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Biosynthesis of Cerebroside by *Termitomyces clypeatus* Using Serine as a Precursor

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Abstract

Cerebrosides A and B (A-B) are sphingolipids with high neuro protective activities. The present study aimed to validate the role of serine as a precursor for the biosynthesis of cerebrosides A-B by *Termitomyces clypeatus* CTM-1. Mycelium culture of *T. clypeatus* CTM-1 was carried out on a rotary shaker, using media with different serine doses in the range of 0 to 1.0 g/L. At the serine dose of 0.75 g/L, the cerebroside a content of mycelium reached a maximum of (0.30 ± 0.02)%, while the cerebroside B content increased to (0.10 ± 0.01)% in 9 days. The cerebroside levels were about 36% and 11% higher than those at a serine dose of 0, respectively. It was clear that a moderate level of serine in mycelium-culture medium might be used as a precursor to enhance significantly the biosynthesis of cerebrosides A-B.

Keywords: Bioactive compounds; Cerebroside; *Termitomyces clypeatus*; Mycelium; Biosynthesis; Precursor

Introduction

Cerebrosides A and B (A-B) are novel bioactive sphingolipids recently found in the edible mushroom *Termitomyces albuminosus*. As shown in Figure 1, both cerebrosides A-B are glucosylceramides (i.e., a glucose links with a ceramide in each), and have a unique C19 hydroxylated sphingosine base with branching around the middle. On the other hand, cerebroside A possesses a C16 α-hydroxy fatty acid, while cerebroside B possesses a C18 α-hydroxy fatty acid [1]. It is demonstrated that cerebrosides A-B have high neuro protective activities. Cerebrosides A-B can open large-conductance Ca^{2+} -activated K^{+} channels [2]. A treatment with cerebrosides A-B for middle cerebral artery occlusion reduces the cerebral infarction dose-dependently, and it for global cerebral ischemia significantly attenuates the death of pyramidal cells in the hippocampal CA1 area [3,4,10]. Furthermore, the administration of cerebrosides A-B can significantly relieve the pain reactions induced by heat and chemical stimuli [5].

Wild mushroom *T. albuminosus* is rear throughout the world. In addition, it is difficult to be cultivated artificially due to its symbiotic relationship with termites. In our previous work, a stain *T. clypeatus* CTM-1 (CCTCC NO: M2014185) was developed by means of tissue isolation, purification and screening, using the edible mushroom *T. clypeatus* as raw material [6]. Submerged culture of *T. clypeatus* CTM-1 on a rotary shaker at 150 rpm and 28°C for 7 days yielded 5.08 g/L of biomass, and the mycelium contained 0.15% of cerebroside A and 0.07% of cerebroside B on dry basis. It suggests that the submerged culture of *T. clypeatus* CTM-1 is an effective way to produce the ingredient of functional foods and even cerebrosides A-B.

In microorganisms, cerebrosides are synthesized via a series of enzyme-catalyzed reactions using serine as the precursor [7,8]. In these reactions, enzymes such as serine-palmitoyltransferase (+PLP), 3-ketosphinganine reductase (+NADPH), dihydroceramide desaturase, cermide glucosyltransferase and glucosylceramidase are involved, while 3-ketosphinganine, D-erythro-dihyedrcermide and ceramide are produced as intermediates. On the other hand, cerebrosides form cell- and species-specific profiles at the cell surfaces that characteristically change in development, differentiation. These features of cerebroside biosynthesis pathway provide a theoretical base for the Precursor-Directed Biosynthesis (PDB) of cerebrosides A-B by *T. clypeatus* CTM-1. The present study aimed to validate serine's role as a precursor for the biosynthesis of cerebrosides A-B by *T. clypeatus* CTM-1. Mycelium culture of *T. clypeatus* CTM-1 was carried out on a rotary shaker, using media with different serine doses. The effects of serine on mycelium growth and cerebroside biosynthesis were investigated.

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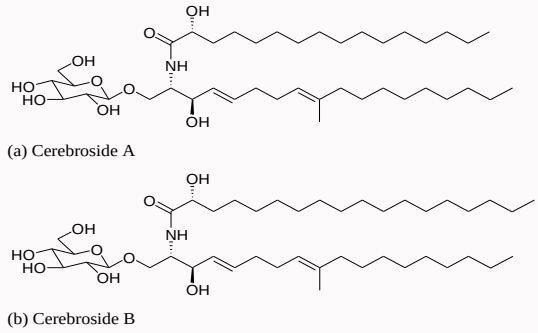

Figure 1: Structures of cerebrosides A and B [1].

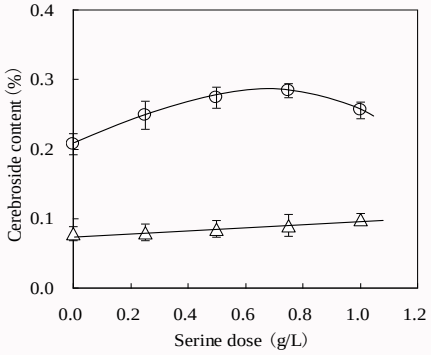

Figure 2: Change of the cerebroside content of mycelium with serine dose in 7-d mycelium culture (○ Cerebroside A; Δ Cerebroside B).

Materials and Methods

Strain and inoculum preparation

The strain *T. clypeatus* CTM-1 was maintained on slants of PDAY solid medium at 4°C and transferred monthly. The PDAY solid medium was composed of (g/L): potato extract 15, glucose 20, agar 20, and yeast extract 2. Its pH was adjusted to 5.0 with 1 M citric acid solution.

For inoculum preparation, three pieces of strain colony (with a diameter of about 0.5 cm^2 each) were precultured in a 500 mL Erlenmeyer flask containing 200 mL of seed-culture medium. The flask was kept on a rotary shaker at 28°C and 150 rpm for 3 days. The seed-culture medium was composed of (g/L): glucose 20, peptone 10, $MgSO_4$ 0.75, KH_2PO_4 1.5. It was adjusted to pH 5.0 with 1 M citric acid and sterilized at 115°C for 30 min.

Mycelium culture

Mycelium-culture media were prepared by adding 0.25, 0.50, 0.75 and 1.00 g/L of serine respectively to a basal mycelium-culture medium. The basal mycelium-culture medium was composed of (g/L): glucose 27, peptone 11, $MgSO_4$ 0.75, KH_2PO_4 1.5, vitamin B_1 0.05 and vitamin B_6 0.05. It had an optimal carbon/nitrogen ratio for the mycelium growth of *T. clypeatus* CTM-1 [9]. In order to maintain the optimal carbon/nitrogen ratio, peptone in the basal mycelium-culture medium was reduced by the equivalent nitrogen amount of added serine. The mycelium-culture media were adjusted to pH 5.0 with 1 M citric acid and sterilized at 115°C for 30 min.

Mycelium culture was carried out with a 500-mL Erlenmeyer flask containing 200 mL of a mycelium-culture medium. 20 mL of prepared inoculum was transferred into the flask to start the culture. The flask was kept on a rotary shaker at 28°C and 150 rpm.

Measurement of biomass

At a certain time of mycelium culture, mycelium was collected by filtration, and dried at 105°C for 4 hr. Biomass was defined as the mass of dry mycelium per liter of culture medium.

Analysis of cerebrosides A-B within mycelium

The cerebrosides A-B contained in mycelium were quantitated using a LC-MS/MS system (liquid chromatography: LC-10AD, Shimadzu Corporation, Japan; mass spectrograph: API 3000, AB Sciex Pte. Ltd., USA) [4]. The cerebrosides A-B were extracted with a dichloromethane-methanol solution overnight at the room temperature after grinding and ultrasonic treatments. Cerebroside A and Cerebroside B preparations with purity of 99.5% each [10-12] were used as the reference materials. The cerebroside content and the cerebroside B content of mycelium was expressed as the mass percentage of cerebroside A and cerebroside B in dry mycelium, respectively.

Analysis of serine within mycelium and culture broth

For mycelium, 0.5 g of each sample was ground with mortar and pestle, and then mixed with 5 mL deionized water. The serine was extracted with the assistance of ultrasonic wave at 60°C for 40 hr. After centrifugation (10 min at 5,000 rpm and 4°C), the supernatant was adjusted to 50 mL with deionized water and used as test solution. For culture broth, each sample was centrifuged, and the supernatant was used as test solution.

Serine in the test solution was quantitated with an amino acid analyzer (L-8800, Hitachi, Ltd., Japan) coupled with a 2622-type cation-exchange column (46 mm × 4.6 mm). The test solution were filtered through Sep Pak C18 filters (Millipore) before injected into the analyzer. A standard solution containing 18 amino acids (Merck Chemicals (Shanghai) Co., Ltd., China) was used as the reference material. The serine content of mycelium was expressed as the mass percentage of serine in dry mycelium, and the serine concentration of culture broth was expressed as the grams of serine per liter of culture broth.

Analysis of glucose within culture broth

Ten milliliter of culture broth was centrifuged for 10 min at 5,000 rpm and 4°C. Glucose contained in the supernatant was analyzed using a bio-sensing system (SBA-40E) Biology Institute, Shandong Academy of Sciences, China). Glucose concentration was expressed as the grams of glucose per liter of culture broth.

Statistical analysis

Each mycelium culture and chemical analysis was performed in triplicate. The result was expressed as mean ± Standard Deviation (SD).

Results and Discussion

Effect of serine on cerebroside biosynthesis

In order to investigate the effect of serine on cerebroside biosynthesis, mycelium culture was carried out for 7 days using the mycelium-culture media. The cerebroside content of mycelium changed with serine dose as shown in Figure 2. At the serine dose of 0 (i.e. no serine was added to the basal mycelium-culture medium), the cerebroside A content was (0.21 ± 0.02)%, and the cerebroside B content was (0.08 ± 0.01)%. The cerebroside content increased with increasing serine dose in the range of 0 to 0.75 g/L, and decreased

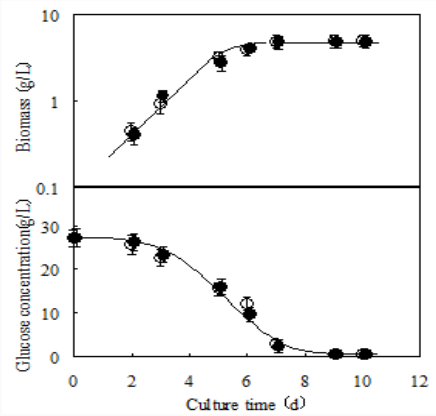

Figure 3: Mycelium growth and glucose consumption during mycelium culture (○ at the serine dose of 0; ● at the serine dose of 0.75 g/L).

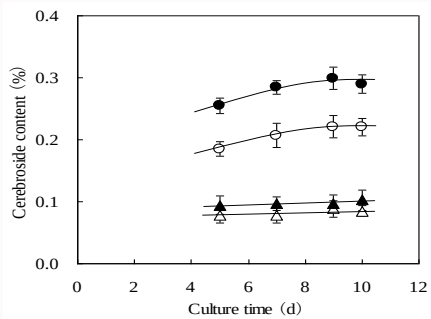

Figure 4: Change of the cerebroside content of mycelium during mycelium culture (○ Cerebroside A at the serine dose of 0; △ Cerebroside B at the serine dose of 0; ● Cerebroside A at the serine dose of 0.75 g/L; ▲ Cerebroside B at the serine dose of 0.75 g/L).

with increasing serine dose in the range of 0.75 to 1.0 g/L. The cerebroside B content increased linearly with increasing serine dose in the range of 0 to 1.00 g/L. At the serine dose of 0.75 g/L, the cerebroside A content reached a maximal value of (0.28 ± 0.01)%, and the cerebroside B content increased to (0.09 ± 0.02)%. It was obvious that in *T. clypeatus* CTM-1, the synthesis of cerebroside A was more advanced than that of cerebroside B, and a moderate level of serine in mycelium-culture medium might be used as a precursor to enhance significantly the synthesis of cerebrosides A-B.

Precursor-Directed Biosynthesis (PDB) is a common method for producing uncommon and unusual derivatives, since a proper level of precursors in culture broth may get into the enzymatic process, thus leading to an improvement in the metabolisms of organisms [13-15]. For instance, in the Ubiquinone-10 (CoQ10) production by *Pseudomonas diminuta* NCIM 2865, carrot and tomato juice enhanced CoQ10 yield from 15.58 mg/L to 29.22 mg/L and 24.35 mg/L, respectively. The reason was that carrot and tomato provided the precursors for synthesizing CoQ10 and carotenoids [16]. Feeding some organic acids as precursors to *Saccharothrix algeriensis* NRRL B-24137 resulted in the directed biosynthesis of new dithiolopyrrolone analogs. When cinnamic-acid concentration rose from 0 to 1.25 mM, benzoyl-pyrrothine production was increased significantly from 0 to 15.4 mg/g-DCW. However, a cinnamic-acid concentration higher than 5 mM sharply reduced the production [14].

Mycelium growth during the culture

Figure 3 shows mycelium growth and glucose consumption

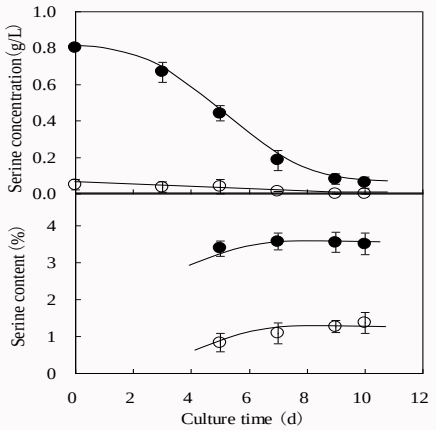

Figure 5: Changes of the serine concentration of culture broth and the serine content of mycelium during mycelium culture (○ at the serine dose of 0; ● At the serine dose of 0.75 g/L).

during mycelium culture. Serine dose in mycelium-culture media was 0 and 0.75 g/L, respectively. At the two serine doses, mycelium had the same growth curve, including an exponential phase in the period of 2 d to 6 d, and a stationary phase after that. In addition, glucose at the two serine doses was also consumed similarly. The glucose concentration decreased fast in the period of 2 d to 7 d, and depleted at 10 d. In the stationary growth phase, glucose was utilized for maintaining the growth and the metabolic activities of mycelium. This phenomenon was also observed at other serine doses in the range of 0 to 1.00 g/L. Biomass was almost stable at 4.89 ± 0.78 g/L after the mycelium culture for 7 d.

Cerebroside biosynthesis during the culture

Figure 4 shows the change of the cerebroside content of mycelium during mycelium culture at the serine dose of 0 and 0.75 g/L. Cerebrosides A-B were synthesized not only in the exponential growth phase, but also in the stationary growth phase (during the period of 6 d to 10 d). At the serine dose of 0.75 g/L, the cerebroside A content reached a maximum of (0.30 ± 0.02)%, while the cerebroside B content increased to (0.10 ± 0.01)% in 9 days. The cerebroside levels were about 36% and 11% higher than those at a serine dose of 0, respectively. In wild mushroom *T. clypeatus*, the stipe contains 0.27% of cerebroside A and 0.09% of cerebroside B, and the cap contains 0.25% of cerebroside A and 0.08% of cerebroside B [4]. The cerebroside levels in the mycelium produced by the PDB method were comparable to those in the wild mushroom.

In order to understand how serine was used for the biosynthesis of cerebrosides A-B, the changes of the serine concentration of culture broth and the serine content of mycelium during mycelium culture were investigated. As shown in Figure 5, the culture broth had an initial serine concentration of 0.05 g/L and 0.80 g/L at the serine dose of 0 and 0.75 g/L, respectively. During mycelium culture, the serine concentration decreased in close correspondence to the glucose consumption (Figure 3), indicating that all nutrients within the culture broth were effectively utilized. Correspondingly, a large difference in the serine content of mycelium was observed when serine dose varied. In the period of 5 d to 10 d, the serine content increased from (0.83 ± 0.25)% to (1.37 ± 0.29)% at the serine dose of 0 g/L, while it increased from (3.39 ± 0.20)% to (3.53 ± 0.30)% at the serine dose of 0.75 g/L. It was obvious that *T. clypeatus* CTM-1 had the abilities of utilizing serine in culture media and producing serine *in vivo*, and

a high serine level in mycelium contributed to the improvement of cerebroside biosynthesis. Using 0.75 g/L of serine in culture medium led to high levels of serine (Figure 5) and cerebrosides A-B (Figure 4) in mycelium, because the enzymatic process of *T. clypeatus* CTM-1 was stimulated and its metabolism was improved [13-15].

In general, strain improvement and process control are two main approaches for enhancing the biosynthesis of chemical compounds. Comparing with the strain improvement, the process control is more time-saving and direct for the cultivation process [17]. In our previous study, pH, temperature and culture-medium composition were optimized for the mycelium growth of *T. clypeatus* CTM-1 [6,9]. The present study indicated for the first time the effective use of serine as a precursor to enhance the biosynthesis of cerebrosides A-B by *T. clypeatus* CTM-1.

Conclusion

Mycelium culture of *T. clypeatus* CTM-1 was carried out using media with different serine doses in the range of 0 to 1.0 g/L, and the effects of serine on mycelium growth and cerebroside biosynthesis were investigated. The experimental results could be concluded as follows:

(1) Serine affected significantly the content of cerebrosides A-B in mycelium. On the other hand, biomass in the culture did not change with serine dose.

(2) At the serine dose of 0.75 g/L, the cerebroside A content of mycelium reached a maximum of (0.30 ± 0.02)%, while the cerebroside B content increased to (0.10 ± 0.01)% in 9 days. The cerebroside levels were about 36 % and 11% higher than those at a serine dose of 0, respectively. Cerebrosides A-B was synthesized not only in the exponential growth phase, but also in the stationary grow phase.

(3) *T. clypeatus* CTM-1 had the abilities of utilizing serine in culture media and producing serine *in vivo*. A high serine level in mycelium contributed to the improvement of cerebroside biosynthesis.

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