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Effect of Signal Peptides on Secretory Expression and Enzymatic Properties of Leucine Dehydrogenase in Bacillus subtilis: Postprint

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Abstract

Based on the N-terminal charge of signal peptides, signal peptides from both Sec and Tat pathways were selected to construct Bacillus subtilis shuttle plasmids, achieving for the first time the secretory expression of the Bacillus cereus-derived leucine dehydrogenase gene in Bacillus subtilis. The Tat pathway signal peptide PhoD demonstrated the best performance in promoting protein secretion, with extracellular enzyme activity reaching 20.25 U/ml, which is 2.2-fold higher than that without signal peptide addition. The higher N-terminal charge of the signal peptide may be beneficial for the secretion of multimeric proteins. The expressed product was purified and enzymatically characterized. The results showed that the specific activity of the purified enzyme was 13 U/mg; when L-Leucine was used as substrate, the Km of the enzyme was 6.17 mM and Vmax was 14.49 mol·min^{-1}·mg^{-1}; substrate specificity studies revealed that the enzyme exhibited the highest affinity for its natural substrate L-Leucine, showed activity toward some aliphatic amino acids, but was inactive toward the aromatic amino acid L-Phenylalanine; the optimal pH for the enzyme was 10.5–12, with a pH stability range of 5.0–11.0; the optimal reaction temperature was 55 °C; temperature-dependent circular dichroism scanning of changes in the enzyme' s secondary structure showed that the α-helix content gradually decreased with increasing temperature; differential scanning calorimetry (DSC) determined the enzyme' s unfolding temperature (Tm value) to be 64.13 °C, indicating that the enzyme possesses good thermostability.

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Full Text

Effect of Signal Peptides on the Secretory Expression of Leucine Dehydrogenase in *Bacillus subtilis* and Study of Its Enzymatic Properties

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Abstract

Based on the N-terminal charge number of signal peptides, we selected both Sec and Tat pathway signal peptides to construct shuttle plasmids for *Bacillus subtilis*, achieving for the first time the secretory expression of the leucine dehydrogenase gene from *Bacillus cereus* in *B. subtilis*. The Tat pathway signal peptide PhoD demonstrated the most effective protein secretion, with extracellular enzyme activity reaching 20.25 U/ml—2.2 times higher than that without a signal peptide. The higher N-terminal charge number of the signal peptide may facilitate the secretion of multimeric proteins. The expressed product was purified and its enzymatic properties were characterized. Results showed that the specific activity of the purified enzyme was 13 U/mg; the Km and Vmax values for L-leucine as substrate were 6.17 mM and 14.49 mol · min^{-1} · mg^{-1}, respectively. Substrate specificity studies revealed that the enzyme exhibited the highest affinity for its natural substrate L-leucine, showed activity toward some aliphatic amino acids, but was inactive toward the aromatic amino acid L-phenylalanine. The optimal pH was 10.5–12.0, with stability maintained in the range of 5.0–11.0. The optimal reaction temperature was 55°C. Circular dichroism temperature scanning revealed changes in the enzyme' s secondary structure, with α-helix content gradually decreasing as temperature increased. Differential scanning calorimetry (DSC) determined the enzyme' s unfolding temperature (Tm value) to be 64.13°C, indicating good thermostability.

Keywords: Signal peptides; Leucine dehydrogenase; *Bacillus subtilis*; Enzymatic properties

Leucine dehydrogenase (EC 1.4.1.9, LeuDH) utilizes NAD^+ as a coenzyme to reversibly catalyze the conversion of L-leucine and some branched-chain amino acids to their corresponding keto acids and analogs, and can also catalyze the reduction of α-keto acids to chiral amino acids [1]. Leucine dehydrogenase finds application in the biocatalytic synthesis of chiral amino acids for pharmaceutical intermediates [2] and in clinical biochemical diagnostics, where it can be coupled with urease to determine serum urea levels for auxiliary detection of kidney diseases [3] and for analyzing serum leucine aminopeptidase [4].

Currently, most studied leucine dehydrogenases originate from *Bacillus* species, typically forming homomultimers of 6–8 subunits. Researchers have constructed

genetically engineered strains using single-subunit genes. The leucine dehydrogenase from *Bacillus cereus* DSM626 expressed in *E. coli* showed crude enzyme activity of 16 U/mg, with active enzyme accounting for 28% of soluble protein [5]. The enzyme from *Bacillus cereus* ATCC14579 achieved high-level expression in *E. coli*, with recombinant crude enzyme specific activity of 7.59 U/mg [6]. The enzyme from *Bacillus sphaericus* IFO 3525 expressed in *E. coli* JM109 exhibited specific activity of 10.6 U/mg, 25 times higher than the original strain [7]. These studies have primarily focused on heterologous expression in *E. coli*. Given the pharmaceutical applications of leucine dehydrogenase, higher safety requirements necessitate the selection of safer expression hosts. Compared with *E. coli*, *B. subtilis* offers advantages including reduced inclusion body formation, direct secretion of expressed products into the extracellular medium, and convenient downstream separation and purification, providing a solid foundation for industrial production applications.

Appropriate signal peptide selection is an effective strategy for achieving efficient secretory expression of heterologous proteins in *B. subtilis*. Currently, due to limited understanding of the compatibility between signal peptides and target proteins, optimal signal peptides can only be obtained through extensive screening. The Sec and Tat pathways have been extensively studied in *B. subtilis*. Signal peptides for both pathways share similar structures: (1) a positively charged N-terminus, (2) a hydrophobic core H-region, and (3) a C-region containing signal peptidase recognition sites. The Tat pathway features a distinctive twin-arginine motif (SRRxΦΦ, where Φ represents hydrophobic residues and x represents any amino acid residue) [8]. Signal peptide structure and secretion pathway significantly affect protein secretion. Sagiya et al. [9] increased the N-terminal charge number to achieve tuna growth hormone (tGH) production of 240 mg/L, representing a 10-fold increase in yield. Gabriela Flores et al. [10] constructed a single-chain penicillin G acylase (PGA) from a heterodimer; functional single-chain variants were produced using Sec pathway leader peptides, while no active protein was obtained with Tat pathway peptides, demonstrating that signal peptide secretion pathway and structure are critical for optimal secretory expression of recombinant proteins.

In this study, we expressed the leucine dehydrogenase gene from *B. cereus* ATCC14579 in *B. subtilis* and selected four Sec pathway signal peptides (LipB, NprE, AmyQ, and SacB) and three Tat pathway signal peptides (LipA, YwbN, and PhoD) based on N-terminal charge number to construct secretory expression plasmids. We compared the effects of different signal peptides on leucine dehydrogenase secretion and characterized the enzymatic properties of the obtained enzyme, providing a research foundation for industrial production of leucine dehydrogenase from *B. cereus*.

1.1.1 Strains and Plasmids

E. coli JM109, *B. subtilis* 168, expression plasmids pMA5 and pHT43 were preserved in our laboratory. The leucine dehydrogenase gene (*leudh*) was syn-

thesized by GenScript (Nanjing).

1.1.2 Reagents

Restriction endonucleases, T4 DNA ligase, rTaq DNA polymerase, Protein Low Marker, and DNA Marker were purchased from TaKaRa (Dalian). DNA gel extraction kit was from Axygen. Gram-positive bacterial DNA extraction kit, peptone, and yeast extract were from Sangon Biotech (Shanghai). All other reagents were purchased from Sinopharm Chemical Reagent Co., Ltd.

1.1.3 Culture Media

LB medium (g/L): peptone 10 g, yeast extract 5 g, NaCl 10 g. TB medium (g/L): peptone 12 g, yeast extract 24 g, glycerol 5 g, $K_2HPO_4 \cdot 3H_2O$ 16.43 g, KH_2PO_4 2.3 g.

1.2.1 Construction of Recombinant Plasmid pMA5-leudh

Using the synthesized *leudh* gene as template, PCR was performed with primers F1/R1 (introducing a 6×His tag at the C-terminus) to incorporate BamHI/MluI restriction sites. Both the PCR product and plasmid pMA5 were double-digested, gel-purified, and ligated overnight with T4 DNA ligase to obtain recombinant plasmid pMA5-leudh. The ligation product was transformed into *E. coli* JM109, and positive transformants were sequenced by Suzhou Hongxun Biotechnology Co., Ltd.

1.2.2 Construction of Recombinant Plasmids with Different Signal Peptides

Using *B. subtilis* 168 genomic DNA as template (extracted according to the kit protocol), signal peptide sequences were amplified by PCR with designed primers incorporating NdeI/BamHI sites. The signal peptide sequences and pMA5-leudh were double-digested, gel-purified, ligated, and transformed into *E. coli* JM109. Positive transformants were verified by colony PCR and sequencing. The template for signal peptide AmyQ was plasmid pHT43.

1.2.3 Culture Conditions for Recombinant Strains

Recombinant plasmids were transformed into *B. subtilis* 168 using the method described in Dr. Xia Yu' s doctoral thesis from Jiangnan University [11]. Single colonies were inoculated into LB medium (100 g/ml kanamycin) and cultured overnight, then transferred to 50 ml TB medium (100 g/ml kanamycin) at 5% inoculum and cultured for 48 h. The culture was centrifuged at 4°C, 8000 rpm for 10 min, and the fermentation supernatant was collected.

Table 1 Primer sequences

Primer	Sequence
F1	CGCGGATCCATGACCCTGGAGATCTCCGAAT
R1	CGACGCGTTTAATGATGATGATGATGATGACGACGGCTAATGATATCGTGACCG
LipA-F	GGGAATTCCATATGAAATTTGTAAAAAGAAGGATC
LipA-R	CGCGGATCCGGCTTTTGCTGACGGCTG
SacB-F	GGGAATTCCATATGAACATCAAAAAGTTTGCAAAAC
SacB-R	CGCGGATCCCGCAAACGCTTGAGTTGCGCCT
AmyQ-F	GGGAATTCCATATGATTCAAAAACGAAAGCGGACAG
AmyQ-R	CGCGGATCCTACGGCTGATGTTTTTGTAATC
LipB-F	GGGAATTCCATATGGTGAAAAAAGTACTTATGGCAT
LipB-R	CGCGGATCCAGCTTTTGCGCCAGACGGCGGAG
NprE-F	GGGAATTCCATATGGGTTTAGGTAAGAAAT
NprE-R	CGCGGATCCACCTTCAGCAGCCTGAAC
PhoD-F	GGGAATTCCATATGGCATACGACAGTCGTTTTG
PhoD-R	CGCGGATCCGGCCCCAACCGACTGGGCAATC
YwbN-F	GGGAATTCCATATGAGCGATGAACAAAAAAAGCC
YwbN-R	CGCGGATCCCGCAACGGCTGCCCCCGCCAT

Note: Underlined sequences indicate restriction enzyme sites.

1.2.4 Activity Assay and SDS-PAGE Analysis of Recombinant LeuDH

The reaction system (3 ml) contained 20 mM L-leucine in 0.2 M glycine-KCl-KOH buffer (pH 10.5) with 12.5 mM NAD^+ solution. After equilibration at 37°C for 5 min, enzyme solution was added and mixed. A control without enzyme was prepared simultaneously, and absorbance changes at 340 nm were measured over 3 min. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 mol NADH per minute under these conditions. Recombinant enzyme was analyzed by SDS-PAGE for apparent molecular weight estimation, and protein concentration was determined by the Bradford method [12].

1.2.5 Purification of Recombinant LeuDH

Fermentation broth was centrifuged at 4°C, 8000 rpm for 30 min, and the supernatant was collected. The supernatant was filtered through a 0.45 m membrane and loaded onto a HisTrap HP 1 ml affinity chromatography column using an ÄKTA avant system.

1.2.6 Analysis of Enzymatic Properties

1.2.6.1 Determination of Kinetic Parameters

L-leucine solutions of varying concentrations (0.2–20 mM) were prepared in 0.2 M glycine-KCl-KOH buffer, and enzyme activities were measured at each

concentration. Km and Vmax values were calculated using the Lineweaver-Burk double reciprocal plot method.

1.2.6.2 Substrate Specificity Determination

Different substrates (L-leucine, L-valine, L-isoleucine, L-norvaline, L-methionine, L-phenylalanine, L-alanine) were dissolved at 20 mM in 0.2 M glycine-KCl-KOH buffer. Equal concentrations of enzyme were added, and relative activities were determined with L-leucine activity defined as 100%.

1.2.6.3 Optimum Temperature and Thermal Stability

Enzyme activity was measured across 30–85°C to determine the optimal reaction temperature. For thermal stability, enzyme solutions were incubated in 25 mM phosphate buffer at 30, 37, 40, 50, 55, 60, 65, and 70°C for 20 min, followed by residual activity measurement.

1.2.6.4 Optimum pH and pH Stability

L-leucine-glycine-KCl-KOH solutions were prepared at pH 8.0–12.0 for optimal pH determination. For pH stability, enzyme solutions were diluted with buffers of pH 4.0–12.0, incubated at 25°C for 16 h, and residual activity was measured.

1.2.6.5 Circular Dichroism Analysis of Secondary Structure

Enzyme solution (0.08 mg/ml in aqueous buffer) was subjected to temperature-dependent secondary structure analysis. Conditions: scanning wavelength 190–220 nm, 1 cm cuvette path length. Ellipticity ( ) was measured at 25, 45, 55, 65, and 85°C, and secondary structure was predicted using the online server http://dichroweb.cryst.bbk.ac.uk/html/process.shtml.

1.2.6.6 Determination of Tm Value by Nano DSC

Enzyme solution was dialyzed against PB buffer for 8 h to reduce salt concentration, then concentrated to 0.2–1 mg/ml by ultrafiltration. The Tm value of the purified enzyme was determined by Nano DSC, and data were analyzed and fitted using Launch NanoAnalyze software.

2.1 Expression and Identification of Recombinant LeuDH

Using the synthesized *leudh* gene from *B. cereus* ATCC14579 as template, PCR amplification yielded a product with size consistent with theoretical predictions. The target gene was cloned with pMA5 to construct expression vector pMA5-leudh, which was verified by double digestion (Figure 1 [Figure 1: see original paper]). The recombinant plasmid was transformed into the host to construct recombinant strain *B. subtilis* 168/pMA5-leudh, which showed extracellular activity of 9.04 U/ml after fermentation. SDS-PAGE analysis revealed the leucine dehydrogenase molecular weight to be approximately 44 kDa (Figure 2 [Figure 2: see original paper]).

2.2 Effect of Different Signal Peptides on LeuDH Secretory Expression

Based on different N-terminal positive charges, seven signal peptides from Sec and Tat pathways were selected for *B. subtilis* 168/pMA5-leudh. Among them, AmyQ, SacB, LipB, and NprE belong to the Sec pathway with N-terminal charges of 4, 3, 2, and 2, respectively; PhoD, YwbN, and LipA belong to the Tat pathway with N-terminal charges of 6, 5, and 4, respectively. Seven signal peptides were inserted upstream of the *ldh* gene via NdeI/BamHI sites on plasmid pMA5 to construct secretory expression plasmids, as shown in Figure 3 [Figure 3: see original paper].

Table 2 Comparison of LeuDH activity guided by different signal peptides

Results demonstrated that PhoD from the Tat pathway achieved the highest extracellular expression level with activity of 20.25 U/ml. In the Sec pathway, AmyQ and SacB showed better expression effects with extracellular activities of 16.80 U/ml and 14.09 U/ml, respectively. Overall, Tat pathway signal peptides outperformed Sec pathway peptides (Table 2). The Tat pathway is particularly suited for transporting folded proteins and multimeric proteins [13–14]. Since *B. cereus*-derived LeuDH forms homomultimers, its optimal secretion under PhoD guidance suggests the Tat pathway is more suitable for LeuDH secretory expression. Analysis of Sec pathway signal peptides revealed that LipB and NprE with N-terminal charges of 2 showed significantly lower secretion than SacB and AmyQ with charges of 3 and 4. In the Tat pathway, PhoD with a charge of 6 showed optimal secretion, while LipA with a charge of 4 performed poorly. Both pathways exhibited the same trend: higher N-terminal charge numbers correlated with higher protein secretion levels, demonstrating that N-terminal charge number influences protein secretion and that increased charge promotes secretion. Low secretion levels with signal peptides YwbN, LipA, LipB, and NprE may be attributed to altered structural domain arrangements in the peptides affecting mRNA transcription and translation, resulting in reduced activity. SDS-PAGE analysis of each recombinant strain is shown in Figure 4 [Figure 4: see original paper].

2.3 Purification of Recombinant LeuDH

Fermentation supernatant from the recombinant strain was centrifuged, filtered, and loaded onto a nickel affinity column. LeuDH began to elute at 350 mM imidazole concentration, with 428 mM being the optimal elution concentration. After purification, specific activity reached 13 U/mg, and SDS-PAGE yielded a single band (Figure 5 [Figure 5: see original paper]).

2.4.1 Determination of Kinetic Parameters

The Km value is a characteristic constant of enzymes that reflects enzyme properties and plays an important role in practical applications. The Km and Vmax

values of leucine dehydrogenase for substrate L-leucine were determined to be 6.17 mM and 14.49 mol·min^{-1}·mg^{-1}, respectively (Figure 6 [Figure 6: see original paper]). These Km values indicate that L-leucine is a suitable substrate.

2.4.2 Determination of Substrate Specificity

Based on previous reports [15], different substrates were selected for specificity analysis (Table 3). Results showed that leucine dehydrogenase exhibited activity toward various amino acids, with relatively high activity toward most aliphatic amino acids. The branched-chain structure of amino acids affected enzyme activity, while no activity was observed toward some aromatic amino acids.

2.4.3 Optimum pH and pH Stability

pH affects enzyme activity primarily by influencing enzyme structural stability and the ionization states of certain groups on both enzyme and substrate molecules. The optimal reaction pH and pH stability of the recombinant enzyme were investigated across pH 4.0–12.0. The enzyme showed optimal activity at pH >10.5 and maintained high activity in the range of pH 5.0–11.0, with activity decreasing significantly below pH 5.0 and above pH 11.0 (Figure 7 [Figure 7: see original paper]). This pH stability profile (pH 6.0–11.0 at 25°C for 16 h) is similar to that of a commercially available leucine dehydrogenase from Shanghai Lanyuan.

2.4.4 Optimum Temperature and Thermal Stability

Both low and high temperatures affect enzyme activity, with optimal activity occurring only at the ideal temperature. As shown in Figure 8 [Figure 8: see original paper], the optimal reaction temperature for the recombinant enzyme was 55°C, above which enzyme denaturation caused activity decline. This represents a significant improvement in thermostability compared with the same enzyme expressed in *E. coli*, which had an optimal temperature of 37°C [6]. When enzyme solution was incubated at 30-70°C for 20 min, over 80% residual activity was retained at 60°C, demonstrating excellent stability.

2.4.5 Secondary Structure Changes

Temperature-dependent secondary structure analysis of LeuDH (Figure 9 [Figure 9: see original paper]) showed intact absorption peaks at lower temperatures. At 85°C, the absorption peaks were severely disrupted, with most of the enzyme in an unfolded state and α-helix content gradually decreasing to only 11.4% (Table 4), indicating that intact α-helical structure contributes to enzyme stability. The significant fluctuations in β-sheet and turn content at 65°C may result from dissociation of homomultimers, exposing additional structural elements.

2.4.6 Determination of Tm Value

The Tm value represents the temperature at which half of the DNA double helix unwinds and is an important parameter for primers. Nano DSC determined the Tm value to be 64.13°C with ΔH = 877.5 kJ/mol, displaying a single peak for the homomultimeric protein (Figure 10 [Figure 10: see original paper]). Compared with a commercial *Bacillus sp.* LeuDH from TOYOBO with thermal stability below 60°C, this enzyme demonstrates superior thermostability.

3 Conclusion

The leucine dehydrogenase gene from *B. cereus* was successfully transformed into *B. subtilis* 168, achieving the first expression of LeuDH in *B. subtilis*. To further improve extracellular LeuDH activity, seven signal peptides from Sec and Tat pathways were selected based on N-terminal charge number. In the Sec pathway, signal peptides AmyQ, SacB, LipB, and NprE with N-terminal charges of 4, 3, 2, and 2 showed extracellular activities of 16.80 U/ml, 14.09 U/ml, 0.23 U/ml, and 0.13 U/ml, respectively. In the Tat pathway, signal peptides PhoD, YwbN, and LipA with charges of 6, 5, and 4 showed extracellular activities of 20.25 U/ml, 1.91 U/ml, and 0.78 U/ml, respectively.

Both pathways demonstrated the same trend: higher N-terminal charge numbers resulted in higher protein secretion levels, confirming that N-terminal charge number influences protein secretion and that increased charge promotes secretion. PhoD is currently the only signal peptide that strictly follows the Tat pathway for secretory expression [16], and it showed the best secretion efficiency and activity. The Tat pathway may be more suitable for LeuDH secretory expression. Signal peptide screening and optimization enhanced the secretion efficiency and expression activity of LeuDH in *B. subtilis*.

Enzymatic characterization revealed a Km of 6.17 mM and Vmax of 14.49 mol $\cdot$ $min^{-1} \cdot mg^{-1}$ for L-leucine, indicating good substrate affinity. The enzyme exhibited broad substrate specificity, showing activity toward some aliphatic amino acids in addition to its natural substrate L-leucine. The optimal pH was 10.5–12.0 with stability maintained at pH 5.0–11.0, demonstrating good pH tolerance. The optimal reaction temperature was 55°C, with over 80% residual activity retained after 20 min at 60°C, indicating good thermal stability. Circular dichroism temperature scanning of secondary structural changes showed that intact α-helical structure contributes to enzyme stability. The Tm value of 64.13°C further confirmed high thermostability, providing a valuable reference for industrial production of leucine dehydrogenase.

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