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The Silkworm Mutant *lemon* (*lemon lethal*) Is a Potential Insect Model for Human Sepiapterin Reductase Deficiency*

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Yan Meng[‡], Susumu Katsuma[‡], Takaaki Daimon[‡], Yutaka Banno[§], Keiro Uchino[¶], Hideki Sezutsu[¶], Toshiki Tamura[¶], Kazuei Mita[‖], and Toru Shimada[‡1]

From the [‡]Department of Agricultural and Environmental Biology, Graduate School of Agricultural and Life Sciences, University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan, the [§]Institute of Genetic Resources, Graduate School of Bioresource and Bioenvironmental Science, Kyushu University, Hakozaki 6-10-1, Higashi-ku, Fukuoka 812-8581, Japan, and the [¶]Transgenic Silkworm Research Center and [‖]Insect Sciences Department, National Institute of Agrobiological Sciences, Owashi 1-2, Tsukuba, Ibaraki 305-8634, Japan

Tetrahydrobiopterin (BH4) is an essential cofactor for aromatic acid hydroxylases, which control the levels of monoamine neurotransmitters. BH4 deficiency has been associated with many neuropsychological disorders. An inherited defect in BH4 biosynthesis is caused by the deficiency of sepiapterin reductase (SPR), which catalyzes the biosynthesis of BH4 from guanosine triphosphate at the terminal step. The human SPR gene has been mapped at the PARK3 locus, which is related to the onset of Parkinson disease. In this study, we report that mutant strains, *lemon* (*lem*) and its lethal allele *lemon lethal* (*lem^l*) with yellow body coloration, of the silkworm *Bombyx mori* could be used as the first insect model for human SPR deficiency diseases. We demonstrated that mutations in the SPR gene (*BmSpr*) were responsible for the irregular body coloration of *lem* and *lem^l*. Moreover, biochemical analysis revealed that SPR activity in *lem^l* larvae was almost completely diminished, resulting in a lethal phenotype that the larvae cannot feed and that die immediately after the first ecdysis. Oral administration of BH4 and dopamine to *lem^l* larvae effectively increased their survival rates and feeding abilities. Our data demonstrate that BmSPR plays a crucial role in the generation of BH4, and monoamine neurotransmitters in silkworms and the *lem* (*lem^l*) mutant strains will be an invaluable resource to address many questions regarding SPR and BH4 deficiencies.

Tetrahydrobiopterin (BH4)[2] is an essential cofactor for a number of enzymes, such as phenylalanine hydroxylase, tyrosine hydroxylase, tryptophan hydroxylase, and nitric-oxide synthase. These enzymes play an important role in the metabolism of aromatic amino acids and monoamine neurotransmitter biosynthesis (Fig. 1*B*) (1). Previous reports have shown that BH4 deficiency is associated with numerous metabolic syndromes and neuropsychological disorders (2, 3). BH4 is synthesized from GTP through a cascade of three enzymes (*i.e.* GTP cyclohydrolase I (EC 3.5.4.16), 6-pyruvoyl-tetrahydropterin (PTP) synthase (EC 4.6.1.10), and sepiapterin reductase (SPR; EC 1.1.1.153)) (Fig. 1*A*).

SPR catalyzes the conversion of PTP to BH4 at the terminal step in the presence of reduced NADPH. It also catalyzes the reduction of sepiapterin (SP) to form BH4 by subsequent catalysis of dihydrobiopterin reductase (EC 1.6.99.7) (Fig. 1*A*). Mammalian SPRs have been a focus of study in recent years. The major symptoms of SPR deficiency are mental retardation, dystonia, spasticity, and movement disorder (1, 3). Diagnosis and therapy of SPR and/or BH4 deficiency-dependent genetic diseases, such as recessive DOPA-responsive dystonia and phenylketonuria, have been developed recently (3–5). Takazawa *et al.* (6) showed that the human SPR gene could be a causative gene for PARK3, the original reported pedigree of Parkinson disease (7). However, these findings are insufficient for developing treatments because patients from different families or regions exhibit distinct physiological and metabolic disorders due to SPR/BH4 deficiencies. Therefore, it is necessary to find or develop suitable models in other animals to obtain a better understanding of related human diseases. Recently, two groups generated *Spr* knock-out mice and concluded that these mice could be invaluable resources to address the issues regarding SPR/BH4 deficiencies (6, 8).

SPR has been previously purified from the silkworm *Bombyx mori*, and its activity in the fat body of normal larvae has been characterized (9, 10). However, the SPR gene (*BmSpr*) has not been identified yet in *B. mori*. *lemon* (*lem*) is a body color mutant of *B. mori*, which is regulated by a single recessive gene called *lem* (11). The *lem* silkworms display yellow body coloration during larval developmental stages, especially during molting, which is markedly different from that of wild-type strains (Fig. 2*A*). *lemon lethal* (*lem^l*) is a homozygous lethal allele of *lem*. The *lem^l* larvae grow normally in the first instar. After the first ecdysis, the *lem^l* larvae stop feeding, shake their heads frequently, and die within 3 days (Fig. 2, *B* and *C*) (12). *lem* (*lem^l*) was mapped onto the proximal end of the third linkage group of *B. mori* (11); however, the candidate gene itself remains unknown to date. Previous studies have shown that a large amount of yellow pteridines, SP, and sepialumazine accumu-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB465548-AB465551.

[1] To whom correspondence should be addressed. Tel.: 81-3-5841-5057; Fax: 81-3-5841-5089; E-mail: shimada@ss.ab.a.u-tokyo.ac.jp.

[2] The abbreviations used are: BH4, tetrahydrobiopterin; PTP, 6-pyruvoyl-tetrahydropterin; SP, sepiapterin; SPR, sepiapterin reductase; ORF, open reading frame; RT, reverse transcription; nt, nucleotide(s); aa, amino acid(s).

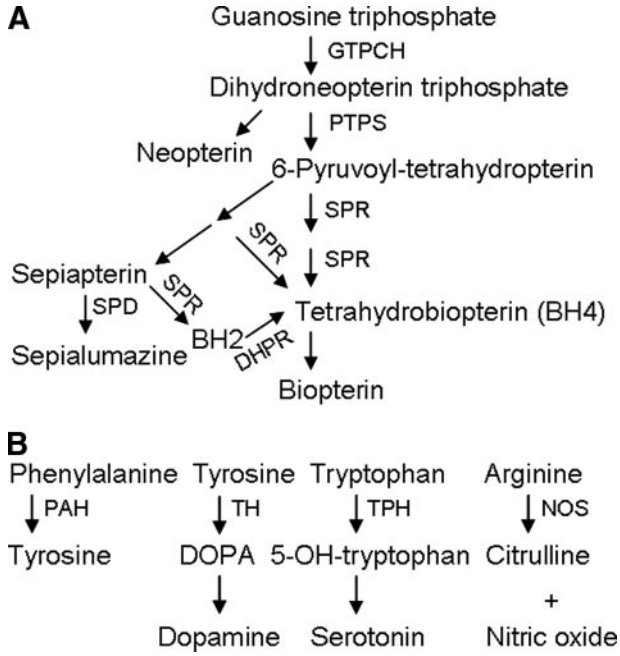

FIGURE 1. *A*, the biosynthesis pathway of BH4 and pteridines from GTP. *GTPCH*, GTP cyclohydrolase; *PTPS*, PTP synthase; *SPD*, sepiapterin deaminase; *DHPR*, dihydrobiopterin reductase; *BH2*, dihydrobiopterin. *B*, physiological functions of BH4 as cofactors for aromatic acid hydroxylases and nitric-oxide synthase. *TH* and *TPH*, tyrosine hydroxylase and tryptophan hydroxylase, rate-limiting enzymes for the production of dopamine and serotonin, respectively. *PAH*, phenylalanine hydroxylase; *NOS*, nitric-oxide synthase.

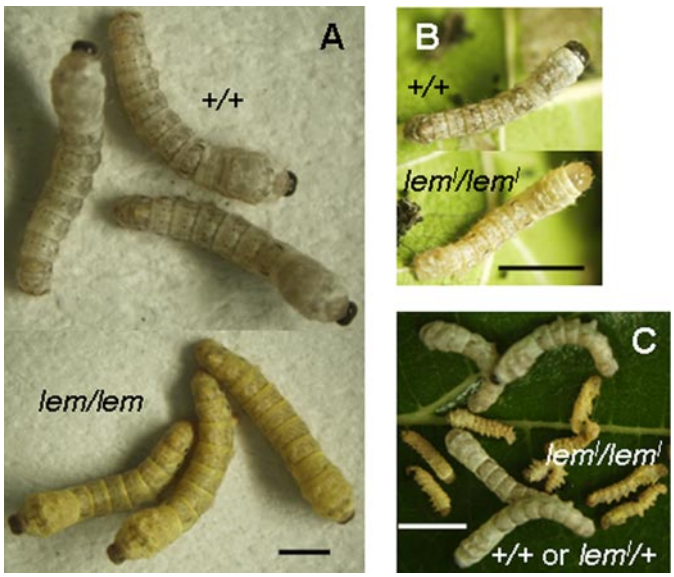

FIGURE 2. *A*, larval body color comparison between normal (*top*, *white*) and *lem* (*bottom*, *yellow*) strains in the third molting. *B*, larval body color comparison between normal (*top*, *white*) and *lem*^{l} (*bottom*, *bright yellow*) strains at the beginning of the second instar. *C*, lethality of *lem*^{l} homologous larvae. They die from day 2 of the second instar, whereas +/+ or *lem*^{l}/+ larvae of the same strain grow normally. *Scale bar*, 5 mm.

lated in the integument of the *lem* larvae (13, 14). In addition, SPR activity was absent in the *lem* mutant silkworms (9). Lack of SPR activity accelerates the accumulation of SP and sepialumazine (Fig. 1*A*). Collectively, these results suggest that SPR was related to irregular body coloration in the *lem* (*lem*^{l}) mutant.

In this study, we characterized and identified the mutations in *BmSpr* of *lem* and *lem*^{l} mutants. Linkage analysis showed that *BmSpr* is the candidate gene for the *lem* (*lem*^{l}) mutant. In addition, biochemical studies revealed that a decrease in BmSPR activity is responsible for the abnormal coloration, and a loss of BmSPR activity leads to the lethality of *lem*^{l} larvae. Furthermore, we succeeded in increasing the survival rates of the *lem*^{l} larvae by oral inoculation of BH4 and dopamine. Together, we conclude that BmSPR plays a crucial role in the generation of BH4 and monoamine neurotransmitters in *B. mori*, similar to that in mammals. The utility of the *lem* (*lem*^{l}) mutant as a potential insect model for human SPR deficiency has been discussed in this study.

EXPERIMENTAL PROCEDURES

Silkworm Strains—Five *B. mori* mutant *lem* strains (e36, l70, f40, r04, and b602) and one *lem*^{l} strain (a65) used in this study were obtained from Kyushu University (SilkwormBase; available on the World Wide Web). Normal strain 772 was obtained from the National Institute of Agrobiological Sciences. Normal strains of p50T and Sho-on were maintained in our laboratory. All larvae were fed fresh mulberry leaves under normal conditions (12 h light/12 h dark, 25 °C).

Genomic PCR and Reverse Transcription (RT)-PCR—Genomic DNA was prepared using a DNeasy blood and tissue kit (Qiagen). Genomic PCR was performed using TaKaRa Ex Taq (TaKaRa). Total RNA was extracted from whole body or tissues, as described previously (15). Expression profiles of *BmSpr* in different stages or tissues were analyzed by RT-PCR using a TaKaRa RNA PCR kit (TaKaRa). The PCR conditions were set up as recommended by the suppliers. The PCR primers used in the experiments are listed in Table 1 or available upon request.

Cloning of B. mori Sepiapterin Reductase Gene—To identify whether the *B. mori* gene was homologous to the SPR gene, we screened expressed sequence tag data bases (16) and genome sequences (17, 18) using the BLAST program. By sequencing cDNA and genomic clones, we obtained the full-length *BmSpr* sequence that encodes a putative SPR and determined its genomic structure.

Protein Extract from Whole Body—Total proteins were extracted from each of the five individual pools at the beginning of the second larval instar. Whole bodies were homogenized in 10 mM phosphate-buffered saline (pH 7.0) containing a mixture of proteinase inhibitors (Roche Applied Science). The supernatant of the homogenates was collected by centrifugation (15,000 rpm, 4 °C, 20 min) and subjected to the PD-10 column (Amersham Biosciences) for desalting and buffer exchange with phosphate-buffered saline (pH 6.4; 200 mM). The resultant solution was concentrated using Amicon Ultra centrifugal filter devices (Millipore). The protein concentration was estimated using a Coomassie Plus protein assay reagent kit (Pierce) with bovine serum albumin as a standard.

Bacterial Expression and Purification of Recombinant BmSPR—The coding regions of the wild-type, *lem* mutant type, and *lem*^{l} mutant type *BmSpr* with a His_6 tag sequence at the C terminus were amplified by PCR from the corresponding cDNA templates. The primers used are listed in Table 1. PCR products were digested with EcoRI and ligated into a pET24b vector (Novagen), resulting in three recombinant expression

TABLE 1
Main primers used in this study
Artificial EcoRI sites are underlined. Attached His_6 tag sequences are shown in boldface type.

Name	Sequence (5′–3′)	Purpose
BmSprORF-fw	GGAGGACAAACCGGAGTCTG	RT-PCR and linkage analysis for *lem*
BmSprORF-re	CCCGCAAGGGGATACGAAGA	RT-PCR and linkage analysis for *lem*
BmSprMfw	CGTAACAAAGATCAGCTCGCCAT	Linkage analysis for lem^l
BmSprMre	CGAGCCTATCCTCTATCTCATC	Linkage analysis for lem^l
pETBmSPR-fw	CGGAATTCGATGGCTATGTCGTCTAGCATC	Expression in *E. coli* for three proteins
pETBmSPR-re1	CGGAATTCTCA**GTGGTGGTGGTGGTGGTG**TTCGTCATCGAAATAGTCGAC	Expression in *E. coli* for normal and lem^l mutant type proteins
pETBmSPR-re2	CGGAATTCTTA**GTGATGGTGATGGTGATG**GTCGACGTGCTCTGCCGGACT	Expression in *E. coli* for *lem* mutant type protein

TABLE 2
Linkage analysis

lem			lem^l			
Phenotype (genotype)	SNP in *BmSpr* ORF (nt 786)		Phenotype (genotype)	PCR marker in *BmSpr* ORF		
	A/T	A/A		325 bp only	325 bp and 352 bp	352 bp only
Normal (*lem*/+)	50	0	Normal (+/+)	35	0	0
Yellow (*lem*/*lem*)	0	140	Normal (lem^l/+)	0	56	0
			Yellow and lethal (lem^l/lem^l)	0	0	96

vectors, pET/BmSPR, pET/BmmtSPR, and pET/BmmtSPR[1]. They were transformed into *Escherichia coli* BL21 (DE3) competent cells. BmSPR expression was induced by 1 mM isopropyl-1-thio-β-D-galactopyranoside. The transformants were cultured overnight at 15 °C following isopropyl-1-thio-β-D-galactopyranoside induction. The cells were collected by centrifugation and suspended in a B-PER bacterial protein extraction reagent (Pierce) containing a mixture of proteinase inhibitors (Roche Applied Science), and the supernatant was collected by centrifugation. His-tagged BmSPRs were purified using a His GraviTrap nickel affinity column (GE Healthcare) according to the manufacturer's instructions. Finally, the eluate was desalted and concentrated. Protein concentration was determined as described above.

Immunoblot Analysis—Expression of recombinant BmSPRs were analyzed by immunoblot analysis using anti-His antibody (Qiagen), as described previously (19). After SDS-PAGE (20), the proteins were electrophoretically transferred to a polyvinylidene fluoride membrane (Immobilon-P; Millipore) using a blotting apparatus (Atto). The membrane was exposed to anti-His antibody (1:5000 dilution) and then to the secondary antibody, goat anti-mouse IgG-horseradish peroxidase conjugate (Zymed Laboratories Inc.) (1:5000 dilution). The blot was visualized using Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences) and a LAS 1000 imaging system (Fuji Film).

Enzyme Assay—SPR activity was assayed according to the method reported by Katoh (21) with slight modifications. Ten micrograms of protein from whole body or 0.1–0.5 μg of recombinant BmSPR was used in the assessment. The standard reaction mixture consisted of 100 μM NADPH (Sigma), 50 μM sepiapterin (Sigma), 100 mM potassium phosphate buffer (pH 6.4), and the enzyme in a final volume of 200 μl. The reaction was initiated by the addition of the enzyme and kept at 37 °C for 5–30 min. SPR activity was determined by measuring the rate of decrease in absorbance at 420 nm using a model 680 microplate reader (Bio-Rad). Reaction without the addition of the enzyme was used as a control. One unit of enzyme was defined as the amount of the enzyme/μg of protein that catalyzed the reduction of 1 nmol of sepiapterin/min (nmol/min/μg), using an extinction coefficient for sepiapterin of 1.04×10^4/mol/cm at 420 nm. The data were analyzed by one-way analysis of variance followed by Dunnett's test to localize the significant difference. A p value of less than 0.01 was considered significant.

To analyze the effect of pH on SPR activity, the final concentration of 20 mM Britton-Robinson buffer (pH 2.0–12.0) was used. To analyze the effect of temperature on SPR activity, each reaction mixture was kept at temperatures ranging from 20 to 90 °C for 5 min. The effect of the substrate concentration on SPR activity was analyzed by varying sepiapterin concentrations from 2.5 to 160 μM in the presence of 0.1 μg of protein and saturating amounts of 250 μM NADPH. The reaction was performed at 37 °C for 5 min. Kinetic parameters of maximal velocity (V_{max}) and Michaelis constant (K_m) were estimated using the double reciprocal (Lineweaver-Burk) plot (22). Inhibition of *B. mori* SPR by melatonin and *N*-acetylserotonin (Sigma) was investigated using 0.2 μg of protein at 37 °C for 10 min. Reactions with 1% ethanol were used as controls.

Linkage Analysis—Normal strain p50T, *lem* strain l70, and lem^l strain a65 were used in genetic linkage analysis. The F1 male moths of p50T and l70 were backcrossed with l70 females. Single nucleotide polymorphism at nt 786 in the *BmSpr* ORF of BC1 individuals was investigated by genomic PCR and direct sequencing of the PCR products (Table 2). The F1 moths of p50T and a65 (lem^l/+) were sibling-mated. Genotypes of F2 individuals randomly sampled from several broods were determined by a PCR marker in *BmSpr* ORF (Table 2). Genomic DNA was isolated using the Wizard SV 96 genomic DNA purification system (Promega), as described in the recommended protocol. The primers used are listed in Table 1. DNA sequences were determined using the ABI Big Dye Terminator Cycle Sequencing Ready Reaction kit version 3.1 (Applied Biosystems) and an ABI Prism 3130 genetic analyzer (Applied Biosystems).

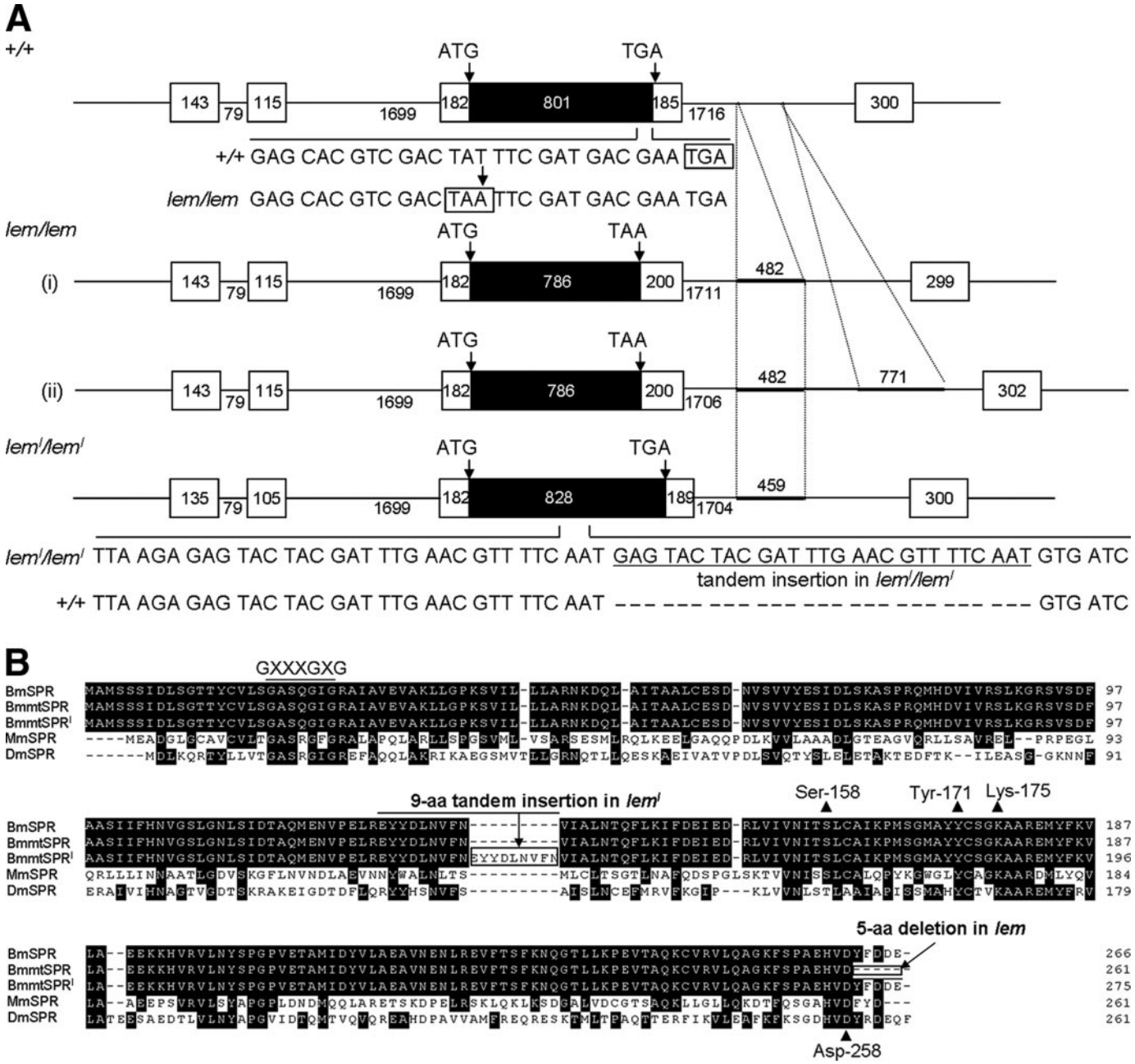

FIGURE 3. **Schematic representation of the *BmSpr* gene structure and alignment of amino acid sequences.** *A*, the *top* gene structure is the normal type (strains p50T and Sho-on). The *middle two* are structures in *lem* strains (*i*, strains l70, e36, r04, and b602; *ii*, strain f40), and the *bottom* structure is in *lem*^{l} strain (a65). The *boxes* indicate exons in *black* for the coding region and *white* for the noncoding region. *Numerals* show the length of exons or introns. Start and stop codons are indicated by *arrows*. The single nucleotide substitution (T → A) at the end of the *BmSpr* ORF in *lem* strains led to an abnormal forward stop codon (TAA, *boxed*). Tandem insertion of 27 nt into *BmSpr* ORF is *underlined*, which caused a 9-aa increase in the *lem*^{l} strain. Genomic insertions found in the third intron of mutant types of *BmSpr* are indicated as *thick bars*. *B*, three types of *B. mori* SPRs were aligned with mouse SPR (*Mus musculus*; GenBank™ accession number Q64105) and *Drosophila* SPR (*Drosophila melanogaster*; GenBank™ accession number NP_727265) using the ClustalX program. Amino acid residues conserved among more than four SPR sequences are highlighted. Five amino acid deletions in *lem* (-YFDDE) and 9-aa tandem insertions in *lem*^{l} (-EYYDLNVFN-) are *aligned* and *boxed*. Important residues for mammalian SPR activity, such as G*XXX*G*X*G (Rossmann fold, for NADPH binding), Ser^{158}-Tyr^{171}-*XXX*K^{175} triad (catalytic site), and Asp-258 (an aspartate anchor, for pterin substrate) are also shown.

BH4 and Monoamine Feeding Experiment—To investigate the rescue effect of BH4 on the *lem*^{l} larvae, 10-fold diluted concentrations (0.03–30 mM) of BH4 (Wako) were orally supplied to newly hatched *lem*^{l}/*lem*^{l} larvae of the a65 strain. Fresh mulberry leaves were chopped and completely permeated in freshly prepared BH4 solutions. The leaves were dried in air and fed to the larvae every day. Survival rates were recorded from the beginning of second instar (day 0 in Fig. 6*A*). Oral administration of two monoamines, dopamine (Sigma) or serotonin (Wako), at a final concentration of 50 mM was performed against third instar *lem*^{l} larvae, which were survived by supplying 30 mM BH4 until the second ecdysis. Survival rate curves were recorded from day 1 to day 4. Feeding abilities of the living larvae were examined by counting their feces (more than 10 feces, high food intake; 1–10 feces, low food intake; no feces, nonfeeding). Distilled water-wetted leaves were used as a negative control.

RESULTS

Characterization of BmSpr Gene in Normal and Mutant Strains—Previous studies showed that SPR deficiency is involved in abnormal body coloration of the *lem* (*lem*^{l}) mutant (9, 13, 14). To examine whether the *B. mori* SPR gene (*BmSpr*) corresponds to *lem* (*lem*^{l}), we first determined a full-length cDNA sequence of *BmSpr* using expressed sequence tag data bases (16). The sequence is located on Bm_scaf63, which is a newly integrated scaffold near the end of chromosome 3 (KAIKObase; available on the World Wide Web).

We determined the gene structures of *BmSpr* in normal and mutant strains by genomic primer walking and identified the mutations in *BmSpr* open reading frames (ORFs) of the mutants (Fig. 3*A*). *BmSpr* comprised an ORF of 798 bp, which encodes a 266-aa protein with a predicted molecular mass of 29.2 kDa (Fig. 3*B*). In the *BmSpr* ORF of five *lem* strains, a single nucleotide mutation was identified at the same position (T786A), which formed an abnormal forward stop codon and resulted in a 5-aa deletion (-YFDDE) at the C terminus. In the *lem*^{l} strain, 27 nt were inserted in tandem in the middle of the *BmSpr* ORF, which resulted in an addition of 9 aa (-EYYDLNVFN-) (Fig. 3, *A* and *B*). Moreover, one or two genomic insertions were found in the third intron of the mutant *BmSpr* (Fig. 3*A*).

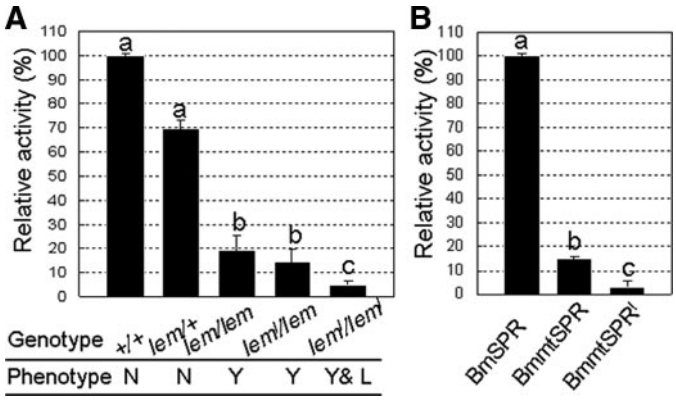

FIGURE 4. **Enzyme assays for BmSPR.** *A*, comparison of SPR activity among different genotypes. Total proteins were extracted from whole bodies of five individuals at the beginning of the second larval instar. Ten micrograms of proteins were used. The reaction was carried out as a standard enzyme assay at 37 °C for 30 min. Corresponding phenotypes are shown *under* each genotype. *N*, normal; *Y*, yellow; *L*, lethal. *B*, comparison of SPR activity among the three types of recombinant BmSPR proteins. Recombinant proteins (0.5 μg) were added to the reaction mixture. The reaction was carried out at 37 °C for 20 min. The *bars* indicate the mean ± S.D. ($n = 3$). Values indicated using different *letters* are significantly different ($p < 0.01$).

Enzyme Activity of BmSPR—We investigated the SPR activities in different genotypic larvae at the beginning of the second instar (Fig. 4*A*). When compared with the SPR activities in normal phenotypes, the activities in *lem* mutant silkworms were significantly low, and almost no activity was detected in the *lem*^{l} mutant silkworms. To investigate the enzymatic properties of BmSPR and compare the activities between normal and mutant proteins, we produced the three recombinant proteins using a bacterial expression system. Expression and purification of recombinant BmSPRs were analyzed by SDS-PAGE and immunoblot analysis (Fig. 5, *A* and *B*). Our analyses showed that BmSPR was a typical NADPH-dependent enzyme (Fig. 5*C*), which exhibited suitable enzymatic parameters to the substrate of SP with K_m of 28.3 μM and V_{max} of 14.5 nmol/min/μg (Table 3). Increase in activity was observed at pH 4–6 with pH 5 being an optimal condition (data not shown). The most suitable reaction temperature for BmSPR was 50 °C (data not shown). Moreover, two potent inhibitors of mammalian SPRs, melatonin and *N*-acetylserotonin, significantly inhibited BmSPR activity, with IC_{50} of 100 and 200 μM, respectively (data not shown). Comparison among the three proteins showed that the enzyme activity of BmmtSPR (*lem* type) and BmmtSPR^{l} (*lem*^{l} type) was 15 and 3%, respectively, as compared with that of the normal BmSPR (Fig. 4*B*). The values were consistent with SPR activities measured in the second instar larvae of the normal and *lem* and *lem*^{l} mutants (Fig. 4*A*). These data show that reduced SPR activity correlates with abnormal coloration and mortality in the mutant strains.

Linkage Analysis—To determine the consistency between the *lem* or *lem*^{l} phenotype and the *BmSpr* genotype, we performed linkage analysis between the normal and *lem* or *lem*^{l} using p50T, l70, and a65 strains, respectively. We backcrossed F1 male moths of the normal and *lem* with *lem* females. Single nucleotide polymorphism of a total of 190 individuals from (*lem/lem* × *lem/+*) was sequenced in *BmSpr* ORF at nt 786 (Table 2). Moreover, we analyzed the genotypes of 187 F2 individuals of the normal and *lem*^{l} by a PCR marker in *BmSpr* ORF (Table 2). The results showed that phenotypes of all of the individuals were identical with their genotypes (*i.e.* no recombination between *BmSpr* and *lem* or *lem*^{l} was detected among their progenies). Based on these results, we concluded that the candidate gene *BmSpr* corresponds to *lem* (*lem*^{l}).

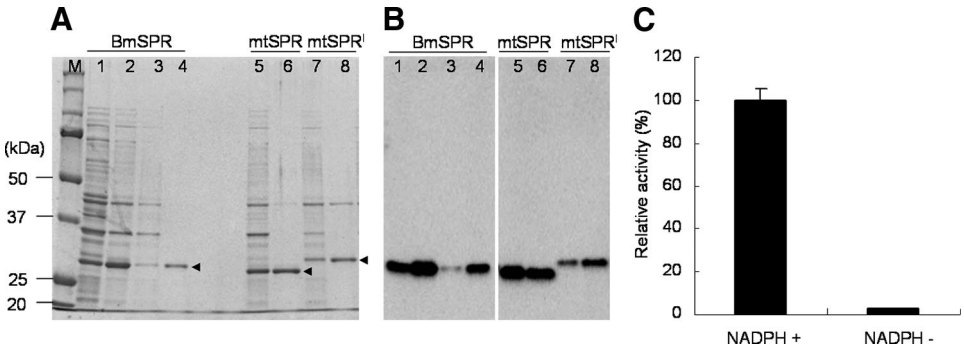

FIGURE 5. **Expression of recombinant BmSPR proteins and enzymatic property of normal BmSPR.** Three recombinant proteins, normal type BmSPR, and two mutant types, BmmtSPR (*lem*) and BmmtSPR^{l} (*lem*^{l}), were expressed using a bacterial expression system and purified using nickel chromatography columns. Expression and purification of recombinant BmSPRs were analyzed by SDS-PAGE (*A*) and immunoblot analysis (*B*). *Lane 1*, total cell proteins before isopropyl-1-thio-β-D-galactopyranoside induction; *lanes 2*, *5*, and *7*, total cell proteins induced by 1 mM isopropyl-1-thio-β-D-galactopyranoside; *lane 3*, proteins not binding to the nickel column; *lanes 4*, *6*, and *8*, eluted proteins using a buffer containing 20 mM sodium phosphate, 500 mM NaCl, and 200 mM imidazole (pH 7.4). *M*, protein standard. *C*, effect of cofactor NADPH on BmSPR activity. The reaction was carried out as a standard enzyme assay using 0.5 μg of enzyme with (+) or without (−) 100 μM NADPH. The reaction was carried out at 37 °C for 30 min. The data indicate the mean ± S.D. ($n = 3$).

Therapeutic Effects of BH4 and Monoamine Administration—The *lem*^{l} larvae, in which SPR activity was almost completely diminished, do not eat and die immediately after the first ecdysis. To verify whether SPR deficiency-induced lack of BH4 is responsible for the lethality of the *lem*^{l} mutant, we performed BH4 administration experiments by oral inoculation. We found that BH4 administration effectively improved the growth and development of the *lem*^{l} larvae in a dose-dependent manner (Fig. 6, *A* and *B*). Larvae fed with lower concentrations of BH4 ate fewer mulberry leaves and developed more slowly than those fed with 30 mM BH4, which showed body size similar to that of the wild type (Fig. 6*B*). Although a majority of the larvae fed with 30 mM BH4 died during the larval stage, about 8% individuals grew normally with a 7–11-day-longer larval period and successfully accomplished the morphological transition from larvae to pupae (Fig. 6*A*). Furthermore, we performed oral administration experiments with two monoamines, dopamine and serotonin. The results clearly showed that, similar to BH4, dopamine administration effectively increased the survival rates of *lem*^{l} larvae, because this treatment drastically improved their feeding abilities (Fig. 6*C*). In contrast, serotonin administration did not show any positive effects on survival rates and feeding behavior of the *lem*^{l} larvae (Fig. 6*C*). Taken together, these results suggest that BH4 deficiency results in the lethal phenotype observed in the *lem*^{l} mutant, which is mainly due to the lack of dopamine.

TABLE 3
Comparison of enzymatic properties of SPRs from *B. mori* and other organisms

Protein/organism	Enzyme property			Source/reference
	K_m	V_{max}	Optimal pH	
	μM	nmol/min/μg		
BmSPR	28.3	14.5	5	Recombinant protein in this study
Bombyx	10.2	21.4	5.2	Iino *et al.* (10)
Drosophila	153			Ruiz-Vazquez *et al.* (31)
Rat	15.4	21.7	5.5	Sueoka and Katoh (32)
Horse	21		5.5	Katoh (21)

DISCUSSION

In this paper, we report the identification and characterization of *BmSpr* and conclude that *BmSpr* corresponds to the yellow body color mutant *lem* (*lem*^{l}) of the silkworm *B. mori*. Using genetic and biochemical approaches, we demonstrated that the mutations in *BmSpr* significantly reduced SPR activity

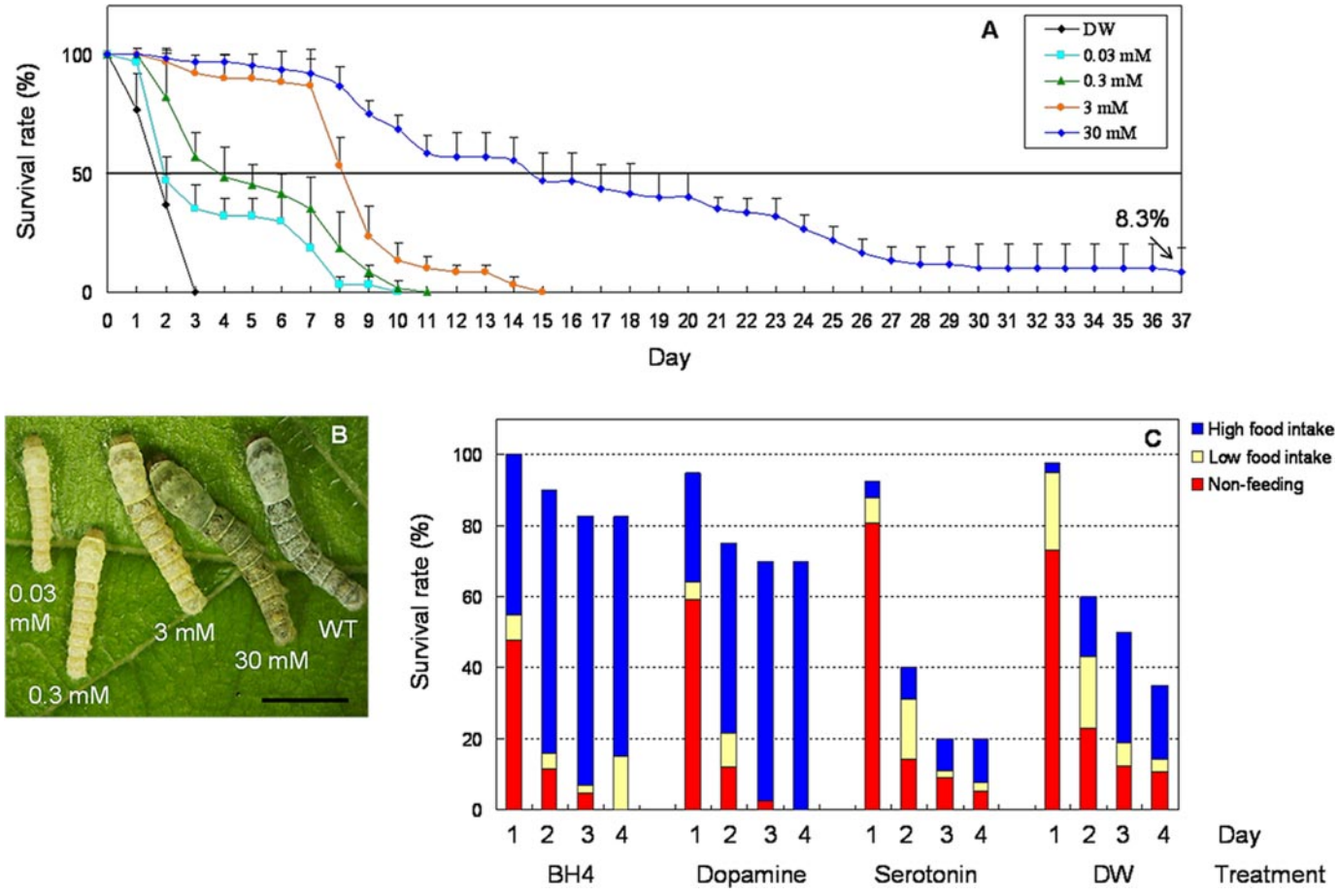

FIGURE 6. **Rescue of the *lem*^{l} lethal larvae by BH4 and monoamine administration.** *A*, survival rate curves by different doses of BH4. Eggs of a65 strain were randomly divided into five parts. Mulberry leaves wetted by various concentrations of BH4 were fed daily to newly hatched larvae. From the beginning of the second instar (day 0), 40 *lem*^{l}/*lem*^{l} larvae were grouped and used in each treatment. Survival rate curves were plotted daily throughout the larval stage. Distilled water (*DW*) was used as a negative control. The points indicate the mean ± S.D. ($n = 3$). *B*, the phenotypes of the *lem*^{l} larvae in each BH4 treatment on day 2 of the third instar. *WT*, wild type. *Scale bar*, 1 cm. *C*, survival rates and feeding abilities of *lem*^{l} larvae with oral administration of dopamine and serotonin. Final concentration of monoamines was 50 mM. Forty *lem*^{l}/*lem*^{l} larvae at the beginning of third instar survived by 30 mM BH4 from first instar were used in each group. Survival rate curves were recorded from day 1 to day 4. Feeding abilities of the living larvae were examined by counting their feces: more than 10 feces, high food intake; 1–10 feces, low food intake; no feces, nonfeeding. BH4 (30 mM) and distilled water were used as control reagents.

both in the *lem* (*lem^l*) larvae and in mutant BmSPR proteins. Moreover, oral administration of BH4 and dopamine successfully increased the survival rates of the *lem^l* larvae, suggesting that BH4 deficiency induced by loss of BmSPR activity leads to the lethality of *lem^l* larvae, probably due to the lack of dopamine. Therefore, we propose that the *lem* (*lem^l*) mutant can be regarded as a useful insect model for human SPR-deficient diseases.

Various pteridine derivates synthesized from GTP are the primary components in insect body coloration (Fig. 1*A*). The larval body color in *B. mori* is determined by the concentrations of melanin in the cuticle and of xanthommatin, sepialumazine, sepiapterin, and uric acid in the epidermis (23–25). Synthesis of pteridine from GTP is possible when a number of enzymes, including SPR (EC 1.1.1.153) and sepiapterin deaminase (EC 3.5.4.24) (Fig. 1*A*), cooperate. Our data indicated that the 5-aa deletion at the end of BmSPR in *lem* and the 9-aa insertion in the middle of BmSPR in *lem^l* caused a marked decrease in SPR activity, although important motifs for SPR activity are highly conserved in *lem* and *lem^l* (Figs. 3*B* and 4) (26, 27). These data proved that mutations in *BmSpr* are responsible for the abnormal accumulation of yellow pteridines in the integuments of *lem* (*lem^l*) (13, 14).

Park *et al.* (28) proposed that in the case of complete SPR defect, BH4 biosynthesis from PTP could be compensated by carbonyl and/or aldose reductases in humans. However, Bonafe *et al.* (1) showed that the compensation might be true for some peripheral tissues but not for the brain. Similarly, in the silkworm, Iino *et al.* (29, 30) discovered two carbonyl enzymes in the fat body and integument, which could reduce PTP to form BH4. However, the BH4 forming activity in the *lem* mutant was 10-fold lower than in the normal strain (29). These studies indicate that the salvage pathways cannot provide sufficient BH4, unlike the BH4 biosynthesis pathway catalyzed by SPR. Biochemical analysis showed that BmSPR exhibited enzymatic properties more similar to those of mammalian SPRs than to *Drosophila* SPR (Table 3) (21, 31, 32), which was consistent with a previous report (10). Collectively, the biosynthetic pathway of BH4 and the enzymatic properties of SPR are similar between *Bombyx* and mammals, suggesting that the silkworm is a suitable animal for studying human SPR/BH4 deficiency.

In mammals, BH4 exhibits various physiological functions, such as acting as a cofactor for aromatic hydroxylases and nitric-oxide synthase. Therefore, appropriate levels of BH4 are necessary for the metabolism of phenylalanine and the production of monoamine neurotransmitters (Fig. 1*B*) (8). In general, patients with BH4 deficiency present progressive neuronal deterioration, convulsions, abnormal movements, and difficulty in swallowing (2). Abnormal symptoms observed in the *lem^l* larvae, such as head shaking and feeding inability after the first ecdysis, are strikingly similar. Oral inoculation of BH4 effectively improved the feeding ability of the *lem^l* larvae and enabled them to grow normally through the larval developmental stage (Fig. 6, *A* and *B*), suggesting that loss of BmSPR activity reduced BH4 to a lethal level in the *lem^l* larvae. Further, we observed that oral inoculation of dopamine also effectively improved the survival rate and feeding ability of *lem^l* larvae (Fig. 6*C*). These results demonstrate that SPR deficiency-induced lack of dopamine results in the abnormal behavior observed in the *lem^l* larvae.

SPR deficiency in patients was frequently misdiagnosed as dihydrobiopterin reductase deficiency (3). Although diagnosis and therapy of SPR/BH4 deficiencies have advanced in recent years (1, 3–6), it is necessary to develop appropriate animal models to obtain better understanding the complexity of these diseases. Several invertebrates, such as *Caenorhabditis elegans* and *Drosophila*, have been used as animal models for human diseases (33–35). Together with these invertebrate animal models, the silkworm larvae have a number of advantages as an animal model; they are genetically tractable, easily maintained in laboratories throughout the year using artificial diets, and can be reared on a large scale at low cost. Moreover, the advantage of the large body size of silkworm larvae, which makes handling easier when injecting drugs and microorganisms but can be a practical problem in small animals, has made *B. mori* a useful model for infection with human pathogenic microorganisms (36–38). Based on the present results, we propose the *lem* (*lem^l*) mutant as the first insect model for human SPR deficiency. Recent studies have shown that *Spr*-null mice display greatly decreased amounts of BH4, severe monoamine deficiencies, and growth retardation. Also, a majority of mice died within 1–2 months (6, 8). Similar to the rescue effects of BH4 and dopamine feeding on the *lem^l* larvae, oral administration of BH4 and neurotransmitters completely rescued dwarfism and phenylalanine metabolism (6, 8). Together with the present results, we hope that the silkworm *lem* (*lem^l*) mutant can provide useful information for clinical diagnosis, for therapy options, and for screening therapeutic agents or new drug candidates for human SPR deficiency.

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REFERENCES

1. Bonafe, L., Thony, B., Penzien, J. M., Czarnecki, B., and Blau, N. (2001) *Am. J. Hum. Genet.* **69,** 269–277
2. Blau, N., Barnes, I., and Dhondt, J. N. (1996) *J. Inherit. Metab. Dis.* **19,** 8–14
3. Blau, N., Bonafe, L., and Thony, B. (2001) *Mol. Genet. Metab.* **74,** 172–185
4. Abeling, N. G., Duran, M., Bakker, H. D., Stroomer, L., Thöny, B., Blau, N., Booij, J., and Poll-The, B. T. (2006) *Mol. Genet. Metab.* **89,** 116–120
5. Friedman, J., Hyland, K., Blau, N., and MacCollin, M. (2006) *Neurology* **67,** 2032–2035
6. Takazawa, C., Fujimoto, K., Homma, D., Sumi-Ichinose, C., Nomura, T., Ichinose, H., and Katoh, S. (2008) *Biochem. Biophys. Res. Commun.* **367,** 787–792
7. Wszolek, Z. K., Cordes, M., Calne, D. B., Muenter, M. D., Cordes, I., and Pfeifer, R. F. (1993) *Nervenarzt* **64,** 331–335
8. Yang, S., Lee, Y. J., Kim, J.-M., Park, S., Peris, J., Laipis, P., Park, Y. S., Chung, J. H., and Oh, S. P. (2006) *Am. J. Hum. Genet.* **78,** 575–587
9. Matsubara, M., Tsusué, M., and Akino, M. (1963) *Nature* **199,** 908–909
10. Iino, T., Dohke, K., and Tsusué, M. (1992) *Zool. Sci.* **9,** 119–125
11. Banno, Y., Fujii, H., Kawaguchi, Y., and Yamamoto, K. (2005) *A Guide to the Silkworm Mutants 2005: Gene Name and Gene*, Institute of Genetic Resources, Kyushu University, Fukuoka, Japan
12. Tsujita, M. (1955) *Jpn. J. Genet.* **30,** 107–117
13. Tsusué, M., and Akino, M. (1965) *Zool. Mag.* **74,** 91–94
14. Goto, M., Konishi, M., Sugiura, K., and Tsusué, M. (1966) *Bull. Chem. Soc.*

Japan **39,** 929–932
15. Meng, Y., Omuro, N., Funaguma, S., Daimon, T., Kawaoka, S., Katsuma, S., and Shimada, T. (2008) *Arch. Insect Biochem. Physiol.* **67,** 9–19
16. Mita, K., Morimyo, M., Okano, K., Koike, Y., Nohata, J., Kawasaki, H., Kadono-Okuda, K., Yamamoto, K., Suzuki, M. G., Shimada, T., Goldsmith, M. R., and Maeda, S. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 14121–14126
17. Mita, K., Kasahara, M., Sasaki, S., Nagayasu, Y., Yamada, T., Kanamori, H., Namiki, N., Kitagawa, M., Yamashita, H., Yasukochi, Y., Kadono-Okuda, K., Yamamoto, K., Ajimura, M., Ravikumar, G., Shimomura, M., Nagamura, Y., Shin, I. T., Abe, H., Shimada, T., Morishita, S., and Sasaki, T. (2004) *DNA Res.* **11,** 27–35
18. Xia, Q., Zhou, Z., Lu, C., Cheng, D., Dai, F., Li, B., Zhao, P., Zha, X., Cheng, T., Chai, C., Pan, G., Xu, J., Liu, C., Lin, Y., Qian, J., Hou, Y., Wu, Z., Li, G., Pan, M., Li, C., Shen, Y., Lan, X., Yuan, L., Li, T., Xu, H., Yang, G., Wan, Y., Zhu, Y., Yu, M., Shen, W., Wu, D., Xiang, Z., Yu, J., Wang, J., Li, R., Shi, J., Li, H., Li, G., Su, J., Wang, X., Li, G., Zhang, Z., Wu, Q., Li, J., Zhang, Q., Wei, N., Xu, J., Sun, H., Dong, L., Liu, D., Zhao, S., Zhao, X., Meng, Q., Lan, F., Huang, X., Li, Y., Fang, L., Changfeng Li, D., Sun, Y., Zhang, Z., Yang, Z., Huang, Y., Xi, Y., Qi, Q., He, D., Huang, H., Zhang, X., Wang, Z., Li, W., Cao, Y., Yu, Y., Yu, H., Li, J., Ye, J., Chen, H., Zhou, Y., Liu, B., Wang, J., Ye, J., Ji, H., Li, S., Ni, P., Zhang, J., Zhang, Y., Zheng, H., Mao, B., Wang, W., Ye, C., Li, S., Wang, J., Wong, G. K.-S., and Yang, H. (2004) *Science* **306,** 1937–1940
19. Daimon, T., Taguchi, T., Meng, Y., Katsuma, S., Mita, K., and Shimada, T. (2008) *J. Biol. Chem.* **283,** 15271–15279
20. Laemmli, U. K. (1970) *Nature* **227,** 680–685
21. Katoh, S. (1971) *Arch Biochem. Biophys.* **146,** 202–214
22. Lineweaver, H., and Burk, D. (1934) *J. Am. Chem. Soc.* **56,** 658–666
23. Mazda, T., Tsusué, M., and Sakate, S. (1980) *Insect Biochem.* **10,** 357–362
24. Ohashi, M., Tsusué, M., Yoshitake, N., Sakate, S., and Kiguchi, K. (1983) *J. Seric. Sci. Jpn.* **52,** 498–504
25. Kato, T., Sawada, H., Yamamoto, T., Mase, K., and Nakagoshi, M. (2006) *Pigment Cell Res.* **19,** 337–345
26. Auerbach, G., Herrmann, A., Gütlich, M., Fischer, M., Jacob, U., Bacher, A., and Huber, R. (1997) *EMBO J.* **16,** 7219–7230
27. Fujimoto, K., Hara, M., Yamada, H., Sakurai, M., Inaba, A., Tomonura, A., and Katoh, S. (2001) *Chem. Biol. Interact.* **130**, 825–832
28. Park, Y. S., Heizmann, C. W., Wermuth, B., Levine, R. A., Steinerstauch, J., Guzman, J., and Blau, N. (1991) *Biochem. Biophys. Res. Commun.* **175,** 738–744
29. Iino, T., Sawada, H., Tsusué, M., and Takikawa, S. (1996) *Biochim. Biophys. Acta* **1297,** 191–199
30. Iino, T., Takikawa, S. I., Yamamoto, T., and Sawada, H. (2000) *Arch. Biochem. Biophys.* **373,** 442–446
31. Ruiz-Vazquez, P., Silva, F. J., and Ferre, J. (1996) *Comp. Biochem. Physiol. B* **113,** 131–136
32. Sueoka, T., and Katoh, S. (1982) *Biochim. Biophys. Acta* **717,** 265–271
33. Mahajan-Miklos, S., Tan, M. W., Rahme, L. G., and Ausubel, F. M. (1999) *Cell* **96,** 47–56
34. Wu, Y., Bolduc, F. V., Bell, K., Tully, T., Fang, Y., Sehgal, A., and Fischer, J. A. (2008) *Proc. Natl. Acad. Sci. U. S. A.* **105,** 12399–12404
35. Liu, Z., Wang, X., Yu, Y., Li, X., Wang, T., Jiang, H., Ren, Q., Jiao, Y., Sawa, A., Moran, T., Ross, C. A., Montell, C., and Smith, W. W. (2008) *Proc. Natl. Acad. Sci. U. S. A.* **105,** 2693–2698
36. Kaito, C., Akimitsu, N., Watanabe, H., and Sekimizu, K. (2002) *Microb. Pathog.* **32,** 183–190
37. Hamamoto, H., *et al.* (2004) *Antimicrob. Agents Chemother.* **48,** 774–779
38. Orihara, Y., Hamamoto, H., Kasuga, H., Shimada, T., Kawaguchi, Y., and Sekimizu, K. (2008) *J. Gen. Virol.* **89,** 188–194