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Gene Cloning and Characterization of *Pseudomonas putida* L-Methionine-α-deamino-γ-mercaptomethane-lyase[1]

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ABSTRACT

Methionine dependency has been reported in cancer cell lines and primary tumors. Thus, L-methionine deprivation might have potential value for the treatment of human cancers with a methionine requirement. L-Methionine-α-deamino-γ-mercaptomethane-lyase has been reported to decrease plasma methionine levels and to inhibit tumor growth in experimental animals but has not been studied extensively because sufficient homogeneous enzyme was not available. In this study, we cloned the L-methioninase gene from *Pseudomonas putida* and isolated pure and abundant recombinant enzyme. Both L-methionine and L-cysteine in culture medium were completely degraded by 1 unit/ml purified enzyme. Two hundred and fifty units/kg L-methioninase administered i.v. to mice yielded 0.7 unit/ml of plasma concentration and lowered total plasma sulfur-containing amino acids by more than 75%. Although sensitivity to enzymatic methionine depletion differed among cell lines, leukemia cell lines were generally more sensitive than solid tumor cell lines. The availability of pure recombinant L-methioninase will allow *in vivo* studies on the antitumor activity and the potential toxicity of enzymatic methionine depletion.

INTRODUCTION

Normal and malignant cells may differ in their amino acid requirements (1, 2). In consequence, dietary restrictions of amino acids have been used successfully to treat tumor-bearing animals (3, 4). However, the most efficient approach to amino acid depletion is irreversible enzymatic degradation. L-Asparaginase derived from bacterial species has been used in the treatment of acute lymphoblastic leukemia. Many human malignant cell lines and primary tumors have absolute requirements for L-methionine (5–9). Under normal circumstances, methionine comes from dietary proteins. Most normal tissues can also synthesize methionine from either homocysteine or methylthioadenosine (Fig. 1). The biochemical mechanism for methionine dependency in cancer cells has been studied extensively (10–12), but the fundamental mechanism remains unclear.

A therapeutic approach to enzymatic methionine depletion was first reported in 1973 (13). L-Methioninase,[3] in the presence of pyridoxal 5′-phosphate, catalyzes the α, γ-elimination of methionine to α-ketobutyrate, methanethiol, and ammonia. The enzyme isolated from *Clostridium sporogenes* (14), in combination with D-homocysteine or L-homocysteine thiolactone, was reported to inhibit the growth of leukemia cells with an absolute methionine requirement but did not affect normal fibroblasts capable of using homocysteine in place of methionine for growth (8). The semipurified enzyme from *C. sporogenes* significantly inhibited Walker carcinosarcoma 256 of rats without significant toxicity (13). For effective enzymatic degradation of L-methionine, L-methioninase with a lower Michaelis constant (K_m) was searched in various bacteria species. L-Methioninase isolated from *Pseudomonas putida* was reported to exhibit a K_m of 1 mM (15), whereas L-methioninase from *C. sporogenes* showed a K_m of 90 mM (16). However, purification of the enzyme from these bacteria species has yielded a very limited amount that is insufficient for further *in vivo* studies (17, 18). To overcome this problem, we cloned the L-methioninase gene from *P. putida* genomic DNA and expressed large amounts of recombinant enzyme in *Escherichia coli*. We chose *P. putida* for the gene cloning because L-methioninase from this bacteria species has a low K_m and the partial peptide sequences of the enzyme have already been reported (19). The cloned gene contains a 1194-bp open reading frame that encodes a polypeptide of 398 amino acids. The purified recombinant enzyme displayed biochemical properties similar to those reported previously for the natural protein (15). Pure recombinant L-methioninase selectively lowered sulfur-containing amino acid levels in cell culture medium and in mice. In general, leukemia cell lines were more sensitive to enzymatic methionine depletion than cell lines of nonhematological origin, except for one lung cancer cell line. The availability of pure and abundant L-methioninase will allow application of *in vivo* enzymatic degradation of plasma L-methionine to experimental cancer therapy.

MATERIALS AND METHODS

Cell Lines. *P. putida* was purchased from the American Type Culture Collection (Rockville, MD). Cancer cell lines were either from the American Type Culture Collection or were a gift from Dr. M. O. Diaz, the University of Chicago, Chicago, IL.

Isolation of Chromosomal DNA from *P. putida*. *P. putida* was grown overnight at 30°C as described previously (15). One hundred ml of bacterial culture were harvested by centrifugation, resuspended in 10 ml SET buffer [20% sucrose, 50 mM Tris-HCl (pH 7.6), and 50 mM EDTA], frozen, thawed, and resuspended in 2 ml SET buffer. The suspension was treated with 0.2 ml of lysozyme [5 mg/ml in TEN buffer consisting of 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, and 10 mM NaCl] and 0.1 ml RNase A at 10 mg/ml in 0.1 M sodium acetate (pH 4.8) and 0.3 mM EDTA and then incubated on ice for 15 min. After adding 0.05 ml of 25% SDS, the mixture was incubated at 37°C with gentle shaking for 6 h. Then, 0.3 ml proteinase K (2 mg/ml in TEN buffer) and 1.5 ml chloroform:isoamyl alcohol (24:1) were added, followed by incubation overnight at 37°C with gentle shaking. The suspension was diluted with 1 ml distilled water and was extracted three times with 10 ml chloroform:isoamyl alcohol. After the final extraction, DNA was precipitated with ethanol from the aqueous phase and was washed with 70% ethanol. After drying, DNA was resuspended in TE buffer [10 mM Tris-HCl (pH 8) and 1 mM EDTA].

PCR Amplification of the Partial Sequence of the L-Methioninase Gene from *P. putida* DNA. We synthesized sense primers (1S1 and 1S2; 2S1 and 2S2) and antisense primers (1AS and 2AS) as listed in Table 1 based upon two partial peptide sequences reported previously (designated peptide 1, GAITSTLWTLLRPGDEVLLGNTLYGCTFAFLHHGIGEFGVKLRHVD; and peptide 2, ADIAGVAKIARKHGATVVVDNTYQTPYLQRPLELGADLVVXSATKTL; Ref. 19). The PCR was used to amplify the partial sequence of the L-methioninase gene from *P. putida* genomic DNA. An internal oligonucleotide 1INT (Table 1) was synthesized from peptide 1 as a probe for Southern blot analysis of the PCR products. The PCR reaction was carried out in 50 μl reaction volume containing 1 μg genomic DNA, 1× PCR buffer [10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5

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[3] The abbreviations used are: L-methioninase, L-methionine-α-deamino-γ-mercaptomethane-lyase; IPTG, isopropyl-β-thio-galactopyranoside; PBL, peripheral blood lymphocyte; MTAP, methylthioadenosine phosphorylase; MTA, methylthioadenosine.

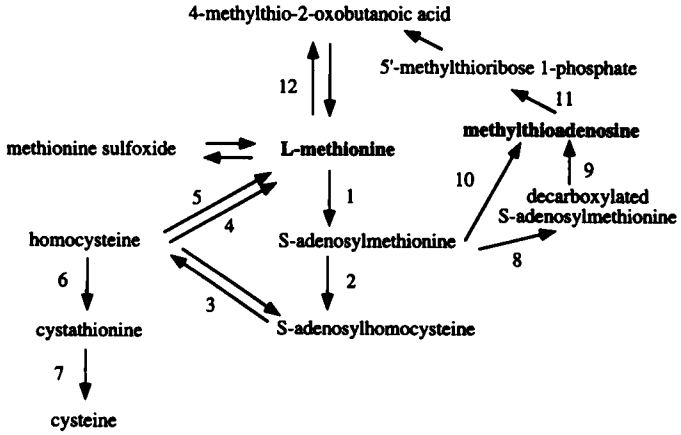

Fig. 1. Mammalian methionine metabolism. L-Methionine is mainly supplied exogenously from dietary proteins but is also synthesized from homocysteine and 4-methylthio-2-oxobutanoic acid converted from MTA and methionine sulfoxide. MTA is catalyzed to adenine and 5'-methylthioribose 1-phosphate by MTAP. L-Cysteine is synthesized from L-methionine through several enzymatic reactions. The enzymes involved are: *1*, methionine adenosyltransferase; *2*, *S*-adenosylmethionine methyltransferases; *3*, adenosylhomocysteinase; *4*, betaine-homocysteine methyltransferase; *5*, 5-methyltetrahydrofolate-homocysteine methyltransferase; *6*, cystathionine β-synthase; *7*, cystathionine γ-lyase; *8*, adenosylmethionine decarboxylase; *9*, spermidine synthase; *10*, adenosylmethionine cyclotransferase; *11*, MTAP; and *12*, transaminases.

mM $MgCl_2$, and 0.001% gelatin], 400 μM of each deoxynucleotide triphosphate, 100 ng each of sense and antisense primers, and 2.5 units of Taq polymerase (Stratagene, La Jolla, CA). Thirty-five cycles were performed with the Programmable Cyclic Reactor (ERICOMP, San Diego, CA). Each cycle consisted of denaturation (92°C for 1 min), annealing (50°C for 1 min), and extension (72°C for 2 min). In the last cycle, the extension was increased to 7 min. The PCR products were separated by agarose gel electrophoresis and were visualized by ethidium bromide staining.

Southern Blot Analysis of the PCR Products and *P. putida* DNA. The PCR products resolved by agarose gel electrophoresis were transferred to a nylon membrane (Hybond-N^+; Amersham, Arlington Heights, IL) that was then probed with the radiolabeled 1INT oligonucleotide. The labeling of the 1INT oligonucleotide and hybridization were carried out as described (20). For genomic Southern blotting, *P. putida* DNA was digested with various restriction endonucleases, separated in 0.8% agarose gels, and transferred to a nylon membrane. The membrane was probed with the PCR product that was amplified using primers 1S1 and 2AS and was labeled by the random priming method (21). After washing, the membrane was exposed to an X-ray film at −80°C.

Screening of a Phage Library Constructed from *P. putida* DNA with the PCR Product. Based upon the results of genomic Southern blot analysis, we used size-selected *Bgl*II fragments ranging from 4 to 6 kb and followed the supplier's instructions to construct a *P. putida* genomic DNA library with a λFIX II/*Xho*I partial fill-in vector kit (Stratagene). Briefly, the *Bgl*II-digested DNA fragments were recovered from the gel slices, were subjected to a partial fill-in reaction in the presence of dATP and dGTP, and then were ligated to the λFIX II vector that was digested with *Xho*I and partially filled-in with dTTP and dCTP. The ligation mixture was packaged with Gigapack II Gold packaging extract (Stratagene). An aliquot (2.5×10^5 plaque-forming units) of this library was screened using the radiolabeled PCR product that was prepared as described above. After three screening cycles, two clones were obtained. Phage DNA from these clones was purified and was digested with *Not*I to rescue the inserts. The resulting inserts were subcloned into the *Not*I-digested pBluescript II SK(+) vector (Stratagene). Plasmid DNA was purified from these subclones and was subjected to restriction enzyme mapping and DNA sequencing.

Expression of L-Methioninase Gene. To facilitate overexpression of enzyme protein in an expression vector, the cloned gene was modified as follows. The sense primer 3S containing an *Eco*RI site, a two-ribosome binding site in tandem with a single adenine base overlapped (AGGAGGA), and the first codon and the antisense primer 3AS containing the *Bam*HI site (Table 1) were used to amplify a 1368-bp fragment from the cloned DNA. The *Eco*RI/*Bam*HI fragment was cloned into the *Eco*RI/*Bam*HI-digested pKK223–3 expression vector (Pharmacia Biotech, Piscataway, NJ). The plasmid was transformed into the host strain JM105. The recombinant protein was induced with 1 mM IPTG in several recombinant clones. The clone with the highest expression of L-methioninase was used for additional experiments.

Enzyme Purification. The transformed bacteria were grown in Luria-Bertani medium containing 50 μg/ml ampicillin. At the density (absorbance) of 0.4 OD_{600}, IPTG at 1 mM was added to the culture. After incubation at 37°C for 3 h with vigorous shaking, the bacteria were collected by centrifugation, and the resulting pellet was washed with 5 volumes of PBS. An acetone powder of the bacteria was prepared as described (22) and stored at −20°C until use. One hundred mg of acetone powder, prepared from 400 ml of the bacterial culture, were suspended in 10 ml buffer A consisting of 20 mM potassium phosphate buffer (pH 7.4), 20 μM pyridoxal 5'-phosphate, 15 μM β-mercaptoethanol, and 1 mM EDTA. The suspension was sonicated and centrifuged at 50,000 × *g* for 30 min. To purify the enzyme, the supernatant was heated at 60°C for 15 min and centrifuged at 50,000 × *g* for 30 min as described (18). One-ml aliquots of the supernatant were clarified by filtration through a 0.22 μm filter and injected onto a Mono Q HR 5/5 anion exchange column (Pharmacia Biotech, Inc.) pre-equilibrated with buffer B containing 350 mM sodium chloride in buffer A and then with buffer A. Proteins were eluted with a linear gradient with 0–350 mM potassium chloride in buffer A (23). The fractions containing enzyme activity were pooled and applied to a 9-ml column of ACTICLEAN ETOX (Sterogen, Arcadia, CA) to remove endotoxin. The column was treated with 45 ml of 1 M sodium hydroxide and 150 ml pyrogen-free water before sample application. All purification steps after heat treatment were carried out at 4°C. The purified enzyme was concentrated by ultrafiltration through an XM10 membrane filter (Amicon, Beverly, MA) and stored at −20°C in 10 mM potassium phosphate buffer (pH 7.4) containing 20 μM pyridoxal 5'-phosphate and 50% glycerol.

Table 1 *Oligonucleotides used for cloning and expression of L-methioninase gene*

Name of primer	Sequence
Primers to amplify the partial sequence	
Sense primers	
1S1	5'-GGTGCTATCACCTCCACCCT-3'
1S2	5'-TGGAC(C/T)CTGCTGCGTCCGGG-3'
2S1	5'-GA(C/T)ATCGC(T/A/G)GG(T/C)GT(T/A)GC(T/A/G)AA-3'
2S2	5'-ATCGC(T/A/G)CGTAAACACGG(T/C)GC-3'
Antisense primers	
1AS	5'-CCGAATTC(A/G)CCGAT(A/G)CCGTG-3'
2AS	5'-ACCAG(G/A)TC(A/T/C)GC(A/G)CCCAGTTC-3'
An internal oligonucleotide for Southern blot analysis of PCR products	
1INT	5'-AACAC(C/T)CTGTACGG(T/C)TG(C/T)AC(C/T)TTCGCTTTCCTGCAC-3'
Primers to modify the cloned gene for overexpression	
Sense primer	
3S	5'-AAGAATTCAAAATAAGGAGGAAAAAAA*ATG*CGCGA-3'
Antisense primer	
3AS	5'-TTGGATCCTGTTCTGATTTGCGATCG-3'

Enzyme Assay and Characterization of Enzyme Properties. Enzyme assay for L-methioninase was carried out in 1 ml of 100 mM potassium phosphate buffer (pH 7.4) containing 10 μM pyridoxal 5'-phosphate, 30 mM L-methionine, and the enzyme solution as described (15). The reaction was started by adding the enzyme. After incubation for 10 min at 37°C, the reaction was terminated by adding 125 μl of 50% trichloroacetic acid. The amount of α-ketobutyrate produced was determined spectrophotometrically in a solution containing 250 μl reaction mixture, 500 μl of 1 M sodium acetate (pH 5.0), and 200 μl of 3-methyl-2-benzothiazolone hydrazone hydrochloride. One enzyme unit was defined as the production of 1 μmol α-ketobutyrate per minute. The protein concentration was determined with the BCA protein assay kit (Pierce Chemical Co., Rockford, IL) using BSA as a standard.

The K_ms for L-methionine and L-cysteine were determined by Lineweaver-Burk plots. Substrate specificity was determined with various sulfur-containing compounds in the reaction described above, except that in reaction mixtures, substrate concentration was 15 mM and the purified enzyme was used at 0.7 unit/ml. The results are expressed as the relative production of 2-oxo acid compared to L-methionine.

Endotoxin Assay. The amount of endotoxin was chromogenically measured by the Limulus Amebocyte Lysate test (QCL-1000; BioWhittaker, Walkersville, MD) following the supplier's instructions with *E. coli* endotoxin (Associates of Cape Cod, Woods Hole, MA) as a standard.

Pharmacokinetic Analysis. The *in vitro* effect of enzyme was assessed by the addition of purified L-methioninase at 1 unit/ml into DMEM (Life Technologies, Inc., Gaithersburg, MD) containing 10% dialyzed horse serum (Life Technologies, Inc.). *In vivo* pharmacokinetics were analyzed in CDF1 mice (Harlan-Sprague-Dawley, Inc., Indianapolis, IN) fed with Rodent Diet 8604 (Harlan-Sprague-Dawley). Animals received 250 units/kg of purified L-methioninase i.v. and were bled at various times thereafter. Residual L-methioninase activities were measured spectrophotometrically as described earlier. L-Methionine *in vitro* was measured with a Beckman Model 6300 High Performance Analyzer. L-Cysteine *in vitro* and total sulfur-containing amino acids *in vivo* were measured after enzymatic conversion to thiols and 2-oxo acids, which were then determined with the spectrophotometric method described above and a Spectroquant Hydrogen Sulfide kit (EM Science, Gibbstown, NJ), respectively.

Effects of L-Methioninase on Cancer Cell Lines and Peripheral Blood Lymphocytes. Ten cancer cell lines of various origins (leukemias: CEM, MOLT4, MOLT16, K562, and HL60; lung cancer: A549, A427, and CaLu6; glioma: T98G; and breast cancer: MCF7) and normal PBL from a healthy donor were cultured in DMEM or RPMI 1640 (Life Technologies, Inc.) containing 10% dialyzed horse serum in 96-well plates at an initial density of 1×10^5 cells/ml for cancer cell lines and 1×10^6 cells/ml for PBL. The cells were treated with the purified enzyme at 0 to 2.5 units/ml. The viability of cultured cell lines and PBL was measured spectrophotometrically with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (24) and with erythrocin B dye exclusion method, respectively, after a 4-day incubation. The growth of cells treated with different concentrations of the enzyme was expressed as the percentage of growth compared to that in cells without treatment.

Chemicals. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

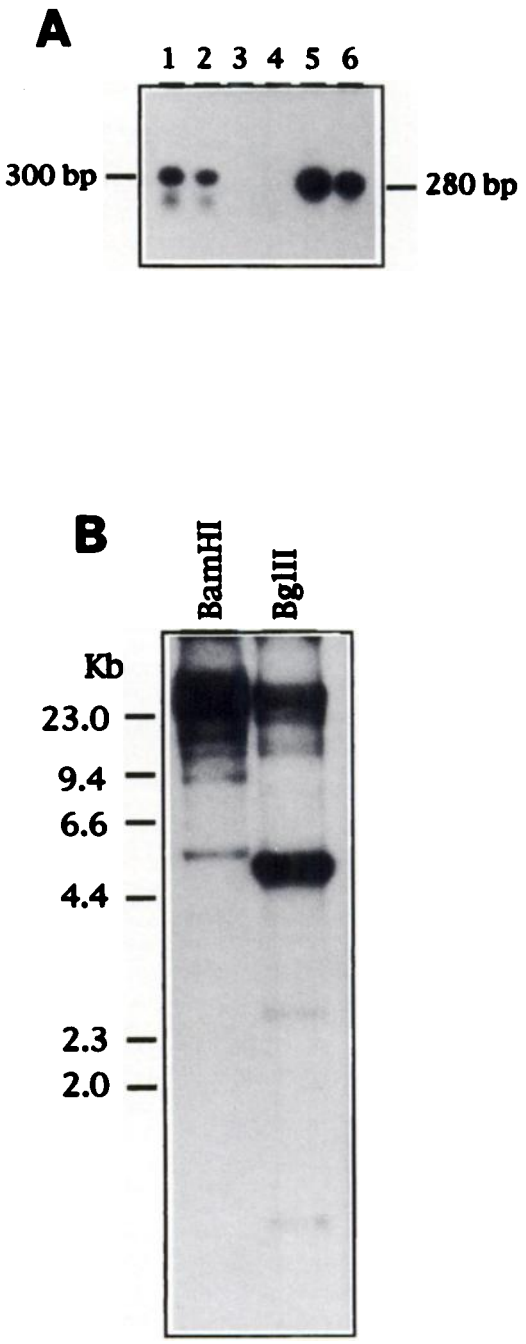

Fig. 2. Southern blot analysis of the PCR products (*A*) and *P. putida* DNA (*B*). *A*, the L-methioninase gene was amplified by PCR from *P. putida* DNA as described in "Materials and Methods." The products were resolved in 2% agarose gels and analyzed by Southern blotting with a radiolabeled internal oligonucleotide, 1INT. The lanes and the primers used are: *Lanes 1* and *2*, 1S1 and 2AS; *Lane 3*, 2S1 and 1AS; *Lane 4*, 2S2 and 1AS; and *Lanes 5* and *6*, 1S2 and 2AS. *B*, *Pseudomonas* DNA (10 μg) was digested with the indicated restriction enzyme, separated on a 0.8% gel, transferred to a nylon membrane, and hybridized with the radiolabeled PCR product.

RESULTS

Cloning of the L-Methioninase Gene. Various combinations of sense and antisense primers were tested to amplify fragments from *P. putida* genomic DNA because the order of the two peptide sequences was unknown. The reactions using primer sets of 1S1 and 2AS, or 1S2 and 2AS, amplified DNA fragments that hybridized with the radiolabeled 1INT oligonucleotide in Southern blots (Fig. 2*A*). These results indicate that peptide 1 is located upstream of peptide 2 (Fig. 3). The fragment amplified with 1S1 and 2AS was purified and used as a probe for genomic Southern blot analysis of *P. putida* DNA. The Southern blot analysis showed a major 5.4-kb *Bgl*II band (Fig. 2*B*). With *Bam*HI digestion, the major hybridizing band was larger than 23 kb. Another band detected with *Bgl*II or *Bam*HI digestion may be due to low stringency washing conditions or to partial digestion. In blots of DNA digested with *Hin*dIII, *Eco*RI, *Sac*I, *Sal*I, or *Xba*I, either a single large band (>10 kb) or multiple bands of an equal intensity were detected (data not shown). Therefore, a library was constructed using the size-selected *Bgl*II fragment of *P. putida* DNA as described in "Materials and Methods." After three screening cycles with the radiolabeled PCR product, two positive clones were obtained that were identical by restriction enzyme mapping. The insert was rescued with *Not*I digestion, subcloned, and sequenced.

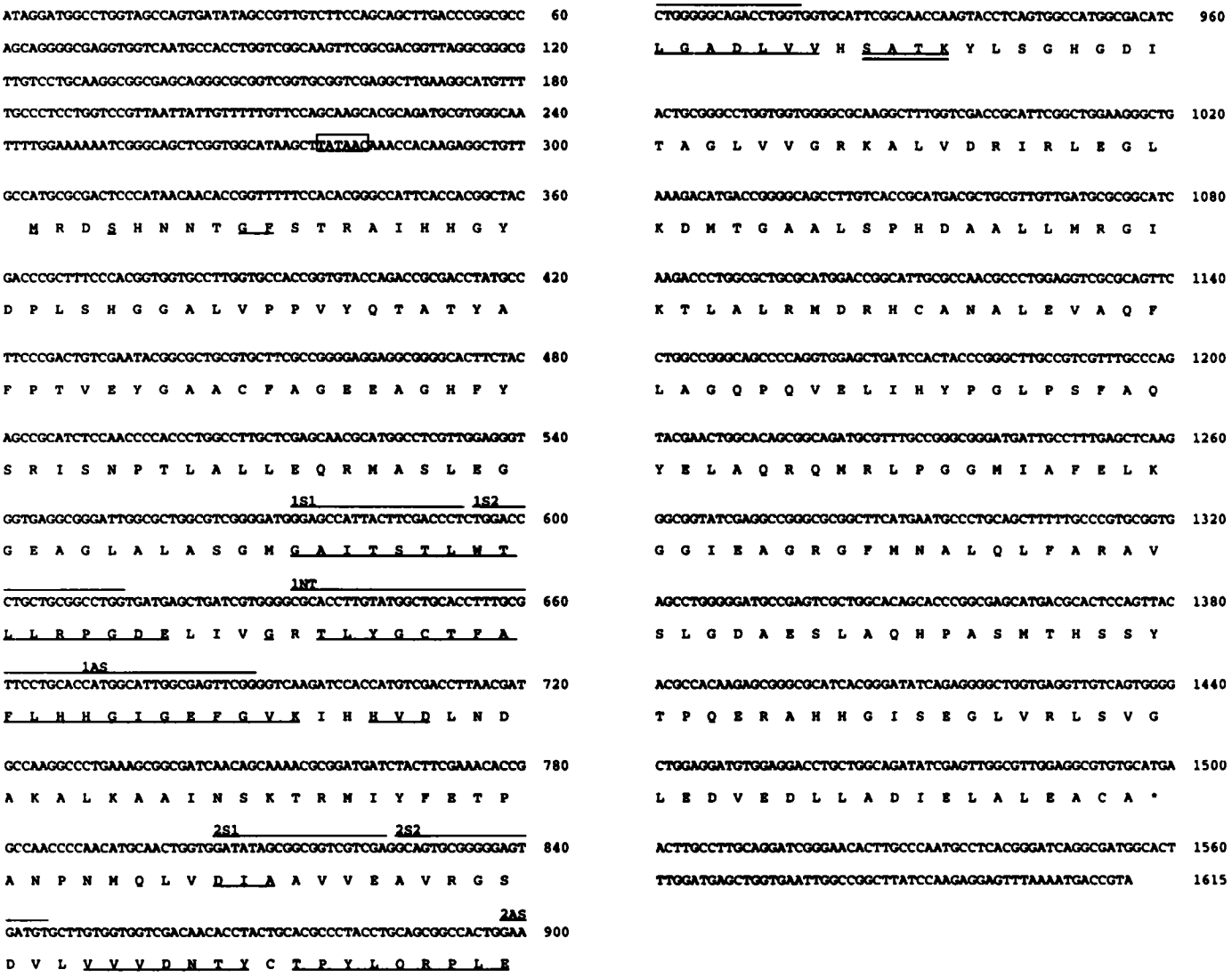

Fig. 3. The nucleotide sequence of the L-methioninase gene and its deduced peptide sequence. The peptide sequences homologous to the reported partial sequences are *underlined* with interruptions indicating mismatches. The *double underlined* sequence (SATK) is a consensus sequence of pyridoxal 5'-phosphate-dependent enzymes. The *boxed* sequence TATAAC is a potential promoter region (Pribnow box). The *overlined* sequences correspond to oligonucleotides used for PCR amplification of the partial gene and Southern blots of PCR products. The major enzyme restriction sites are *Hin*dIII (272), *Xho*I (512), *Sal*I (708, 856, and 994), *Pst*I (887 and 12), *Sac*I (1301), and *Sma*I (1185).

The obtained nucleotide sequence[4] contains a 1194-bp open reading frame (Fig. 3). This sequence contains the oligonucleotides 1S1, 1S2, 2AS, and 1 INT (overlined in Fig. 3). The deduced peptide sequence has 398 amino acids with a molecular mass of 42,720 daltons and contains regions that are 70–80% homologous to the partial peptide sequences used for synthesis of PCR primers (underlined in Fig. 3). However, the homology to an amino terminal peptide sequence of the purified native enzyme reported by Nakayama *et al.* (19) is not significant (36%; 4 matched of 11 amino acids; underlined in Fig. 3). The sequence SATK (double underlined in Fig. 3) matches the consensus sequence SXXK that was reported to be highly conserved in enzymes using pyridoxal 5'-phosphate as a cofactor, such as *E. coli* cystathionine γ-synthase, cystathionine β-lyase, *P. aeruginosa O*-succinylhomoserine sulfhydrylase, and human and rat cystathionine γ-lyase (19, 25–28). The potential Pribnow sequence TATAAC (boxed in Fig. 3) was found 21 bases upstream of the first base of the first codon. The amino acid composition of the deduced peptide sequence

Table 2 *Amino acid composition of L-methioninase obtained from the deduced peptide sequence of the cloned gene*

Amino acid composition shown here was identical to the reported data with hydrolysis of the enzyme (19).

Residue	Number	Mol %
Ala	51	12.8
Cys	5	1.2
Asp	18	4.5
Glu	24	6.0
Phe	13	3.3
Gly	39	9.8
His	18	4.5
Ile	17	4.3
Lys	9	2.2
Leu	50	12.6
Met	12	3.0
Asn	10	2.5
Pro	17	4.3
Gln	13	3.3
Arg	21	5.3
Ser	21	5.3
Thr	21	5.3
Val	25	6.3
Trp	1	0.2
Tyr	13	3.3
Total	398	100

[4] The nucleotide sequence reported in this report has been deposited at GenBank (accession no. L43133).

Table 3 *Purification of L-methioninase*

Step	Total volume (ml)	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg protein)	Recovery of activity (%)	Endotoxin (ng/mg protein)
I. Crude extract from 100 mg acetone powder	5	20.4	366.4	17.9	100.0	
II. Heat treatment (60°C for 15 min)	5	12.8	333.2	26.0	90.9	
III. Mono Q	80	5.2	249.1	47.9	68.0	64.0
IV. ACTICLEAN ETOX	150	4.1	185.6	45.3	55.7	0.47

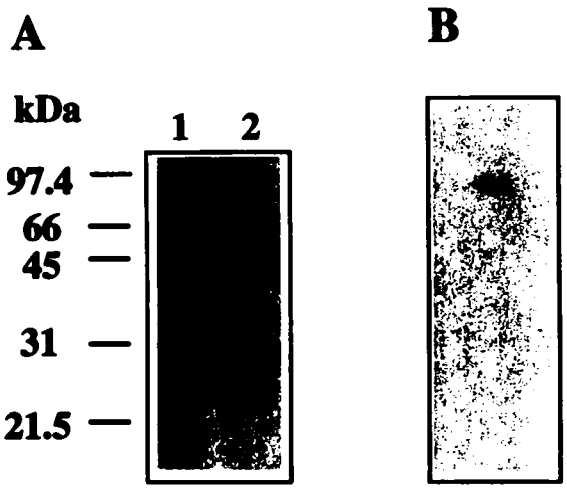

Fig. 4. Gel electrophoresis of recombinant L-methioninase. *A*, proteins were analyzed by denatured PAGE after a 3-h incubation with and without 1 mM IPTG. *Lane 1*, crude extract (10 μg) without IPTG induction; *Lane 2*, crude extract (10 μg) with IPTG induction. *B*, enzyme protein was purified as described in "Materials and Methods." The final preparation was subjected to native PAGE.

shown in Table 2 was similar to that obtained after hydrolysis of the purified enzyme (19).

Expression and Purification of the Recombinant L-Methioninase. The vector pKK223-3, which contains the strong *tac* promoter, was used for overexpression of L-methioninase. The 5′ upstream and 3′ downstream regions of the cloned gene were modified by PCR to facilitate subcloning. In a pilot study, expression of enzyme protein was most efficiently induced after a 3-h incubation with 1 mM IPTG (Fig. 4*A*). Table 3 represents the result of a typical purification of L-methioninase, starting with 100 mg of bacterial acetone powder. Only two purification steps were required to obtain homogeneous protein, as judged by native PAGE (Fig. 4*B*). The final preparation exhibited a specific activity of 47.9 units/mg protein, and the total recovery was approximately 70%. Passage over the ACTICLEAN ETOX column reduced endotoxin from 64 to 0.47 ng/mg protein, a level reported to cause no pyrogenic reactions in mice (18).

Properties of L-Methioninase. The substrate specificity of recombinant L-methioninase was determined by measuring the production of 2-oxo acids. DL-Homocysteine was a 3-fold better substrate than L-methionine and *S*-adenosylmethionine. *S*-Adenosylhomocysteine, L-methionine sulfoxide, cystathionine, and cysteine were poor substrates (Table 4). The K_ms for L-methionine and L-cysteine were 1 and 10.9 mM, respectively. Optimal conditions for the storage of purified L-methioninase were experimentally determined. In the presence of 30–50% glycerol and 20 μM pyridoxal 5′-phosphate in 10 mM potassium phosphate buffer (pH 7.4), no decrease in the enzyme activity was observed during storage for 3 months at −20°C.

***In Vitro* and *in Vivo* Degradation of Sulfur-containing Amino Acids by Recombinant L-Methioninase.** The *in vitro* effect of recombinant L-methioninase on degradation of sulfur-containing amino acids was assayed in DMEM containing 100 μM L-methionine and 200 μM L-cysteine. After the addition of recombinant enzyme at 1 unit/ml to DMEM, the concentration of L-methionine or L-cysteine was determined at different time points. L-Methionine decreased to 29.2 μM after 5 min and decreased to 5.5 μM after 30 min (Fig. 5*A*). L-Cysteine decreased to 47.3 μM after 5 min and decreased to 5.7 μM after 30 min. Both amino acids were not detectable by 60 min. The *in vitro* elimination half-time of enzyme activity was 5 h.

In experimental animals, L-methioninase activity reached a peak plasma level of 0.7 unit/ml after a single i.v. injection of 250 units/kg and disappeared within 3 h (Fig. 5*B*). The elimination half-time was 60 min. Sulfur-containing amino acids in plasma, which were present at a mean concentration of 202 μM before treatment, decreased by 75% by 60 min after the injection and remained at less than 80 μM for 12 h.

The *in vivo* degradation of L-methionine by the recombinant enzyme was comparable to that by semipurified enzyme from *C. sporogenes* reported previously (13).

Sensitivity of Cancer Cell Lines to L-Methioninase. Five leukemia cell lines tested were all sensitive to growth inhibition by L-methioninase. The concentration of enzyme that inhibited cell growth by 50% (IC_{50}) was 0.5 unit/ml or lower in each leukemia cell line (Fig. 6*A*). Solid tumor cell lines demonstrated variable sensitivity (Fig. 6*B*). The IC_{50} in a non-small cell lung cancer cell line CaLu6 was comparable to that in five leukemia cell lines. However, in two other non-small cell lung cancer cell lines A-427 and A-549, the IC_{50} was 0.8 unit/ml. The glioma cell line T98G and the breast cancer cell line MCF-7 were less sensitive to L-methioninase, with IC_{50} values of 1.5 units/ml. With L-methioninase at 2.5 units/ml, the growth of MCF-7 cells was inhibited by 55%, whereas all other cell lines tested were inhibited by 80% or greater. The viability of quiescent PBL was not affected by these concentrations of L-methioninase (Fig. 6*A*).

DISCUSSION

Many cancer cell lines and primary tumors require methionine for growth (5–9). Semipurified bacterial L-methioninase has been reported to inhibit the *in vitro* and *in vivo* growth of tumors with a methionine requirement (8, 9, 13). However, the prior unavailability of a pure and abundant source of enzyme has hindered the clinical application of L-methioninase chemotherapy. Therefore, we cloned

Table 4 *Substrate specificity of L-methioninase*

Substrate	Relative activity (%)[a]	K_m (mM)[b]
L-methionine	100	1.0
D-methionine	0	ND[c]
DL-homocysteine	338	ND
S-adenosylmethionine	108	ND
S-adenosylhomocysteine	0	ND
L-methionine sulfoxide	19	ND
L-cystathionine	13	ND
L-cysteine	8	10.9

[a] The results are expressed as the production of 2-oxo acid with the indicated substrate relative to that with L-methionine in the reaction containing 15 mM substrate and the purified enzyme at 0.7 unit/ml as described in "Materials and Methods." Values are the mean of three determinations.

[b] K_ms are determined by Lineweaver-Burk plots.

[c] ND, not determined.

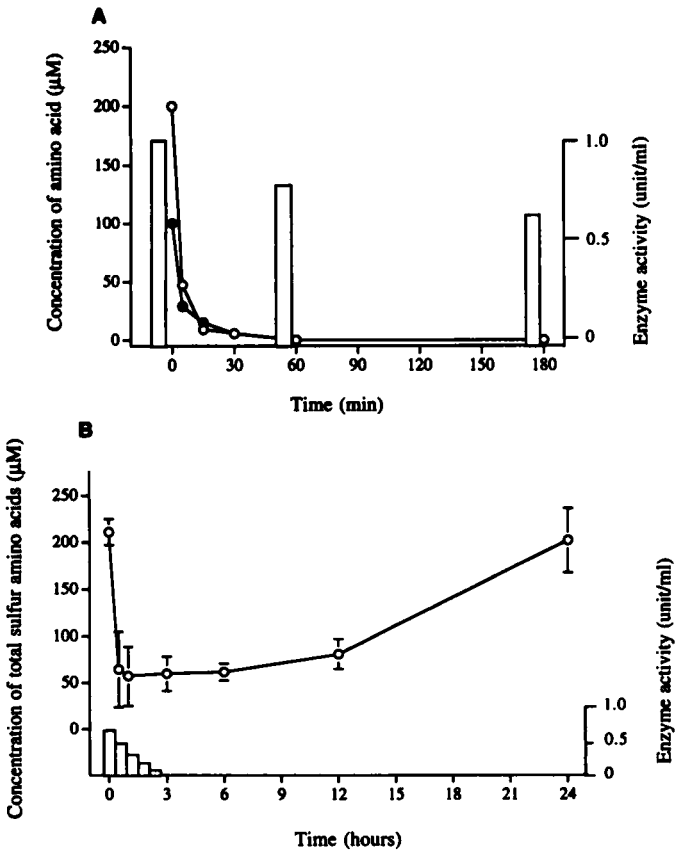

Fig. 5. Activities of L-methioninase. *A, in vitro* degradation of L-methionine and L-cysteine by L-methioninase. Levels of sulfur-containing amino acids and residual enzyme activities in medium were determined as described in "Materials and Methods" following the addition of recombinant enzyme at 1 unit/ml. The results are the mean of three determinations. ●, L-methionine; ○, L-cysteine; □, residual enzyme activities. *B, in vivo* degradation of sulfur-containing amino acids in CDF1 mice given 250 units/kg L-methioninase i.v. The results are the means of three experiments; *bars*, SD. ○, sulfur-containing amino acids; □, enzyme activities.

and expressed the gene for L-methioninase from *P. putida*. The deduced peptide sequence of the cloned gene contains 398 amino acids with a molecular mass of 42,720 daltons, comparable to the reported weight of a homotetrameric subunit (29). The peptide sequence contains 70–80% homologous regions to the two partial peptide sequences used in this study, although it is not significantly homologous to an amino terminal peptide sequence reported previously by Nakayama (19). Because the same nucleotide sequence was obtained in independent clones and the recombinant protein had the same catalytic activity as that reported for L-methioninase (15), we are certain that the sequence is correct. In support of this conclusion, the amino acid composition of the deduced recombinant polypeptide was similar to that obtained after hydrolysis of the enzyme (19). Furthermore, the cloned L-methioninase included the consensus sequence (SXXK) with the active lysine (K) residue that was highly conserved in other pyridoxal 5′-phosphate dependent enzymes from different species (25–28).

L-Methioninase has been isolated from various prokaryotes. However, the yield of pure enzyme was very low. Even with the improved purification procedure reported recently (18), 819 units of pure enzyme were isolated from 30 g of acetone powder from *P. putida*, whereas a total of 249 units of enzyme were isolated from 100 mg of acetone powder from the recombinant bacteria in this study. Thus, enzyme purification from the recombinant bacteria is 91-fold more efficient than from the original *P. putida* strain. The crude extract from recombinant bacteria contained specific enzyme activity approximately 100-fold higher than that obtained from *P. putida*.

Although the cytotoxic effects of L-methioninase have been attributed to depletion of L-methionine and its precursors, our results suggest that depletion of L-cysteine as well may play a role in cell growth inhibition. L-Cysteine is a nonessential amino acid only if L-methionine is present in sufficient concentrations to meet the total sulfur-containing amino acid requirement (30) because the sulfur moiety in L-cysteine derives mainly from L-methionine under physiological conditions. Depletion of L-cysteine may have inhibitory effects on cell growth under the conditions of L-methionine limitation, during which L-cysteine behaves as an essential amino acid. L-Methioninase treatment may cause L-cysteine deprivation partly by direct degradation of L-cysteine and mainly by depletion of L-methionine, the precursor of L-cysteine. Growth inhibition by L-methioninase was partially rescued by adding L-cysteine at 24 h after L-methioninase[5]. Ammonia and thiols produced from sulfur-containing amino acids by L-methioninase may also contribute to toxicity of the enzyme.

The results of *in vitro* cell growth inhibition indicate that leukemia cell lines are particularly sensitive to L-methioninase. The IC_{50} in leukemia cell lines was less than 0.5 unit/ml, which can be achieved in mice by a single i.v. administration of L-methioninase. Therefore, leukemia may be a promising target for L-methioninase treatment. Normal PBL were insensitive to L-methioninase, probably because of their nonproliferative state. The results of *in vivo* pharmacokinetics suggest that L-methioninase should be given by continuous infusion or by repeated injections with an interval of 12 h to continuously suppress plasma sulfur-containing amino acids.

In normal human cells, methionine is synthesized from homocysteine and from MTA (30). L-Methionine can be replaced with hom-

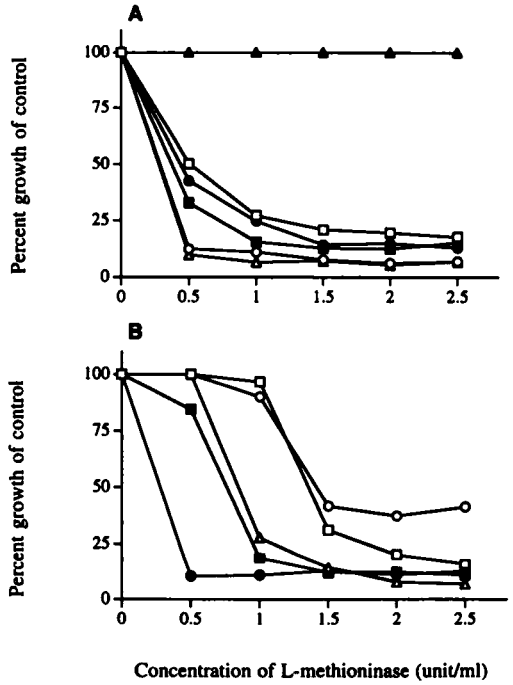

Fig. 6. Sensitivity of cancer cell lines to L-methioninase. Five leukemia cell lines and normal lymphocytes (*A*), and five solid tumor cell lines (*B*) were cultured for 4 days in medium with L-methioninase at the indicated concentrations. The growth of cells treated with different concentrations of the enzyme was expressed as the percentage of growth compared to that in cells without treatment. Each value is the mean of eight determinations. Leukemia cell lines used in *A* are: T-cell acute lymphoblastic leukemias, CEM (□), MOLT4 (○), and MOLT16 (△); acute myelogenous leukemia, HL60 (■); chronic myelogenous leukemia in blastic crisis, K562 (●); and normal lymphocytes (▲). Solid tumor cell lines used in *B* are: glioma, T98G (□); breast cancer, MCF7 (○); and non-small cell lung cancers, A549 (△), A427 (■), and CaLu6 (●).

ocysteine to support the growth of normal cells but not of tumor cells (5, 6). However, some methionine-auxotrophic malignant and transformed cells synthesize methionine from methyl-tetrahydropteroyl-L-glutamine and homocysteine (11). We reported previously that MTAP-negative cancer cells were killed selectively in methionine-free medium supplemented with MTA, whereas MTAP-positive cells were rescued by adding MTA (31). MTAP-positive normal cells may produce L-methionine endogenously from MTA, even when plasma L-methionine is depleted by L-methioninase, whereas MTAP-negative cancer cells cannot use MTA to synthesize methionine. Therefore, chemotherapy with L-methioninase and MTA, which is not a substrate of L-methioninase,[5] may be selective for MTAP-negative tumors with a L-methionine requirement.

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