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QUARTERLY

Sequence determination and analysis of *S*-adenosyl-L-homocysteine hydrolase from yellow lupine (*Lupinus luteus*)★✪

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The coding sequences of two *S*-adenosyl-L-homocysteine hydrolases (SAHases) were identified in yellow lupine by screenig of a cDNA library. One of them, corresponding to the complete protein, was sequenced and compared with 52 other SAHase sequences. Phylogenetic analysis of these proteins identified three groups of the enzymes. Group A comprises only bacterial sequences. Group B is subdivided into two subgroups, one of which (B1) is formed by animal sequences. Subgroup B2 consist of two distinct clusters, B2a and B2b. Cluster B2b comprises all known plant sequences, including the yellow lupine enzyme, which are distinguished by a 50-residue insert. Group C is heterogeneous and contains SAHases from Archaea as well as a new class of animal enzymes, distinctly different from those in group B1.

S-Adenosyl-L-homocysteine hydrolase (AdoHcy hydrolase, SAHase, *S*-adenosylhomocysteinase, EC 3.3.1.1) catalyzes the reversible breakdown of *S*-adenosyl-L-homocysteine (AdoHcy) to adenosine and homocysteine (Fig. 1). AdoHcy is formed in methylation reactions that utilize *S*-adenosyl-L-methionine (AdoMet). AdoMet is a methyl donor in

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Abbreviations: AdoHcy, *S*-adenosyl-L-homocysteine; AdoMet, *S*-adenosyl-L-methionine; NAD^+, nicotinamide adenine dinucleotide; NCBI, National Center for Biotechnology Information; ORF, open reading frame; pfu, plaques forming units; SAHase, *S*-adenosyl-L-homocysteine hydrolase; SDS/PAGE, denaturing polyacrylamide gel electrophoresis.

methylation of many biomolecules ranging from small neurotransmitters (norepinephrine, histamine) to macromolecules (nucleic acids, lipids, polysaccharides, proteins) (Giovanelli, 1987). By removing the AdoHcy byproduct, SAHase serves as a regulator of AdoMet-dependent biological methylation reactions. Inhibition of SAHase results in cellular accumulation of AdoHcy, which reduces the rate of transmethylation, including reactions involved in viral mRNA replication. Therefore, SAHase is a potential target for antiviral therapy. About fifty SAH hydrolase sequences from various sources have been deposited in the genomic databases. These sequences contain a domain responsible for non-covalent binding of NAD^+, which is a cofactor of the enzymes. In this paper, we have determined the nucleotide and amino-acid sequence of SAHase from a legume plant, yellow lupine (*Lupinus luteus*). The experiments described here were carried out with yellow lupine in the symbiotic state, with roots infected with nitrogen fixing bacteria, *Bradyrhizobium lupini.*

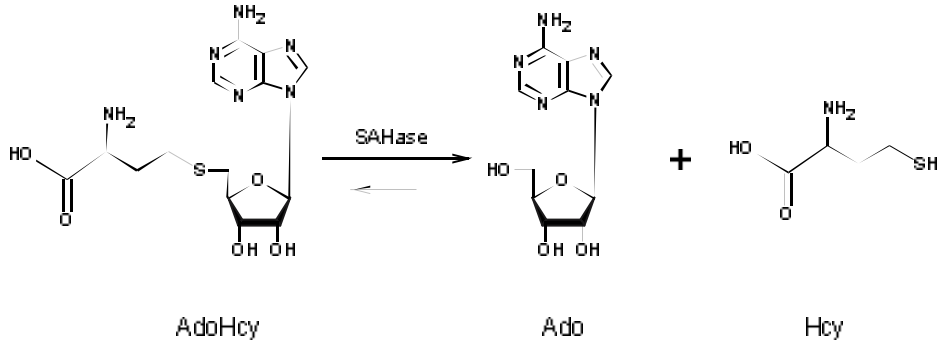


Figure 1. Reversible breakdown of *S*-adenosyl-L-homocysteine (AdoHcy) to adenosine (Ado) and homocysteine (Hcy) catalyzed by *S*-adenosyl-L-homocysteine hydrolase (SAHase).

MATERIALS AND METHODS

Screening of cDNA library. A cDNA library from *Lupinus luteus* roots with young and mature nodules (Sikorski *et al*., 1999) was screened with a probe prepared by PCR amplification. Two PCR primers: RJ1F 5′-GTYACCAAGAGCAAGTTTGA-3′ and RJ1R 5′-ACAATGGCATTGTTCTTCAT-3′ were designed using the aligned sequences of SAHases from *Catharanthus roseus* (Schroder *et al*., 1994), *Medicago sativa* (Abrahams *et al*., 1995), *Nicotiana sylvestris* (Mitsui *et al*., 1993), and *Petroselinum crispum* (Kawalleck *et al*., 1992) (see Fig. 2 for localization of primers and Fig. 3 for plant phylogenetic relations). RJ1F, with two-fold degeneration, corresponds to the region between nucleotides 697–716, and RJ1R to the region 1015–1034 of the above sequences. Five ng of the above cDNA library was used as a template in PCR amplification with Taq DNA polymerase. The amplicon was 338 nucleotides long and showed 87% sequence identity at nucleotide level and 92% identity at amino-acid level in comparison with the corresponding fragment of *Medicago sativa* SAHase. A probe was prepared with 100 ng of the amplicon, 0.033 mmoles [α^{32}P]dCTP (3000 Ci/mmol), random primers, and Klenow enzyme. It was subsequently purified with QIAquick PCR purification kit (QIAGEN, cat. no. 28106) and used for library screening. About 1.5×10^5 pfu of the cDNA library was screened with the probe yielding about 200 hybridizing signals. cDNA inserts of five purified λ clones were subcloned into the pSK Bluescript vector by *in vivo* excision following the Stratagene UNI-ZAP cDNA library protocol. Plasmid DNA isolation by alkaline lysis was done according to Sambrok *et al*. (1989). The clones

were sequenced with the fmol DNA Cycle Sequencing Kit (Promega, cat. no. Q4100). Clones pR1, pR2 and pR3 contained a 271-nucleotide insert (GenBank accession number AF252255) corresponding to the coding region 610–880 of *Medicago sativa* SAHase. Two other clones, pR4 and pR5, were identical and contained a 1806 bp insert corresponding to the complete SAHase coding sequence (Fig. 2) (GenBank accession number AF185635). In the common region they differ from pR1, pR2 and pR3 (88% identity at nucleotide level, 97% at amino-acid level). The longest ORF of pR4 and pR5 shows 84% identity at nucleotide level and 91% identity at amino-acid level to *Medicago sativa* SAHase. The pR4 and pR5 inserts have 45 nucleotides upstream of the start codon and a 303-nucleotide 3′ untranslated region.

Hybridization experiments. Samples of 10 μg of total RNA from *Lupinus luteus* roots, roots infected with *Bradyrhizobium lupini*, leaves, and seeds were used to prepare Northern blots. RNA isolation was according to Chirwing *et al*. (1979). Hybridization under high stringency conditions with a radioactively labeled insert of pR4 revealed the presence of a single hybridization band in all samples. The length of the hybridizing RNA is about 1800 nucleotides which is in agreement with the size of the pR4 and pR5 inserts.

Samples of 5 μg of *Lupinus luteus* genomic DNA digested with restriction enzymes were used for Southern blot analysis. Genomic DNA preparation was performed as in Crespi *et al*. (1994). The blot was hybridized under high stringency conditions with the same probe as above. Two hybridizing genomic DNA fragments (about 8.2 and 2.8 kb) were obtained with *Xba*I, three fragments (12.0, 5.0, and 2.0 kb) with *Eco*RI, and one (8.0 kb) with *Bam*HI.

Cloning and expression. The longest ORF of the pR4 clone was ligated into the pET-15b expression vector using restriction sites for *Nde*I and *Bam*HI introduced into the 5′ and 3′ ends of the *Lupinus luteus* SAHase coding sequence by PCR amplification with appropriate primers. BL21 *Escherichia coli* cells transformed with the above vector produced a new protein after induction with IPTG.

Protein sequence analysis. Multiple protein sequence alignment was performed using the CLUSTAL W program (Thompson *et al*., 1994). The amino-acid sequence of *Lupinus luteus* SAHase was compared with 52 other sequences present in the NCBI and SWISS PROT genomic databases and annotated as SAHases. The aligned sequences were used to construct a phylogenetic tree in the TreeView program (Page, 1996).

RESULTS AND DISCUSSION

The sequences of the cDNA clones pR1–pR5 attest to the presence of two SAHase homologues in the *Lupinus luteus* genome. The clones pR1, pR2, and pR3 reveal an incomplete coding sequence of one of those enzymes, LlSAHase-1. The fragment of LlSAHas-1 is identical in all three clones and corresponds to the region 655–925 bp of LlSAHas-2 as shown in Fig. 2. Two other clones, pR4 and pR5, correspond to the complete coding sequence of LlSAHase-2 (Fig. 2). The 1806 bp inserts of clones pR4 and pR5 are 100% identical at nucleotide level and have an identical structure, i.e. a 1458-bp-long coding sequence surrounded by a 45-bp 5′-UTR and a 303-bp 3′-UTR (Fig. 2). Such sequence and structure identity of two independently isolated cDNA clones strongly suggests that they represent the complete transcript of the LlSAHase-2 gene. LlSAHase-1 and LlSAHase-2 reveal 88% identity at nucleotide level and 97% identity at amino-acid level. The phylogenetic status of LlSAHase-2 is shown in Fig. 3 and is in agreement with *Lupinus luteus* phylogeny (the identity between LlSAHase-2 and *Medicago sativa* SAHase is 84% at nucleotide level and 91% at amino-acid level). In the case of LlSAHase-1, the known fragment is too short for phylogenetic analysis with ade-

-45		ATC	TAT	CTA	TCT	TTC	TCT	TCT	CTT	GAT	TCA	CAG	AAT	CAA	TCA	AGA
1	ATG	GCA	TTG	CTA	GTA	GAG	AAA	ACC	ACA	AGT	GGT	CGT	GAA	TAC	AAG	GTG
1	M	A	L	L	V	E	K	T	T	S	G	R	E	Y	K	V
49	AAG	GAC	ATG	TCC	CAA	GCA	GAC	TTC	GGT	CGT	CTA	GAA	ATA	GAG	TTA	GCA
17	K	D	M	S	Q	A	D	F	G	R	L	E	I	E	L	A
97	GAA	GTT	GAA	ATG	CCT	GGG	TTG	ATG	GCT	TCA	AGA	TCT	GAA	TTT	GGT	CCC
33	E	V	E	M	P	G	L	M	A	S	R	S	E	F	G	P
145	TCT	CAG	CCA	TTC	AAA	GGA	GCT	AAG	ATC	ACT	GGC	TCC	CTT	CAC	ATG	ACT
49	S	Q	P	F	K	G	A	K	I	T	G	S	L	H	M	T
193	ATC	CAA	ACT	GCA	GTC	CTG	ATT	GAA	ACC	CTC	ACT	GCC	CTT	GGT	GCT	GAA
65	I	Q	T	A	V	L	I	E	T	L	T	A	L	G	A	E
241	GTC	AGA	TGG	TGT	TCA	TGC	AAC	ATC	TTC	TCC	ACT	CAG	GAC	CAT	GCT	GCT
81	V	R	W	C	S	C	N	I	F	S	T	Q	D	H	A	A
289	GCT	GCC	ATT	GCA	CGT	GAC	AGT	GCT	GCT	GTG	TTT	GCA	TGG	AAG	GGT	GAG
97	A	A	I	A	R	D	S	A	A	V	F	A	W	K	G	E
337	ACC	CTC	CAG	GAG	TAT	TGG	TGG	TGC	ACT	GAG	CGT	GCC	CTT	GAT	TGG	GGT
113	T	L	Q	E	Y	W	W	C	T	E	R	A	L	D	W	G
385	CCT	GGT	GGT	GGC	CCT	GAC	CTC	ATT	GTT	GAT	GAT	GGT	GGT	GAC	ACC	ACG
129	P	G	G	G	P	D	L	I	V	D	D	G	G	D	T	T
433	TTG	TTG	ATC	CAT	GAA	GGG	GTT	AAG	GCT	GAG	GAG	ATT	TAT	GAG	AAG	AGT
145	L	L	I	H	E	G	V	K	A	E	E	I	Y	E	K	S
481	GGC	CAG	TTC	CCT	GAC	CCT	GAT	TCA	ACT	GAT	AAT	GCT	GAG	TTT	AAG	ATT
161	G	Q	F	P	D	P	D	S	T	D	N	A	E	F	K	I
529	GTG	TTG	TCT	ATT	ATA	AAG	GAA	GGG	TTG	AAG	ACA	GAT	CCT	AAG	AGG	TAT
177	V	L	S	I	I	K	E	G	L	K	T	D	P	K	R	Y
577	CAT	AAG	ATG	AAG	GAT	AGA	GTT	GTT	GGT	GTT	TCG	GAA	GAG	ACA	ACT	ACT
193	H	K	M	K	D	R	V	V	G	V	S	E	E	T	T	T
625	GGT	GTT	AAG	AGG	TTG	TAT	CAG	ATG	CAG	GCT	AAT	GGA	ACT	CTC	TTG	TTC
209	G	V	K	R	L	Y	Q	M	Q	A	N	G	T	L	L	F
673	CCT	GCT	ATC	AAT	GTC	AAT	GAC	TCT	GTC	ACC	AAG	AGC	AAG	TTT	GAT	AAC
225	P	A	I	N	V	N	D	S	V	T	K	*S*	*K*	F	D	N
721	TTA	TAT	GGA	TGC	CGC	CAC	TCT	CTC	CCC	GAT	GGA	CTG	ATG	AGA	GCC	ACT
241	L	Y	G	C	R	H	S	L	P	D	G	L	M	R	A	T
769	GAT	GTT	ATG	ATT	GCC	GGC	AAG	GTC	GCA	GTT	GTT	GCT	GGA	TAT	GGA	GAT
257	D	V	M	I	A	G	K	V	A	V	V	A	G	Y	G	D
817	GTT	GGA	AAG	GGT	TGT	GCT	GCT	GCA	TTG	AAA	CAA	GCT	GGT	GCT	CGT	GTC
273	V	G	K	G	C	A	*A*	A	L	K	Q	A	G	A	R	V
865	ATA	GTA	ACC	GAG	ATT	GAT	CCA	ATC	TGT	GCC	CTT	CAA	GCT	ACA	ATG	GAA
289	I	V	T	E	I	D	P	I	C	A	L	Q	A	T	M	E
913	GGT	CTT	CAA	GTT	CTA	ACA	TTG	GAA	GAT	GTT	GTC	TCC	GAG	GCT	GAT	ATC
305	G	L	Q	V	L	T	L	E	D	V	V	S	E	A	D	I
961	TTT	GTT	ACC	ACC	ACA	GGT	AAC	AAG	GAC	ATC	ATC	ATG	CTT	GAT	CAC	ATG
321	F	V	T	T	T	G	N	K	D	I	I	M	L	D	H	M
1009	AAG	AAA	ATG	AAG	AAC	AAT	GCC	ATT	GTC	TGC	AAC	ATT	GGT	CAC	TTC	GAC
337	K	K	M	K	N	N	A	I	V	C	N	I	G	H	F	D
1057	AAT	GAA	ATC	GAC	ATG	CTT	GGC	CTT	GAG	ACA	CAC	CCT	GGT	GTC	AAG	CGC
353	N	E	I	D	M	L	G	L	E	T	H	P	G	V	K	R
1105	ATC	ACA	ATC	AAG	CCT	CAA	ACT	GAT	AGG	TGG	GTC	TTC	CCT	GAG	ACC	AAC
369	I	T	I	K	P	Q	T	D	R	W	V	F	P	E	T	N
1153	ACT	GGC	ATC	ATT	ATA	TTG	GCA	GAG	GGT	CGT	TTA	ATG	AAC	TTG	GGT	TGT
385	T	G	I	I	I	L	A	E	G	R	L	M	N	L	G	C

1201	GCC	ACA	GGA	CAC	CCA	AGT	TTT	GTT	ATG	TCA	TGT	TCA	TTC	ACC	AAC	CAG
401	A	T	G	H	P	S	F	V	M	S	C	S	F	T	N	Q
1249	GTT	ATT	GCT	CAG	CTT	GAG	TTG	TGG	AAT	GAG	AAG	AGT	TCT	GGA	AAG	TAT
417	V	I	A	Q	L	E	L	W	N	E	K	S	S	G	K	Y
1297	GAG	AAG	AAG	GTT	TAT	GTT	CTG	CCT	AAG	CAC	CTT	GAT	GAG	AAG	GTT	GCT
433	E	K	K	V	Y	V	L	P	K	H	L	D	E	K	V	A
1345	GCT	CTT	CAC	CTT	GAA	AAG	CTT	GGA	GCT	AAG	CTC	ACY	AAG	CTT	AGC	AAG
449	A	L	H	L	E	K	L	G	A	K	L	T	K	L	S	K
1393	GAC	CAA	GCT	GAT	TAT	ATC	AGT	GTC	CCT	GTT	GAG	GGT	CCA	TAC	AAG	CCT
465	D	Q	A	D	Y	I	S	V	P	V	E	G	P	Y	K	P
1441	TTC	CAC	TAT	AGG	TAC	TGA	TAT	GAT	ATC	AAT	CAT	GAT	GAT	ACT	GAG	GGA
481	F	H	Y	R	Y	STOP										
1489	AAA	GAA	AGT	CAT	TTT	TAT	GAT	ATC	AAT	CAT	GAT	GAT	ACT	GAG	GGA	AAA
1537	AGA	AAA	GTC	ATT	TTT	GTC	ATT	TTT	ATC	TTG	AAA	CTG	GAT	TTT	TTC	TAA
1585	TTA	CTA	TAT	TTT	TCA	GAT	TTG	TGG	TAG	GGT	GGT	AGT	TTT	ATG	ATA	TTT
1633	TTG	TTG	GAT	GTT	TTA	TTC	CAT	TGG	GTT	GGG	AGG	GTA	AGA	GCA	AAA	ACA
1681	AAT	CTA	ATG	GTC	TTT	GCA	GAA	ATG	AGA	CCA	AAT	AAT	GGG	TTT	TTG	AAT
1729	AAG	GCT	TTG	ATT	GAG	GTT	GTG	TGG	GTT	ATG	ATT	TTG	ATT	TAT	GTT	TTG
1777	TTA	ATT	CAC	CAT	TTA	CTA	TCA	TAC	TTT	GGT	CTC					

Figure 2. Nucleotide and amino-acid sequence of *S*-adenosyl-L-homocysteine hydrolase from yellow lupine (LlSAHase-2).

Sequences corresponding to the PCR primers RJ1F and RJ1R are underlined. The plant-specific insert is shown in green. It separates the catalytic and NAD^+-binding SAHase domains. The fragment printed in red corresponds to the partially sequenced LlSAHase-1 (not shown). The deduced amino-acid residues that would be different in the LlSAHase-1 protein are shown in *italics*.

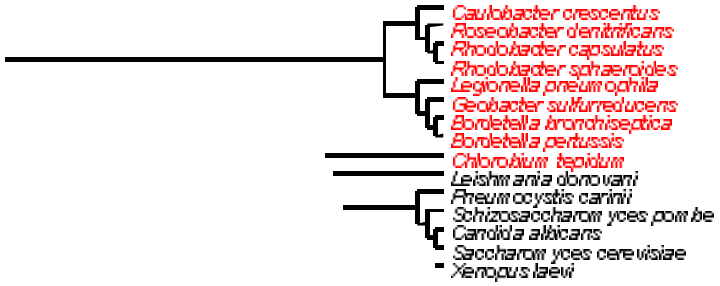
quate resolution. It remains, therefore, an open question whether the two LlSAHase homologues are simply paralogues occupying distinct loci (a more probable hypothesis) or whether they represent two alleles. Moreover, because of the paleopolyploidy of the *Lupinus luteus* genome, even orthology of LlSAHase-1 and LlSAHase-2 cannot be excluded.

The length of yellow lupine SAH hydrolase mRNA estimated by Northern blotting is around 1800 nucleotides, in good agreement with the length of the pR4 and pR5 insert. The Southern blot data reveal the presence of one (about 8.0 kb) *Bam*HI fragment, two *Xba*I fragments (8.2 and 2.8 kb), and three *Eco*RI fragments (12.0, 5.0, and 2.0 kb) in genomic DNA which hybridize with the LlSAHase-2 probe. The pattern of restriction-enzyme digestion suggests that there is a single gene for each of the SAHase homologues in the *Lupinus luteus* genome. The probe cross-hybridization with the LlSAHase-1 and LlSAHase-2 genes could be expected because of their high similarity.

The size of the transgenic protein (about 57 kDa determined by SDS/PAGE electrophoresis) produced by *Escherichia coli* cells transformed with the plasmid bearing the insert from pR4, is in agreement with the calculated mass of *Lupinus luteus* SAHase incremented by the additional histidine tag encoded by the pET-15b vector. It is also consistent with the mass (55 kDa) of SAHase isolated from lupine seeds (Guranowski & Pawełkiewicz, 1977).

The amino-acid sequence of yellow lupine LlSAHase-2 contains a specific insert, characterizing plant enzymes, located between amino-acid residues 149 and 200 (Fig. 2). Multiple protein sequence alignment indicates that all known *S*-adenosyl-L-homocysteine hy-

drolases can be divided into three groups: group A comprising only (but not all) bacterial enzymes, group B formed primarily by eukaryotic proteins plus a few bacterial sequences, and group C dominated by Archaea. The enzymes in group A are typically 400–425 amino acids long (molecular mass 45–47 kDa). Group B can be divided into two subgroups. Subgroup B1 contains only animal enzymes except for one bacterial sequence. Subgroup B2 is clearly subdivided into two clusters. Cluster B2a contains only bacterial sequences and one protozoan sequence, while cluster B2b is comprised exclusively of plant enzymes. It should be stressed that all known plant sequences without exception are grouped in cluster B2b. The enzymes derived from animals, typified by human SAHase I (Coulter-Karis & Hershfield, 1989), are somewhat longer (430–440 amino acids, 47–49 kDa) than the bacterial proteins in group A. Plant SAHases are still longer and consist of 485 residues (55 kDa). This size difference corresponds to the extra segment of about 50 amino acids inserted between residues around 150 and 200, and may be related to different subunit composition of the plant enzymes (Ogawa *et al*., 1987). Finally, the main



group C comprises enzymes from different king doms, but not from plants, and is the only group where the enzymes from Archaea are found. Interestingly, in this group there are also animal enzymes, from *Homo sapiens* (GenBank accession number U82761) and *Drosophila melanogaster* (Mar tin *et al*., 1995), with closer homology to the Archaea se-quences than to their homologues in group B1. These enzymes are about 80 amino acids longer than human SAHase I but sequence alignments indicate that the extension is at the N-terminus and is not re lated to the in sert present in the plant enzymes.

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