Cloning of Bombyx mori phenylalanyl-tRNA synthetase and the generation of its mutant with relaxed amino acid specificity

Hidetoshi Teramoto* and Katsura Kojima

Silk-Materials Research Unit, Division of Insect Sciences, National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305-8634, Japan

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The genes encoding two subunits (α and β) of phenylalanyl-tRNA synthetase (PheRS), which catalyzes aminoaacylation of tRNA<sup>phe</sup> with L-phenylalanine (Phe), were cloned from the silk glands of the domesticated silkworm, Bombyx mori, and their full-length cDNA sequences were identified. The deduced amino acid sequences of the B. mori PheRS (BmPheRS) showed a high similarity with its counterparts from other organisms. A comparison with the previous mutation studies suggested that the Ala residue at the amino acid position 450 in the α-subunit of BmPheRS would be one of the key determinants for discriminating Phe from its amino acid competitors. To relax the amino acid specificity of BmPheRS, Ala to Gly mutation was introduced at the residue 450 of its α-subunit to generate the αA450G BmPheRS mutant. In vitro activity assay demonstrated that the recombinant αA450G BmPheRS mutant catalyzed the aminoaacylation of the synthesized B. mori tRNA<sup>phe</sup> with p-chloro- and p-bromo-substituted Phe analogs which were not recognized by the wild-type BmPheRS. This finding opens up the possibility of developing a novel type of genetically-modified silkworm which can utilize unnatural amino acids as protein building blocks.

**Keywords**: Bombyx mori, phenylalanyl-tRNA synthetase, aminoaacylation, amino acid specificity, unnatural amino acids

**INTRODUCTION**

The domesticated silkworm, Bombyx mori, synthesizes a large amount of silk proteins in its silk glands during fifth instar and subsequently processes them into fiber to construct a robust cocoon. This characteristic has made B. mori one of the important animals in industrial applications. Recent success in the genetic modification of B. mori (Tamura et al., 2000) has drastically increased its importance as a host of protein production: it became possible to obtain silk fiber with a modified primary structure for specific applications (Kojima et al., 2007; Yanagisawa et al., 2007) and to produce useful proteins such as for pharmaceutical applications (Tomita et al., 2003; Royer et al., 2005). The uniqueness of B. mori as a host animal is characterized by its ability to produce large amounts of recombinant proteins including highly-repetitive fibrous ones and by its proximity to humans in the manner of post-translational modifications.

In the field of protein engineering, unnatural amino acid mutagenesis has become a powerful methodology to obtain engineered proteins in the past two decades (Budisa, 2006). Various amino acids bearing unnatural functional groups have been incorporated into proteins in vivo in place of or in addition to 20 natural amino acids. This methodology is now widely employed especially in the Escherichia coli expression system such as to label proteins in specified cells (Ngo et al., 2009), to probe the protein structure and function (Fleissner et al., 2009), and to generate proteins with new properties (Yoo et al., 2007; Liu et al., 2008). Moreover, recent progress has made it possible to incorporate unnatural amino acids into proteins produced by eukaryotic organisms such as yeast (Chin et al., 2003) and mammalian cells (Liu et al., 2007). In this context, the expansion of this methodology into B. mori would provide a quite versatile tool to produce various kinds of proteins with novel characteristics in large scale.

In vivo incorporation of the unnatural amino acids into proteins has been achieved through several different strategies (Budisa, 2006). One of them employs the relaxation of substrate (amino acid) specificity of aminoaacyl-tRNA synthetase (aaRS) (Link and Tirrell, 2005). The aaRSs catalyze the charging of specific tRNAs with their cognate amino acids (aminoaacylation of tRNA) under strict substrate specificity, thereby guaranteeing the accuracy of the genetic code. In this strategy, the incorporation of unnatural amino acids is achieved by enlarging the size of the amino acid binding pockets of aaRSs by appropriate point mutations. Such aaRS mutants show relaxed amino acid specificity toward structurally-similar amino acid analogs. Several mutants of methionyl-tRNA synthetase (MetRS) and phenylalanyl-tRNA synthetase (PheRS) have previously been generated and a wide variety of the analogs of L-methionine (Met) (Link et al., 2006), L-phenylalanine (Phe) (Kirshenbaum et al., 2002), and L-tryptophan (Trp) (Kwon and Tirrell, 2007) have been successfully incorporated into proteins in vivo in place of natural amino acids. The key process of this strategy is to discover the appro-
appropriate point mutation to relax the amino acid specificity of aaRSs toward the desired amino acid analogs.

In our attempts to apply the above strategy to *B. mori*, Phe was chosen as a target amino acid to be replaced with its unnatural analogs because of the following three reasons: (1) Phe is an essential amino acid of *B. mori* (Arai and Ito, 1965), which makes it possible to control its uptake by feeding, (2) a wide range of knowledge around the structure-function relationships of PheRS has been accumulated (Ibba et al., 1994; Kotik-Kogan et al., 2005; Ling et al., 2007), and (3) a variety of Phe analogs are commercially available. PheRS consists of two subunits, α and β, and functions as (αβ)4 heterotetramer, which is the widely-conserved organization of cytoplasmic PheRSs. The catalytic core domain for the aminoacylation of tRNA_Phe including the amino acid binding pocket is located within the PheRS α-subunit while the β-subunit provides additional functions such as the tRNA anticodon binding and editing of mis-aminoacylated species (Roy et al., 2004; Roy and Ibba, 2006). In this study, as a first step to realize the in vivo incorporation of the Phe analogs into proteins synthesized by *B. mori*, the genes encoding the PheRS α- and β-subunits were cloned from the silk glands of *B. mori* and a mutant of the *B. mori* PheRS (BmPheRS) with relaxed amino acid specificity was generated by point mutation at its predicted amino acid binding pocket within the α-subunit. The generated mutant was found to catalyze the aminoacylation of the synthesized *B. mori* tRNA_Phe with p-chloro- and p-bromo-substituted Phe analogs in vitro.

**MATERIALS AND METHODS**

**Materials**

All chemicals used in this study are reagent grade and were used as received. All synthetic oligonucleotides were from Hokkaido System Science (Sapporo). All PCR experiments were performed using KOD plus polymerase (TOYOBO; Osaka). However, ExTaQ polymerase (Takara Bio; Otsu) was used to amplify the DNA probes for Southern blotting. All restriction endonucleases were from Takara Bio except *BsaI*, which was from New England Biolabs (Ipswich, MA, USA). Mg-ATP was from Biochemicals (Cambridge, MA, USA). L-Phenylalanine was from Nacalai Tesque (Kyoto). *p*-Fluoro-, *p*-chloro-, *p*-bromo-, and *p*-iodo-L-phenylalanine were from Bachem (Bubendorf, Switzerland).

**cDNA cloning of the BmPheRS α- and β-subunit genes**

Silk glands of the fifth-instar larvae of *B. mori* (Daizo) were collected and washed in a ringer solution (148 mM NaCl, 1.3 mM KCl, 3.0 mM CaCl2, 1.7 mM NaHPO4, and 1.7 mM KH2PO4). The total RNA was isolated from the silk glands using ISOGEN (Nippon Gene; Tokyo) according to the manufacturer’s instruction. Two micrograms of the isolated total RNA was reverse-transcribed into single-stranded cDNAs with an oligo(dt) primer using an Omniscript RT Kit (Qiagen; Hilden, Germany) according to the manufacturer’s instruction. The cDNA encoding the BmPheRS α-subunit was amplified by the nested PCR method with the following oligonucleotide primer sets: 5’-GGTGAGGCTGGAGCTTGGTTT-3’ and 5’-CAACATACAGAAGATTAAAA-3’ (1st PCR), and 5’-GGGCGGAAGACTAAAATAA-3’ and 5’-CAACAGTTTATTATTATATTGAG-3’ (2nd PCR). The cycling parameter was: 94°C for 2 min followed by 5 cycles of 98/55/68°C for the 1st PCR, and 94°C for 2 min followed by 30 cycles of 98/60/68°C for the 2nd PCR. The amplified DNA fragment was purified by extracting it from agarose gel after an electrophoretic separation using the S.N.A.P. Gel Purification Kit (Invitrogen; Carlsbad, CA, USA), ligated into pCR4Blunt-TOPO (Invitrogen), and sequenced. The obtained plasmid was designated as pCR4-BmPheRS_α. The cDNA encoding the BmPheRS β-subunit was similarly amplified with the following primer sets: 5’-CCTGTTTTTAAATTATATTAT-3’ and 5’-GGTGACATCCATTGCTAAAT-3’ (1st PCR), and 5’-CAACAGTTTATTATTATATTAG-3’ and 5’-CATTTACAGAATGTTTCA-3’ (2nd PCR). The cycling parameter was: 94°C for 2 min followed by 25 cycles of 98/58/68°C for the 1st PCR, and 94°C for 2 min followed by 26 cycles of 98/50/68°C for the 2nd PCR. The amplified DNA fragment was purified by extracting it from agarose gel after electrophoretic separation using the S.N.A.P. Gel Purification Kit, ligated into pCR4Blunt-TOPO, and sequenced. The obtained plasmid was designated as pCR4-BmPheRS_β.

The 5’- and 3’-rapid amplification of cDNA ends (RACE) was performed using a GeneRacer Kit (Invitrogen) according to the manufacturer’s instruction. The 5’ and 3’ cDNA ends of the BmPheRS α-subunit were amplified by a nested PCR method with the primers provided by the kit and the following gene-specific primers: 5’-GG TACACACGTGTCTGTATACCTT-3’ and 5’-GCTTTG GGGGTACTGAGTTCAGGACACGATAC-3’ and 5’-CATTTACAGAATGTTTCA-3’ (1st and 2nd PCR in 5’-RACE, respectively), and 5’-GGGCGGAAGACTAAAATAA-3’ and 5’-CAACAGTTTATTATTATATTGAG-3’ (1st and 2nd PCR in 3’-RACE, respectively). The cycling parameter for 5’-RACE was: 94°C for 2 min followed by 5 cycles of 98/72°C for 10/120 sec, 5 cycles of 98/70°C for 10/120 sec, 20 cycles of 98/65/68°C for 10/30/120 sec, and 1 cycle of 68°C for 10 min for the 1st PCR, and 94°C for 2 min followed by 25 cycles of 98/68°C for 10/120 sec.
for 10/60 sec for the 2nd PCR. The cycling parameter for 3’-RACE was: 94°C for 2 min followed by 5 cycles of 98/72°C for 10/120 sec, 5 cycles of 98/70°C for 10/120 sec, 20 cycles of 98/65/68°C for 10/30/120 sec, and 1 cycle of 68°C for 10 min for the 1st PCR, and 94°C for 2 min followed by 20 cycles of 98/65/68°C for 10/30/120 sec for the 2nd PCR. The amplified DNA fragments were purified by extracting them from agarose gel after electrophoretic separation using S.N.A.P. Gel Purification Kit or by ethanol precipitation, ligated into pCR4Blunt-TOPO or pCR-BluntII-TOPO (Invitrogen), and sequenced. The 5’ and 3’ cDNA ends of the BmPheRS β-subunit were similarly amplified with the following gene-specific primers: 5’-CGTTAGGTGTAAAAGTAGTTACGTTCCTTTCC-3’ and 5’-CAGCATCTGCTTTGTCACCTTGGTCACCTTGTTC-3’ (1st and 2nd PCR in 5’-RACE, respectively), and 5’-GCTGCTGAAAACCATGGCTCCTGGAACACTAATAGC-3’ and 5’-CGAGATCAGCGATGTTGTCATACTGCTTGGTCACCTTGTTC-3’ (1st and 2nd PCR in 3’-RACE, respectively). The cycling parameter for 5’- and 3’-RACE was: 94°C for 2 min followed by 5 cycles of 98/72°C for 10/120 sec, 5 cycles of 98/70°C for 10/120 sec, 20 cycles of 98/65/68°C for 10/30/120 sec, and 1 cycle of 68°C for 10 min for the 1st PCR, and 94°C for 2 min followed by 25 cycles of 98/65/68°C for 10/30/60 sec for the 2nd PCR. The amplified DNA fragments were purified using a Wizard SV Gel and PCR Clean-Up System (Promega; Madison, WI, USA), ligated into pCR-BluntII-TOPO, and sequenced.

The full-length cDNA sequences for the BmPheRS α- and β-subunits have been deposited in DDBJ under the accession numbers (Acc. No.) AB546861 and AB546862, respectively.

**Southern blotting**

Genomic DNA of *B. mori* (Daizo) was obtained from the silk glands of the fifth-instar larvae. Ten micrograms each of the genomic DNA were digested with restriction enzymes, *Bam*HI, *Hind*III, *Kpn*I, *Pst*I, *Sac*I, and *Xba*I. Five micrograms of each digested DNA were separated on a 1.0 % agarose gel. After electrophoresis, the fragments were blotted onto a Hybond-N+ nylon membrane (GE Healthcare; Little Chalfont, UK). Hybridization was performed using the AlkPhos Direct Labelling and Detection System (GE Healthcare). The signals from the hybridized probes were detected using a LAS-3000 image analyzer (Fujifilm; Tokyo). The probes were prepared as follows: the DNA fragments in the 5’ regions, 271 bp for the BmPheRS α-subunit gene (227-497 bases in Acc. No. AB546861) and 523 bp for the BmPheRS β-subunit gene (950543-950021 bases in Acc. No. DF090326) (Fig. 2), were respectively amplified from pCR4-BmPheRS_α and *B. mori* genomic DNA with the following primer sets: 5’-CGGGCGGAAGAATCCTAAAAATG-3’ and 5’-GGTACAACCACGTGTCTGGATACTTC-3’ for the α-subunit gene and 5’-ATGCCCAATTTTCTTTAAAG-3’ and 5’-TTCCCTTGGAACACTAATAG-3’ for the β-subunit gene. The amplified DNA fragments were purified by extracting them from agarose gel after electrophoretic separation using QIAquick Gel Extraction Kit (Qiagen), ligated into pCR-BluntII-TOPO, and sequenced. The DNA fragments of 515 and 767 bp were amplified from the obtained plasmids with the universal primers, M13 Forward (-20) and M13 Reverse, purified with QIAquick PCR Purification Kit (Qiagen) followed by ethanol precipitation, and used as probes for the BmPheRS α- and β-subunit genes, respectively.

**Plasmid construction for BmPheRS expression**

An expression plasmid encoding the BmPheRS α- and β-subunits in one operon was constructed as follows. The ORF of the BmPheRS α-subunit was amplified from pCR4-BmPheRS_α with the following primer set bearing *Bam*HI and *Sac*I sites (designated by underlines): 5’-GGA TCCATGGAAATTCGAAGAAG-3’ and 5’-GAGCTCTT CATTATCGAGCCTTG-3’. The amplified DNA fragment was purified by extracting it from agarose gel after electrophoretic separation using the Wizard SV Gel and PCR Clean-Up System, ligated into pCR-BluntII-TOPO, and sequenced. The obtained plasmid was designated as pCR-BmPheRS_α-BS. In a similar way, the ORF of the BmPheRS β-subunit was amplified from pCR4-BmPheRS_β with the following primer set bearing *Sac*I and *Pst*I sites (designated by underlines) with a 14-nt intergenic sequence (described in bold) from the *E. coli* pheST operon (Mechulam et al., 1985) inserted to express the BmPheRS α- and β-subunits as an operon as reported previously (Sanni et al., 1990): 5’-GAGCTCGGCCAG GAAATAGATTATGCCACAA-3’ and 5’-CCTCTAG CATTTACGAGCCTTG-3’. The amplified DNA fragment was purified using the QIAquick PCR Purification Kit by extracting it from agarose gel after electrophoretic separation, ligated into pCR-BluntII-TOPO, and sequenced. The obtained plasmid was designated as pCR-BmPheRS_α-BS. The plasmids, pCR-BmPheRS_α-BS and pCR-BmPheRS_β-SP, were digested with *Bam*HI and *Sac*I, and *Sac*I and *Pst*I, respectively. The digested DNA fragments were simultaneously ligated into the *Bam*HI and *Pst*I-digested pQE-30 (Qiagen) to obtain the expression plasmid, pQE30-BmPheRS_α_β. The other expression plasmid, pQE30-BmPheRS_α, which bears only the BmPheRS α-subunit gene, was constructed by inserting the *Bam*HI and *Sac*I-digested fragment of pCR-BmPheRS_α-BS into the *Bam*HI and *Sac*I-digested pQE-30.

The introduction of Ala to Gly mutation at the amino acid position 450 of the BmPheRS α-subunit was performed by converting a partial DNA fragment (from the
Fig. 1. The full-length cDNA and deduced amino acid sequences of the BmPheRS α- (A) and β-subunits (B). The numbers at the left and right of the sequences represent DNA and amino acid positions, respectively. The alternative 5' cDNA ends detected by 5'-RACE analysis are shown by dots above the sequences. The extra ATG codons and up-stream ORFs at 5'UTRs are designated in bold and by underlines, respectively. Possible polyadenylation signals are marked by boxes.
Expression and purification of BmPheRS

The expression and purification of the recombinant BmPheRS were performed based on the protocol reported previously (Kwon et al., 2006). The expression plasmids, pQE30-BmPheRS_α, pQE30-BmPheRS_αA450G_β, and pQE30-BmPheRS_αA450G_β, encoding the A450G α-subunit mutant and the wild-type β-subunit, was constructed as above using pCR-BmPheRS_α-BS-A450G instead of pCR-BmPheRS_α-BS. The constructed three expression plasmids above, pQE30-BmPheRS_α_β, pQE30-BmPheRS_α, and pQE30-BmPheRS_αA450G_β, attach a hexa-histidine tag on the N-terminus of the BmPheRS α-subunit (wild-type or A450G mutant) to facilitate purification.

Preparation of the In vitro transcribed B. mori tRNA\(^ {\text{Pre}}\)

The plasmid, pUC19-BmtRNA\(^ {\text{Pre}}\), which encodes the B. mori tRNA\(^ {\text{Pre}}\) (76-mer; Acc. No. K00347) (Keith and Dirheimer, 1980), was constructed as follows. First, the B. mori tRNA\(^ {\text{Pre}}\) gene was amplified by PCR from three partially complementary synthetic oligonucleotides, 5'-GGGGGATATGTAATACGACTCACTATAAGCCGGAATTGCTCAGGTTG-3' (contains a Ndel site and a T7 promoter designated by an underline and in bold, respectively), 5'-CCAGGGACCTTTAGATCTTCTACATAGCTCCTCAAC-3', and 5'-GGGACATTCTTTGAGTTCTCTGCTGCT-3' (contains EcoRI and BsaI sites designated by an underline and in bold, respectively). Then, the amplified DNA fragment was digested with Ndel and EcoRI and inserted into the Ndel and EcoRI-digested pUC19 (Invitrogen). pUC19-BmtRNA\(^ {\text{Pre}}\) was linearized by BsaI digestion, which was used as a template to synthesize the B. mori...
tRNA<sub>Phe</sub> by the <i>in vitro</i> run-off transcription with MEGAscript T7 Kit (Ambion; Austin, TX, USA) according to the manufacturer’s instruction. The 76-mer transcript was purified according to the protocol described previously (Kwon <i>et al.</i>, 2006) as follows: The transcript was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and the organic layer was re-extracted with water. The combined aqueous layer was extracted with chloroform/isoamyl alcohol (24:1). The aqueous layer was then mixed with an equal volume of 2-propanol, stored overnight at −20°C, centrifuged at 10,000 × g for 10 min, washed with 70% ethanol, dried, and redissolved in nuclelease-free water. Unreacted nucleotides were eliminated using CHROMA SPIN-30 DEPC-H<sub>2</sub>O Columns (Clontech Laboratories; Mountain View, CA, USA). Concentration of the transcript was determined by a UV absorbance at 260 nm. The obtained <i>in vitro</i>-transcribed <i>B. mori</i> tRNA<sub>Phe</sub> was annealed just before use by heating at 85°C for 4 min with an annealing buffer (60 mM Tris-HCl (pH 7.5) and 2 mM MgCl<sub>2</sub>) followed by a slow cooling to 25°C at 0.1°C/sec.

**Aminoacylation assay**

Aminoacylation reactions were performed in 10 μL volume in the standard reaction mix (0.1 M Na-HEPES (pH 7.5), 30 mM KCl, 10 mM MgCl<sub>2</sub>, 2 mM Mg-ATP, and 2.5 μM <i>in vitro</i>-transcribed <i>B. mori</i> tRNA<sub>Phe</sub>) with 25 μM Phe or one of Phe analogs and 0.01 A<sub>280</sub> units of the Ni-NTA-purified recombinant BmPheRS (wild-type or αA450G mutant) for 10 or 30 min at the specified temperatures (10, 20, 30, 40, or 50°C) and pHs (5.5-9.0). Here, one A<sub>280</sub> unit is defined as the protein amount that gives an A<sub>280</sub> value of 1 when dissolved in 80 μL of 6 M guanidine hydrochloride and is measured in a 1-cm path length. For pH control, the buffer in the above standard reaction mix was replaced with the following buffers: 0.1 M Na-MES (pH 5.5, 6.0, and 6.5), 0.1 M Na-HEPES (pH 7.0 and 7.5), or 0.1 M Tris-HCl (pH 8.0, 8.5, and 9.0). As control experiments, the aminoacylation activities of the Ni-NTA-purified proteins from pQE30-BmPheRS α and the blank vector (0.01 and 0.005 A<sub>280</sub> units, respectively) were assayed at 30°C by a 10 min reaction at pH 7.5.

Aminoacylation reactions were stopped by adding 16.7 μL of quenching solution, which is a 4:1 mixture of acid-urea PAGE sample buffer (0.1 M NaOAc (pH 5.0), 8 M urea, 0.05% xylene cyanol, and 0.05% bromophenol-blue) and 80% glycerol, to each 10 μL reaction solution followed by vortexing. The unreacted and aminoacylated <i>B. mori</i> tRNA<sub>Phe</sub> in the mixtures were separated by electrophoresis on a 10% acid-urea polyacrylamide gel in Fig. 2.

**Fig. 2.** Schematic models of the genes encoding the BmPheRS α- (A) and β-subunits (B) in the genome. The genomic sequences from the assemble results (Build 2, Ver. 3) of the Japanese and Chinese WGS sequencing data (The International Silkworm Genome Consortium, 2008) are presented by horizontal lines with their Acc. No. and sizes below. The numbers below the genomic sequences show their base positions. Exons are described by filled boxes below the genomic sequences and 5' and 3'UTRs are described by open boxes. A sequence gap is depicted by slashes. The oligonucleotide probes designed for Southern blotting are shown by gray bars. The positions of restriction sites close to the probes are indicated by arrows. The asterisk (*) depicts a restriction site detected in the contig (Acc. No. BAAB01103040) from the assemble results of the Japanese WGS sequencing data (Mita <i>et al.</i> 2004).
0.1 M NaOAc (pH 5.0) as reported previously (Kohrer and Rajbhandary, 2008) at 4°C for 21-23 h at 9 V/cm. The separated tRNAs were visualized with GelRed (Biotium Inc.; Hayward, CA, USA).

RESULTS AND DISCUSSION
Cloning of the BmPheRS α- and β-subunit genes

By PCR amplification using oligonucleotide primers designed based on the predicted cDNA sequences of the BmPheRS α- and β-subunits, which were constructed by BLAST searches against the EST and genome databases of B. mori (KAIKObase) (Shimomura et al., 2009) using the amino acid sequences of PheRSs from other organisms, we obtained the cDNA clones containing the coding regions whose deduced amino acid sequences are identical with the predicted ones. 5'- and 3'-RACE analyses further revealed the full-length cDNAs of the BmPheRS α- and β-subunits (Fig. 1A and 1B, respectively). Since the 5'-ends of these cDNAs were detected as varied lengths, the sequences of the longest clones were shown and the other 5'-ends were indicated by dots. The coding sequences for the BmPheRS α- and β-subunits start from the ATG codons at base position 1 and respectively extend to the termination codons at base position 1474 and 1750, encoding proteins of 491 and 583 amino acids, which were followed by the potential polyadenylation signals at base position 1481 and 1808, respectively. Extra ATG codons were found in the 5' untranslated region (UTR) of both subunits (Fig. 1A and 1B; bold). They are all immediately followed by in-frame termination codons to construct small ORFs (Fig. 1A and 1B; underlines). Such small ORFs in 5'UTR are known as upstream ORFs (Mignone et al., 2002).

The observed variation in the lengths of the 5'-ends suggested that the transcription of the genes encoding the BmPheRS α- and β-subunits might also be organ-specifically regulated because it was previously reported that the glycyl-tRNA synthetase (GlyRS) from B. mori has two presumptive transcription start sites and that one of them is used preferentially in posterior silk glands (Nada et al., 1993). The existence of upstream ORFs in the 5'UTR raised doubts about the functionality of the cloned cDNAs. However, since the longer transcript of the B. mori GlyRS also contains an upstream ORF in its 5'UTR, the existence of upstream ORFs does not necessarily show that such genes are non-functional. In fact, upstream ORFs have been found in several eukaryotic mRNAs, which has been speculated to be involved in controlling the translation efficiency of mRNAs (Mignone et al., 2002).

Based on the cDNA sequences, the predicted gene structures of the BmPheRS α- and β-subunits in the genome were constructed by searching the genome database of B. mori containing the whole genome shotgun (WGS) sequencing data (Mita et al., 2004; The International Silkworm Genome Consortium, 2008). The search retrieved one set of contigs and scaffolds containing all queried cDNA sequences for each subunit and no other homologous genes were detected. The BmPheRS α- and β-subunit genes were found to consist of six and twelve exons, respectively. These exons were mapped over three contigs and one scaffold with one large gap for the α-subunit and over one scaffold for the β-subunit (Fig. 2A and 2B). To experimentally confirm these predicted gene structures, DNA probes complementary to almost all regions of the exon 1 of the α-subunit gene and to the exons 1, 2, and 3 with the first and second introns of the β-subunit gene were prepared for Southern blotting analyses. Southern blotting with the DNA probe for the α-subunit against the genomic DNA digested with HindIII, Kpnl, PstI, and SacI gave signals of 1.7, 4, 9, and 2 kbp (Fig. 3A), which were all consistent with the predicted sizes (>1.2, >0.9, >3.5,
and 2.0 kbp, respectively). Southern blotting with the DNA probe for the β-subunit against the genomic DNA digested with BamHI, PstI, SacI, and XbaI gave signals of 7.5, > 10, 7.5, and 10 kb (Fig. 3B), which were also all consistent with the predicted sizes (7.2, 19, 7.2, and 11 kb, respectively). These analyses showed that the BmPheRS α- and β-subunit genes are both single copies in the genome.

The deduced amino acid sequences of the BmPheRS α- and β-subunits were aligned with those of other PheRSs from eukaryotes (Drosophila melanogaster, Homo sapiens, and Saccharomyces cerevisiae) and prokaryotes (E. coli and Thermus thermophilus) (Fig. 4A and 4B). The alignments showed that the BmPheRS α- and β-subunits have high sequence similarities with those of the eukaryotic PheRSs: 81, 80, and 68% similarity with the α-subunits and 78, 74, and 60% similarity with the β-subunits from D. melanogaster, H. sapiens, and S. cerevisiae, respectively. On the other hand, relatively low sequence similarities were observed with prokaryotic PheRSs: 49 and 47% similarity with the α-subunits and 42 and 42% similarity with the β-subunits from E. coli and T. thermophilus, respectively. Each subunit of PheRS is characterized by the presence of several conserved sequences. PheRS α-subunit contains three sequence motifs involved in forming the catalytic core of PheRS (Eriani et al., 1990; Mosyak et al., 1995). The BmPheRS α-subunit was found to contain sequences corresponding to these three motifs (Fig. 4A; arrows). In the PheRS β-subunit, two consensus sequences, (P/A)NRxDx(L/M) and RxDx5D(x/E)D, are considered to be essential for the catalytic activity of PheRS (Moor et al., 2003). These sequences are conserved in the BmPheRS β-subunit (Fig. 4B; dots). In addition, the N-terminal four residues, MPT(V/I), which are commonly found in the eukaryotic PheRS β-subunit (Moor et al., 2003), are also conserved...
Fig. 4. Amino acid sequence alignments among the α- (A) and β-subunits (B) of PheRSs from B. mori (Bm), D. melanogaster (Dm), H. sapiens (Hs), S. cerevisiae (Sc), E. coli (Ec), and T. thermophilus (Tt). Asterisk (*) denotes that the marked sequence was predicted to be PheRS by annotation. Alignment was performed using MultAlin (Corpet, 1988). Amino acids identical for all six sequences are shaded black with white letters. The three sequence motifs in the α-subunit are specified by arrows, where the identification of the motifs was based on the crystal structure of T. thermophilus PheRS (Mosyak et al., 1995). Ala 450 of the α-subunit, which was mutated to Gly in this study, is shown by an inverted triangle. The two consensus sequences in the β-subunit are shown by dots. The N-terminal four residues conserved among eukaryotic PheRS β-subunit are depicted in bold. The amino acid sequences were obtained from the NCBI database (Acc. No. Q9W3J5 and Q9VCA5 for D. melanogaster; Acc. No. NP_004452 and NP_005678 for H. sapiens; Acc. No. NP_116631 and NP_013161 for S. cerevisiae; Acc. No. AP_002334 and AP_002333 for E. coli; Acc. No. CAA78104 and CAA78105 for T. thermophilus).
in the BmPheRS β-subunit (Fig. 4B; bold).

From the above analyses on the cloned full-length cDNAs sequences and their deduced amino acid sequences, it was expected that the obtained cDNAs encode functional PheRS α- and β-subunits. This expectation was strongly supported by the results, which revealed that both cloned genes exist as single copies in the genome with no homologous genes. Furthermore, the results revealed that their deduced amino acid sequences exhibit sequence similarity with other eukaryotic and prokaryotic PheRSs, and that they possess the same well-defined sequence characteristics as the PheRS.

**Aminoacylation activity of the recombinant BmPheRS**

To assess the function of the cloned BmPheRS genes experimentally, the BmPheRS α- and β-subunits were expressed in *E. coli* and subjected to enzyme assay. To obtain the recombinant BmPheRS as an active complex, the α- and β-subunits were simultaneously expressed from the expression vector (pQE30-BmPheRS_α_β) bearing both subunit genes in one operon with a hexa-histidine tag on the N-terminus of the α-subunit and were partially-purified over Ni-NTA resin as reported previously (Kwon *et al.*, 2006). For control experiments, the same expression and purification procedures were applied to the blank vector (pQE-30) without any insert DNA and the expression vector bearing only the α-subunit gene (pQE30-BmPheRS_α). The SDS-PAGE analysis of the product from the blank vector revealed that a lot of proteins were contaminated through the Ni-NTA purification procedure (Fig. 5; upper panel; lane 1). The product from pQE30-BmPheRS_α gave two abundant protein bands at 54 and 62 kDa (Fig. 5; upper panel; lane 2) which were not observed in the product from the blank vector. The lower molecular weight protein was verified to be the BmPheRS α-subunit by a MALDI-TOF-MS analysis after an in-gel digestion with trypsin (data not shown), whose estimated molecular weight was close to the expected one (57.5 kDa). The higher molecular weight band was an unidentified protein, which was probably co-purified with the BmPheRS α-subunit. An additional protein band was detected at 66 kDa in the product from pQE30-BmPheRS_α_β (Fig. 5; upper panel; lane 3). This protein band was verified to be the BmPheRS β-subunit by MALDI-TOF-MS analysis (data not shown), whose estimated molecular weight was close to the expected one (65.2 kDa). Since a hexa-histidine tag was only added to the N-terminus of the α-subunit, co-purification of the β-subunit revealed the complex formation among these two subunits.

The enzyme activities of the above three protein products to aminoaclate the synthesized *B. mori* tRNA\(^{\text{Phe}}\) with Phe were then assayed. After the aminoaclation reactions for 10 min at a physiological condition (30°C and pH 7.5), unreacted tRNA\(^{\text{Phe}}\) and its aminoaclated derivative (aa-tRNA\(^{\text{Phe}}\)) were separated on an acid-urea polyacrylamide gel as described previously (Kohrer and Rajbhandary, 2008). The reactions with the protein products from the blank vector and pQE30-BmPheRS_α gave no detectable bands of aa-tRNA\(^{\text{Phe}}\) by the acid-urea PAGE (Fig. 5; lower panel; lanes 1 and 2, respectively). On the other hand, a reaction with the protein product from pQE30-BmPheRS_α_β exclusively gave a band of aa-tRNA\(^{\text{Phe}}\) (Fig. 5; lower panel; lane 3), demonstrating that the synthesized *B. mori* tRNA\(^{\text{Phe}}\) was aminoaclated with Phe.

The above results demonstrated that the proteins encoded by the cloned BmPheRS α- and β-subunit genes possess the expected function to catalyze the aminoaclation of the *B. mori* tRNA\(^{\text{Phe}}\) and that the coexistence of the two subunits is essential for enzyme functioning. Therefore, the partially-purified protein product expressed by pQE30-BmPheRS_α_β in *E. coli* can be used to investigate the characteristics of BmPheRS.

The optimum reaction condition of the recombinant BmPheRS was then investigated at different temperatures and pHs. First, aminoaclation assays were performed at 10, 20, 30, 40, and 50°C at pH 7.5 by a 10 min reaction (Fig. 6A). At 10°C, most of the tRNA\(^{\text{Phe}}\) was detected as the unreacted form while a faint band of aa-tRNA\(^{\text{Phe}}\) was observed. At higher temperatures between 20-40°C, most of the tRNA\(^{\text{Phe}}\) was aminoaclated. At 50°C, no detectable band of aa-tRNA\(^{\text{Phe}}\) was observed. This was confirmed to be due to the denaturation of the enzyme by the experimental result that the pre-heated enzyme at 50°C or higher for 30 min gave no detectable bands of aa-tRNA\(^{\text{Phe}}\) after a 10 min aminoaclation reaction at 30°C and pH 7.5 while the pre-heated enzyme at 40°C or lower retained aminoaclation activity (data not shown). Next, aminoaclation assays were performed at pH 5.5-9.0 at 30°C by a 10 min reaction (Fig. 6B). At the pH range between 6.5-8.0, most of the tRNA\(^{\text{Phe}}\) was aminoaclated. On the other hand, at the pH below 6.0 and above 8.5, the aminoaclation reaction was impeded. Hence, the optimum reaction condition of the recombinant BmPheRS is in temperature range between 20-40°C and in the pH range between 6.5-8.0, which is in good accordance with the native PheRS previously isolated from the larvae of *D. melanogaster* (Christopher *et al.*, 1971).

**Relaxing the amino acid specificity of BmPheRS by point mutation**

In order to relax the amino acid specificity of BmPheRS, the amino acid residues responsible for discriminating Phe from the other amino acids was first predicted. The previously reported crystal structure of the *T. thermophilus* PheRS complexed with Phe showed that the side chains of Val 261 and Ala 314 in its α-subunit form the back
Relaxing AA specificity of *B. mori* PheRS

...wall of the amino acid binding pocket and prevent the penetration of amino acids deeper inside the protein (Reshetnikova et al., 1999). The importance of these residues as the determinants of amino acid specificity was further demonstrated by mutation studies: point mutation to Gly at Ala 294 of the *E. coli* PheRS α-subunit, which is equivalent to Ala 314 of the *T. thermophilus* PheRS α-subunit, relaxed its amino acid specificity toward various *p*-substituted Phe analogs (Ibba and Hennecke, 1995; Kirshenbaum et al., 2002). The sequence alignment showed that Ala 450 of the BmPheRS α-subunit (Fig. 4A) is equivalent to Ala 294 of the *E. coli* PheRS α-subunit. Therefore, the Ala to Gly mutation was introduced at amino acid position 450 of the BmPheRS α-subunit to generate its mutant with relaxed amino acid specificity.

The recombinant BmPheRS comprised of the A450G α-subunit mutant and the wild-type β-subunit (αA450G BmPheRS mutant) was expressed and partially-purified along with its wild-type counterpart, and its aminoacylation activity toward *p*-substituted Phe analogs were investigated. Fig. 7 shows the result of acid-urea PAGE after aminoacylation reactions at 30°C and pH 7.5 for 30 min with Phe and the four kinds of Phe analogs, *p*-fluoro-*L*-phenylalanine (F-Phe), *p*-chloro-*L*-phenylalanine (Cl-Phe), *p*-bromo-*L*-phenylalanine (Br-Phe), and *p*-ido-*L*-phenylalanine (I-Phe). The wild-type BmPheRS catalyzed aminoacylation of the *B. mori* tRNA<sub>Phe</sub> with Phe and F-Phe but not with Cl-Phe, Br-Phe, or I-Phe. On the other hand, the αA450G BmPheRS mutant catalyzed the aminoacylation of the *B. mori* tRNA<sub>Phe</sub> with Cl-Phe and Br-Phe in addition to Phe and F-Phe but not with I-Phe. These results clearly demonstrated that the amino acid specificity of BmPheRS was relaxed enough to accept Phe analogs bearing larger atoms than fluorine at the *para*-position of the phenyl ring.

The above results are in good accordance with the previous results in the *E. coli* PheRS: the wild-type *E. coli* PheRS recognized Phe and F-Phe whereas its αA294G mutant recognized Cl-Phe and Br-Phe, and perhaps even I-Phe (Ibba et al., 1994). They showed that the aminoacylation kinetics are dependent on the van der Waals’ radius of atoms at the *para*-position. Although the results obtained in this study are not quantitative, the observed clear differentiation between Br-Phe and I-Phe by the enzyme strongly suggested that the aminoacylation kinetics of the αA450G BmPheRS mutant is also determined by the van der Waals’ radius of atoms.

In this study, we cloned the full-length cDNAs encoding the α- and β-subunits of BmPheRS and discovered an appropriate point mutation (Ala 450 in the α-subunit to...
Fig. 7. Structures of Phe analogs and the aminocacylation of the in vitro-transcribed B. mori tRNA\textsuperscript{Phe} with Phe or one of the Phe analogs by the recombinant wild-type BmPheRS (left panel) or the αA450G BmPheRS mutant (right panel). The reactions were carried out in the presence of L-phenylalanine (Phe), p-fluorophenylalanine (F-Phe), p-chlorophenylalanine (Cl-Phe), p-bromophenylalanine (Br-Phe), and p-iodophenylalanine (I-Phe) at 30°C for 30 min at pH 7.5. The unreacted tRNA\textsuperscript{Phe} and its aminocacylated derivative (aa-tRNA\textsuperscript{Phe}) were separated on a 10% acid-urea polyacrylamide gel.

Gly) to relax its amino acid specificity toward p-substituted Phe analogs, Cl-Phe and Br-Phe. Since an aryl bromide functional group is a good substrate for Suzuki-Miyaura coupling under a mild reaction condition (Chalker et al., 2009), Br-Phe could be a unique residue in proteins for in vitro or in vivo assay systems is necessary for the next step. This would further lead to the generation of a novel type of a genetically-modified silkworm which can incorporate various Phe analogs into silk proteins and other recombinant proteins.

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