The sequence of the coding strand of the 944-bp insert of the plasmid pMBM1 is shown without the terminal *Eco*RI linkers (GGAATTCC) that were added to the cDNA library. The 690-bp *pcm* cDNA initiates at the ATG codon at position 118 and terminates at the TGA codon at position 808.

which are also shared among the wheat, *E. coli*, and human polypeptides. Similarity elsewhere in these polypeptide chains could then reflect the specific requirements for binding the methyl-accepting L-isoaspartyl proteins. Considering that the wheat and *E. coli* methyltransferases do not appear to catalyze the methylation of D-aspartyl peptides in vitro, amino acid residues specific to the human methyltransferase in these regions of identity may be the putative structural elements involved in the recognition of D-aspartyl residues. Overall, such conservation of the L-isoaspartyl methyltransferase in bacteria, plants, and animals with characteristically similar functions suggests that this enzyme is a direct descendant of an ancient protein (Doolittle et al., 1986).

DISCUSSION

The deterioration of seeds that occurs as a consequence of prolonged storage is a mark of the aging process in plants. Here, cellular damage can accumulate and reduce seed viability as a function of time, temperature, and moisture content (Nooden & Leopold, 1988). The primary detrimental effects of aging in seeds are chromosomal aberrations and lesions in cellular membranes, although it is becoming more evident that damage to proteins also contributes significantly to the aging process (Nooden & Leopold, 1988). For example, Jones and Gersdorff (1941) showed that the solubility properties of proteins in wheat seeds change over periods as short as several months, reflecting alterations in protein structure. More recently, it has been documented that a variety of proteins progressively lose enzymatic activity as a seed ages, including DNA polymerase, α-amylase, cytochrome oxidase, malate dehydrogenase, alcohol dehydrogenase, glutamate dehydrogenases, catalases, peroxidase, and several hydrolytic enzymes (Nooden & Leopold, 1988; Bhattacharyya & Sen-Mandi, 1985; Agrawal & Kharlukhi, 1987). Loss of any one

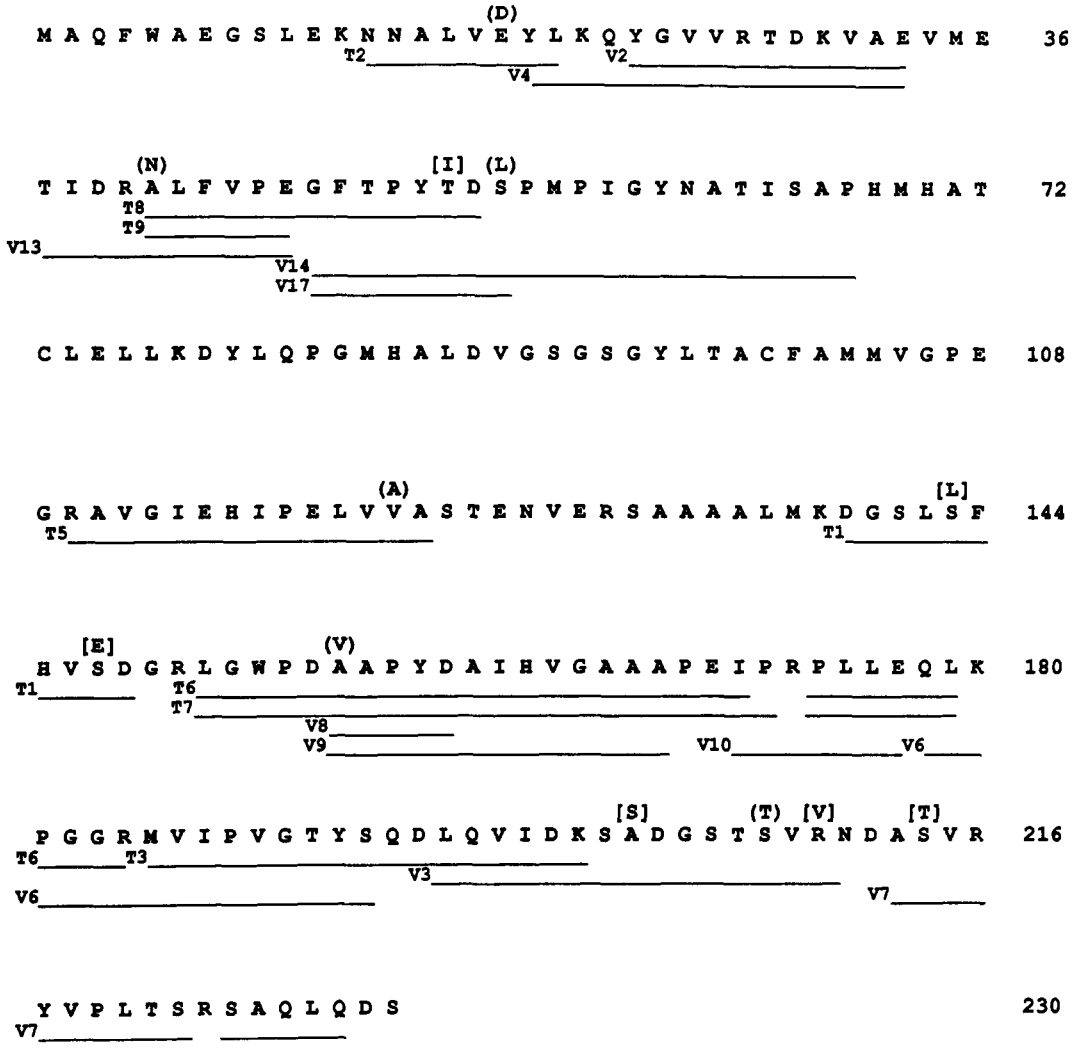

FIGURE 9: Alignment of sequenced peptide fragments of L-isoaspartyl methyltransferase from wheat germ and its predicted amino acid sequence from pMBM1. Following digestion with trypsin (T) and *S. aureus* V8 protease (V), peptides were recovered by reverse-phase HPLC and sequenced by automated Edman degradation as described in the Experimental Procedures. Peptide sequences of fragments numbered in the order of elution are shown by lines in comparison to the deduced cDNA sequence. The presence of a space indicates that unambiguous identification of the amino acid residue could not be made in this cycle. Additional residues identified in a particular cycle are indicated in parentheses above. Residues above in brackets denote an amino acid substitution at this position where no evidence was found for the cDNA-encoded residue. At position 41, A was found in T8 and V13; N was found in T9. At position 52, I was found in T8, V14, and V17. At position 54, only L was found in V17. At position 156, A was found in T6 and V8; V was found in T7 and V9.

of these enzymatic activities might be expected to have a detrimental effect on the germination and development of an aged seed. For instance, it has been shown that embryos isolated from nongerminating, aged seeds having unusually low levels of α-amylase activity are able to develop into normal, mature plants when supplied with simple sugars (Bhattacharyya & Sen-Mandi, 1985). This demonstrated that the embryo itself was still viable, but was unable to germinate because utilizable substrates for the germination and growth of the embryonic tissues were not available. The researchers speculated that the inability to mobilize food reserves in the seed was caused in part by a loss of α-amylase activity as a consequence of aging.

It is clear that protein damage occurs in seeds subjected to both endogenous and exogenous stresses. Yet, how is it that seeds are able to remain viable for decades and sometimes centuries (Bewley & Black, 1985)? Is repair of damaged proteins possible in a dormant seed? Villiers (1975) has suggested that repair and turnover of proteins could occur in a dormant seed as long as it is not completely dry (the critical moisture content is approximately 15–28% depending on the species), provided that oxygen is present to support normal metabolism, albeit at a very low rate. Interestingly, it has been shown that when dormant seeds are imbibed, they possess a highly developed metabolism including respiration, turnover of membrane lipids and proteins, RNA synthesis, and active polysomes (Priestley, 1986). Thus, it appears that the existence of such metabolic functions in the hydrated seed permits extensive repair to take place. Whether repair occurs during dormancy or upon germination, it is likely that internal repair mechanisms are present in seeds and function to extend the lifespans of their proteins.

We propose that one internal repair system for damaged proteins in seeds is mediated by the L-isoaspartyl methyltransferase described here. All of the plants assayed in this survey possess an enzymatic activity that catalyzes the specific methylation of L-isoaspartyl-containing peptide substrates. Interestingly, the seeds of these plants generally contained the highest specific activities of the L-isoaspartyl methyltransferase. We suspect that the problem of spontaneous protein degradation may be particularly severe in seeds that must survive for extended periods of time in a relatively inactive

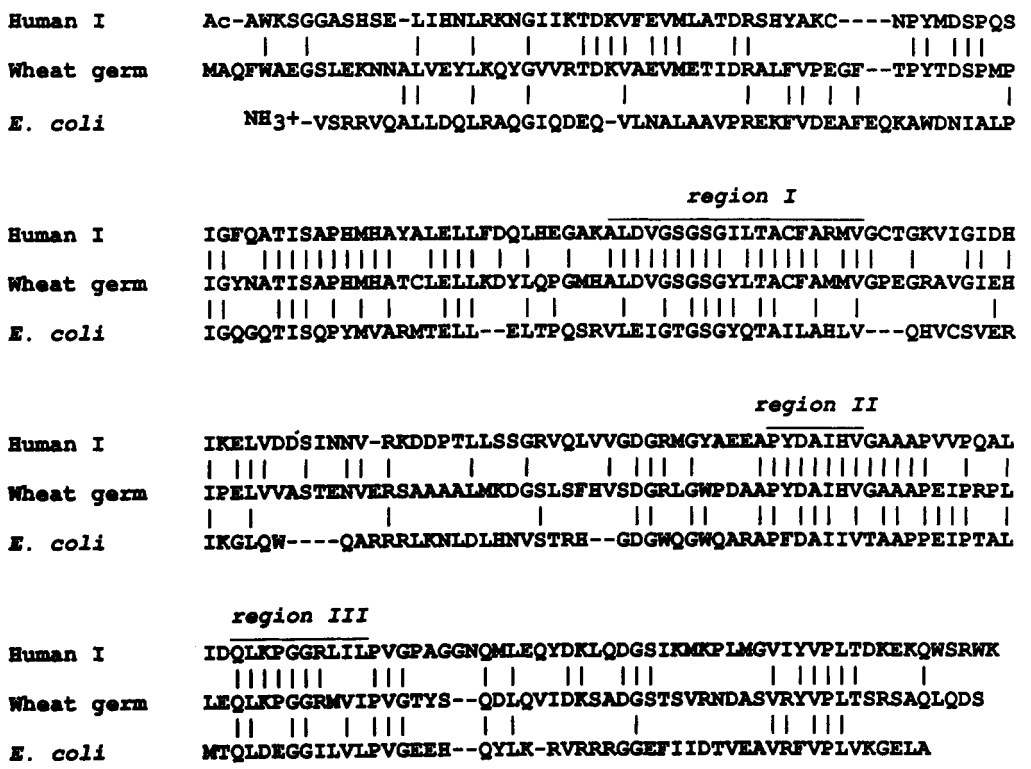

FIGURE 10: Amino acid sequence comparison of L-isoaspartyl methyltransferase from wheat and *E. coli* with that of L-isoaspartyl/D-aspartyl methyltransferase from human erythrocytes. The protein sequences of isozyme I of the human erythrocyte enzyme (Ingrosso et al., 1989), the deduced sequence of the wheat enzyme from pMBM1, and the deduced sequence for the *E. coli* enzyme (Fu et al., 1991) have been aligned, with dashes denoting the insertion of a gap. A line between residues represents sequence identity. Three regions (regions I, II, and III) known to be conserved among methyltransferases are underlined (Ingrosso et al., 1989). The initiator methionine residue of the *E. coli* enzyme is removed in the mature protein; N-terminal modification has not been defined for the wheat germ protein, but the N-terminus does appear to be blocked to Edman degradation (data not shown).

state and then rapidly develop into a functional plant. We hypothesize that dormant seeds accumulate racemized and isomerized aspartyl residues in their proteins as a consequence of aging. The rate of accumulation of these altered residues would depend on the environmental temperature and perhaps the water content of the seed. Once germination begins, the presence of proteins containing altered aspartyl residues might be expected to be detrimental to the development of the embryo, because they may prevent full utilization of storage proteins and directly inhibit catalytic enzymes. On the basis of the localization and unique specificity of the L-isoaspartyl methyltransferase, we propose that one role of the methyltransferase in a plant is to prevent the accumulation of damaged aspartyl residues within a seed or in other long lived tissue. Variation in the level of the methyltransferase in the seeds of different plant species may reflect the inherent susceptibility of that species to protein deterioration and exogenous stresses. Significantly, we also find evidence for the methyltransferase in other plant tissues, especially roots. This suggests that protein repair can occur in both germ-line and somatic tissues.

Genetic evidence to support the proposed role of the L-isoaspartyl methyltransferase in the seeds of plants comes from recent work done in the bacterium *E. coli*. Li and Clarke (1992b) have shown that *E. coli* mutants lacking an L-isoaspartyl methyltransferase (*pcm*$^-$) are able to grow apparently normally in the logarithmic phase but survive poorly in the extended stationary phase compared to a *pcm*$^+$ parent strain. Presumably, these mutant *E. coli* cells exhibit wild-type growth characteristics in the logarithmic phase because aberrant proteins containing altered aspartyl residues can be rapidly replaced by normal proteins and/or because the altered proteins are stable under nonstress conditions. However, in the stationary phase when the protein turnover is significantly reduced, mutant *E. coli* cells lacking the methyltransferase apparently cannot prevent the accumulation of such aberrant proteins. The loss of viability of these mutant *E. coli* cells under conditions of limited protein synthesis is the first genetic evidence to support the role of the L-isoaspartyl methyltransferase in the repair and metabolism of aged proteins containing L-isoaspartyl residues. On the basis of this evidence, we propose that the L-isoaspartyl methyltransferase plays an analogous role in plants in the maintenance of proteins in aging seeds.

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