

A Novel Biocatalyst and Its Optimized Process for Preparing L-theanine

LI Jia-you^{1*} GUO Li-yun² JIAO Qing-cai²

(1 College of Biological and Chemical Engineering, Jiaxing University, Jiaxing 314001, China)

(2 State Key Laboratory of Pharmaceutical Biotechnology, Nanjing University, Nanjing 210093, China)

Abstract L-theanine (γ -glutamylethylamide) is the main free amino acid component of tea and its favorable physiological effects on mammals have been reported. An enzymatic method for optically pure L-theanine production with a new L-aminoacylases-production fungi *Cunninghamella echinulata* 9980 was developed. The optimum conditions for enzymatic catalysis were at pH 6.5 with 50 mmol/L N-Acyl-DL-theanine and 40 mmol/L CoCl_2 . After 12-h incubation at 50°C, 22.5 mmol/L L-theanine was obtained, the conversion rate against N-Acyl-L-theanine being 90%. *Cunninghamella echinulata* and the aminoacylase were applied in preparation of L-theanine.

Key words L-theanine N-Acyl-DL-Theanine *Cunninghamella echinulata* L-aminoacylase Optical resolution

L-theanine (γ -glutamylethylamide), which was first isolated and identified in the late 1940s, is the main free amino acid component of tea. Regarding the physiological functions of L-theanine, many researchers have reported significant reduction in blood pressure in spontaneously hypertensive rats after some amounts of tea extract were ingested^[1] and inhibition of the convulsive action^[2] and spontaneous activity^[3] caused by caffeine administration. It has also been shown that the oral intake of L-theanine causes a feeling of relaxation in human volunteers^[4] and is effective against on alcoholic liver injury in mice^[5]. Its enhancing effects on the antitumor activity of adriamycin (doxorubicin) has also been reported^[6~8]. L-theanine has been used as a new food additive.

Methods for the synthesis of L-theanine were developed. Chemical methods had been reported largely before 1993^[9]. In the last decade enzymatic methods were favorable in researchers and manufacturers. 120 mmol/L

theanine was obtained after 200 mmol/L Gln and 1.5 methylamine were incubated with bacterial gamma-glutamyltranspeptidase (GGT) for 2 h^[10], then an improved reaction mixture containing 200 mmol/L sodium glutamate, 1.2 M ethylamine, 300 mmol/L glucose, 50 mmol/L potassium phosphate buffer (pH 7.0), 5 mmol/L MnCl_2 , 5 mmol/L AMP, 100 units/ml glutamine synthetase (GS), and 60 mg/ml yeast cells was developed with approximately 170 mmol/L theanine synthesized in 48 h^[11]. Aminoacylases (AC, N-acyl-L-amino acid amidohydrolase, E.C. 3.5.1.14), a readily available and inexpensive enzyme are mainly used in the industrial production of enantiopure L-amino acids from their N-acetyl derivatives. In light of this and due to the commercial importance of L-theanine, a great deal of L-aminoacylase-producing microorganisms were tested. The strain called *Cunninghamella echinulata* 9980 displayed the highest intracellular L-aminoacylase activity without D-aminoacylase activity. It is also the first time that aminoacylases, regardless of its source, were applied in DL-

theanine resolution.

1 Materials and methods

1.1 Chemicals

All reagents were analytical grade while DL-theanine and N - acyl - DL - theanine were synthesized in our laboratory.

1.2 The acylation of DL-theanine

N-acetyl-DL-theanine was prepared by acylation of DL-theanine with acetic anhydride in an alkaline aqueous solution. Thus, 16.5 ml of acetic anhydride was added dropwise to the aqueous solution, containing 0.1 mol of DL-theanine at pH 8~9 and 4°C. The mixture was stirred for about 1.5h and then the pH value was adjusted to 2~3. The reaction system was evaporated to dryness under vacuum, desalted with absolute ethyl alcohol, then evaporated alcohol again and added acetone late. The precipitated product was filtered and dried. The yields were in the range of 80%~85% (w/w).

1.3 Microorganism

Cunninghamella echinulata 9980 used as the microbial source of the aminoacylase was isolated from a soil sample. 5% (v/v) cell suspension of 2d culture (28°C) was used to inoculate. Culture medium containing per 1: 20 g of glucose, 10 g of polypeptone, 1 g of yeast extract, 2 g of N-acetyl-DL-theanine, 2 g of K_2HPO_4 , 0.5 g of $MgSO_4$. The pH of the medium was adjusted to 6.5~7.0, tation was carried out at 28°C with 100 ml medium in a 250 ml Erlenmeyer flask. After 36h, the pellets of mycelium were harvested by filtration and washed by distilled water twice, then were kept for use.

1.4 Test of aminoacylase activity

The aminoacylases activity of a defined amount of resting cells was measured as the L-theanine production (μmol) with 1 g wet mycelium in the initial one hour. Standard substrate solution (100 ml) contained 10mmol/L N-acyl-DL-theanine. The pH of the biocatalytic system was 7.0. The enzymatic reaction was carried out in a batch reactor provided with an overhead mechanical stirrer at 40°C stably. In test the factor investigated was in various levels while others were kept in set level. The reaction mixture was then centrifuged and the supernatant was

analysed for L-theanine produced.

1.5 Measurement of L-theanine

The concentration of L-theanine was measured with spectrophotometry. A mixture of 1ml centrifugal supernatant of biocatalytic system, 1ml of acetate buffer (pH 5.4) and 1ml of ethanolic ninhydrin solutions was incubated at 100°C for 15 min. After reaction, the absorption spectrum of 570 nm was obtained to find out the relationship of Abs and concentration. The concentration of L-theanine were calculated from the calibration curve obtained by this method.

2 Results

2.1 Effects of carbon and nitrogen resources on aminoacylase activity

Growth and metabolism of microorganisms are affected by culture conditions. *Cunninghamella echinulata* 9980 was cultivated with different carbon and nitrogen resource to investigated the fluctuation of aminoacylase activity. The result showed in Fig. 1a and Fig. 1b indicated that glucose was the best carbon source and polypeptone was the best nitrogen source in the substrates.

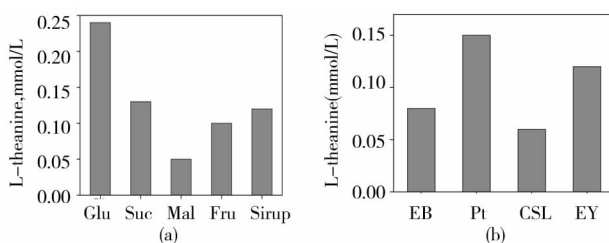


Fig. 1 Effects of carbon sources and nitrogen sources on aminoacylase activity

(a) Carbon sources Glu; glucose; Suc; sucrose; Mal; maltose; Fru; fructose (b) Nitrogen sources EB; extract of beef; Pt; polypeptone; CSL; corn slurry liquid; EY; extract of yeast

2.2 Effect of temperature and pH on aminoacylase activity of resting cells

Enzyme activity of biocatalysis can be affected by environmental factors, of which the temperature and pH value are usually considered in study and industrial process^[12].

For investigation on the effect of temperature on aminoacylase, resting cells were incubated under

biocatalytic assay conditions as described above, but with varying temperature from 30°C to 60°C. Fig. 2a describes the effect of temperature on the activity of aminoacylase in *Cunninghamella echinulata* 9980 resting cells. A temperature of 50°C was found to be optimal with the maximum activity for N-acyl-L-theanine to convert into L-theanine. For the study of thermostability, the activities of aminoacylase in *Cunninghamella echinulata* 9980 resting cells, which were incubated for 6h at different temperatures, were investigated. The enzymatic special activity was assumed as 100% when the resting cells was incubated for 0h. From Fig. 2b, the optimal temperature for producing L-theanine industrially was 40°C, because the enzyme of ADI in resting cells operated for a 6h incubation at 50°C and 60°C deactivated remarkably.

To optimize the pH condition, reactions were tested in a range of mixture solutions between pH 4.0 and 9.0. The stability of ADI was studied after the resting cells had been incubated for 24h in various pH, and the enzymatic special activity was assumed as 100% if the resting cell was not incubated before test. The results obtained were shown in Fig. 2c and Fig. 2d. The aminoacylase reaction gave a maximum yield and stability at pH 7.0.

Above data show that the optimal temperature for bioconversion will usually be lower than the point at which enzymatic activity is maximum because of the protein denaturation of heat, and enzyme is stablest and most effective at the same pH value.

2.3 Effect of metal ions on aminoacylase activity in resting cells

Metal ions are necessary to some enzymes^[13]. Aminoacylase was reported to exhibit metal dependence^[14]. We investigated the effect of various metal ions (Co^{2+} , Mn^{2+} , Mg^{2+} , Zn^{2+}) with concentrations from 0 to 80mmol/L on the production of L-theanine by aminoacylase in *Cunninghamella echinulata* 9980. In experiments, solutions of metal ions were added directly to the reaction mixtures. Co^{2+} at the concentration of 40mmol/L resulted in an increase in aminoacylase activity remarkably (Fig. 3). Mn^{2+} could promote the catalysis triflingly at a low concentration. Enzymatic activity was not affected notably at the tested concentrations of Zn^{2+} and

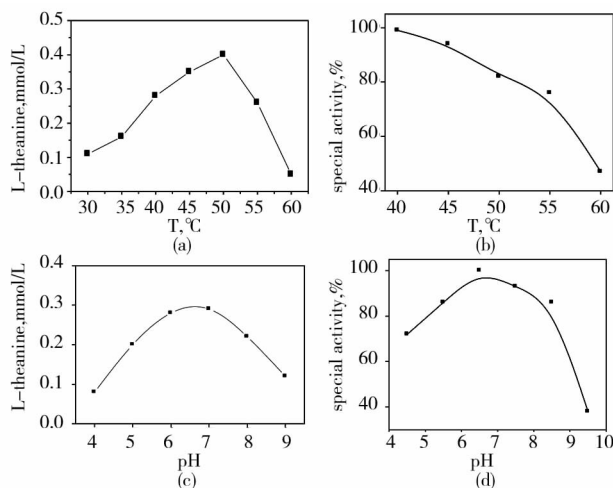


Fig. 2 Determination of optimal conditions for production of L-theanine by *Cunninghamella echinulata* 9980 resting cells

(a) Temperature (b) Thermostability (c) pH (d) Stability of pH

Mg^{2+} .

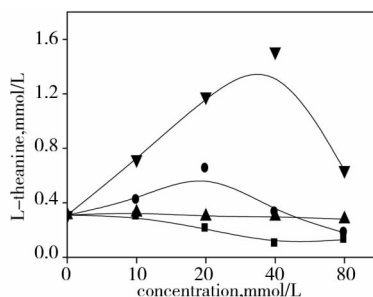


Fig. 3 Effect of addition of metal ions on aminoacylase activity in *Cunninghamella echinulata* 9980 resting cells

—▲— Mg^{2+} ; —▼— Co^{2+} ; —■— Zn^{2+} ; —●— Mn^{2+}

2.4 Effect of substrate and product concentration on aminoacylase activity in *Cunninghamella echinulata* 9980

Since substrate inhibition may be an important limitation in the development of an industrial process, we investigated the effects of initial concentration of N-acyl-L-theanine on the conversion to L-theanine by *Cunninghamella echinulata* 9980 resting cells. Fig. 4a shows that the production of L-theanine was changed with the concentration of substrate, in which the conversional rate was highest at 50mmol/L. When the concentration of substrate was up to 90mmol/L, the inhibition of substrate was observably.

Product inhibition is normal and usual in the biocatalytic assays. To disclose the detail in the bioconversion of N-acyl-L-theanine by aminoacylase, the effect of addition of L-theanine to the reaction mixture was tested. The production of L-theanine was measured in each case (by subtraction of the added amount from the final amount). A clear trend of decreasing L-theanine production was observed when the concentration of L-theanine added was increased (Fig. 4b). In fact the product of L-theanine is removed from the reactor immediately to decrease the negative effect of product inhibition on the enzymatic catalysis.

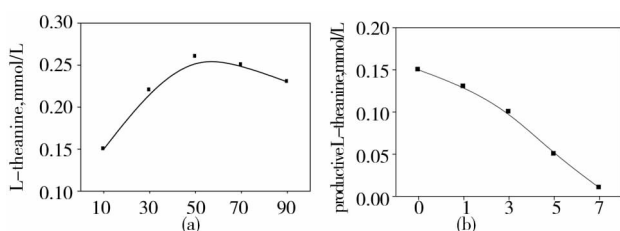


Fig. 4 Effect of initial substrate concentrations and amount of additional L-theanine on aminoacylase activity in *Cunninghamella echinulata* 9980 resting cells

(a) Concentration of N-acyl-L-theanine, mmol/L

(b) Additional L-theanine, mmol/L

3 Conclusion and discussion

Chemical synthesis of theanine is simple and industrial manufacture. But theanine produced by chemical methods is apt to become optically inactive racemic mixtures of L- and D- isomers. L-theanine acts physiologically while D-theanine does not work. Aminoacylase is widely developed in various racemic amino acids. The present study has established a basis for developing an industrial process to produce optically pure L-theanine using *Cunninghamella echinulata* 9980, a new L-aminoacylase-producing fungi. The method of coupling with chemical and enzymatic synthesis in preparing L-theanine seems not to have been established. The valuable methods mentioned previously involved the enzymes extracted from cells. In our process the use of free cells avoids enzyme extraction and purification, which are expensive and time-

consuming steps. Operation in an Erlenmeyer flask gives good results. It is therefore possible to anticipate the advantages, which will arise from scaling up.

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L-茶氨酸新型酶法制备及其催化工艺优化

李加友^{1*} 郭丽芸² 焦庆才²

(1 嘉兴学院生物与化学工程学院 嘉兴 314001)

(2 南京大学医药生物技术国家重点实验室 南京 210093)

摘要 L-茶氨酸是茶叶中游离氨基酸的主要组成部分,关于其良好的生理活性已有广泛报道。首次报道了来源于 *Cunnighamella echinulata* 9980 的 L-氨基酰化酶用于高光学纯度的 L-茶氨酸的酶法制备。该酶在 pH6.5,底物 N-乙酰-DL-茶氨酸浓度为 50mmol/L,且有 40mmol/L CoCl₂ 时催化效果较好。结果表明,在上述条件下,50℃ 作用 12h 得 L-茶氨酸 22.5mmol/L,转化率 90%。

关键词 L-茶氨酸 N-乙酰-DL-茶氨酸 *Cunnighamella echinulata* L-氨基酰化酶 光学拆分

中图分类号 Q55