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Enzymatic synthesis of *N*-methyl-L-phenylalanine by a novel enzyme, *N*-methyl-L-amino acid dehydrogenase, from *Pseudomonas putida*

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Abstract—An enzymatic system for the synthesis of *N*-methyl-L-phenylalanine from phenylpyruvic acid and methylamine with a novel enzyme, *N*-methyl-L-amino acid dehydrogenase from *Pseudomonas putida* ATCC12633, using $NADP^+$ and glucose dehydrogenase from *Bacillus subtilis* as a co-factor-recycling system is described. Analysis of the product on a laboratory preparative scale process revealed *N*-methyl-L-phenylalanine in 98% yield and over 99% ee. *N*-Methyl-L-phenylalanine can be used as chiral building blocks for the synthesis of several products with pharmacological activity.

1. Introduction

Optically active *N*-methyl-α-amino acids occur as functionally critical components of several biologically active natural peptides, such as dolastatins[1] and didemnins.[2] These peptides have been studied because they inhibit certain aspects of viral proliferation, tumor cell growth, and proliferative immune responses.[1,2] Therefore, enantiomerically pure *N*-methyl-α-amino acids are useful building blocks in enantiospecific syntheses of medicines and pesticides. Several methods for the preparation of optically active *N*-methyl amino acids have already been reported.[3–8] However, each procedure has its drawbacks, which include an excessive number of steps needed to prepare the substrate,[5] problems of racemization,[6] harsh reaction conditions,[7] and the instability of acid-sensitive substrates.[8] Thus, there is no satisfactory method available for industrial production of enantiomerically pure *N*-methyl amino acids under mild conditions. Although bacteria producing *N*-methyl-L-phenylalanine[9] and enzymes, which synthesize *N*-methyl-L-alanine[10] and *N*-methyl-L-glutamic acid,[11] have been reported, none have been adapted for the modification of amino acids with other *N*-alkyl groups. The ability to synthesize a variety of enantiomerically pure *N*-alkyl-α-amino acids from a common intermediate using enzymatic catalysis would be useful in the development of pharmaceutical agents.

Recently, we identified a gene encoding a novel *N*-methyl-L-amino acid dehydrogenase (NMAADH) in *Pseudomonas putida* ATCC12633 and succeeded in the functional expression of the enzyme in host *Escherichia coli* BL21(DE3) cells.[12] The enzyme converts an α-keto acid and methylamine into the corresponding *N*-methyl-L-amino acid in an NADPH-dependent manner (Scheme 1). NADPH is a very expensive reagent however and so the economic viability of this reaction requires limiting the use of this co-enzyme to catalytic amounts. Thus, a convenient method to recycle the co-enzyme is of fundamental importance.[13,14] In anticipation of possible scale-up, we decided to demonstrate the feasibility of a NADPH regeneration system in an enzyme-coupled system employing glucose dehydrogenase (GDH) from *Bacillus subtilis*[15] (Scheme 2). Herein, we report the use of NMAADH in the synthesis of optically active *N*-methyl-L-phenylalanine from phenylpyruvic acid and methylamine.

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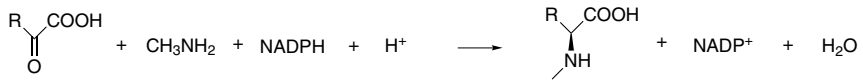
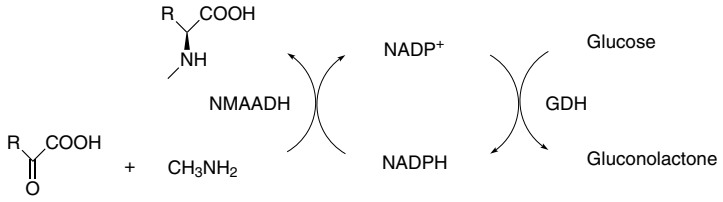


Scheme 1. Reaction catalyzed by *N*-methyl-L-amino acid dehydrogenase.



Scheme 2. The NMAADH-catalyzed production of *N*-methyl-L-amino acid in combination with co-factor-recycling catalyzed by GDH.

2. Results and discussion

Recombinant *P. putida* NMAADH can be efficiently produced in host *E. coli* BL21(DE3) cells induced with isopropyl-β-D-thiogalactopyranoside (IPTG).[12] The purified NMAADH converted the following α-keto acids into their corresponding *N*-methyl-L-amino acids in the presence of methylamine and NADPH, as determined by photometric assay monitoring the oxidation of NADPH at 340 nm using a Shimadzu UV2450 spectrophotometer (relative activity in parentheses): pyruvic acid (100%) > α-ketohexanoic acid (52%) > phenylpyruvic acid (30%) > α-ketobutyric acid (30%) > fluoropyruvic acid (27%) > α-ketovaleric acid (16%) > α-ketoisocaproic acid (9.0%). The broad substrate specificity of the enzyme indicates that the enzyme would be useful in the production of a wide variety of *N*-methyl-L-amino acids. Furthermore, the enzyme was able to catalyze reactions using ethylamine, isopropylamine, diethylamine, cyclopropylamine, and *n*-propylamine, although the activities for these amines were less than 2% of that for methylamine. Therefore, the enzyme could be used for the synthesis of various *N*-alkyl-L-amino acids. We have confined our bioconversion studies here to the production of *N*-methyl-L-phenylalanine, because no enzyme affecting this catalysis has yet been reported.

Recombinant *E. coli* BL21(DE3) cells harboring the genes encoding NMAADH and GDH were grown in a Luria–Bertani medium containing 100 μg/mL ampicillin and 25 μg/mL chloramphenicol at 37 °C for 16 h. Three hours after the induction of gene expression with 1 mM IPTG at 37 °C for 3 h, the cells were harvested by centrifugation, re-suspended in 20 mM Tris–HCl (pH 7.0) and washed twice with the same buffer. The resulting cells were used directly for enzymatic synthesis. The standard reaction mixture for the *N*-methyl-L-phenylalanine synthesis contained 700 mM methylamine–HCl, 100 mM phenylpyruvic acid, 100 mM glucose, 0.2 mM $NADP^+$, and washed cells (OD_{660} = 20) at pH 9.0. Aliquots of the reaction mixture were removed for analysis of the product and the substrate. Both *N*-methyl-L-phenylalanine and phenylpyruvic acid were detected by high-performance liquid chromatography (HPLC) (Waters Alliance; Waters, Milford, MA) with an ULTRON ES-PhCD column (6.0×150 mm, Shinwa Chemical Industries, Kyoto, Japan) and 20 mM KH_2PO_4–H_3PO_4–CH_3CN (2000:1:500, v/v) as eluent at a flow rate of 0.85 mL/min at 40 °C, monitored at 210 nm. The concentration of glucose was determined by a glucose assay kit (Glucose CII-Test Wako; Wako Pure Chemical Industries, Osaka, Japan).

Initially, we attempted to synthesize *N*-methyl-L-phenylalanine in a 10 mL standard reaction mixture at 30 °C with reciprocal shaking. In order to prevent a decrease in pH during the reaction, the reaction mixture was adjusted to pH 8.0–9.0 with 10 M NaOH. After a 24 h reaction, 75 mM (13 g/L) of *N*-methyl-L-phenylalanine was produced, with a yield of 75%. Phenylpyruvic acid and glucose were almost completely depleted after 10 h (Fig. 1A).

Next, a large-scale production was performed in a 100 mL standard reaction mixture at pH 8.0–9.0 at 30 °C with stirring. After a 7 h reaction, 91 mM (16 g/L) of *N*-methyl-L-phenylalanine was produced, with a yield of 98%. Phenylpyruvic acid and glucose were added again at 7 h, but the rate of the formation of *N*-methyl-L-phenylalanine had significantly decreased after this point, largely due to the inactivation of GDH during the reaction: GDH activity was completely lost after 15 h, whereas NMAADH was still active. After 22 h of reaction, the overall yield of *N*-methyl-L-phenylalanine was 54% (Fig. 1B).

The product was isolated from a 24 h reaction mixture (100 mL) prepared with a protocol identical to that employed for the reaction shown in Figure 1A, as follows. Cells were removed by centrifugation, and the supernatant adjusted to pH 10.0 with 10 M NaOH and concentrated about 2.5-fold with an evaporator. The solution was adjusted to pH 7.0 with 12 M HCl and left overnight at 4 °C. The solids formed were collected by filtration and dissolved in 50 mL 0.7 M NaOH. The solution was then adjusted to pH 7.0 with 12 M HCl. Crystals, which had formed by addition of acetone, were collected, washed with 20 mL acetone, and dried under vacuum to obtain 1.1 g of *N*-methyl-L-phenylalanine. ^1H NMR (400 MHz, D_2O) spectrum of the product showed the

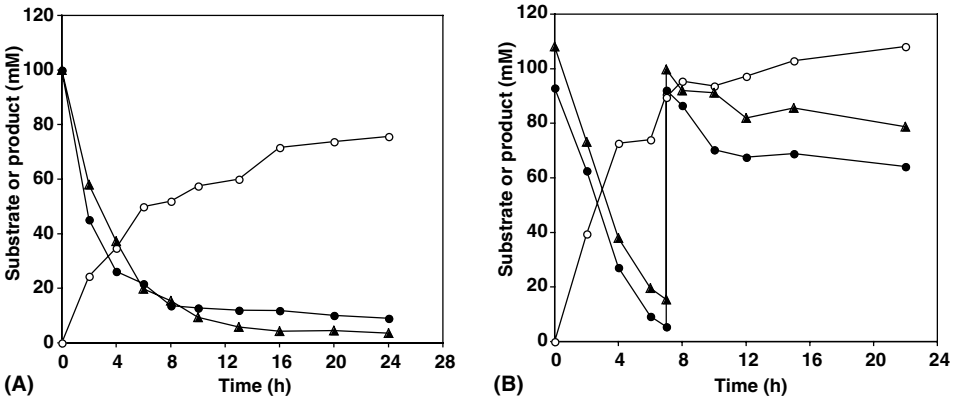


Figure 1. Time course of the small-scale (A) and large-scale (B) production of *N*-methyl-L-phenylalanine by *N*-methyl-L-amino acid dehydrogenase (NMAADH) from *P. putida* ATCC12633. The reactions were carried out at 30 °C at pH 9.0 in 700 mM methylamine–HCl, 100 mM phenylpyruvic acid, 100 mM glucose, 0.2 mM $NADP^+$ and the *E. coli* cells (OD_{660} = 20) expressing NMAADH and glucose dehydrogenase. *N*-Methyl-L-phenylalanine (○) and phenylpyruvic acid (●) were determined by HPLC. Glucose (▲) was determined with a glucose assay kit.

following signals: δ = 2.55 (s, 3H); 3.09 (d, 2H, J = 6.3 Hz); 3.72 (t, 1H, J = 6.3 Hz); 7.14–7.21 (m, 2H); 7.21–7.33 (m, 3H), which agree with those of authentic *N*-methyl-L-phenylalanine.

The enantiomeric excess (ee) of the product obtained by a 7 h reaction in the large-scale production, was determined by HPLC with a CHIRALPAK WE column (4.6×250 mm, Daicel Chemical Industries, Tokyo, Japan) and 2 mM $CuSO_4$ as eluent at a flow rate of 0.75 mL/min at 50 °C, monitored at 254 nm. The retention time of *N*-methyl-L-phenylalanine was 21.5 min, while that of *N*-methyl-D-phenylalanine was 41.2 min. *N*-Methyl-L-phenylalanine was obtained with a satisfactorily high enantiomeric excess (>99% ee).

3. Conclusion

In conclusion, we have demonstrated the coupled enzymatic synthesis of *N*-methyl-L-phenylalanine with good yield and high ee by using NMAADH from *P. putida* and GDH from *B. subtilis*. Thus, due to its catalytic efficiency and broad substrate specificity, NMAADH may be an attractive reagent in the development of biocatalytic routes to *N*-substituted L-amino acids for use as pharmaceutical intermediates.

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