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Molecular cloning and bacterial expression of cDNA encoding a plant cysteine synthase

[*Spinacia oleracea* L./genetic complementation/*O*-acetylserine(thiol)-lyase/sulfur assimilation/pyridoxal phosphate]

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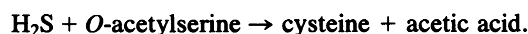
ABSTRACT **Cysteine synthase (CSase) [*O*-acetyl-L-serine acetate-lyase (adding hydrogen sulfide), EC 4.2.99.8] catalyzes the formation of L-cysteine, the key step in sulfur assimilation in plants, from *O*-acetyl-L-serine and hydrogen sulfide. We report here the isolation and characterization of cDNA clones encoding cysteine synthase from spinach (*Spinacia oleracea* L.). Internal peptide sequences were obtained from V8 protease-digested fragments of purified CSase. A λgt10 cDNA library was constructed from poly(A)$^+$ RNA of young green leaves of spinach. Screening with two synthetic mixed nucleotides encoding the partial peptide sequences revealed 19 positively hybridized clones among 2 × 10^5 clones. Nucleotide sequence analysis of two independent cDNA clones revealed a continuous open reading frame encoding a polypeptide of 325 amino acids with a calculated molecular mass of 34,185 Da. Sequence comparison of the deduced amino acids revealed 53% identity with CSases of *Escherichia coli* and *Salmonella typhimurium*. Sequence homology was also observed with other metabolic enzymes for amino acids in bacteria and yeast and with rat hemoprotein H-450. A bacterial expression vector was constructed and could genetically complement an *E. coli* auxotroph that lacks CSases. The accumulation of functionally active spinach CSase in *E. coli* was also demonstrated by immunoblotting and assaying enzymatic activity. Southern hybridization analysis showed the presence of two to three copies of the cDNA sequence in the genome of spinach. RNA blot hybridization suggested constitutive expression in leaves and roots of spinach.**

Most inorganic sulfate assimilated by plants appears ultimately in cysteine and methionine in proteins (1). Cysteine is the principal starting metabolite for the synthesis of other sulfur-containing metabolites, such as the sulfur moiety of methionine (2). The *de novo* synthesis of cysteine is one of the key reactions in biology, being comparable in importance to CO_2 assimilation in photosynthesis because animals require a dietary source of methionine for sulfur metabolism to inorganic sulfate and plants, in turn, assimilate inorganic sulfate back to cysteine. Cysteine synthase (CSase) [*O*-acetyl-L-serine acetate-lyase (adding hydrogen sulfide), EC 4.2.99.8], therefore, plays a central role in the sulfur cycle in nature. This pyridoxal phosphate (PLP)-dependent enzyme catalyzes the formation of cysteine from *O*-acetylserine and hydrogen sulfide through the following reaction (2, 3):



$$H_2S + O\text{-acetylserine} \rightarrow \text{cysteine} + \text{acetic acid.}$$

This enzyme is also responsible for the biosynthesis of some heterocyclic β-substituted alanines, some of the important secondary plant products (4, 5). These nonprotein amino acids—e.g., mimosine (6) and quisqualic acid (7), are formed through the actions of particular isoforms of CSase from *O*-acetylserine and heterocyclic compounds instead of hydrogen sulfide. For further molecular biological investigation of the biosynthesis of these amino acids, it was imperative to obtain cDNA encoding CSase from plants.

Our recent study indicated that there are two isoforms of cysteine synthase, CSases A and B, in green leaves of spinach. The subcellular localizations of these isoforms are different (K.S., unpublished data). In this paper, we report the isolation of spinach cDNA clones encoding CSase A by means of a strategy involving the microsequencing of peptide fragments followed by synthetic oligonucleotide screening of a library. We have sequenced and characterized the isolated cDNA clones.§ The expression of the cDNA in a cysteine-auxotroph *Escherichia coli* could genetically complement the cysteine requirement.

MATERIALS AND METHODS

Microsequencing of Peptide Fragments of CSase A. The amino acid sequences of internal peptide fragments were determined as described (8). Briefly, the purified CSase A (K.S., unpublished data) was dissolved in 20 μl of load buffer [0.125 M Tris·HCl (pH 6.8)/0.1% (wt/vol) SDS/20% (wt/vol) glycerol]. Ten microliters of *Staphylococcus aureus* V8 protease (Pierce) (0.1–0.2 μg/μl) in water was overlaid with 10 μl of load buffer containing 0.001% (wt/vol) bromophenol blue. Electrophoresis was done until the sample was stacked in the upper gel and then interrupted for 30 min to digest the protein. Electrophoresis was continued, and the separated digests were electroblotted onto the poly(vinylidene difluoride) membrane (Immobilon P, Millipore). The amino acid sequence was analyzed by a gas-phase protein sequencer (model 477A, Applied Biosystems), according to the standard program recommended by the supplier.

Construction and Screening of a cDNA Library. Poly(A)$^+$ RNA was isolated from 10-week-old leaves of *Spinacia oleracea* cv. parade (Sakata, Yokohama, Japan) by guanidine hydrochloride/phenol extraction followed by oligo(dT)-cellulose column chromatography (9). Double-stranded cDNAs were prepared by the method of Gubler and Hofmann (10). After ligation with an *Eco*RI adaptor, size fractionation, and phosphorylation, the cDNAs were inserted into the *Eco*RI site of λgt10 by using a kit supplied by Amersham.

Approximately 2 × 10^5 nonamplified plaques were screened with two synthetic oligonucleotide probes encoding the internal peptide fragments, V8-1 and V8-2. Probe V812 encoding V8-1 was a 50-mer in length and exhibited 16-fold degeneracy, containing 11 deoxyinosines, 3′-TGI TGI CCI GGI CTT(C) TAI ACC TTT(C) CCI TGI CCI CCI TTT(C)

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Abbreviations: CSase, cysteine synthase; PLP, pyridoxal phosphate.
†To whom reprint requests should be addressed.
§The sequence reported in this paper has been deposited in the GenBank data base (accession no. D10476).

TAI CTG(A) TAI AA-5′. Probe V822 encoding V8-2 was a "guessmer" (11), 56-mer in length, and exhibited 8-fold degeneracy, containing 9 deoxyinosines, 3′-CAI GAC TAI CTT(C) GGC TGG TCG CCI TTG(A) TGI CCI TAA CCI GAC CGI AAG(A) TAI CGI CG-5′. Duplicate filters (Hybond-N+, Amersham) were obtained, and each filter was hybridized with the two ^{32}P-labeled probes, respectively. The final washes on posthybridization were done in 6× standard saline citrate (SSC)/0.1% (wt/vol) SDS at 42°C. Nineteen doubly positive clones were isolated for both V812 and V822.

Subcloning and DNA Sequence Analysis. Among the 19 clones, two, designated as λCS2 and λCS4 and possessing ≈1.3-kilobase (kb) -length inserts, were subcloned into pUC19 and M13mp18 for nucleotide sequencing (12). The DNA sequence was determined on both strands by the dideoxynucleotide chain-termination method and a series of synthetic primers with a 7-deaza Sequenase kit version 2.0 (United States Biochemical). Sequence comparison was analyzed by DNASIS (version 7.0) -DBREF 56 (version 10) system, with the sequence libraries of GenBank (R68.0), the National Biomedical Research Foundation–Protein Information Resource (R28.0), the European Molecular Biology Library (R27.0), and Swiss–Prot (R18.0).

Expression in a Cysteine-Auxotroph, *E. coli* NK3. A *Nco* I restriction site was introduced at the initiation ATG region of the cDNA by using an oligonucleotide-directed *in vitro* mutagenesis system (Amersham). The prokaryotic expression vector pKM1 was constructed by insertion of the mutagenized coding region of the cDNA clone λCS2 into a unique *Nco* I site of pTV118N (Takara Shuzo, Kyoto). In pKM1, the coding region of cDNA started at 8 base pairs (bp) under the Shine–Dalgarno sequence of the *lacZ* promoter. A cysteine-auxotroph, *E. coli* NK3 (*ΔtrpE5 leu-6 thi hsdR hsdM$^+$ cysK cysM*), was transformed with pKM1 or with pTV118N as a control. For genetic complementation of the cysteine requirement, the transformed *E. coli* was cultured on an M9 agar plate (9) supplemented with 0.02% leucine and tryptophan. For immunoblotting and enzyme assaying, *E. coli* was grown in LB medium (9) containing carbenicillin (100 mg/liter) for 2.5 hr at 37°C; isopropyl β-D-thiogalactoside was added to 1 mM, and the incubation was continued for 1.5 hr. Bacteria were harvested by centrifugation at 4°C, and total protein was extracted as described (7).

Immunoblot Analysis and Enzyme Assaying. Immunoblot and immunostaining analyses were done on an Immobilon P membrane with 3,3′-diaminobenzidine as the developer as reported (13). The rabbit primary antibody against CSase A of spinach (K.S., unpublished data) was used at 1:400 dilution. The enzymatic activity of CSase was determined as described (7). Protein was quantified by the method of Bradford (14).

Nucleic Acid Hybridization Analysis. For DNA blot hybridization, total DNA was prepared from 10-week-old *Sp. oleracea* cv. parade leaves as described (15). DNA (20 μg) was digested with restriction enzymes, separated in a 0.8% agarose gel, transferred to a nylon filter (Hybond-N+), and then hybridized with the random-primer-labeled cDNA as a ^{32}P probe as reported (15).

For RNA gel blots, total RNA was isolated from leaves and roots of 4- and 10-week-old spinach by a reported method (13). Twenty micrograms of total RNA was denatured and separated in a formaldehyde/agarose (1.2%) gel, followed by transfer to a Hybond-N+ filter. Hybridization was done in 5× standard saline phosphate/EDTA (SSPE) (1 × SSPE = 0.18 M NaCl/0.01 M sodium phosphate, pH 7.7/1 mM Na_2EDTA)/50% (vol/vol) formamide/5× Denhardt's solution/0.5% (wt/vol) SDS/denatured salmon sperm DNA (100 μg/ml) at 42°C with the ^{32}P-labeled cDNA insert as a probe.

For both DNA and RNA blot hybridization, the final washes of the filters were in 0.1× SSPE/0.1% (wt/vol) SDS at 65°C for 15 min.

RESULTS

Determination of Internal Peptide Sequences and Isolation of cDNA Clones Encoding CSase A. The N-terminal amino acid of the purified CSase A was blocked and, thus, gave no information on the amino acid sequence, even after the acid treatment to remove the N-formyl group (16) or the pyroglutamyl peptidase treatment (17). Thus, internal amino acid sequences were determined after digestion with *St. aureus* V8 protease. Four oligopeptide sequences were elucidated up to 20 amino acid residues (Fig. 1). Two synthetic mixed oligonucleotides, V812 and V822, were designed for hybridization screening of a λgt10 library. These probes contained deoxyinosine at ambiguous positions and exhibited 16- and 8-fold degeneracies, respectively. On screening of the non-amplified library comprising 2 × 10^5 plaques, 19 clones were found doubly positive to two probes and isolated. Two clones, λCS2 and λCS4, with an ≈1.3-kb-length insert were selected for further analysis because this length of insert well-matched that expected from the molecular mass (35 kDa) of a subunit of CSase A determined by SDS/PAGE (K.S., unpublished data).

Sequence of CSase A cDNA. Sequence determination of λCS2 and λCS4 clones revealed the same open reading frame of 975 bp encoding 325 amino acids (Fig. 1). The calculated molecular mass of the encoded peptide was 34,185 Da, which was coincident with that of purified CSase A determined by SDS/PAGE. All four determined peptide sequences were completely identical with the sequences predicted from cDNA, except for 3 equivocal residues in V8-5. The insert of λCS2 had a shorter 5′-leader sequence of 5 bp and a different upstream position of poly(A)-tail attachment. Two putative signal sequences of poly(A) addition were identified at 23 bp and 22 bp upstream of the poly(A) tails of λCS4 and λCS2, respectively. The sequence around the initiation ATG codon, CAAAATGGT, closely matched the proposed consensus sequence (18) for plant-gene initiation codons, AACAATGGC, indicating this ATG as the proper start methionine codon. This interpretation was also supported by the fact that a TAA stop codon is placed at 7 bp upstream of the ATG codon.

Comparison of the Deduced Amino Acid Sequences with Those of Other Proteins. Comparison of the deduced amino acid sequences revealed 53% identity and 70% maximum homology, taking into account gaps and equivalent amino acids, with CSase A of *E. coli* and *Salmonella typhimurium* (19, 20), and 45% identity and 64% maximum homology with CSase B of *E. coli* (21) (Fig. 2). The amino acid sequence also exhibited substantial homology, 40–50% maximum homology taking into account gaps and equivalent amino acids, with those of amino acid-metabolizing enzymes (22, 23) of bacteria and yeast and that of rat hemoprotein H-450 (24).

Expression of Spinach CSase cDNA and Genetic Complementation in Cys$^-$ *E. coli*. To confirm the identity of the isolated clones encoding CSase, a cysteine-auxotroph mutant, *E. coli* NK3, was genetically complemented by the expression of spinach CSase cDNA under control of the *lacZ* promoter. *E. coli* NK3 transformed with an expression vector, pKM1, could grow in the minimal medium without cysteine, whereas *E. coli* transformed with control vector pTV118N could not (Fig. 3). The expression of functionally active CSase in *E. coli* NK3 transformed with pKM1 was also demonstrated by immunoblot analysis and the enzymatic activity of CSase (Fig. 4). These results confirmed that this cDNA clone encodes CSase that is functionally active in *E. coli*.

Hybridization Analysis. Southern blot analysis of genomic DNA indicated the presence of at least two to three copies of homologous sequences of CSase A cDNA in spinach (Fig.

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        ▽
1    TCA GTC TCG ATT CTT CTC AGA TTT CTA TCT CTT TTA AGT ATA TAA CCC AAA    51

52   ATG GTT GAG GAG AAG GCC TTC ATT GCT AAA GAT GTG ACT GAA TTG ATT GGG    102
1     M   V   E   E   K   A   F   I   A   K   D   V   T   E   L   I   G     17
                  V8-3
103  AAA ACG CCA TTG GTA TAT CTC AAC ACT GTC GCC GAT GGT TGT GTT GCT CGT    153
18    K   T   P   L   V   Y   L   N   T   V   A   D   G   C   V   A   R     34

154  GTT GCT GCA AAG CTG GAA GGA ATG GAA CCT TGC TCT AGT GTT AAA GAC AGG    204
35    V   A   A   K   L   E   G   M   E   P   C   S   S   V   K   D   R     51

205  ATT GGG TTC AGT ATG ATT ACT GAT GCT GAA AAA AGC GGG CTT ATT ACA CCT    255
52    I   G   F   S   M   I   T   D   A   E   K   S   G   L   I   T   P     68

256  GGA GAG AGT GTC CTG ATT GAG CCC ACC AGT GGA AAT ACT GGC ATT GGA TTA    306
69    G   E   S   V   L   I   E   P   T   S   G   N   T   G   I   G   L     85
          V8-2
307  GCC TTC ATC GCA GCA GCT AAA GGT TAC AAG CTC ATC ATT ACG ATG CCA GCA    357
86    A   F   I   A   A   A   K   G   Y   K   L   I   I   T   M   P   A     102

358  TCA ATG AGT CTT GAG CGG AGG ACT ATT CTC AGG GCC TTT GGT GCT GAG CTT    408
103   S   M   S   L   E   R   R   T   I   L   R   A   F   G   A   E   L     119

409  ATC CTT ACT GAT CCA GCA AAA GGT ATG AAA GGG GCT GTT CAG AAG GCT GAG    459
120   I   L   T   D   P   A   K   G   M   K   G   A   V   Q   K   A   E     136

460  GAG ATC CGT GAC AAA ACT CCT AAT TCA TAT ATA CTA CAA CAG TTT GAA AAC    510
137   E   I   R   D   K   T   P   N   S   Y   I   L   Q   Q   F   E   N     153

511  CCT GCC AAC CCA AAG GTT CAT TAT GAA ACA ACT GGA CCA GAA ATT TGG AAA    561
154   P   A   N   P   K   V   H   Y   E   T   T   G   P   E   I   W   K     170
                                          V8-1
562  GGC ACA GGT GGA AAA ATT GAT ATA TTC GTC TCT GGA ATA GGG ACT GGA GGT    612
171   G   T   G   G   K   I   D   I   F   V   S   G   I   G   T   G   G     187

613  ACA ATA ACA GGT GCA GGA AAA TAC CTA AAG GAA CAA AAC CCG GAT GTT AAG    663
188   T   I   T   G   A   G   K   Y   L   K   E   Q   N   P   D   V   K     204

664  CTA ATT GGC CTG GAA CCA GTG GAA AGT GCT GTA TTG TCT GGA GGA AAA CCT    714
205   L   I   G   L   E   P   V   E   S   A   V   L   S   G   G   K   P     221

715  GGC CCA CAT AAG ATT CAA GGA CTT GGA GCT GGA TTC ATA CCT GGT GTT CTG    765
222   G   P   H   K   I   Q   G   L   G   A   G   F   I   P   G   V   L     238

766  GAT GTG AAT ATT ATC GAT GAA GTG GTT CAG ATA TCC AGT GAA GAA TCT ATT    816
239   D   V   N   I   I   D   E   V   V   Q   I   S   S   E   E   S   I     255
                                  V8-5
817  GAA ATG GCC AAA TTG CTC GCC CTC AAG GAA GGT CTA CTG GTT GGG ATT TCA    867
256   E   M   A   K   L   L   A   L   K   E   G   L   L   V   G   I   S     272

868  TCT GGT GCT GCT GCT GCC GCT GCC ATT AAA GTG GCA AAG AGG CCT GAA AAT    918
273   S   G   A   A   A   A   A   A   I   K   V   A   K   R   P   E   N     289

919  GCT GGA AAA CTC ATC GTC GCT GTC TTT CCC AGC TTT GGC GAA CGA TAT TTA    969
290   A   G   K   L   I   V   A   V   F   P   S   F   G   E   R   Y   L     306

970  TCC TCG GTG TTG TTT GAT TCA GTG AGG AAG GAG GCA GAG AGC ATG GTT ATT    1020
307   S   S   V   L   F   D   S   V   R   K   E   A   E   S   M   V   I     323

1021 GAG TCC TAA GTT CGA GTT CTC CTT TAA TGG TCT TGG ATA CTT CAC AGG CTT    1071
324   E   S   *                                                            326

1072 TGG TCT TTT CAT GTG GTC TAA ATC CTA GTT TTC TTC TGT TTT CCT TTT CTT    1122

1123 TTC TCG TAG ACA TGC AAA TCG TGG CCC TTC TGT ATT AGA ACA ATG TCA GTT    1173

1174 GTG TCC TTG ATG TTT GGT CAT CAT GCA CTG GCA ACT ATG GTT TGT GAT GTA    1224
                                   ▼
1225 TAA AAT ACT CTT TCA TGT TCT TCC ATT ACA TAT TGT TGA TGA GGG ATT TCA    1275

1276 TGT GAT AAA AAA AAA AAA AAA AAA AAA A                                  1303
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FIG. 1. Nucleotide sequence and deduced amino acid sequence of a cDNA clone λCS4 encoding CSase A from spinach. The underlined amino acid sequences indicate the partial peptide fragments determined by protein microsequencing for purified CSase A. Dashed underlining in fragment V8-5 indicates three equivocal amino acids. Double underlining indicates putative polyadenylylation signals. ▽, 5′ end of a cDNA clone, λCS2; ▼, alternative poly(A) site of a cDNA clone, λCS2; *, stop codon.

5A). The genes may comprise a small multi-gene family. RNA-blot hybridization showed that an ≈1.6-kb-length transcript was expressed constitutively in leaves and roots of 4- and 10-week-old spinach (Fig. 5B).

DISCUSSION

Full-length cDNA clones of spinach CSase A were isolated by screening a library with synthetic oligonucleotides encoding partial peptide sequences determined by microsequencing. The identity of the clones was confirmed by the matching of peptide sequences and expression of a catalytically active protein product in *E. coli*.

CSase requires PLP as a cofactor for its catalytic activity. The consensus amino acid sequence of the putative PLP-binding region was proposed to be NPHK on the basis of sequence comparison of several PLP-dependent amino acid decarboxylases (25). Among these residues, the PLP-binding site, lysine (K), and the preceding histidine (H) are highly

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  1 : MVEEKAFIAKDVTELIGKTPLVYLNTVADGCVARVAAKLEGMEPCSSVKD
  1 :   MSKIFEDNSLT--..H....R.NRIGNG---RILA.V.SRN.SF...C
  1 :     MSKIYEDNSLT..H....R.NR--IG-NGRILA.V.SRN.SF...C
  1 :          MSTLEQT..N....K.QRMGPDNGSEVWL.L.GNN.AG...D

 51 : RIGFSMITDAEKSGLITPGESVLIEPTSGNTGIGLAFIAAAKGYKLIITM
 49 : .IGAN..WD...R.VLK..VE-.V.P.S.....A..YV..AR..KLTLT.
 47 : .IGAN..WD...R.VLK..VE-.V.P.N.....A..YV..AR..KLTLT.
 43 : .AALS..VE...R.EIK..-DV.I.A.S.....A..MI..LK..RMKLL.

101 : PASMSLERRTILRAFGAELILTDPAKGMKGAVQKAEEIRDKTPNSY-ILQ
 99 : .ET..I...KLLK.L..N.V.TEGAK..K..IQK.E.IVASNPEKYLL.Q
 97 : .ET..I...KLLK.L..N.V.TEGAK..K..IQK.E.IVASDPQKYLL.Q
 93 : .DN..Q...AAMR.Y..E.I.VTKEQ..E..RDL.L.MANRGEGK--L.D

151 : QFENPANPKVHYETTGPEIWKGTGGKIDIFVSGIGTGGTITGAGKYLKEQ
149 : ..S..A..EI.EK.......ED.D.QVDV.IAGV..G..W..VTPYIKGT
147 : ..S..A..EI.EK.......ED.D.QVDV.ISGV..G..L..VTRYIKGT
143 : ..N..D..YA.YT.......QQ.G.RITH.VSSM..T..I..VSRFMREQ

                                          ▼
201 : NPDVKLI--GLEPVESAVL------SGGKPG[PHK]IQGLGAGFIPGVLDVN
199 : KGKTDLISVAVEPTDSPVIAQALAGEEIK..[PHK]IQGIGAGFI.ANLDLK
197 : KGKTDLITVAVEPTDSPVIAQALAGEEIK..[PHK]IQGIGAGFI.GNLDLK
193 : SKPVTIVGL----QPE--------EGSSI..IRR---WPTEYL.GIFNAS

251 : IIDEVVQISSEESIEMAKLLALKEGLLVGISSGAAAAAAIKVAKRPENAG
249 : LV.K.IG.TNEEAISTARR.MEE.GILA.I...A.V.A.LKLQEDESFTN
247 : LI.K.VG.TNEEAISTARR.MEE.VFLA.I...A.V.A.LKLQEDESFTN
243 : LV.E.LD.HQRDAENTMRE.AVR.GIFC.V...G.V.G.LRVAKANPDAV

301 : KLIVAVFPSFGERYLSSVLFDSVRKEAESMVIESX   CSase A (Sp. oleracea)
299 : KNI.VILPSS.E....TAL.ADLFTEKELQQ       CSase A (E. coli)
297 : KNI.VILPSS.E....TAL.ADLFTEKELQQ       CSase A (Sa. typhimurium)
293 : --V.AIICDR.D....TGV.GEEHFSQGAGI       CSase B (E. coli)
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FIG. 2. Comparison of deduced amino acid sequences of CSases from spinach, *E. coli*, and *Sa. typhimurium*. Dots indicate identical amino acid residues in four sequences. Dashes indicate gaps in sequence for the best alignment. The boxed region is the putative PLP-binding domain. ▼, Lysine residue for the putative PLP-binding site.

conserved. The sequences of CSases A of spinach, *E. coli*, and *Sa. typhimurium* contain the same conserved region, PHK (Fig. 2, boxed). The region surrounding this box encompassing 16 amino acid residues is also highly conserved among three CSases. These facts indicate that this lysine residue could be the putative PLP-binding site. Levy and Danchin (20) proposed, on sequence comparison of PLP enzymes, that Lys-137 (Lys-143 in Fig. 2) of CSase A of *E. coli* might be the PLP-binding residue. Because this lysine residue, however, is not conserved in spinach CSase A, this site is unlikely to be the binding residue.

The peptide sequences of CSases of spinach and bacteria show homologies of ≈40–50%, considering gaps and equivalent amino acids, with the sequences of yeast threonine dehydratase (22) and bacterial tryptophan synthase β chain (23). These two enzymes as well as CSase catalyze PLP-

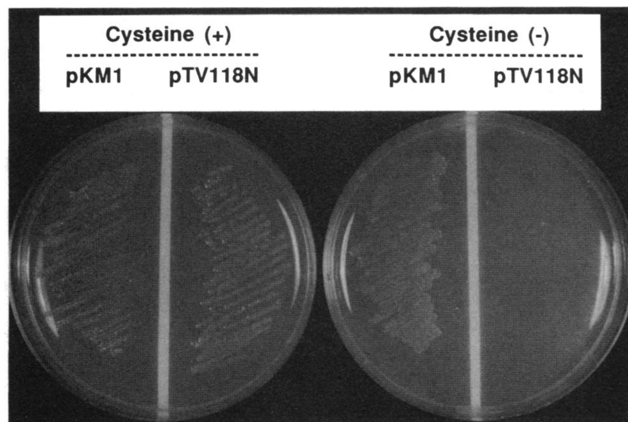

FIG. 3. Genetic complementation of Cys⁻ *E. coli* by transformation with an expression vector, pKM1. A cysteine-auxotroph, *E. coli* NK3 (Δ*trpE5 leu-6 thi hsdR hsdM⁺ cysK cysM*), was transformed with an expression vector, pKM1, carrying the spinach cDNA or a control plasmid, pTV118N. Transformed bacteria were spread on M9 minimal agar plates supplemented with 0.02% leucine and tryptophan plus 0.5 mM cysteine (left plate) or without cysteine (right plate).

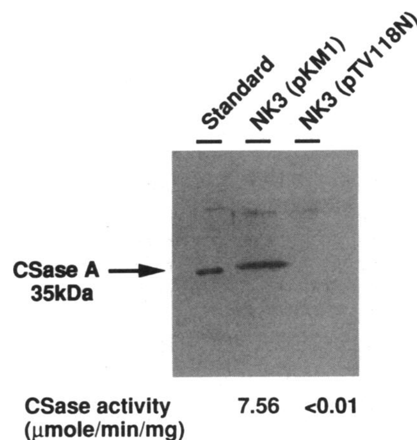

FIG. 4. Expression analysis of spinach CSase A in *E. coli* NK3 by immunoblotting and enzyme assaying. For immunoblotting, the protein was separated by SDS/12% PAGE, transferred onto a nylon filter, and then localized by immunostaining with rabbit anti-CSase A serum. For the enzyme assay, the activities of CSase were determined in cell-free extracts of *E. coli* as reported (7). Lanes: standard, purified spinach CSase A (0.4 μg); NK3 (pKM1), total protein of *E. coli* NK3 transformed with expression vector pKM1 (10 μg); NK3 (pTV118N), total protein of *E. coli* NK3 transformed with control vector pTV118N (10 μg).

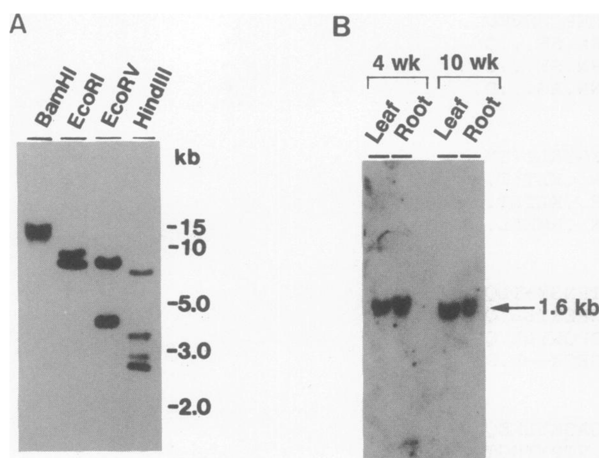

FIG. 5. Nucleic acid hybridization analysis. (*A*) Southern-blot analysis of genomic DNA of spinach. Genomic DNA (20 μg) was digested with appropriate restriction enzymes, separated by agarose gel (0.8%) electrophoresis, transferred onto a nylon filter, and then hybridized with ^{32}P-labeled cDNA of spinach CSase as probe. (*B*) RNA blot analysis of total spinach RNA. Total RNA of leaves and roots of 4- and 10-week-old spinach (20 μg) was electrophoresed on an agarose gel (1.2%), transferred onto a nylon filter, and then hybridized with the ^{32}P-labeled probe.

dependent reactions involving modifications at C_β of α-amino acids. Moreover, the steric courses of the reactions catalyzed by these enzymes all involve retention of the configuration at C_β. These facts suggest that the ancestors of these enzymes were the same and that they have evolved with the same reaction mechanisms. In this context, it is interesting that the sequence of rat hemoprotein H-450 (24) also exhibits extensive similarity with those of amino acid-metabolizing enzymes for reactions involving C_β. Because it was shown that hemoprotein H-450 has no CSase catalytic activity, this protein may possess other functions, such as the binding activity of amino acid analogues.

The expression of spinach cDNA in an *E. coli* mutant could functionally complement the cysteine requirement of the auxotroph. There are three additional examples of genetic complementation through expression of plant amino acid-biosynthetic enzymes in auxotrophic *E. coli*: glutamine synthase (26, 27), dihydrodipicolinate synthase (28, 29) for lysine biosynthesis, and Δ^1-pyrroline-5-carboxylate reductase (30) for proline biosynthesis. The biosynthetic enzymes, at least, for these amino acids are possibly similar in structure and function in plants and bacteria.

The transcript expression of the CSase A gene(s) was rather constitutive in leaves and roots of 4- and 10-week-old spinach. This fact suggests that CSase A has fundamental functions in cell growth, such as sulfur detoxication and assimilation. Recently Lunn *et al.* (31) reported the presence of three isoforms of CSase in spinach leaves. Each isoform separately localizes in chloroplasts, cytosol, and mitochondria. The cDNA clone isolated in the present study presumably encodes the isoform localizing in cytosol because the distribution of CSase A is primarily in cytosol (K.S., unpublished data). This conclusion is also supported by the facts that the mRNA expressed in both green and nongreen tissues and that the cDNA sequence possessed no apparent transit-peptide-encoding sequence for transportation of protein to chloroplasts or mitochondria.

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