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A novel L-leucine 5-hydroxylase from *Nostoc piscinale* unravels unexpected sulfoxidation activity toward L-methionine

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ABSTRACT

Hydroxy amino acids are produced by Fe(II)/αKG-dependent dioxygenases and used widely as medicinal intermediates for chemical synthesis. A novel L-leucine 5-hydroxylase gene from *Nostoc piscinale* (NpLDO) was cloned into pET28a (+), pColdI and pQE-80 L plasmids. Using a two-step purification process (Ni-affinity chromatography and gel filtration), highly purified recombinant NpLDO was obtained. Recombinant NpLDO displayed unexpectedly high sulfoxidation activity toward L-methionine. The reaction products were analyzed by high-performance liquid chromatography. Sequence alignment analysis implied that residues of His150, His236 and Asp152 constitute the catalytic triad of NpLDO, which is completely conserved in the Fe(II)/αKG-dependent dioxygenase superfamily. Biochemical data showed that NpLDO catalyzed regio- and stereoselective hydroxylation of L-leucine and sulfoxidation of L-methionine with Fe(II) and L-ascorbic acid as cofactor, and αKG as cosubstrate, respectively.

1. Introduction

Hydroxylation is a growing research field in biotechnology and molecular biology. Understanding the hydroxylation of free L-amino acids facilitates the discovery of novel pharmaceutical intermediates by biosynthetic processes, e.g., 4-hydroxyisoleucine has physiological activity with pharmacological significance, including antifungal, antibacterial, antiviral and anticancer properties [1]. In the process of hydroxylation, amino acids are usually converted into hydroxy amino acids [2], and because they have several chiral carbons asymmetric synthesis of optically active compounds is feasible [3]. For example, (2*S*, 4R)-4-hydroxyproline is an important intermediate to synthesize chiral pharmaceuticals and chiral antibiotics [4], (2*S*, 3R, 4*S*)-4-hydroxyisoleucine is a promising compound for drugs and functional foods [5] and L-*threo*-β-hydroxyleucine is a promising target material for the preparation of some cyclic depsipeptides [6], which possess useful physiological activities. Thus, hydroxy amino acids possess various physiological activities that could be biosynthesized by dioxygenase rather than chemical synthesis processes, which use more extreme environmentally unfriendly and costly conditions, such as high temperature and atmospheric pressure.

Ferrous iron [Fe(II)] and α-ketoglutarate (αKG)-dependent dioxygenases (Fe/αKG-DOs) are the largest known subgroup of mononuclear non-heme iron enzymes. DOs-mediated hydroxylation also requires dioxygen, where one of the oxygen atoms is incorporated into the substrate to form the hydroxy amino acid while the other oxygen atom is used to oxidize α-KG to form succinate [7]. Fe/αKG-DOs are distributed widely in bacteria, fungi, plants, and vertebrates [7]. These enzymes feature a conserved HXD/EXnH motif that coordinates a Fe(II) center at the active site, called the "facial triad" [8]. DOs catalyze a variety of reactions via C–H bond activation, including hydroxylation, dealkylation, desaturation, epoxidation, epimerisation, halogenation, cyclisation, peroxide formation and ring expansion/contraction reactions [9–12]. Some of these reactions are involved in the biosynthetic pathway of secondary metabolites, such as flavonoids, β-lactam antibiotics, alkaloids and gibberellins. DOs are highly desirable as hydroxylation biocatalysts because they have the advantages of catalyzing reactions in a regio- and stereoselective manner. Commercial applications of dioxygenases began with plant growth retardants and has now extend to a clinically used pharmaceutical compound for

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cardioprotection [13].

The hydroxylation of free L-amino acids is usually catalyzed by specific Fe/αKG-DOs. Several Fe/αKG-DOs have been reported to catalyze the hydroxylation of free amino acids, such as L-isoleucine-4-hydroxylase (IDO) [14], L-asparagine 3-hydroxylase (AsnO) [15], L-arginine 3-hydroxylase (VioC) and L-proline cis-4-hydroxylase (cis-P4H) [16,17]. A L-leucine 5-hydroxylase (LdoA) was reported to catalyze regio- and stereoselective hydroxylation of L-leucine and L-norleucine to (2*S*,4*S*)-5-hydroxyleucine and (2*S*)-5-hydroxynorleucine, respectively [18]. Although many Fe/αKG-DOs have been identified, the substrate recognition mechanism catalyzed by L-Leucine 5-hydroxylase remains unknown [2,18]. *Nostoc piscinale* strain has been reported to have biodegradation potential in the waste of motor oil and could be used in bioconversion of androst-4-en-3 [19,20]. We found that it contained a putative gene from the result of Basic Local Alignment Search Tool (BLAST) search, which shares 72% similarity with LdoA, which was reported to be a novel L-Leucine 5-hydroxylase [18]. Therefore, we presumed that NpLDO was also an L-leucine 5-hydroxylase. NpLDO should be a promising biocatalyst for effective production of bioactive peptides required for industry [14].

In this study, we identified a novel L-leucine 5-hydroxylase from *Nostoc piscinale* which showed both hydroxy and sulfoxidation activities toward L-leucine and L-methionine, respectively (Fig. 1). Research on amino acid hydroxylase enzymes continues to significant enormous commercial interest and our findings provide a new NpLDO enzyme that has potential commercial use for the production of chiral hydroxy amino acids in the future.

2. Materials and methods

2.1. Vector construction and expression of NpLDO

The NpLDO gene (GenBank accession No. WP_062292570.1) was cloned and ligated into the pET28a (+) (Novagen, Madison, WI, USA), pColdI (TaKaRa, Osaka, Japan) and pQE-80 L (Qiagen, Hilden, Germany) vectors between *Bam*H I and *Hin*d III sites with a His_6 tag (HHHHHH) at the N-terminus. The three recombinant plasmids harboring the NpLDO sequence were transformed to *E. coli* BL21 (DE3) cells respectively and were grown in lysogeny broth (LB) at 37 °C. Cells were grown to an OD_{600} value of 0.8 and isopropyl β-D-1-thiogalactopyranoside (IPTG) was then added to a final concentration of 0.5 mM to induce protein overexpression. The cultures were incubated at 16 °C overnight. The cells were harvested by centrifugation at 4000 g and 4 °C for 15 min, and were then resuspended in lysis buffer [20 mM Tris-HCl, pH 8.0, 20 mM imidazole, 0.5 M NaCl and 1 mM dithiothreitol (DTT)]. The suspension was disrupted by sonication using the parameters 1 s pulse, 1 s output and 50% duty cycle for 30 min. The samples were cooled on ice during cell disruption.

2.2. Purification of NpLDO protein

The cell debris was removed by centrifugation at 40,000 g and 4 °C for 30 min. The supernatant was loaded onto a column that had been pre-equilibrated with 20 mL lysis buffer. The His-tag target protein was then trapped on 2 mL of Ni-NTA superflow resin (Qiagen, Hilden, Germany). After washing the resin with buffer (20 mM Tris-HCl, pH 8.0, 40 mM imidazole, 0.5 M NaCl and 1 mM DTT), NpLDO was eluted with 15 mL of elution buffer (20 mM Tris-HCl, pH 8.0, 500 mM imidazole, 0.5 M NaCl and 1 mM DTT). The eluted fractions containing NpLDO were pooled and concentrated by ultrafiltration (10 kDa MWCO, Millipore, Billerica, MA, USA) at 4 °C, and the target protein was further purified by gel filtration using a Superdex 200 HR 10/300 column (GE Healthcare, Uppsala, Sweden) equilibrated with 50 mM Tris–HCl buffer (pH 8.0) containing 100 mM NaCl. Fractions containing recombinant NpLDO were collected. The purified protein was concentrated by ultrafiltration, and used for activity assays.

2.3. Analysis of grayscale value of protein bands

The concentration of the purified protein was determined by the BCA assay, according to the manufacturer's protocol. The NpLDO expression in each vector was analyzed by SDS-PAGE, followed by gel imaging using a gel documentation system with Image Lab Software (Bio-Rad, Hercules, CA, USA). The quantification of target proteins was determined by calculating the relative band intensity in gray-scale images of the proteins.

2.4. CD measurement

Circular dichroism (CD) spectra (190–250 nm) were recorded using a MOS-450 CD spectropolarimeter (Biologic, Claix, Charente, France) with a 1 mm path-length cell at room temperature. The CD spectra were obtained as the average of four scans with a bandwidth of 0.1 nm, a step resolution of 0.1 nm and a scan rate of 1 nm/s. The CD spectrum of NpLDO (0.1 mg/mL) was recorded in 20 mM Tris-HCl (pH 8.0), 0.1 M NaCl and 1 mM DTT. The protein secondary structure was analyzed by the CDSSTR analysis package to generate secondary structure assignments for the NpLDO [16].

2.5. Product analysis using HPLC

For product analysis, 2 μM of purified protein in a 100 μL reaction

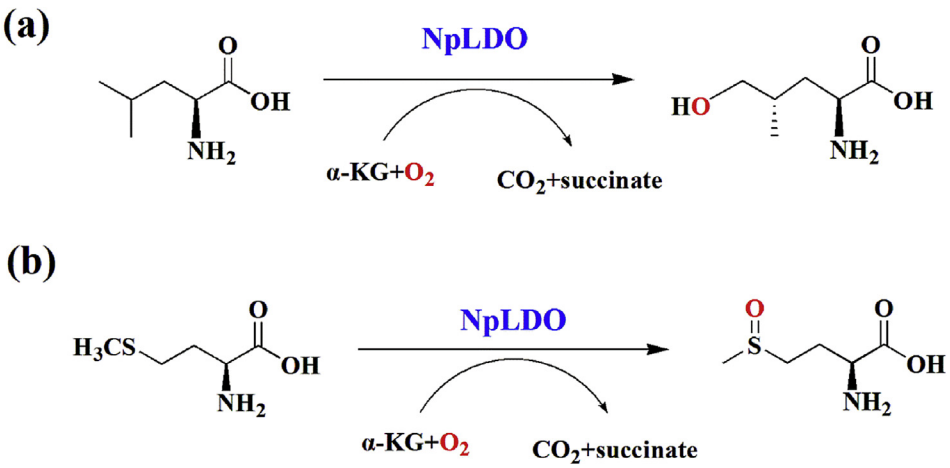

Fig. 1. Enzymatic reaction scheme for the NpLDO hydroxylation of L-leucine and L-methionine.