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# The processing of human mitochondrial leucyl-tRNA synthetase in the insect cells

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**Abstract** A His-tagged full-length cDNA of human mitochondrial leucyl-tRNA synthetase was expressed in a baculovirus system. The N-terminal sequence of the enzyme isolated from the mitochondria of insect cells was found to be IYSATGKWT-KEYTL, indicating that the mitochondrial targeting signal peptide was cleaved between Ser39 and Ile40 after the enzyme precursor was translocated into mitochondria. The enzyme purified from mitochondria catalyzed the leucylation of *Escherichia coli* tRNA<sup>Leu</sup>(CAG) and *Aquifex aeolicus* tRNA<sup>Leu</sup>(GAG) with higher catalytic activity in the leucylation of *E. coli* tRNA<sup>Leu</sup> than that previously expressed in *E. coli* without the N-terminal 21 residues.

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**Key words:** Human mitochondrion; Aminoacyl-tRNA synthetase; tRNA<sup>Leu</sup>; Baculovirus; *Aquifex aeolicus*; *Escherichia coli*

## 1. Introduction

Aminoacyl-tRNA synthetases (aaRSs) play a key role in protein biosynthesis. They catalyze the esterification of amino acids to the 2'- or 3'-hydroxyl group of ribose at the 3'-end of their cognate tRNAs [1,2]. These aaRSs can be divided into Class I and Class II with 10 members in each class according to their conserved sequences and characteristic structural motifs [3]. Leucyl-tRNA synthetase (LeuRS) belongs to Class I, which is characterized by two structural motifs (HIGH and KMSKS) [3]. LeuRS functions to leucylate its cognate tRNA [3], but in some cases it has been shown to mischarge the leucine cognate tRNA with other amino acids in vitro and in vivo [4]. In addition, in vitro, some mutated *Escherichia coli* tRNA<sup>Leu</sup> can be more easily mischarged by *E. coli* LeuRS than the native tRNA<sup>Leu</sup> (unpublished data of our lab).

The cDNA of human mitochondrial leucyl-tRNA synthe-

tase (hmLeuRS) was isolated from an immature myeloid cDNA library and shown to be present in 16 different human tissues [5]. When the cDNA sequence of hmLeuRS was BLASTed with the human genome database, the 22-exon gene was found to be located on chromosome 3p21. Bullard et al. predicted that this enzyme would be targeted into mitochondria and the mitochondrial signal peptide was cleaved after Gly21. The predicted hmLeuRS cDNA was expressed and analyzed in *E. coli* [6]. However, our work here showed that the signal peptide of hmLeuRS was cleaved between Ser39 and Ile40. The direct evidence of the localization of hmLeuRS in mitochondria will be also presented.

This finding is interesting as many neuromuscular disorders are associated with the mutation of mitochondrial tRNA genes [7]. The human mitochondrial tRNA<sup>Leu</sup>(UUR) (hmtRNA<sup>Leu</sup>(UUR)) gene is one of the most frequently mutated genes [8]. The mutations have been associated with mitochondrial encephalomyopathies, mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) [8]. A single replacement of A3243 by G was most frequently found in hmtRNA<sup>Leu</sup>(UUR) mutation related to MELAS [9]. Therefore, further studies on hmLeuRS may lead to better understanding of the mechanisms of hmtRNA<sup>Leu</sup> mutation-related diseases.

Here, we report the cloning of the His-tagged full-length cDNA of hmLeuRS into baculovirus and its expression in *Trichoplusia ni* BTI-Tn-5B1-4 (*Tn5*) insect cells. The processed hmLeuRS was purified from the insect mitochondria to over 95% homogeneity, and its N-terminal sequence and kinetic constants were determined.

## 2. Materials and methods

### 2.1. Chemicals, plasmids, and insect cell line

All chemicals were purchased from Sigma, except otherwise noted. Kinase, ligase, and restriction endonucleases were obtained from New England Biolabs. The Bac-to-Bac baculovirus expression systems including plasmid pFastBac1, MAX EFFENCY DH10BAC competent cells, insect cell *Tn5*, and Sf-900 II serum-free medium (containing L-glutamine) were purchased from Gibco/BRL (Life technologies, Inc.). The hmLeuRS expressed in *E. coli* with the deletion of N-terminal 21 residues (called hmLeuRS-22), was purified as described by Bullard et al. [6]. *E. coli* tRNA<sup>Leu</sup>(CAG) (1066 pmol/A<sub>260</sub>) and *Aquifex aeolicus* tRNA<sup>Leu</sup>(GAG) (900 pmol/A<sub>260</sub>) were isolated in our lab [10,11].

### 2.2. Construction of recombinant baculovirus containing hmLeuRS gene (hmleus) and the expression of hmLeuRS in insect cell *Tn5*

Two complementary oligonucleotides 5'-GGCCGCGCACCATCA-

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**Abbreviations:** aaRS, aminoacyl-tRNA synthetase; LeuRS, leucyl-tRNA synthetase; hmLeuRS, human mitochondrial leucyl-tRNA synthetase; hmtRNA<sup>Leu</sup>, human mitochondrial tRNA<sup>Leu</sup>; *Tn5*, *Trichoplusia ni* BTI-Tn-5B1-4; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

TCACCACCATAGGTAC-3' and 5'-CTAATGGTGGTGATGATGGTGC-3' (encoding Ala<sub>3</sub>-His<sub>6</sub>-Stop) were phosphorylated, annealed and inserted between the *NotI* and *KpnI* sites of the pFastBac1 vector to create pFastBac1H. The full-length cDNA of hmLeuRS without stop codon was polymerase chain reaction (PCR) amplified from pBluescript SK<sup>+</sup> vectors containing *hmleus* (KIAA-0028, accession number D21851, gifted from the Kazusa DNA Research Institute, Japan). The PCR product was inserted between the *SalI* and *NotI* sites of pFastBac1H to create pFastBac1H-*hmleus*, from which the expressed hmLeuRS would be Ala<sub>3</sub>-His<sub>6</sub>-tagged at its C-terminus. The sequence of pFastBac1H-*hmleus* was confirmed by DNA sequencing. pFastBac1H-*hmleus* was used to prepare *hmleus*-recombinant bacmid DNA according to the manufacturer's instructions for Bac-to-Bac baculovirus expression systems. The recombinant bacmid DNA was then used to transfect *Tn5* insect cells, which were grown in Sf-900 II serum-free medium (containing L-glutamine) to express hmLeuRS. The enzyme expressed in the baculovirus system was named hmLeuRS-B.

### 2.3. Purification of hmLeuRS-B

After 4 days, the cells expressing hmLeuRS-B were collected by centrifugation at 500×*g* and washed in phosphate-buffered saline (pH 7.5). The mitochondria, cytoplasm and nuclei/cellular debris were separated [12,13] and hmLeuRS-B was purified from mitochondria with Ni-NTA column chromatography according to the manufacturer's protocol (Qiagen, Germany).

### 2.4. Determination of N-terminal sequence of hmLeuRS-B

The hmLeuRS-B purified from the mitochondria of *Tn5* insect cells was separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred to polyvinylidene difluoride membrane (PVDF, ProBlott<sup>®</sup>, Applied Biosystems, USA), and the first 14 amino acid residues at the N-terminal end were sequenced with protein sequencer 477A (Applied Biosystems) according to the manufacturer's instructions.

### 2.5. Localization of hmLeuRS-B in subcellular fractions using Western blot

Nuclei/cellular debris, cytoplasm and mitochondria were separated as mentioned above. The quality of subcellular fractions was controlled by determining succinate dehydrogenase activity as described [12,13]. The subcellular fractions were analyzed by SDS–PAGE, blotted to nitrocellulose membranes (Bio-Rad, USA). The His-tagged hmLeuRS-B was detected by 0.1 µg/ml monoclonal mouse anti-His-tag antibody and 0.5 µg/ml peroxidase-conjugated goat anti-mouse antibody. The blotting was visualized using the SuperSignal West Pico Chemiluminescence method (Pierce, Inc.).

### 2.6. Determination of kinetic constants

Activities and kinetic constants in ATP-PPi exchange and tRNA<sup>Leu</sup> leucylation were measured at 37°C as described by Bullard et al. [6]. One unit of ATP-PPi exchange activity was defined as the amount of enzyme that catalyzed 1 nmol ATP-PPi exchange in 1 min at 37°C. One unit of leucylation activity was defined as the amount of enzyme needed to catalyze the production of 1 nmol leucyl-tRNA in 1 min at 37°C. Kinetic constants of hmLeuRS were determined using different concentrations of the substrates. Protein concentrations were determined by the Lowry method [14,15].

### 2.7. Fluorescence spectroscopy

Fluorescence spectra were recorded with a Hitachi F4010 fluorescence spectrophotometer (Japan). hmLeuRS-22 was measured in parallel with hmLeuRS-B to find subtle difference in their structures. The emission spectra in 20 mM potassium phosphate buffer (pH 7.5) ex-

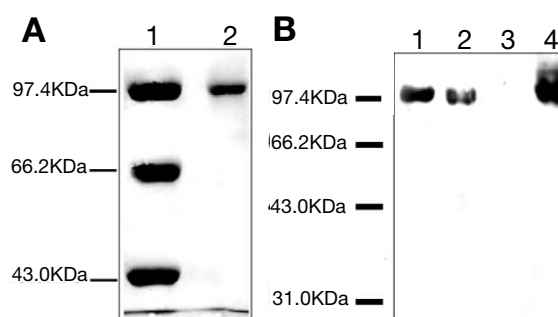


Fig. 1. SDS–PAGE analysis of purified hmLeuRS-B (A) and Western blotting of subcellular fractions from insect cells expressing hmLeuRS-B (B). A: Purified hmLeuRS-B (lane 2, 3 µg) was compared to protein standards (lane 1). B: hmLeuRS-B was present in the mitochondrial (lane 2, 10 µg) and nuclei/cellular debris fractions (lane 4, 40 µg) in order to show trace uncleaved precursor of hmLeuRS-B, while completely absent from cytoplasmic fraction (lane 3, 10 µg). Purified hmLeuRS-B (lane 1, 3 µg) was used as a control.

cited at 295 nm (slit width 20 nm) were recorded in the range of 320–400 nm (slit width 5 nm). The protein concentration was 10 µg/ml.

## 3. Results and discussion

### 3.1. Expression and purification of hmLeuRS-B

Recombinant baculovirus vectors have been widely used to express heterologous genes in cultured insect cells. In most cases, the recombinant proteins will be processed, and targeted to their appropriate cellular locations [16]. The pFastBac1 vector provided with the Bac-to-Bac baculovirus expression system does not offer a C-terminal His-tag needed for purification and detection of hmLeuRS-B. Therefore, we constructed a new vector to express the hmLeuRS with a C-terminal His-tag.

From the mitochondria prepared from  $1.6 \times 10^8$  insect cells, about 120 µg of purified hmLeuRS-B was obtained by Ni-NTA affinity chromatography. The purified hmLeuRS-B was over 95% homogeneous and its molecular mass was about 97 kDa as determined by SDS–PAGE (Fig. 1A, lane 2).

### 3.2. Localization of hmLeuRS-B in vivo

The localization of hmLeuRS in vivo was not determined previously [6], so we investigated the location of hmLeuRS-B in *Tn5* cells in the present work. Lysates of insect cells expressing hmLeuRS-B were fractionated into three fractions: nuclei/cellular debris, cytoplasm, and mitochondria. Succinate dehydrogenase activity is found only in mitochondria [13]. In the present work, a high activity of succinate dehydrogenase was found in the mitochondrial fraction, as compared to the nuclei/cellular debris fraction or the cytoplasm fraction (Table 1). Western blot revealed that the hmLeuRS-B band was present in the mitochondrial fraction, but completely absent

Table 1  
Subcellular fractions of insect cells expressing hmLeuRS-B

Succinate dehydrogenase	Nuclei/cellular debris	Cytoplasm	Mitochondria
Units/( $1 \times 10^7$ cells) <sup>a</sup>	0.434	0	2.05
% units of total	17.4	0 <sup>b</sup>	82.0

All data in this table were the average values with a variation of < 5% from three independent determinations.

<sup>a</sup>Units = Abs<sub>490</sub>/min.

<sup>b</sup>< 1%.

from the cytoplasmic fraction (Fig. 1B, lanes 2 and 3). This result showed that the expression product of *hmleus* gene was translocated into mitochondria in vivo, providing the first direct evidence of the mitochondria localization of this enzyme. Two bands were observed in the nuclei/cellular debris fraction (Fig. 1B, lane 4): one strong band corresponding to hmLeuRS-B, apparently because of the contamination by intact insect cells, and another weaker band above hmLeuRS-B that may be the uncleaved enzyme containing the mitochondrial signal peptide not yet translocated into mitochondria.

### 3.3. Mitochondrial import signal peptide of hmLeuRS-B

The cleaved site of the signal peptide of hmLeuRS-B was determined by amino acid sequencing. The sequence of the first 14 amino acid residues was determined as IYSATG-KWTKEYTL, showing that the signal peptide was cleaved between Ser39 and Ile40. The calculated molecular mass of hmLeuRS-B is 98533.51 Da that approximates to the molecular mass determined by SDS-PAGE (Fig. 1A). The N-terminal sequences of LeuRSs from different sources are aligned in Fig. 2. The processed N-terminal sequence of hmLeuRS-B is very close to that of yeast mitochondrial LeuRS (Fig. 2) [19]. Analysis of the full-length cDNA of hmLeuRS using the predicted amino acid sequence program PSORT indicated that the cleavage of the signal peptide would occur after Gly21 [6]. The difference between the predicted and the expressed mature form in insect cells is most likely due to a miscalculation by the prediction program. Previous expression of mitochondrial proteins in the baculovirus system indicated that the processing of human mitochondria-targeted proteins in baculovirus expression systems was correct [21–23].

### 3.4. Comparison of the fluorescence emission spectra of hmLeuRS-B and hmLeuRS-22

To investigate the subtle conformational differences of hmLeuRS-B and hmLeuRS-22, fluorescence emission spectra were determined. The  $\lambda$  emission of hmLeuRS-B (338.2 nm) was blue shifted slightly in comparison with that of hmLeuRS-22 (338.8 nm), indicating that hmLeuRS-22 has a looser conformation and less hydrophobic regions.

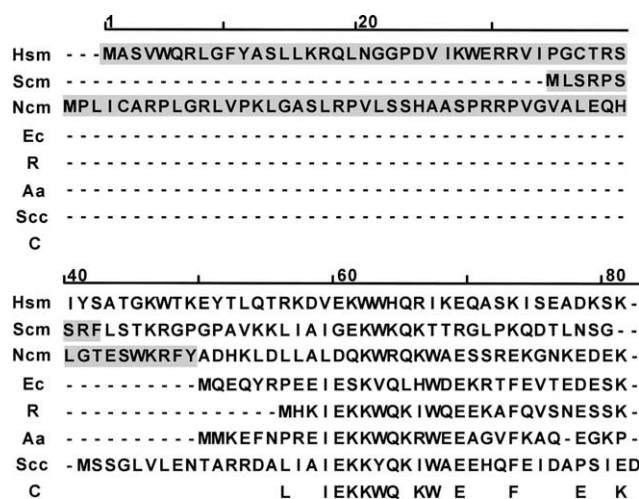


Fig. 2. Alignment of N-terminal sequences of LeuRSs from seven sources. The sequences of LeuRSs from *Homo sapiens* mitochondria (Hsm), *S. cerevisiae* mitochondria (Scm), *Neurospora crassa* mitochondria (Ncm), *E. coli* (Ec), *Rickettsia prowazekii* (R), *A. aeolicus* (Aa) and *S. cerevisiae* cytoplasm (Sc) were downloaded from aaRSs database (<http://rose.man.poznan.pl/aars/>) [17] and aligned by CLUSTAL W [18]. The shadowed sequences represent the cleaved signal peptides of LeuRSs of *H. sapiens* mitochondria, *S. cerevisiae* mitochondria [19], *N. crassa* mitochondria [20]. C represents the consensus sequence of these seven LeuRSs.

### 3.5. Activity assay of hmLeuRS-B

The kinetic constants of hmLeuRS-B in the ATP-PPi exchange and leucylation reactions are summarized in Table 2. In the ATP-PPi exchange reaction,  $k_{cat}$  of hmLeuRS-B is 0.14  $s^{-1}$ , which is lower than that of prokaryotic (*E. coli* 171  $s^{-1}$  [24], and *A. aeolicus* 4.0  $s^{-1}$  [11]), eukaryote cytoplasmic (*Phaeoelus vulgaris* 16  $s^{-1}$  [25]) and plant chloroplastic LeuRSs (*P. vulgaris* 8  $s^{-1}$  [25]). The  $K_m$  of hmLeuRS-B for leucine is 250  $\mu M$ , which is larger than that of *E. coli* LeuRS (52  $\mu M$  [24]) and *A. aeolicus* LeuRS (3.5  $\mu M$  [11]). However, the  $K_m$  for ATP is 127  $\mu M$ , which is smaller than that of *E. coli* LeuRS (750  $\mu M$  [24]) and *A. aeolicus* LeuRS (360  $\mu M$  [11]). These results suggest that in the ATP-PPi exchange reaction,

Table 2  
Kinetic constants for hmLeuRS-B at 37°C<sup>a</sup>

Substrates	Constants	ATP-PPi exchange reaction <sup>b</sup>	Leucylation of tRNA <sup>Leu</sup> reaction <sup>c</sup>
Leucine	$K_m$ ( $\mu M$ )	250	39.7
	$k_{cat}$ ( $s^{-1}$ )	0.14	$5.3 \times 10^{-2}$
	$k_{cat}/K_m$ ( $s^{-1} \mu M^{-1}$ )	$5.6 \times 10^{-4}$	$1.33 \times 10^{-3}$
ATP	$K_m$ ( $\mu M$ )	127	1192
	$k_{cat}$ ( $s^{-1}$ )	0.13	$4.9 \times 10^{-2}$
	$k_{cat}/K_m$ ( $s^{-1} \mu M^{-1}$ )	$1.0 \times 10^{-3}$	$4.1 \times 10^{-5}$
<i>E. coli</i> tRNA <sup>Leu</sup> <sub>1</sub>	$K_m$ ( $\mu M$ )		2.2
	$k_{cat}$ ( $s^{-1}$ )		$4.1 \times 10^{-2}$
	$k_{cat}/K_m$ ( $s^{-1} \mu M^{-1}$ )		$1.85 \times 10^{-2}$
<i>A. aeolicus</i> tRNA <sup>Leu</sup>	$K_m$ ( $\mu M$ )		2.8
	$k_{cat}$ ( $s^{-1}$ )		$1.9 \times 10^{-2}$
	$k_{cat}/K_m$ ( $s^{-1} \mu M^{-1}$ )		$0.68 \times 10^{-2}$

<sup>a</sup>The experiments were performed as described in Section 2. All data in this table were the average values with a variation of < 5% from three independent determinations.

<sup>b</sup>The specific activity of hmLeuRS-B in ATP-PPi exchange reaction was 83 units/mg, as calculated from the molecular mass (98.5 kDa) and average  $k_{cat}$  of leucine and ATP.

<sup>c</sup>The specific activity of hmLeuRS-B in the leucylation of tRNA reaction was 29 units/mg, calculated from the molecular mass (98.5 kDa) and average  $k_{cat}$  of leucine, ATP and *E. coli* tRNA<sup>Leu</sup><sub>1</sub>. The kinetics of leucine and ATP in leucylation of tRNA<sup>Leu</sup> reaction was measured in presence of 20  $\mu M$  of *E. coli* tRNA<sup>Leu</sup><sub>1</sub>.



hmLeuRS-B binds leucine rather weakly, but binds ATP tightly as compared to *E. coli* and *A. aeolicus* LeuRSs.

In the leucylation reaction, for the three substrates of hmLeuRS-B (*E. coli* tRNA<sup>Leu</sup><sub>1</sub> used in the assay), the  $k_{\text{cat}}$  value of hmLeuRS-B is  $4.8 \pm 0.5 \times 10^{-2} \text{ s}^{-1}$ , about 3-fold lower than that in the ATP-PPi exchange reaction. This value is rather low as compared with that of *E. coli* LeuRS ( $3.3 \text{ s}^{-1}$ ) [24], *A. aeolicus* LeuRS ( $0.39 \text{ s}^{-1}$ ) [11], *P. vulgaris* cytoplasmic LeuRS ( $2.05 \text{ s}^{-1}$ ) [25], *Saccharomyces cerevisiae* cytoplasmic LeuRS ( $3.3 \text{ s}^{-1}$ ) [26], plant *P. vulgaris* chloroplastic LeuRS ( $3.15 \text{ s}^{-1}$ ) [27], and *Euglena gracilis* chloroplastic LeuRS ( $2 \text{ s}^{-1}$ ) [28]. The  $K_{\text{m}}$  values for leucine and ATP were 40 and 1192  $\mu\text{M}$ , respectively. In comparison with the ATP-PPi exchange reaction, these results suggest that hmLeuRS-B binds leucine more tightly, but binds ATP only weakly. The  $K_{\text{m}}$  of hmLeuRS-B for leucine is 2.7- and 6.3-fold higher than that of *E. coli* and *A. aeolicus* LeuRSs respectively; that for ATP is 4.25- and 2.16-fold higher, respectively [11,24]. These results show that hmLeuRS-B catalyzes the ATP-PPi exchange and leucylation reactions at a lower rate and binds with its three substrates weakly. Other eukaryotic mitochondrial aaRSs have been found to have lower catalytic activity than their cognate cytoplasmic and prokaryotic aaRSs [12,29,30]. Our present work is in agreement with these reports.

The hmLeuRS-B can catalyze the leucylation of *E. coli* tRNA<sup>Leu</sup><sub>1</sub> and *A. aeolicus* tRNA<sup>Leu</sup> at a lower rate, and the  $K_{\text{m}}$  values for *E. coli* and *A. aeolicus* tRNA<sup>Leu</sup>s are similar, indicating that hmLeuRS-B recognizes the two bacterial tRNA<sup>Leu</sup>s with similar affinity. This result also suggests some evolutionary conservation of catalytic elements for tRNA<sup>Leu</sup>s in hmLeuRS. The catalytic efficiency of hmLeuRS is lower than that observed in the leucylation reaction catalyzed by *E. coli* LeuRS [11,24]. It can be postulated that hmLeuRS catalyzes the leucylation of the natural hmtRNA<sup>Leu</sup> with higher efficiency than that of bacterial tRNA<sup>Leu</sup>s. We cloned the mitochondrial genes encoding the two hmtRNA<sup>Leu</sup> isoacceptors, but were unable to express the genes in *E. coli*.

The mature hmLeuRS-B in insect cells has higher specific leucylation activity (29 units/mg) than hmLeuRS-22 expressed in *E. coli* (3.3 units/mg) [6]. The reason for this may be that the maturation process of mitochondrial proteins in insect cells, such as N-terminal cleavage, refolding of mitochondria-imported proteins and potential modification of amino acid residues, is more suitable for the processing of human mitochondrial proteins in vivo [31]. The more rigid structure of Tn5-derived hmLeuRS-B observed in fluorescence spectra might be the result of such processing. The relative high catalytic efficiency of hmLeuRS-B may be used to investigate the leucylation and mis-aminoacylation properties of mutant hmtRNA<sup>Leu</sup> associated with human mitochondrial diseases.

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