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Expression, Purification, and Characterization of the Protein Repair L-Isoaspartyl Methyltransferase from *Arabidopsis thaliana*

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Protein L-isoaspartate (D-aspartate) *O*-methyltransferase (EC 2.1.1.77) is a repair enzyme that methylates abnormal L-isoaspartate residues in proteins which arise spontaneously as a result of aging. This enzyme initiates their conversion back into the normal L-aspartate form by a methyl esterification reaction. Previously, partial cDNAs of this enzyme were isolated from the higher plant *Arabidopsis thaliana*. In this study, we report the cloning and expression of a full-length cDNA of L-isoaspartyl methyltransferase from *A. thaliana* into *Escherichia coli* under the P_{BAD} promoter, which offers a high level of expression under a tight regulatory control. The enzyme is found largely in the soluble fraction. We purified this recombinant enzyme to homogeneity using a series of steps involving DEAE–cellulose, gel filtration, and hydrophobic interaction chromatographies. The homogeneous enzyme was found to have maximum activity at 45°C and a pH optimum from 7 to 8. The enzyme was found to have a wide range of affinities for L-isoaspartate-containing peptides and displayed relatively poor reactivity toward protein substrates. The best methyl-accepting substrates were KASA-*L-isoAsp*-LAKY (K_m = 80 μM) and VYP-*L-isoAsp*-HA (K_m = 310 μM). We also expressed the full-length form and a truncated version of this enzyme (lacking the N-terminal 26 amino acid residues) in *E. coli* under the T7 promoter. Both the full-length and the truncated forms were active, though overexpression of the truncated enzyme led to a complete loss of activity.

The protein L-isoaspartate (D-aspartate) *O*-methyltransferase (EC 2.1.1.77) is a protein repair enzyme which recognizes abnormal L-isoaspartyl residues and initiates their conversion back into the normal L-form (1–6). Spontaneous deamidation, isomerization, and racemization reactions occur as a result of aging at asparaginyl and aspartyl residues, leading to the formation of L-aspartate, L-isoaspartate, and D-aspartate residues (6–13). Accumulation of such isomerized and racemized residues can affect protein structure and function (14–19). The L-isoaspartyl methyltransferase catalyzes the methyl esterification of L-isoaspartate and, to a lesser degree, D-aspartate residues, using *S*-adenosylmethionine (AdoMet)[2] as the methyl group donor in a reaction representing the first step in the repair of these abnormal residues. This enzyme is widely distributed in bacteria (20, 21), plants (22–28), nematodes (29), flies (30), and mammals, including humans (31, 32), and displays a high degree of sequence conservation (33).

Of all the known L-isoaspartyl methyltransferases studied, the enzymes from *Thermotoga maritima* (21), *Caenorhabditis elegans* (29), rat (34), and human (35) have been overexpressed as recombinant proteins and purified. These studies have allowed the comparison of the biochemical and kinetic properties of the enzyme from different sources. However, none of the plant enzymes have been overexpressed to date. We were thus interested in studying the biochemical properties of the enzyme from the dicot *Arabidopsis thaliana*. In plants, the protein L-isoaspartyl methyltransferase has been found to be localized primarily in seeds (26), suggesting a role in keeping the generally long-lived seed proteins in a functionally active conformation. Considering the fact that seed embryos need to remain viable for ex-

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[2] Abbreviations used: IPTG, isopropyl β-D-thiogalactopyranoside; PMSF, phenylmethylsulfonyl fluoride; [^{14}C]AdoMet, *S*-adenosyl-[*methyl*-^{14}C]L-methionine; Hepes, 4-(2-hydroxyethyl)piperazine-ethanesulfonic acid; DEAE, diethylaminoethyl.

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tended periods of time, it is important to maintain seed proteins in their normal forms to ensure efficient germination and viability (26, 27). Previously, the L-isoaspartyl methyltransferase was purified from the monocot wheat and its cDNA isolated (24). The gene for L-isoaspartyl methyltransferase of *Arabidopsis thaliana* has also been cloned and its genomic organization established (25), but biochemical analysis has been limited by the very small amounts of tissue containing methyltransferase that can be obtained.

In this work, we have purified the enzyme from *Arabidopsis* after overexpressing the protein in bacteria. Although we did not have a full-length cDNA clone of the *Arabidopsis* enzyme, we devised a strategy to isolate a full-length coding sequence and express the protein in *Escherichia coli* using a construct of the pBAD vector (36). This vector was designed to contain a P_{BAD} promoter and the regulator *araC* (37), engineered to provide a tight control and modulation of expression. The P_{BAD} promoter is strongly induced in the presence of L-arabinose and repressed in its absence (38, 36). It is further repressed by glucose, thus offering a controlled expression (39). Newman and Fuqua (40) utilized the P_{BAD} promoter and *araC* regulator to express genes in *A. tumefaciens* and *E. coli* at 22-fold and 400-fold levels, respectively. Bost *et al.* (41) found that expression of a chimeric protein is toxic to the cell only when the P_{BAD} promoter is fully induced, suggesting the importance of being able to regulate the expression of genes at optimal levels. We were able to express large amounts of the enzyme in this way and purify it for biochemical analysis. The results of these studies demonstrate that although the enzymes from different organisms catalyze the same reaction, the recognition of individual substrates does differ and may reflect evolutionary adaptation of the methyltransferases to the types of damaged proteins that they encounter *in vivo*.

MATERIALS AND METHODS

Construction of A. thaliana L-Isoaspartyl Methyltransferase Overexpressing Plasmids

pBAD expression system. A genomic clone (pMBM5) and a partial cDNA clone (pMBM6) of the *A. thaliana* (Ecotype Columbia) L-isoaspartyl methyltransferase gene (*pcm*) have been prepared previously (25). The cDNA clone pMBM6 lacks the first 77 nucleotides of the coding region including exon 1 (6 bp) and the 5′ 71 bp of exon 2 although it has the 3′ untranslated region. In order to clone the full-length cDNA of the *Arabidopsis* methyltransferase, the strategy outlined in Fig. 1 was used. Here, a primer (NT19) was designed corresponding to the first 21 nucleotides of the *Arabidopsis* L-isoaspartyl methyltransferase coding sequence (including exon 1 and the 5′ 15 bp of exon 2) and a *Sal*I site was engineered at the 5′ end. This primer was then used along with a suitable reverse primer in exon 3 (MBM 20, Ref. 25) to amplify a fragment out of the genomic clone (pMBM5) by PCR. This 446-bp fragment has exon 1, exon 2, intron 2, and part of exon 3, including an internal *Nde*I site in exon 2. This fragment was then digested with *Sal*I and *Nde*I to give a 215-bp fragment containing exon 1 and part of exon 2 including all 77 bp of the coding region missing in the pMBM6 clone. The 215-bp fragment was subsequently ligated into the 3.65-kb fragment of pMBM6 cut at the *Sal*I/*Nde*I sites and used to transform *E. coli* DH5α cells. The resulting plasmid is designated as pNT1 (Fig. 2A). DNA sequencing of the plasmid confirmed that the full coding sequence was inserted correctly and that no mutations were introduced during the cloning procedures.

For expression, we chose the pBAD24 vector (36), which has the P_{BAD} promoter of the *ara*BAD operon. This vector has a translation start codon at the *Nco*I site as well as an optimized Shine–Dalgarno sequence (42). To subclone the *Arabidopsis* L-isoaspartyl methyltransferase gene into pBAD24, a specific 5′ primer was designed—NT23 (5′-TTCG**CC<u>ATG</u>*G***AGCAATTCTGGAGTCCAAGT-3′), which has the initiation codon ATG (underlined) followed by the next 21 nucleotides of the coding sequence. To engineer a *Nco*I site (bold), one base was changed (italicized) which would change the second amino acid in the protein from lysine to glutamic acid. A ~924-bp fragment was amplified out of pNT1 using NT23 and a 3′ T3 primer (5′-ATTAACCCTCACTAAAG-3′) by PCR and then cut with *Nco*I and *Xba*I restriction enzymes to yield an ~870-bp fragment which was purified away from the restriction enzymes and small DNA fragments using a GeneClean III kit (Bio101, Inc.) and subsequently ligated into pBAD24 at the *Nco*I and *Xba*I sites. The products of this ligation were used to transform *E. coli* strain JV1068 (43) lacking its native L-isoaspartyl methyltransferase. Colonies yielding the expected 372-bp product using single-colony PCR with primers NT19 and MBM20 were cultured and the plasmids isolated. The plasmid yielding an ~875-bp *Eco*RI product, indicating the presence of the full-length *Arabidopsis* L-isoaspartyl methyltransferase gene, was designated pNT2 (Fig. 2B).

pT7-7 expression system. To clone the truncated *Arabidopsis* L-isoaspartyl methyltransferase cDNA into pT7-7 vector (Stratagene), plasmid pMBM6 was cut with *Eco*RI to yield a 792-bp product (encompassing the partial cDNA), which was purified from an agarose gel as above. This fragment was then ligated into pT7-7 at the *Eco*RI site (predigested with *Eco*RI and dephosphorylated with shrimp alkaline phosphatase, GIBCO BRL). The products of this ligation were used to transform *E. coli* strain DH5α cells, and the initial screen of the positive clones was performed by

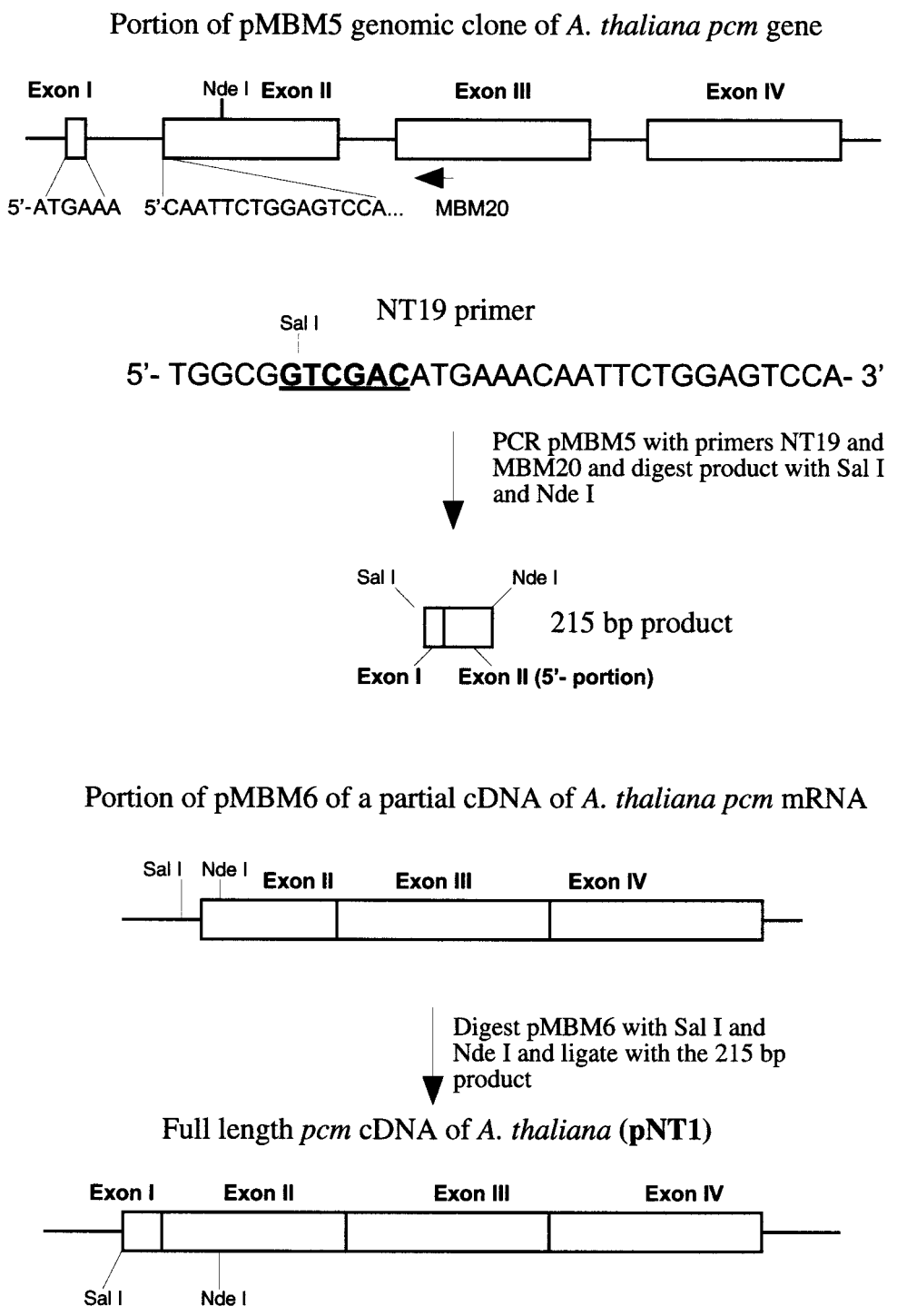
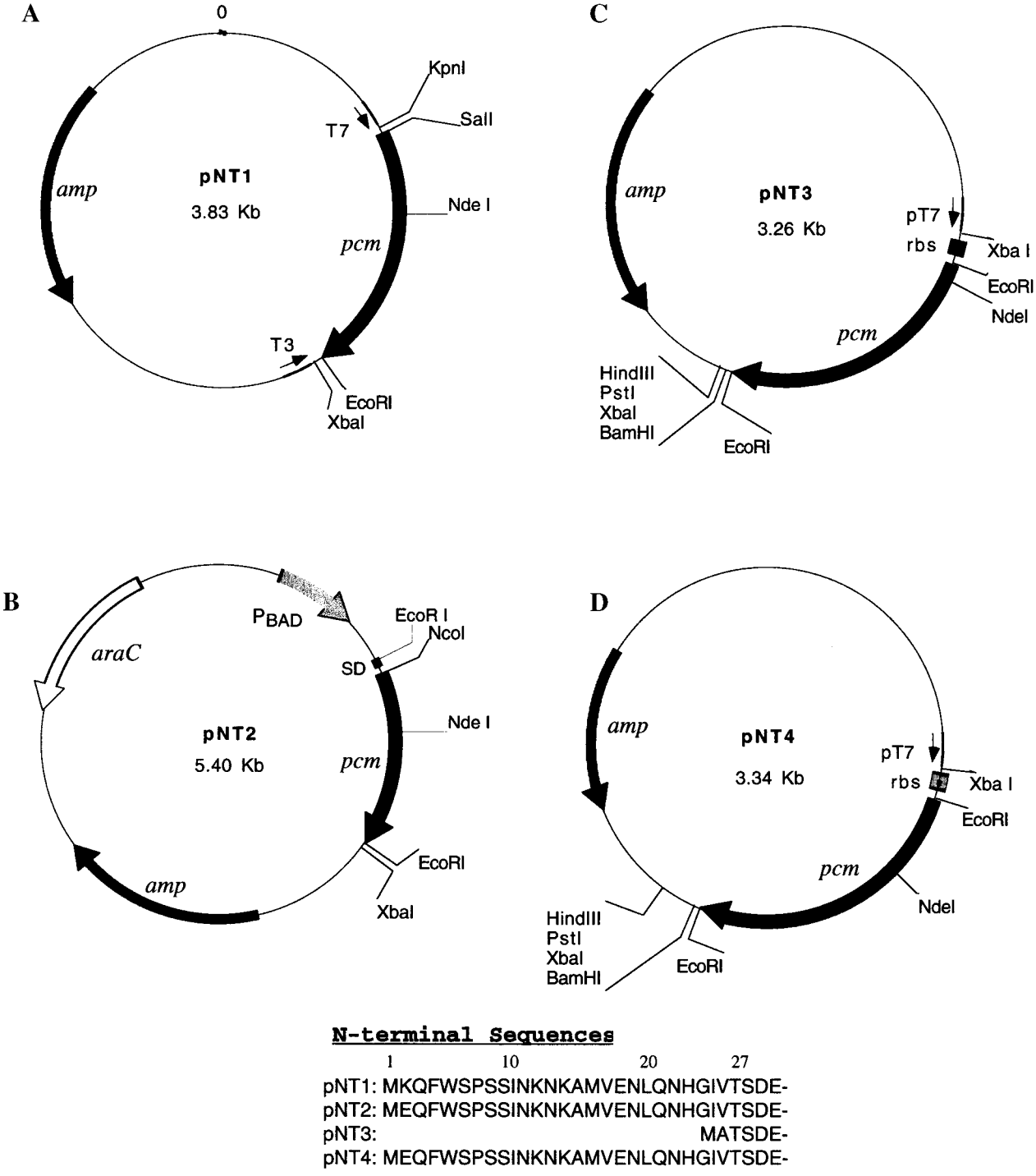


FIG. 1. Scheme for the construction of a full-length cDNA clone of the *Arabidopsis* L-isoaspartyl methyltransferase, described in detail under Materials and Methods.

single-colony PCR followed by restriction enzyme digestion of the plasmids with *Eco*RI. Plasmid yielding the expected 792-bp product was designated pNT3 (Fig. 2C). For expression studies, *E. coli* BL21(DE3) cells (Invitrogen) were transformed with pNT3 plasmid and the positive clones identified by digestion with *Eco*RI. To clone the full-length *Arabidopsis* L-isoaspartyl methyltransferase gene into the pT7-7 vector, the plasmid pNT2 was digested with *Eco*RI and the resulting 875-bp fragment ligated into pT7-7 at the *Eco*RI



N-terminal Sequences

```
          1         10        20   27
pNT1: MKQFWSPSSINKNKAMVENLQNHGIVTSDE-
pNT2: MEQFWSPSSINKNKAMVENLQNHGIVTSDE-
pNT3:                          MATSDE-
pNT4: MEQFWSPSSINKNKAMVENLQNHGIVTSDE-
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FIG. 2. Expression plasmids for the *Arabidopsis* L-isoaspartyl methyltransferase. (A) pNT1 is a pBluescript SK(−) plasmid containing the *Arabidopsis* L-isoaspartyl methyltransferase cDNA inserted at the *Sal*I/*Eco*RI sites, downstream of the T7 promoter. (B) pNT2 is the expression vector pBAD24 containing the *Arabidopsis* L-isoaspartyl methyltransferase cDNA inserted at the *Nco*I/*Xba*I sites, downstream of the P_{BAD} promoter. The vector also has an optimized Shine–Dalgarno sequence (SD). (C) pNT3 is the pT7-7 expression vector containing the N-terminus truncated *Arabidopsis* L-isoaspartyl methyltransferase cDNA (lacking the first 77 nucleotides of the coding region) inserted at the *Eco*RI site, downstream of the T7 promoter. (D) pNT4 is the pT7-7 expression vector containing the full-length *Arabidopsis* L-isoaspartyl methyltransferase cDNA inserted at the *Eco*RI site, downstream of the T7 promoter.

TABLE 1

Plasmids and Strains

Strain/plasmid	Genotype/description	Reference
Strains		
MC1000	Δ(*ara-leu*)7697, *araD*139, Δ(*codB-lac*)3, *galK*16, *galE*15, e14$^-$, *relA*, *rpsL150*, *spoT1*, *mcrB1*, λ$^-$	49
DH5α	*supE*44 Δ*lac*U169 (Φ80 *lac* ZΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	GIBCO–BRL
BL21(DE3)	*hsdS* gal (λc*Its*857 *ind*1 *S*am7 *nin*5 *lac*UV5-T7 gene *1*)	Invitrogen
JV1068	MC1000, Δ*pcm*::Cm, chloramphenicol resistance	43
Plasmids		
pBAD24	Expression vector with pBAD promoter and *ara*C negative regulator, includes Shine–Dalgarno and Kozak sequences as well as ATG for making translational fusions, ampicillin resistance	36
pMBM5	pBSK+ containing a 2.8-kb insert at the *Eco*RI site downstream of the T7 promoter representing the genomic sequence of the *Arabidopsis pcm* gene, ampicillin resistance	25
pMBM6	pBSK+ containing a 792-bp insert at the *Eco*RI site downstream of the T7 promoter representing the partial cDNA sequence of the *Arabidopsis pcm* gene, ampicillin resistance	25
pNT1	Overexpression vector for *Arabidopsis pcm;* contains the *pcm* coding sequence (690 bp) and the 3′ UTR between the *Sal*I and *Eco*RI sites of pBSK−, ampicillin resistance	This study
pNT2	Overexpression vector for *Arabidopsis pcm;* contains the *pcm* coding sequence (690 bp) and the 3′ UTR between the *Nco*I and *Xba*I sites of pBAD24, ampicillin resistance	This study
pNT3	Overexpression vector for the truncated *Arabidopsis pcm;* contains the partial *pcm* coding sequence (missing the N-terminal 77 bp) and the 3′ UTR at the *Eco*RI site of pT7-7, ampicillin resistance	This study
pNT4	Overexpression vector for full-length *pcm;* contains the *pcm* (coding sequence + 3′ UTR) at the *Eco*RI site of pT7-7, ampicillin resistance	This study

site. All subsequent screening steps were conducted in the same fashion as mentioned above and the plasmid containing the full-length sequence was designated pNT4 (Fig. 2D). All plasmids and strains used are indicated in Table 1.

Growth Media and Conditions

E. coli cells were grown in Luria–Bertani medium (44) at 37°C in the presence of the antibiotic ampicillin at 100 μg/ml (Sigma). L-Arabinose and isopropyl β-D-thiogalactopyranoside (IPTG) were from Sigma Chemical Co. (St. Louis, MO).

Preparation of Cytosolic Extracts for Methyltransferase Assays

For control experiments, cells containing the plasmid pNT2 were harvested by centrifugation at 16,000*g* and washed once with chilled extraction buffer A (5 mM sodium phosphate/pH 7.0, 5 mM EDTA, 10% w/v glycerol, 25 μM PMSF, 15 mM 2-mercaptoethanol). Pellets were suspended in one-fifth the volume of buffer as the original culture volume (generally 5 ml). Cells were lysed by sonication on ice (five rounds of five 1-s pulses) with a Branson W-350 sonicator and microtip at a power setting of 4 and the lysates were then centrifuged at 16,000*g* for 15 min at 4°C to obtain a supernatant for methyltransferase activity determination.

Assay of L-Isoaspartyl Methyltransferase Activity

A vapor-diffusion assay (45) was used to determine the methyltransferase activity. The method involves the transfer of radiolabeled methyl groups by the enzyme from *S*-adenosyl-[*methyl*-^{14}C]-L-methionine (57 mCi/mmol, Amersham Life Sciences, Arlington Heights, IL) to a suitable peptide substrate (VYP-*L-isoAsp*-HA) (UCLA Peptide Research Facility). Subsequently, the methyl esters are hydrolyzed and the resulting [^{14}C]methanol is quantified. Typically, the reaction mixture (total of 40 μl) consists of 500 μM of the peptide substrate, 10 μM of [^{14}C]AdoMet, 0.33 M Hepes buffer (pH 7.5), and enzyme (200–300 μg of crude cytosolic extract or 0.05–0.1 μg of purified protein). As a control, the endogenous activity was measured by incubating the enzyme with buffer alone instead of the peptide. The reaction was allowed to proceed at 37°C for 1 h and stopped by quenching with 40 μl of 0.2 N NaOH/1% (w/v) SDS. The contents were vortexed and 60 μl of this mixture was then spotted onto a 1.5 × 8 cm pleated filter paper (Bio-Rad, no. 1650962), which was placed in the neck of a 20-ml scintillation vial containing 5 ml of counting fluor (Safety Solve High Flashpoint cocktail, Research Products International, Mount Prospect, IL). The vials were capped and incubated for 2 h at room temperature. During this period, the resulting [^{14}C]methanol diffuses into the fluor and the unreacted [^{14}C]AdoMet stays on the filter paper. Quantification was done by removal of the paper and counting the vials in a scintillation counter (Beckman LS 100C). Peptide specific activity was calculated by subtracting the endogenous activity from the activity in the presence of the peptide.

TABLE 2

Activity of *Arabidopsis* Recombinant L-Isoaspartyl Methyltransferase from pNT2 Overexpressed in *E. coli* Strain JV1068

Fraction	Total activity[a] (pmol/min)	Specific activity[a] (pmol/min/mg of protein)
Supernatant (soluble form from cytosol)	33,400,000 ± 590,000	870 ± 15
Pellet (inclusion bodies)	14,250 ± 14	396 ± 0.6
Soluble fraction of inclusion bodies in 6 M urea	21,780 ± 130	3025 ± 19
Renatured fraction	2,048 ± 23	439 ± 5

Note. Cells in a 2-liter culture were induced with 0.2% arabinose at an optical density of 0.35 at 600 nm and harvested at a value of 1.2. Pelleted cells were resuspended in 20 ml of chilled extraction buffer A (2 ml/g wet weight), lysed by two passages through a French pressure cell at 1200 psi, and spun at 16,000*g* for 15 min at 4°C. The supernatant was assayed for methyltransferase activity, and the pellet was washed twice, resuspended in 10 ml of extraction buffer, and assayed. The remaining pellet fraction was brought to 6 M urea, vortexed, and spun at 8000*g* for 15 min at 4°C to remove insoluble material. The supernatant was then assayed and the remaining portion then renatured by dialysis at 4°C against a 0.01 M Tris–Cl buffer (pH 7.5) containing a decreasing concentration of urea (6 to 0 M) followed by a final dialysis step against urea-free buffer.

[a] L-Isoaspartyl methyltransferase assays were performed as described under Materials and Methods. Reactions were done in duplicate and the values represent the mean ± range.

Protein Determination

Protein content of the crude extracts was determined by precipitating the protein with 1 ml of 10% (w/v) trichloroacetic acid and using a modification of the Lowry method (46) subsequently. Bovine serum albumin (Sigma) was used for creating a standard curve.

Purification of Arabidopsis Recombinant L-Isoaspartyl Methyltransferase

Preparation of cytosolic extract. Four flasks containing 2 liters of LB supplemented with ampicillin (100 μg/ml) each were inoculated with an overnight culture of JV1068 containing the plasmid pNT2 (0.2% inoculum size) and incubated at 37°C with shaking (250 rpm). Induction was done at an optical density of 0.5 at 600 nm and the incubation was continued further for 5 h until the optical density reached 1.8. All subsequent steps were performed at 4°C unless otherwise indicated. The cells were harvested by centrifugation at 3500*g* for 15 min. The cell pellet (wet weight 25 g) was washed once with chilled extraction buffer A and then suspended in 35 ml of the same buffer by rigorous vortexing. Cell disruption was performed by passing the cell suspension through a French press cell at 20,000 lb/in^2. The contents were then centrifuged at 24,000*g* for 20 min at 4°C and the resulting supernatant was further centrifuged at 100,000*g* for 90 min at 4°C to yield the crude cytosolic extract (25.5 mg/ml protein).

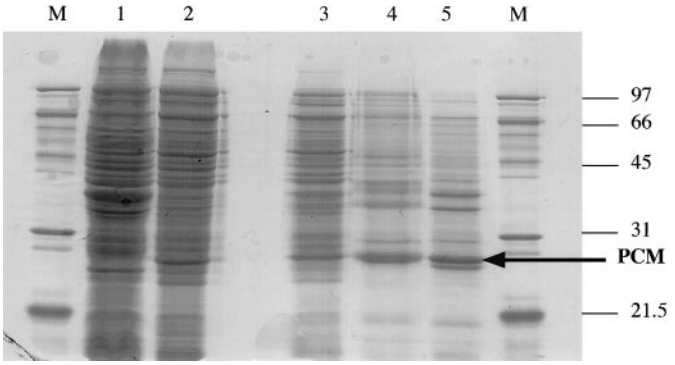

FIG. 3. SDS–polyacrylamide gel electrophoresis analysis of *Arabidopsis* recombinant L-isoaspartyl methyltransferase in bacterial extracts. Extracts were prepared and the proteins fractionated on the gel as described under Materials and Methods. Lane M indicates the Bio-Rad low molecular weight standards (total of 18 μg of protein), including phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.5 kDa). The last standard, lysozyme, is not shown here. The samples analyzed were (1) uninduced cytosolic extract (48 μg), (2) induced cytosolic extract (38.4 μg), (3) induced pellet extract (inclusion bodies, 22 μg), (4) inclusion bodies in 6 M urea (12.5 μg), and (5) renatured inclusion bodies (7.5 μg). The gel was stained with Coomassie brilliant blue and the position of the methyltransferase protein (PCM) is indicated by the arrow.

DEAE–cellulose anion exchange chromatography. Thirty-five milliliters of the crude cytosolic extract was loaded onto a DEAE-52 anion exchange column (Bio-Rad, 2.5-cm internal diameter × 16.5-cm height, 81-ml bed volume) preequilibrated with buffer B (0.05 M Tris–Cl, pH 7.7) at 4°C. The column was washed with 3.7 column volumes of equilibration buffer and the enzyme was then eluted using a linear sodium chloride gradient (0–0.3 M in equilibration buffer, 10 column volumes). The flow rate was 19.6 ml/h and 4.9-ml fractions were collected. The activity eluted as two peaks—peak I at a sodium chloride concentration of 0.08 M (fractions 119–125) and peak II at a sodium chloride concentration of 0.09 M (fractions 128–134). Four milliliters of each of the fractions (119–125) of peak I was pooled (1.4 mg/ml protein) and 4 ml of each of the fractions (128–134) of peak II was pooled (2.6 mg/ml protein). These two peaks were then processed sepa-

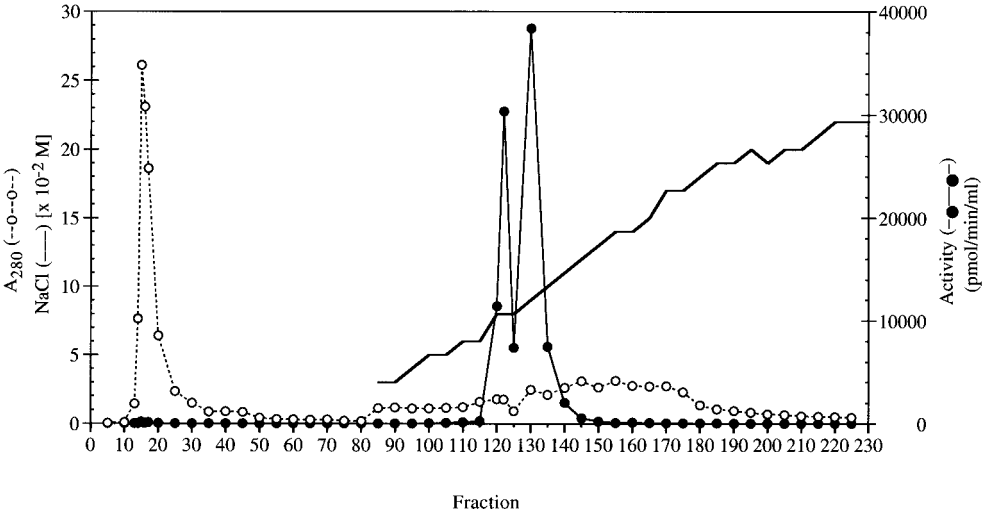

FIG. 4. DEAE–cellulose anion exchange chromatography to purify *Arabidopsis* recombinant L-isoaspartyl methyltransferase. Bacterial cells expressing the enzyme were lysed and the cytosolic fraction was loaded onto a DE52 column. The enzyme was eluted using a sodium chloride gradient as described under Materials and Methods. Fractions were assayed for protein content by measuring the optical density at 280 nm using a 10- to 100-fold dilution of the fractions in water and for methyltransferase activity.

rately and designated as peak I load and peak II load, respectively.

Ammonium sulfate precipitation. Ammonium sulfate was added to each of the above pools at a final saturation level of 80% (w/v) and the contents stirred at 4°C for 1 h. The mixtures were then centrifuged at 11,000*g* for 20 min at 4°C and each of the pellets was suspended in 2 ml of buffer C (35% w/v ammonium sulfate in 0.05 M Tris–Cl/pH 7.7, 12 mM 2-mercaptoethanol) to a final protein concentration of 8 and 16 mg/ml, respectively.

Superdex S-200 chromatography. Two milliliters of each of the redissolved ammonium sulfate pellets was loaded onto Superdex S-200 columns (Amersham Pharmacia Biotech, 1.5-cm internal diameter × 50-cm height, 88-ml bed volume), which were preequilibrated with 0.05 M Tris–Cl (pH 7.7) at 4°C. Elution for peak I load was performed at a flow rate of 12.5 ml/h and 2.5-ml fractions were collected. The activity was found to elute at fractions 25–35; however, upon analysis of these fractions on an SDS–PAGE gel, it was observed that the proteins were not resolved well and the active

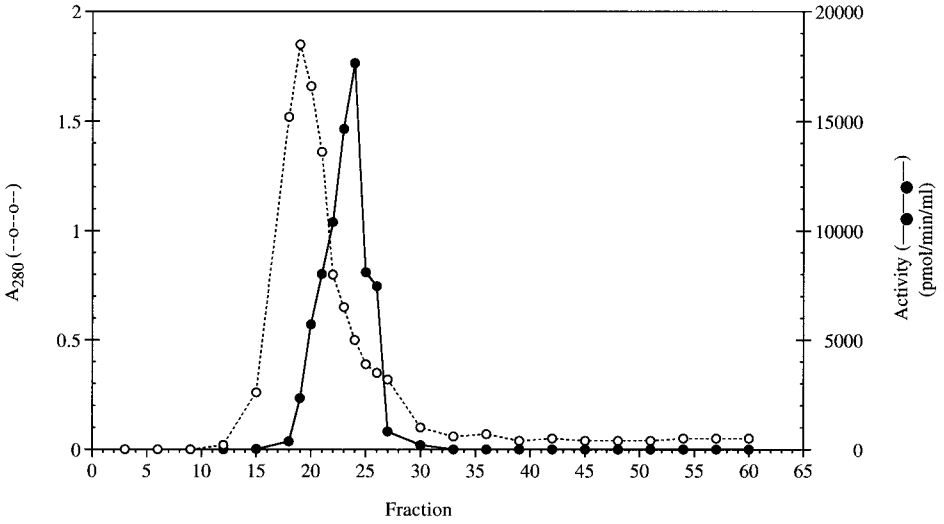

FIG. 5. Superdex S-200 gel filtration chromatography to purify *Arabidopsis* recombinant L-isoaspartyl methyltransferase. The active fractions (128–134) of the DE52 step were pooled and loaded onto an S-200 column as described under Materials and Methods. Every third fraction was assayed for protein content and methyltransferase activity.

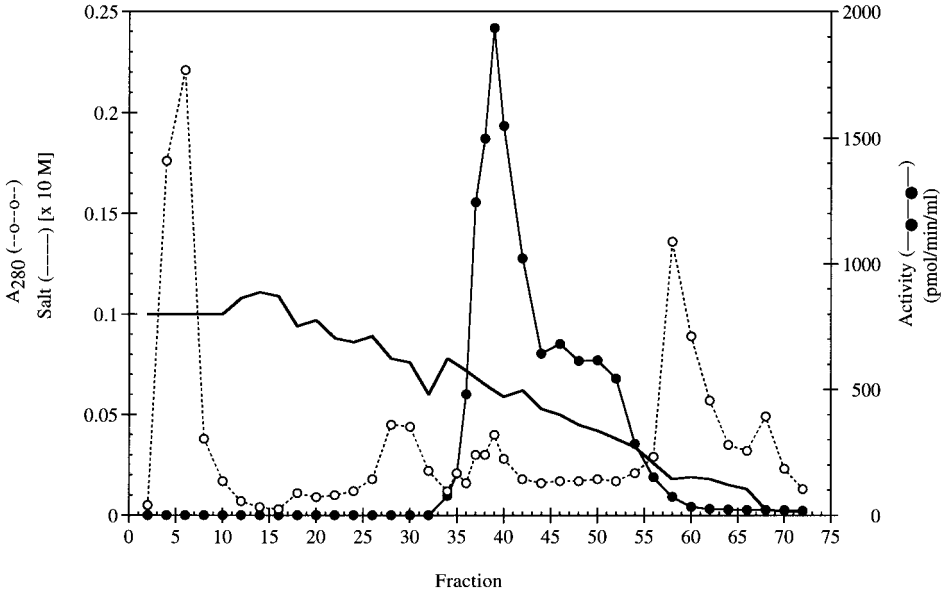

FIG. 6. Phenyl–Sepharose hydrophobic interaction chromatography to purify *Arabidopsis* recombinant L-isoaspartyl methyltransferase. Fractions 21–26 of the S-200 step were pooled and loaded onto a Phenyl–Sepharose column as described under Materials and Methods. Every second fraction was assayed for protein content and methyltransferase activity.

fractions still had a substantial level of contaminating proteins. Thus, peak I was not processed further beyond this step. Elution for peak II load was performed at a flow rate of 18 ml/h and 3-ml fractions were collected. The activity was found to elute at fractions 19–26. For the next step, 2.95 ml of each of the fractions (21–26) was pooled.

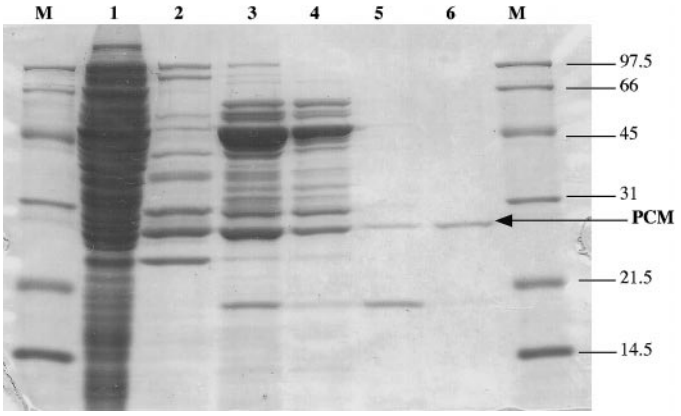

FIG. 7. SDS–polyacrylamide gel electrophoresis analysis of *Arabidopsis* recombinant L-isoaspartyl methyltransferase. Active fractions from each chromatographic step were loaded and the electrophoresis performed using the Laemmli buffer system. The gel was stained with Coomassie brilliant blue. Lane M indicates the Bio-Rad low molecular weight standards (18 μg of protein) as in Fig. 3. The samples analyzed were (1) crude cytosolic fraction (125 μg), (2) peak I of DE52 column (fractions 119–125, 28 μg), (3) peak II of DE52 column (fractions 128–134, 33.3 μg), (4) fractions 21–23 of S-200 column (22.5 μg), (5) fractions 24–26 of S-200 column (6.3 μg), and (6) fraction 38 of Phenyl–Sepharose column (0.6 μg). The position of the methyltransferase protein (PCM) is indicated by the arrow.

Hydrophobic interaction chromatography. To the active pool obtained from the S-200 chromatographic run, ammonium sulfate was added to a final concentration of 1 M and this salted pool was then applied to a Phenyl–Sepharose column (Amersham Pharmacia Biotech, 1-cm internal diameter × 14-cm height, 11-ml bed volume) preequilibrated with buffer D (1 M ammonium sulfate in 0.05 M Tris–Cl, pH 7.8) at 4°C. After loading the sample, the column was washed with 5.5 column volumes of equilibration buffer and the enzyme was then eluted with a linear reverse gradient of ammonium sulfate (1 to 0 M in equilibration buffer). The flow rate was 30 ml/h and 5-ml fractions were collected. The enzyme activity was found to elute between 0.6 and 0.7 M ammonium sulfate concentration (fractions 36–43). These active fractions were stored at −20°C until further use.

SDS–Polyacrylamide Gel Electrophoresis

Gel electrophoresis was performed with the Laemmli buffer system (47), using a 12.5% separating gel. Bio-Rad low molecular weight markers were used as standards. Gels were analyzed by staining with 0.1% Coomassie brilliant blue R-250 (w/v) in 50% (v/v) methanol and 10% (v/v) acetic acid and rapid silver staining (48).

Biochemical Characterization of the Purified Arabidopsis Recombinant Methyltransferase

Fraction 38 of the Phenyl–Sepharose step was chosen as the source of pure recombinant *Arabidopsis* L-isoaspartyl methyltransferase for assays to deter-

TABLE 3

Purification of *A. thaliana* Recombinant L-Isoaspartyl Methyltransferase from *E. coli*

Sample	Protein (mg/ml)	Total protein (mg)	Volume (ml)	Activity (pmol/min/ml)	Total activity (pmol/min)	Specific activity (pmol/min/mg)	Recovery (%)	Purification (fold)
Crude soluble extract	25.5	892	35	35,970	1,260,000	1,410	100	1
DE52 pool								
I (fractions 119–126)	1.65	64.6	39.2	13,700	537,000	8,310	43	5.9
II (fractions 127–134)	2.22	87.2	39.2	23,100	906,000	10,400	72	7.4
S-200	1.22	17.1	21	10,280	216,000	12,600	17	8.9
Phenyl–Sepharose	0.027	1.1	40	1,140	45,700	41,200	3.2	29

mine the optimum temperature, pH, substrate specificity, and kinetic rate constants.

RESULTS AND DISCUSSION

Cloning of a Full-Length Arabidopsis L-Isoaspartyl Methyltransferase cDNA into an Expression Vector

Although various *E. coli* expression systems available presently produce high levels of the cloned gene product, most of them also produce substantial levels of the product in an uninduced state. For this reason, we chose the pBAD expression vector (36) consisting of a P_{BAD} promoter of the arabinose operon. Transcription from the P_{BAD} promoter is induced by the presence of arabinose and repressed when arabinose is absent. However, the uninduced levels are repressed further in the presence of glucose. Thus, the expression system offers tight regulation as well as efficient modulation. The pBAD24 vector utilized in this study specifically contains an optimized Shine–Dalgarno sequence (42) as well as a translation start codon (ATG) at the *Nco*I site.

Cloning of the L-isoaspartyl methyltransferase cDNA into pBAD24 required inserting the sequence at the *Nco*I site in order for it to be in the correct reading frame. The *Arabidopsis* full-length cDNA was amplified out of pNT1 (Fig. 2A) using a specifically designed 5′ primer having an *Nco*I site, digested with *Nco*I and *Xba*I, and cloned into the pBAD24 vector at the *Nco*I/*Xba*I sites. This plasmid is designated as pNT2 (Fig. 2B). The presence of the gene was confirmed by PCR as well as restriction digests. To clone the sequence at the *Nco*I site, we had to modify the second amino acid residue of the protein encoded from lysine to glutamate. Because *E. coli* has an endogenous L-isoaspartyl methyltransferase, encoded by the *pcm* gene, we utilized mutant strain JV1068 as the host where the *pcm* gene is disrupted and active enzyme is not produced (43). This strain would thus offer a clean background for producing the plant methyltransferase as the only L-isoaspartyl methyltransferase.

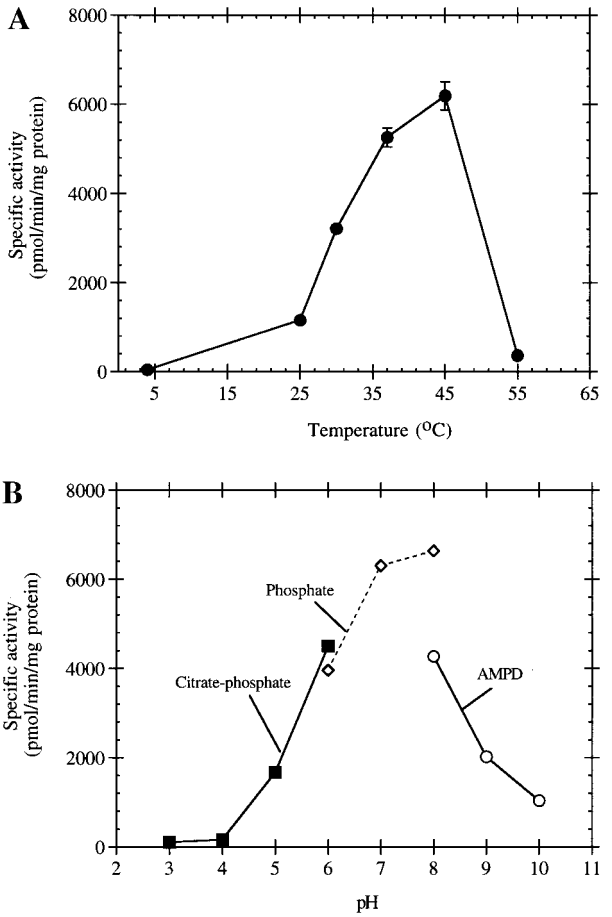

FIG. 8. (A) Effect of temperature on the activity of purified *Arabidopsis* recombinant L-isoaspartyl methyltransferase. The activity of the purified enzyme was measured at temperatures ranging from 4 to 55°C. (B) Effect of pH on the activity of purified recombinant *Arabidopsis* L-isoaspartyl methyltransferase. The activity of the purified enzyme was measured at 37°C in final concentrations of 0.05 M disodium phosphate adjusted to pH with citric acid (pH 3–6), 0.05 M sodium phosphate (pH 6–8), and 0.05 M 2-amino-2-methyl-1,3-propanediol (AMPD) (pH 8–10). For both A and B, reactions were performed as described under Materials and Methods using 0.06 μg of the enzyme except that the time of incubation was 20 min. Reactions were done in triplicate and the error bars represent the standard deviation from the mean value. When no error bar is shown, the error was smaller than the width of the line.

TABLE 4

Activity of Purified *Arabidopsis* Recombinant L-Isoaspartyl Methyltransferase toward Different L-Isoaspartate- and D-Aspartate-Containing Peptide and Protein Substrates

Substrate[a]	Specific activity[b] (pmol/min/mg of protein)
KASA-[L-*isoAsp*]-LAKY	4179 ± 23
VYP-[L-*isoAsp*]-HA	5028 ± 64
VYP-[L-*isoAsp*]-CA	1441 ± 23
VYR-[L-*isoAsp*]-RR	229 ± 12
YVS-[L-*isoAsp*]-GHG	12.7 ± 0.7
KASA-[D-*Asp*]-LAKY	ND[c]
VYP-[D-*Asp*]-PA	ND[c]
Ovalbumin	433 ± 35
γ-Globulin	130 ± 23

[a] All the substrates were used at a concentration of 0.2 mM. The peptides were synthesized by the UCLA Peptide Synthesis Facility; ovalbumin (Grade V, 98% pure) and γ-globulin (Cohn Fraction II, bovine, 99% pure) were purchased from Sigma.

[b] Reactions were performed at 37°C as described under Materials and Methods except that the reaction time was 20 min; 0.06 μg of the enzyme was used. The endogenous activity in the absence of methyl-accepting substrate is subtracted. All reactions were done in duplicate and values represent the mean ± range.

[c] Not detectable.

Overexpression of the Arabidopsis Recombinant L-Isoaspartyl Methyltransferase Enzyme

Control experiments were first conducted to establish optimum conditions for overexpression of the recombinant protein. To determine the optimum concentration of arabinose required for induction, the *E. coli* strain JV1068 harboring the pNT2 plasmid was grown in rich medium to an optical density of 0.5 at 600 nm and was then induced with concentrations of arabinose ranging from 0.05 to 1%. Cells were harvested at a density of 0.9–1.2 absorbance units and cytosolic extracts were assayed for peptide-specific methyltransferase activity. The optimal concentration of the arabinose for maximal induction of methyltransferase activity (1800 pmol/min/mg of protein, representing a 518-fold induction) was found to be 0.2% and a small decrease in the expression was observed when the concentration was raised above this level. A small level of endogenous activity (in the absence of peptide) was detected in induced extracts, which suggests that the recombinant enzyme may be acting on damaged host strain proteins (data not shown). As a control, the JV1068 host strain alone was also induced with 0.2% arabinose—extracts from these cells show negligible levels of methyltransferase.

Glucose is known to be a repressor of the P_{BAD} promoter (39). We thus monitored its effect on the expression by adding glucose alone or in conjunction with arabinose to two sets of cultures containing the pNT2 plasmid at 0.5 optical density units at 600 nm. In the presence of 0.2% glucose alone, the activity was decreased to 0.5% of the induced level. When 0.2% arabinose and 0.2% glucose were used together, strong repression still occurred and the activity was only 0.6% of the induced level. Thus the expression of this protein using the pBAD expression vector can be tightly modulated using a combination of inducer and repressor. Finally, to determine the optimal time for induction, cultures were induced with 0.2% arabinose at fixed time intervals to 6 h after inoculating fresh media with an overnight culture. The optimum time for induction was found to be the early stationary phase (4 h).

To determine whether the overexpressed recombinant protein exists in the soluble fraction or forms inclusion bodies, the cells were induced with 0.2% arabinose, lysed by French press treatment, and separated into a supernatant fraction representing the soluble form and a pellet fraction that would include inclusion bodies. As shown in Table 2, over 99% of the activity is localized in the soluble fraction. Interestingly, when the inclusion bodies were treated with 6 M urea, we found that the resulting soluble fraction was enriched in methyltransferase activity, with a specific activity more than threefold higher than that in the original cytosolic extract (Table 2). This indicates that this enzyme is an exceptionally stable protein, retaining activity even in the presence of a strong denaturant. When the urea was removed in a step dialysis protocol with buffers with decreasing urea concentration (50), there was a significant loss of activity for unknown reasons. Analysis of the protein profile of the recombinant protein on a SDS–PAGE gel indicates the presence of a ~28-kDa protein in the induced extracts (Fig. 3). This agrees well with the expected molecular

TABLE 5

Kinetic Rate Constants of Purified *Arabidopsis* Recombinant L-Isoaspartyl Methyltransferase

Substrate	K_m (mM)	V_{max} (pmol/min/mg of protein)[a]
KASA-[L-*isoAsp*]-LAKY	0.080 ± 0.018	5007 ± 22
VYP-[L-*isoAsp*]-HA	0.31 ± 0.02	5739 ± 203
Ovalbumin	4.95 ± 0.39	5959 ± 440
AdoMet	0.006 ± 0.0002	5763 ± 24

[a] Methyltransferase reactions were performed as described under Materials and Methods in triplicate using varying concentrations of the substrates VYP-L-*isoAsp*-HA and KASA-L-*isoAsp*-LAKY (0–2 mM), ovalbumin (0–3 mM), and [^{14}C]AdoMet (0–20 μM). For the reactions with variable peptide and ovalbumin concentrations, AdoMet was used at a concentration of 10 μM whereas for variable AdoMet concentrations, VYP-L-*isoAsp*-HA was used as a substrate at 0.5 mM. All the reactions were performed at 37°C. The kinetic parameters of the enzyme were calculated by fitting the data to the Michaelis–Menten equation using the DeltaGraph (Version 4.0) software. Values represent the mean ± SD.

TABLE 6

Comparisons of Michaelis–Menten Rate Constants for the Protein L-Isoaspartyl Methyltransferase from Various Organisms

	K_m (μM)			
	VYP-[L-*isoAsp*]-HA	KASA-[L-*isoAsp*]-LAKY	Ovalbumin	AdoMet
T. maritima[a]	4.1 ± 0.71	2.8 ± 0.24	326 ± 56	1.9 ± 0.7
E. coli[b]	11.8	50.6	727	N/A
Arabidopsis[c]	310 ± 20	80 ± 18	4950 ± 390	6 ± 0.2
Wheat germ[d]	51.7	12.7	>1500	N/A
Maize[e]	43 ± 0.6	92 ± 12	2660 ± 30	N/A
C. elegans[f]	19.4 ± 0.5	9.12 ± 0.02	>3000	3.1 ± 0.01
Human[g]	0.29 ± 0.03	0.52 ± 0.08	30	2.2 ± 0.2

[a] Purified *T. maritima* recombinant enzyme (21).
[b] Partially purified native *E. coli* enzyme (52).
[c] Purified *Arabidopsis* recombinant enzyme (this work).
[d] Purified native wheat germ enzyme (24).
[e] Partially purified corn enzyme (unpublished results). Reactions were done at 45°C.
[f] Partially purified *C. elegans* recombinant enzyme (29).
[g] Purified human erythrocyte enzyme (1).

weight of the protein, ~24.6 kDa. The methyltransferase polypeptide from the soluble fractions of the inclusion bodies in 6 M urea appears to be enriched as expected from the high specific activity shown in Table 2.

Purification of the Arabidopsis Recombinant L-Isoaspartyl Methyltransferase Protein

Since the majority of the recombinant enzyme exists in the soluble fraction, we proceeded to purify the enzyme from this material as described under Materials and Methods. Eight liters of a culture of JV1068 cells containing pNT2 plasmid was induced with 0.2% arabinose in the late-logarithmic phase and the cells were harvested and lysed by French press treatment. This lysate was subjected to a low-speed spin (15,000*g*) and the supernatant recovered was then spun at 100,000*g* to obtain the cytosolic fraction. This fraction was used as the starting material for purification. The supernatant was loaded onto an anion exchange column (DE52) and the enzyme was eluted with an increasing sodium chloride gradient (0–0.3 M). We found that the enzyme activity eluted in two peaks—I (fractions 118–126) and II (fractions 127–135) at 0.08 and 0.09 M NaCl, respectively (Fig. 4). The specific activity of the methyltransferase was slightly lower in peak I (8307 pmol/min/mg of protein) as compared to peak II (10,380 pmol/min/mg of protein). SDS–PAGE gel analysis re-

TABLE 7

Activity of the Full-Length and Truncated Versions of the *Arabidopsis* Recombinant L-Isoaspartyl Methyltransferase in *E. coli*

	Activity (pmol/min/mg of protein)[a]			
	Soluble fraction		Pellet fraction	
Strain	Uninduced	Induced	Uninduced	Induced
pNT3 (truncated) in BL21(DE3)	3.68 ± 0.32	0.34 ± 0.07	1.82 ± 0.18	1.76 ± 0.73
pNT4 (full-length) in BL21(DE3)	2.34 ± 0.01	433 ± 1.4	5.54 ± 0.04	69.5 ± 0.9
BL21(DE3) (no plasmid)	0.28 ± 0.15	ND[b]	1.69 ± 0.26	ND[b]

[a] Cells were grown to an optical density of 0.45 at 600 nm and induced with 1 mM IPTG and harvested at 1.2 optical density units. Cell lysates were prepared by sonication as described under Materials and Methods. Lysates were centrifuged at 16,000*g* for 15 min at 4°C and the supernatants were separated and saved. The resulting pellet was washed once with sterile water and then suspended in chilled extraction buffer A (1/20th the volume of the original culture volume). The values represent the peptide-specific activity after subtracting the background endogenous activity. Assays were done four to six times and the errors represent the standard deviation from the mean.
[b] Not done.

vealed that both the peaks had a prominent polypeptide at 28 kDa. The second peak displayed the presence of contaminating high molecular weight proteins whereas the first peak had much lower levels of these proteins. Since both the peaks had significant activity, we decided to process them separately.

Both peak fractions were concentrated by precipitating the protein with ammonium sulfate at a final concentration of 80%. The precipitates were then suspended in a suitable buffer and applied to a gel filtration column (S-200) under conditions where we expected some salt-induced binding of the enzyme to the gel matrix that would be useful in purification (24). The proteins in peak I were not well resolved and the methyltransferase activity eluted as a broad peak (data not shown), and this fraction was not processed further. On the other hand, the proteins of the second peak were better resolved (Fig. 5). Most of the contaminating protein eluted before the methyltransferase activity (fractions 20–26). Fractions 21–26 were then pooled and suspended in a 20% ammonium sulfate containing buffer and loaded onto a hydrophobic Phenyl–Sepharose chromatographic column. Elution of the proteins was done using a reverse gradient with decreasing ammonium sulfate concentration (1 to 0 M). The methyltransferase activity was resolved as a sharp peak distinct from that of the other contaminating proteins (Fig. 6). The specific activity of one of these fractions (fraction 38) was 55,000 pmol/min/mg of protein. Analysis of the protein profile of the peak fractions on a SDS–PAGE gel revealed a single protein band at 28 kDa (Fig. 7). Upon silver staining the gel, a single polypeptide species was also observed (data not shown). Thus, these peak fractions were subsequently used as the source of homogeneously pure enzyme for biochemical and kinetic studies. Final purification was achieved at a level of 29-fold (Table 3).

Characterization of the Purified Arabidopsis Recombinant L*-Isoaspartyl Methyltransferase*

The purified enzyme was assayed at different temperatures to establish optimal conditions for its activity. As observed in Fig. 8A, the enzyme was most active at 45°C. This type of profile was also observed for the plant L-isoaspartyl methyltransferases from wheat, corn, and rice seeds as well as the bacterial extracts and the human enzyme (data not shown). Thus the methyltransferase protein itself displays significant temperature stability. The recombinant enzyme does, however, lose its activity rapidly at 55°C.

The pH optimum for the activity of the purified enzyme was determined over a range of pH of 3–10 using citrate–phosphate (pH 3–6), phosphate (pH 6–8), and 2-amino-2-methyl-1,3-propanediol (pH 8–10) buffers (Fig. 8B). We found that the enzyme was most active in the pH 7–8 range, with half-maximal activity at about pH 5.5 and pH 8.5. We also found that the nature of the buffer itself could affect the enzyme activity. At pH 8, the enzyme was about 50% more active in the phosphate buffer than in the 2-amino-2-methyl-1,3-propanediol buffer.

Next, we analyzed the activity of the pure enzyme toward various synthetic peptides as well as two protein methyl-accepting substrates, ovalbumin and γ-globulin, which are known to be methylated by isoaspartyl methyltransferases *in vitro* (51). The enzyme was found to have the best efficiency for VYP-L-*isoAsp*-HA and KASA-L-*isoAsp*-LAKY; less activity was found for VYP-L-*isoAsp*-CA and VYR-L-*isoAsp*-RR and little or no activity was found for YVS-L-*isoAsp*-GHG (Table 4). These results suggest that the mere presence of an L-isoaspartate residue in a protein does not guarantee its efficient methylation by the methyltransferase enzyme but that effective methylation also depends on the neighboring sequence of amino acid residues. We found no activity toward peptides containing D-aspartate residues and this corroborates the results observed for the methyltransferase enzyme from other nonmammalian enzymes (21, 24, 29). On the other hand, the enzyme did exhibit some activity toward the protein substrates, though not to the same extent as it did toward the L-isoaspartate-containing peptides (Table 4).

The purified enzyme was next analyzed for its kinetic parameters with both peptide and protein substrates (Table 5). The K_m value of 310 μM determined for VYP-L-*isoAsp*-HA was significantly higher than the values measured previously for this peptide with methyltransferases from bacteria (4.1–11.8 μM), worms (19.4 μM), humans (0.29 μM), or other plants (43–52 μM) (Table 6), suggesting that the *Arabidopsis* enzyme recognizes this substrate relatively poorly. As a control, we also measured the K_m of the native *Arabidopsis* methyltransferase from seed extracts prepared as described in Ref. 24. The K_m value obtained (270 ± 20 μM) was found to be very similar to that of the purified recombinant enzyme. As shown in Table 6, relatively poor affinity was also found for the methyl-accepting peptide KASA-L-*isoAsp*-LAKY, where the K_m of the purified *Arabidopsis* enzyme (80 μM) was also higher than the values from the enzymes of other species, with the exception of the enzyme from maize extracts (92 μM). Ovalbumin was also found to be a relatively poor substrate for the purified *Arabidopsis* enzyme, with a K_m value of 5.0 mM (Table 5). This value for the recombinant enzyme can be compared with that of the native enzyme from *Arabidopsis* seed extracts of 1.2 ± 0.6 mM, although the V_{max} value relative to that of the peptide substrate was also lower, indicating a similar catalytic efficiency of the native enzyme and the recombinant enzyme. Relatively high K_m values for ovalbu-

min have also been previously observed for the wheat germ enzyme ($K_m > 1.5$ mM), the maize enzyme (2.6 mM), and the worm enzyme (>3 mM), although the human enzyme recognizes the substrate with relatively high affinity at a K_m value of 30 μM (Table 6). Finally, we measured the K_m for AdoMet (6 μM), the methyl group donor for the methylation reaction, and found it to be similar to that reported for the nematode and human enzymes (Table 6). Thus the enzymes from the three different plant species have differential substrate affinities. It is possible that these enzymes are evolutionarily "tuned" to recognize the major damaged proteins (and possibly peptides) that they encounter *in vivo*.

Analysis of Activity of a Deletion Mutant of Arabidopsis L-*Isoaspartyl Methyltransferase*

The pMBM6 cDNA clone lacks the first 77 nucleotides of the coding sequence (25). The protein encoded by this cDNA would thus be missing the first 26 amino acid residues and have a predicted molecular weight of 20.8 kDa as opposed to the native full-length protein of 230 amino acids with a predicted molecular weight of 24.6 kDa. To determine whether the N-terminus of the protein is essential for its activity, we expressed this truncated cDNA in *E. coli* and monitored the activity of the enzyme. The expression vector we chose was pT7-7 because of the availability of convenient restriction sites for cloning. This vector has a strong ribosome binding site as well as an ATG start codon upstream of the polylinker sequence. This ATG codon would be necessary for expressing the truncated cDNA as it lacks its own start codon. The truncated cDNA was isolated from pMBM6 by digesting the plasmid with *Eco*RI and then cloned into pT7-7 at the *Eco*RI site (which lies just downstream of the ATG codon) (Fig. 2C). As a control, the full-length version of the cDNA was also cloned from pNT2 into the *Eco*RI site of pT7-7 (Fig. 2D). The recombinant plasmids were then used to transform the bacterial strain BL21(DE3), which has the gene for the T7 RNA polymerase on its chromosome under the control of the *lac* promoter, facilitating the induction by IPTG. Cells were grown to an optical density of 0.45 at 600 nm and were then induced with 1 mM IPTG. Cells were harvested after 2 h and crude extracts were prepared by sonicating the cells. Both the supernatant and the pellet were analyzed for activity. As observed in Table 7, both the full-length version and the truncated version of the *Arabidopsis* methyltransferase were active. Under uninduced conditions, a comparable level of basal activity was detected for both the enzymes above the background of that detected in the host strain, BL21(DE3), alone. However, when cells were induced, we observed a high level of activity only for the full-length version but not for the truncated form. In fact, under induced conditions, the activity of the truncated enzyme decreased to the background level found in the host strain. Thus, although the N-terminal truncated enzyme is active, it may aggregate when overexpressed, leading to a complete loss in activity. On the other hand, the full-length version remained soluble when overexpressed.

These results suggest that the N-terminal 26 amino acids of the *Arabidopsis* L-isoaspartyl methyltransferase may not be essential for catalysis but are required for enzyme stability. We note that other types of methyltransferases lacking N-terminal residues also retain full activity. Konishi and Fujioka (53) found that rat liver glycine methyltransferase missing the N-terminal 8 amino acid residues had kinetic parameters identical to those of the native enzyme, Malherbe *et al.* (54) expressed the human soluble catechol-*O*-methyltransferase lacking the first 26 amino acids in *E. coli* and found it to be catalytically active, and Zheng *et al.* (55) demonstrated that N-terminal truncated Rubisco large subunit N^{ϵ}-methyltransferase missing the first 36 amino acids exhibited a 10-fold increase in activity when expressed in *E. coli.* In these latter cases, however, the truncated proteins were stable.

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