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Molecular cloning and expression of the *Streptomyces* coniferyl alcohol dehydrogenase gene in *Escherichia coli*

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ABSTRACT

Coniferyl alcohol dehydrogenase (CADH) is a key enzyme in catabolism of lignin-related aromatic compounds in bacteria. In *Streptomyces* sp. NL15-2K, CADH is a tetramer of identical subunits with an individual molecular mass of 39 kDa. This work describes the cloning and sequencing of the CADH gene from *Streptomyces* sp. NL15-2K, optimization of a protocol for high-level active CADH expression, and purification of recombinant CADH. A BLAST search and motif analyses of the predicted CADH amino acid sequence indicated the enzyme belongs to the medium-chain zinc-dependent alcohol dehydrogenase group. Cell density at heat-shock treatment, temperatures for heat shock and culture, duration of heat shock, concentration of isopropyl-β-D-thiogalactopyranoside (IPTG) as an inducer, and culture time after induction were adjusted for optimal CADH expression. Expression of active CADH under optimized conditions was approximately 4-fold higher than in the absence of heat shock. CADH purified from recombinant *Escherichia coli* was in the tetrameric form, as was natural CADH from NL15-2K.

Introduction

Lignin is a cross-linked phenylpropanoid polymer and is the second-most abundant component in plants, after cellulose. Partial lignin decay yields numerous aromatic compounds with applications in the cosmetics, food, pharmaceutical, and chemical industries. Lignin is also a potentially important source of biomass for the production of chemicals traditionally derived from petroleum. Lignin degradation has been most extensively studied in basidiomycetes, in which a number of enzymes and mechanisms involved in lignin attack have been identified [1]. Bacterial degradation pathways for lignin-related aromatic compounds have been characterized in *Pseudomonas* [2–4], *Sphingomonas* [5], *Acinetobacter* [6], *Corynebacterium* [7], and *Streptomyces* [8–10]. In these bacteria, most compounds are degraded to protocatechuic acid (3, 4-dihydroxybenzoate) or catechol (1, 2-dihydroxybenzene), then broken down further via specific ring cleavage pathways. My coworkers and I previously isolated *Streptomyces* sp. NL15-2K, which degrades coniferyl alcohol (4-hydroxy-3-methoxycinnamyl alcohol) via coniferyl aldehyde (4-hydroxy-3-methoxycinnamyl aldehyde), ferulic acid (4-hydroxy-3-methoxycinnamate), vanillin (4-hydroxy-3-methoxybenzaldehyde), vanillic acid (4-hydroxy-3-methoxybenzoate), and protocatechuic acid [11]. Coniferyl alcohol dehydrogenase (EC 1.1.1.194) catalyzes the oxidation of coniferyl alcohol to coniferyl aldehyde. In the previous study, we purified and characterized 2 isozymes (CADH I and II)[1] with coniferyl alcohol dehydrogenase activity from *Streptomyces* sp. NL15-2K [12]. Substrate specificity studies indicated that CADH I and II are a cinnamyl alcohol dehydrogenase and a coniferyl alcohol dehydrogenase, respectively. To date, only one publication has described the gene sequence and basic enzymatic characteristics of CADH in *Pseudomonas* sp. strain HR199 [13]. This enzyme is a homodimer with a molecular mass of 27 kDa per subunit, optimum pH 10.9, and 42 °C optimum temperature [13]. CADH from *Streptomyces* sp. NL15-2K is a tetramer with a molecular mass of 39 kDa per subunit, optimum pH 8.5, and 40 °C optimum temperature [12]. Therefore, *Streptomyces* CADH is functionally and structurally different from the *Pseudomonas* enzyme. In this study, I describe the molecular characterization and heterologous expression of the CADH gene from *Streptomyces* sp. NL15-2K.

The second purpose of this study was to develop a simple protocol for gene expression of aggregation-prone CADH. Since CADH purified from NL15-2K tends to aggregate and precipitate in solution, it is difficult to store at 4 °C for longer than a few weeks while maintaining enzyme activity. The enzyme also aggregates when thawing from frozen storage. Therefore, overexpression of CADH was expected to produce inclusion bodies. In fact, a preliminary

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[1] *Abbreviations used:* CADH, coniferyl alcohol dehydrogenase; IPTG, isopropyl-β-D-thiogalactopyranoside; MALDI-TOF-MS, matrix-assisted laser-desorption ionization time-of-flight mass spectrometry; MD-FDH, mycothiol-dependent formaldehyde dehydrogenase; MDR, medium-chain dehydrogenase/reductase; OD, optical density; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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experiment suggested the existence of insoluble CADH precipitates in a cell lysate of recombinant *E. coli*. Generally, most such problems are due to misfolding or aggregation of the expressed protein, a problem for which many solutions have been applied: (1) protein refolding after dissolving inclusion bodies with denaturing reagents, (2) expression as a fusion protein to enhance solubility and/or folding, and (3) induction at low temperature. Other reports suggest exposure of *E. coli* to heat stress prior to protein induction enhanced the solubility of recombinant proteins [14,15]. In these cases, native heat-shock proteins, induced by heat stress, are thought to facilitate proper protein folding, enhancing solubility. In this study, I also investigated the solution of inclusion body formation by heat-shock treatment of recombinant *E. coli* carrying the *Streptomyces* CADH gene.

Materials and methods

Bacterial strains, vectors, and cultivation media

Streptomyces sp. NL15-2K, a CADH producer [12], was used for isolation of chromosomal DNA. The spores, formed on YEME medium [16] supplemented with 1.5% agar, were inoculated and cultured in YEME medium supplemented with 17% sucrose and 0.5% glycine at 30 °C. Chromosomal DNA was extracted according to Hopwood et al. [16]. *E. coli* XL-1 Blue MRA (P2) and the lambda FIX II vector (Agilent Technologies) were used to construct a genomic library. *E. coli* DH5α and plasmids pUC19 and pET-28a(+) (Novagen) were used for subcloning and sequencing. *E. coli* BL21(DE3) and pET-28a(+) were used for protein expression. All *E. coli* strains were cultured in Luria broth or on Luria agar supplemented with appropriate antibiotics when necessary.

Cloning and sequencing of CADH gene

Streptomyces sp. NL15-2K genomic DNA was partially digested with *Sau*3AI and separated by electrophoresis on 0.6% agarose gel. DNA fragments from 9 to 23 kb were purified using the QIAEX II Gel Extraction Kit (Qiagen) and a partial fill-in reaction was performed. The DNA fragments were purified by phenol–chloroform extraction and ethanol precipitation, followed by ligation to the lambda FIX II vector. *In vitro* packaging was performed using the Gigapack III Gold packaging extract (Stratagene) to generate the genomic library.

A previous study revealed that the N-terminal amino acid sequence of NL15-2K CADH (AQEVRGVIAPGKDEPVRM) shares over 94% similarity with 4 oxidoreductases from *Streptomyces* spp. [12]. PCR primers were designed based on an internal consensus amino acid sequence, VAAGQCTKVD, and the N-terminal sequence of CADH: 5′-GTC(G/C)ACCTTGGTGCACTGICCGGC(G/C)GCGAC-3′ (where I is deoxyinosine) and 5′-GGIGTIATCGCICCIGGIAAGGACGAGCCIGT-3′. These were used for probe preparation. PCR was carried out using NL15-2K genomic DNA as a template under the following conditions: denaturing for 3 min at 96 °C, 30 cycles of 1 min at 95 °C, 1 min at 60 °C, and 1 min at 72 °C, with final extension at 72 °C for 10 min. The PCR products were separated by electrophoresis on 1.2% agarose gel. The approximately 450-bp fragment was recovered from the gel using the MinElute Gel Extraction Kit (Qiagen) and used as a probe. A genomic library of *Streptomyces* sp. NL15-2K was screened by the plaque hybridization technique. The Hybond-N+ membrane (GE Healthcare) was used for hybridization. Probe labeling, hybridization, and detection were performed with the Gene Images AlkPhos Direct Labeling and Detection System (GE Healthcare) according to the manufacturer protocol. After the phage DNA was isolated from a positive plaque, an approximately 4.1-kb DNA fragment derived from *Streptomyces* sp. NL15-2K was recovered by digestion with *Kpn*I and subcloned into the same site of pUC19 to generate pUC19/CADH. DNA sequencing was performed using an ABI Prism 310 Genetic Analyzer, 3730 DNA Analyzer (Applied Biosystems), and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The open reading frames (ORFs) were predicted using FramePlot ver.2.3.2 [17]. Homology searches were conducted with the BLAST algorithm [18].

Expression of CADH gene

The coding sequence of the CADH gene was amplified by PCR from pUC19/CADH using a sense primer, 5′-AACCATGGCGCAGGAAGTACGCGGCG-3′ (*Nco*I site underlined) and an antisense primer, 5′-AACTCGAGTCACAGCACCACCACCGAGCG-3′ (*Xho*I site underlined). The sense primer was designed to introduce an "ATG" initiation codon (boldfaced) for CADH expression. The amplified gene was digested with *Nco*I/*Xho*I, purified by agarose gel electrophoresis, and inserted into the same sites of pET-28a(+) to yield pET28a/CADH. After the nucleotide sequence of the CADH gene was verified, pET28a/CADH was transformed into *E. coli* BL21(DE3), and the resulting recombinant strain was used for CADH expression.

E. coli BL21(DE3) harboring pET28a/CADH was cultured in Luria broth containing 1% glucose and kanamycin (30 μL/mL) at 37 °C. Overnight cultures were diluted 1:100 into fresh LB broth supplemented with kanamycin and incubated at 30 °C with shaking at 100 rpm to optical density (OD_{600}) 0.8. Cultures of this stage were optimized for soluble expression of CADH. For heat-shock experiments, the culture (10 mL in a 50-mL flask) was pre-incubated at temperatures ranging from 42 °C to 51 °C for 5 min with shaking at 100 rpm, followed by addition of 1 mM IPTG. After the culture was maintained at the same temperature for 20 min, the flask was transferred to a shaker at 30 °C and incubated for 8 h at 100 rpm. To optimize the duration of the heat-shock treatment, the 20 min incubation time after IPTG addition was replaced by a period ranging from 5 to 60 min. The concentrations of IPTG, culture temperature, and time were optimized. The production of recombinant CADH for enzyme purification was performed under optimal conditions, except the culture scale was changed from 10 to 250 mL.

CADH activity

CADH activity was determined by measuring the amount of coniferyl aldehyde formed from coniferyl alcohol substrate [12]. The formation of coniferyl aldehyde was monitored spectrophotometrically at 400 nm. One unit was defined as the amount of enzyme that led to the formation of 1 μmol coniferyl aldehyde per min under assay conditions. An absorption coefficient of 20.7 mM^{-1} cm^{-1} at 400 nm was used for coniferyl aldehyde. The specific activity was expressed as units per mg of protein, where the protein concentration was determined using a Bio-Rad Protein Assay kit (Bio-Rad) with bovine serum albumin as a standard. To determine the kinetic parameters of the purified CADH, initial velocities at various substrate concentrations were determined by the same assay procedures. The K_m and k_{cat} values were calculated from a Hanes–Woolf plot [19].

High-performance liquid chromatography (HPLC)

CADH activity in cell-free extracts was also confirmed by HPLC analysis. The reaction mixture (0.3 mL) containing 0.1 M Tris–HCl (pH 8.5), 1.5 mM coniferyl alcohol, 2.5 mM NAD^+, and a cell-free extract from *E. coli* cells harboring pET-28a vector or pET28a/CADH was incubated at 30 °C for 30 min. After incubation, the mixture