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ORIGINAL ARTICLE

Biosynthesis of ^{15}NL-phenylalanine by phenylalanine ammonia-lyase from *Rhodotorula glutinis*

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Abstract Catalyzed by phenylalanine ammonia-lyase from *Rhodotorula glutinis*, 2% *trans*-cinnamic acid and 0.5 mol/l $(^{15}NH_4)_2SO_4$ was bioconverted to ^{15}NL-phenylalanine. The yield and the purity of ^{15}NL-phenylalanine reached 71 and 99.3%, respectively. The results showed that 96% of ^{15}N was labeled on the L-phenylalanine and 88% of $(^{15}NH_4)_2SO_4$ was recovered. The present paper provides a new and economic way for biosynthesis of ^{15}NL-phenylalanine.

Keywords ^{15}NL-phenylalanine · Phenylalanine ammonia-lyase · $(^{15}NH_4)_2SO_4$ · *Rhodotorula glutinis*

Introduction

^{15}N, a stable isotope of nitrogen, is a safe tracer and has been used widely in scientific research, such as organic chemistry, biochemistry, pharmacy, and agricultural science. Hachey (1994) used ^{15}N as a tracer to detect the nutrient dynamics during pregnancy and lactation period. In 1995, Yudkoff (Marc and Itzhak 1995) studied the metabolic rate in neonates by ^{15}N and ^{13}C to find protein synthesis pathway.

It is well known that protein synthesis research is essential in life science, in which ^{15}N amino acids play a critical role. Therefore, the ^{15}N amino acids production is prospective and has potential economic values. With the scientists' efforts, some ^{15}N labeled amino acids have been prepared, such as ^{15}N-glycin, ^{15}N-lysine, ^{15}N-alanine and ^{15}N-leucine (Rulin 1986); however, the synthesis of ^{15}NL-phenylalanne has not been reported by now, some isotope labeled L-phenylalanines were prepared by chemical synthesis or bioconversion (LeMaster and Cronan 1982; Tachibana and Ando 1983; Kenjiro et al. 1984) as the substitute, such as L-[3,4-$^{13}C_2$]phenyl-[1-^{13}C]alanine, L-phenyl-[2,3,3-$^{2}H_3$]alanine, D-phenyl-[2,3-$^{2}H_2$]alanine.

There are some factors to be considered in the route selection of ^{15}NL-phenylalanne synthesis, including ^{15}N source availability, labeled products' purity, synthetic efficiency, preparation cost and product yield. In addition, the unconverted ^{15}N sources should be recovered easily. There are four methods to produce L-phenylalanine, i.e., extraction, chemical synthesis, fermentation and enzymatic biotransformation. Among the four preparation methods, the enzymatic-catalyzed process has become more popular in preparation of labeled L-phenylalanine. The reaction catalyzed by phenylalanine ammonia-lyase (PAL) is an effective and economic route to label L-phenylalanine. In this route, the ^{15}N source, $(^{15}NH_4)_2SO_4$, is easily accessible, commercially available and easy to be recovered. In addition, the L-phenylalanine can be synthesized stereoselectively by PAL.

In this work, $(^{15}NH_4)_2SO_4$ and t-Ca were catalyzed to synthesize ^{15}NL-phenylalanne by PAL from the cell of *Rhodotorula glutinis* (Fig. 1), then the cell was removed and the t-Ca was acidified, filtrated and separated from the supernatant containing ^{15}NL-phenylalanine. ^{15}NL-phenylalanne was isolated by adsorption resin and then

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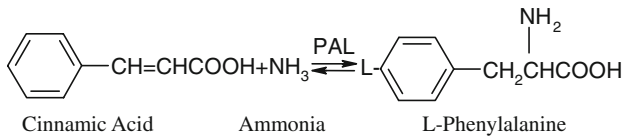

Fig. 1 Biosynthesis of L-phenylalanine by PAL

crystallized to obtain final product. The unconverted $(^{15}NH_4)_2SO_4$ was recovered by a specific apparatus.

Method and materials

Chemicals

$(^{15}NH_4)_2SO_4$ was provided by Shanghai Research Institute of Chemical Industry. L-Phe with biochemical-grade and all other chemicals with analytical-grade were purchased from Beijing Chemical Reagent Company. Yeast extract, beef peptone and glucose were provided by Beijing Shuangxuan Microbial Production Company (Beijing, China).

Microorganism

The yeast, *R. glutinis* AS2.102, obtained from China General Microbiological Culture Collection Center (CGMCC), and was used as a microbial source of PAL in this work.

Culture media

Stock medium contained (g/l): malt extract 10 °Be, agar 20.

The medium was sterilized for 30 min at 121°C.

Seed medium contained (g/l): yeast extract 10, beef peptone10, glucose 5, NaCl 5, K_2HPO_4 1.

PAL induction medium contained (g/l): yeast extract10, beef peptone10, glucose 5, NaCl 5, K_2HPO_4 1, L-Phe 0.5.

Both of the two media were sterilized for 20 min at 121°C.

Assay of PAL activity

PAL activity unit (U) here is defined as the amount of enzyme required to biotransform 1 μmole L-Phe to t-Ca per minute at 30°C. The PAL specific activity is therefore expressed as the number of U in per milligram of dry cell (U/mg). The activity is assayed by the Hodgins' (1968) method. Substrate solution containing 2.5 ml of 50 mmol/l L-Phe, 0.5 ml of 0.05% cetylpyridinium chloride and 1.75 ml of 25 mmol/l Tris–HCl buffer (pH 8.8) are mixed with 0.25 ml of cell suspension. After 10 min of reaction at 30°C, the reaction mixture is centrifuged and the t-Ca concentration in the supernatant is determined at 278 nm with a UNICO (Shanghai, China) UV-2000 spectrophotometer.

Analyses of L-Phe

L-Phe was measured with a method based on reversed-phase HPLC (HITACHI, Japan), using a precolumn derivatization technique with a gradient program. The column used was a C_{18} (250 × 4.6 mm, 5 μm, Diamodsil^{TM}), and the temperature was 30°C. The peaks were detected with an ultraviolet (UV) detector at 360 nm and processed using the data processor of the system.

Cultivation of cell containing PAL by fermentation

The cells on the slant stock medium were cultivated for 24 h at 30°C, and were inoculated into 50 ml seed medium in a 250 Erlenmeyer flask and cultivated on a 150 rpm reciprocal shaker for 24 h at 30°C. Then, 5 ml seed culture was inoculated into a 250 Erlenmeyer flask with 50 ml PAL induction medium and cultivated on a reciprocal shaker for 21 h at 150 rpm and 30°C.

The culture broth was harvested by centrifugation at 5,100×*g* (Centrifugal TDL-5, Shanghai, China) for 10 min. After washing with 0.9% sterilized saline and water, a resting cell suspension containing approximately 25 mg cell dry weight/ml with the PAL specific activity of 18×10^{-3}–20×10^{-3} U/mg. dry cell was prepared in 25 mmol/l Tris–HCl buffer, pH8.8, and used as the source of PAL in the enzymatic reaction.

Enzymatic reaction

The enzymatic reaction solution contained 2% t-Ca, 0.5 mol/l $(^{15}NH_4)_2SO_4$, and 1 mol/l NaOH. Making 0.5 mol/l $(^{15}NH_4)_2SO_4$ and 1 mol/l NaOH reacted to get off ammonia, then added 2% t-Ca and the resting cell suspensions into the reaction solution. The bioconversion was carried out on a reciprocal shaker for 24 h at 150 rpm and 30°C.

Isolation and purification of ^{15}NL-phenylalanne

The cells were removed by centrifugation at 5,100×*g* for 10 min. The resulting supernatant was adjusted to pH 4.0 with sulfate acid. In this process, the $^{15}NH_3$ became $(^{15}NH_4)_2SO_4$, the unconverted t-Ca was acidified and then deposited with some heavy ions and proteins containing in the .supernatant. After removing the deposition by centrifugation and filtration, the supernatant was dealt with by non-polar adsorption resin. The labeled L-phenylalanine could be adsorbed on the resin, and the unconverted $(^{15}NH_4)_2SO_4$ flew out and was collected. The ^{15}NL-phenylalanne

fractions were collected after the column was eluted with deioned water. Some non-polar colored matter had been removed by the adsorption resin, and the remaining polar colored matter could be removed by ethanol in the following step. The collected ^{15}NL-phenylalanne fractions were condensed in vacuo and dissolved in hot 80 % ethanol at 65°C. ^{15}NL-phenylalanne was obtained after crystallizing and freeze-drying. Identity of the product was confirmed by mass spectrometry.

Recovery of unconverted $(^{15}NH_4)_2SO_4$

The collected $(^{15}NH_4)_2SO_4$ fractions were reacted with 30% NaOH under heating. The obtained $^{15}NH_3$ was absorbed by 4% sulfate acid. The remaining $^{15}NH_3$ in the reaction apparatus was driven to the sulfate acid solution by nitrogen gas. The addition amount of sulfate acid was controlled by pH. The final $(^{15}NH_4)_2SO_4$ was freeze-dried. The recovery yield of the $(^{15}NH_4)_2SO_4$ was above 88%.

Results and discussion

Two percent of t-Ca and 0.5 mol/l $(^{15}NH_4)_2SO_4$ (5.34% ^{15}N) were bioconverted to 3.0 g/l ^{15}NL-phenylalanne (5.10% ^{15}N) by the PAL from *R. glutinis*, and 2.1 g/l crystalazed ^{15}NL-phenylalanine was obtained with the purity of above 99% and the yield of 70% after isolation and purification. 96% of ^{15}N was labeled in final product, and the dilution of ^{15}N (from 5.34% ^{15}N in $(^{15}NH_4)_2SO_4$ to 5.10% ^{15}N in ^{15}NL-phenylalanne product) is due to other isotope of N in L-phenylalanne coming from PAL induction medium and from cell metabolites in the culture broth.

In the present paper, the yield and the purity of ^{15}NL-phenylalanine are higher than the reported (29 and 62.9%) (Hadener and Tamm 1987). Compared with reported biosynthesis method of unlabeled L-phenylalanine, the purity of ^{15}NL-phenylalanine (above 99%) in this work is as high as that of the reported unlabeled L-phenylalanine. The yield of 70% is close to that of the unlabeled L-phenylalanine reported (Onishi et al. 1987). The productivity of the labeled L-phenylalanine (3 g/l) is lower than that of the reported unlabeled one (11 g/l) (Yamada et al. 1981), which is due to low concentration of $(^{15}NH_4)_2SO_4$ (0.5 mol/l), and the strain with lower PAL activity in the research. Because of the high price of $(^{15}NH_4)_2SO_4$, we reduced the reported concentration of NH_4^+ from 8 to 1 mol/l in order to decrease the cost of production.

Eighty-eight percent of unreacted $(^{15}NH_4)_2SO_4$ could be recovered using a specific equipment in this work. Because the ^{15}N source is expensive, its recovery is critical for the industrial production. Usually, ion-exchanged resin is used for the isolation of unlabeled L-phenylalanine, but it is difficult to separate ^{15}NL-phenylalanine from $(^{15}NH_4)_2SO_4$. In this work, the non-polar adsorption resin was used to isolate ^{15}NL-phenylalanine from $(^{15}NH_4)_2SO_4$ and other components in the supernatant, which a satisfied results was obtained. Then, another recovery equipment was applied to separate $(^{15}NH_4)_2SO_4$ from other components in non-polar adsorption resin.

The route of production of ^{15}NL-phenylalanine in this paper is effective and economic, and suitable for industrial production. The ^{15}N resource is easily available and recovered. Furthermore, the ^{15}N is easy to be labeled on the L-phenylalanine, because the $(^{15}NH_4)_2SO_4$ is the only nitrogen resource and L-phenylalanine is the only final product. In the proposed route, all ^{15}N in ^{15}NL-phenylalanine comes from $(^{15}NH_4)_2SO_4$, ^{15}NL-phenylalanine is the only target product for the ^{15}N in $(^{15}NH_4)_2SO_4$ and the labeled ^{15}NL-phenylalanine is of high purity, high yield and lower ^{15}N dilution rate. In conclusion, the results in the paper offer an effective and economic route to biosynthesis [1-^{15}N] L-phenylalanine.

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