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# Molecular cloning, expression, and functional analysis of the copper amine oxidase gene in the endophytic fungus *Shiraia* sp. Slf14 from *Huperzia serrata*

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ABSTRACT

Huperzine A (HupA) is a drug used for the treatment of Alzheimer's disease. However, the biosynthesis of this medicinally important compound is not well understood. The HupA biosynthetic pathway is thought to be initiated by the decarboxylation of lysine to form cadaverine, which is then converted to 5-aminopentanal by copper amine oxidase (CAO). In this study, we cloned and expressed an SsCAO gene from a HupA-producing endophytic fungus, *Shiraia* sp. Slf14. Analysis of the deduced protein amino acid sequence showed that it contained the Asp catalytic base, conserved motif Asn-Tyr-Asp/Glu, and three copper-binding histidines. The cDNA of SsCAO was amplified and expressed in *Escherichia coli* BL21(DE3), from which a 76 kDa protein was obtained. The activity of this enzyme was tested, which provided more information about the SsCAO gene in the endophytic fungus. Gas Chromatograph-Mass Spectrometry (GC-MS) revealed that this SsCAO could accept cadaverine as a substrate to produce 5-aminopentanal, the precursor of HupA. Phylogenetic tree analysis indicated that the SsCAO from *Shiraia* sp. Slf14 was closely related to *Stemphylium lycopersici* CAO. This is the first report on the cloning and expression of a CAO gene from HupA-producing endophytic fungi. Functional characterization of this enzyme provides new insights into the biosynthesis of the HupA an anti-Alzheimer's drug.



## 1. Introduction

*Huperzia serrata* (Thunb. ex Murray) Trev., also known as Qian Ceng Ta, is a pteridophyte that belongs to the family Huperziaceae. *Huperzia serrata* has been traditionally used in China to treat a number of ailments, such as schizophrenia, myasthenia gravis, contusions, and swellings [1]. Huperzine A (HupA) is a sesquiterpene alkaloid isolated from *H. serrata*. It is a highly active acetylcholinesterase (AChE) inhibitor, and is a valuable therapeutic option for the treatment of Alzheimer's disease (AD). Alzheimer's disease is a brain disorder characterized by a progressive dementia that occurs in middle or late life. The pathologic characteristics are degeneration of specific nerve cells, and the presence of neuritic plaques and neurofibrillary tangles [2]. Today, over 46 million people live with dementia worldwide and this number is estimated to increase to 131.5 million by 2050 [3]. HupA has a low toxicity, is highly selective, shows reversibility, and has a long duration time compared to other drugs used to treat AD [4]. Furthermore, HupA has also shown anti-inflammatory activity and seems to be effective in the treatment of cerebrovascular type dementia and benign senescent forgetfulness [5,6].

At present, the HupA found in herbal supplements and medicine is mainly extracted from Chinese club moss *H. serrata* and some other species in the Huperziaceae. However, the amount of HupA in *H. serrata* is very small (ca. 0.007%) and *H. serrata* has a limited distribution and a slow growth rate. Furthermore, the complex extraction process from plants and the high costs of downstream

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purification of HupA has impeded its use. Consequently, large quantities of *H. serrata* are required in order to yield practical, usable amounts of HupA. Therefore, new ways to mass produce HupA need to be found in order to protect plant resources from overharvesting and to reduce the cost of HupA-containing medicine. Chemical synthesis of HupA has been attempted by many research groups, but the synthesized HupA was a racemic mixture, which showed much less potent activity than natural HupA. Alternatively, researchers have found that some endophytic fungi in *H. serrata* can produce HupA. However, production of HupA by these endophytes is hindered by the low yields and the loss of biosynthetic capability after several generations. It is thus desirable to overexpress the biosynthetic enzymes for HupA in a heterologous host if stable and efficient production is to be achieved [7–9]. The biosynthetic genes for HupA need to be investigated and the enzymes involved in HupA production need to be identified. Although the biosynthesis of HupA remains poorly understood, various studies revealed that it is initiated by the decarboxylation of lysine to generate cadaverine, with the subsequent formation of 5-aminopentanal and $\triangle^1$-piperideine [10]. Conversion of cadaverine to $\triangle^1$-piperideine is thought to be catalyzed by a copper amine oxidase (CAO) [10] (Fig. 1).

CAOs (primary-amine: oxygen oxidoreductase; EC 1.4.3.21) are found in both eukaryotes and prokaryotes, including plants, mammals, bacteria, yeasts, and fungi. They catalyze the oxidative deamination of primary amines to the corresponding aldehydes and reduce molecular oxygen to hydrogen peroxide [11–13]. CAOs are homodimers. The molecular mass of each subunit is about 70–90 kDa and they contain both $Cu^{2+}$ and 2,4,5-trihydoxyphenylalanine quinone, or topaquinone (TPQ) as cofactors at the active site of each subunit [14–16]. Furthermore, CAOs play important roles in various physiological processes [17–19].

Sun et al. [10] cloned CAO genes in *H. serrata* and revealed some CAO characteristics during the biosynthesis of the lycopodium alkaloids in *H. serrata*. However, there are no reports on CAOs from microorganisms that are involved in the biosynthesis of HupA [20]. We report the cloning and expression of a CAO gene (SsCAO) from the endophytic fungus *Shiraia* sp. Slf14, which has been isolated from *H. serrata*. This fungal strain is capable of producing HupA [7,8]. The enzyme was expressed in *Escherichia coli* and functionally characterized. This work should enable the further characterization of the HupA biosynthetic pathway in this endophytic fungus.

## 2. Materials and methods

### 2.1. Chemicals, strains and plasmids

*Shiraia* sp. Slf14 was isolated from *H. serrata* that was found in Lushan Botanical Garden, Jiangxi Province, China. *E. coli* DH5α and *E. coli* BL21(DE3) were purchased from TIANGEN BIOTECH Co., Ltd. (Beijing, China). The pMD®19-T (simple), Ex Taq, T4 DNA Ligase, QuickCut™ *Nco*I, *Xho*I, *Nde*I, DL2000 DNA marker, protein molecular weight marker (Low), RNAiso Plus, and PrimeScript™ Reagent Kit with gDNA Eraser were obtained from TAKARA BIOTECH Co., Ltd. (Dalian, China). The E.Z.N.A cycle-pure kit, Plasmid Mini Kit I, and the Gel Extraction Kit were from OMEGA, and HisTrapTM FF was obtained from GE Healthcare. The hydrogen Peroxide Assay kit was purchased from Nanjing Jiancheng Bioengineering Institute. All other chemicals were purchased from Sigma.

### 2.2. RNA isolation and cDNA synthesis

Mycelia from *Shiraia* sp. Slf14, which had been cultured for 5 days, were collected and immediately ground with a sterile mortar in liquid nitrogen. Total RNA was extracted from the mycelia using RNAiso Plus (TaKaRa) and was reverse transcribed using the PrimeScript™ Reagent Kit with the gDNA Eraser (TaKaRa). The obtained cDNA was used as the template for subsequent PCR reactions.

### 2.3. Cloning the gene encoding copper amine oxidase

The SsCAO gene in *Shiraia* sp. Slf14 was predicted based on the complete genome sequence of *Shiraia* sp. Slf14 (GenBank No. AXZN00000000) and conserved sequences of known CAOs. RT-PCR

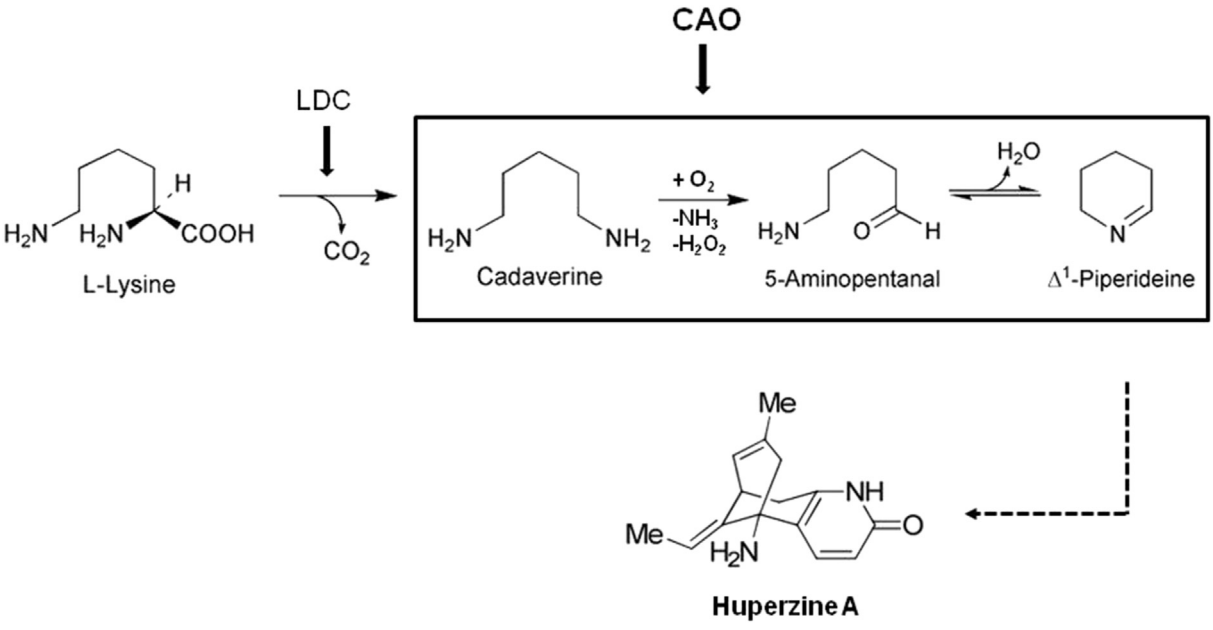


**Fig. 1.** Proposed biosynthetic pathway for Hup A. The steps from lysine to △1-piperideine are shown.