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Journal of Molecular Catalysis B: Enzymatic 12 (2001) 71–75

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

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Separative preparation of the four stereoisomers of β-methylphenylalanine with *N*-carbamoyl amino acid amidohydrolases

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Received 16 August 1999; received in revised form 22 September 1999; accepted 25 September 1999

Abstract

The selective preparation of the four stereoisomers of β-methylphenylalanine (Mphe) from mixtures of the four stereoisomers of *N*-carbamoyl-β-methylphenylalanine (NCMphe) with *N*-carbamoyl amino acid amidohydrolases (carbamoylases) was developed. D-Carbamoylase specifically hydrolyzed *threo*-D-NCMphe with a little side activity toward *erythro*-D-NCMphe, thus *threo*-D-Mphe was produced with high optical purity from a mixture of the four stereoisomers of NCMphe. L-Carbamoylase specifically produced *threo*-L-Mphe from a mixture of the four stereoisomers of NCMphe. The *erythro*-D-Mphe was obtained from *erythro*-DL-NCMphe which was prepared through diastereomer resolution by separative crystallization of benzoyl Mphe with a little side activity of D-carbamoylase toward *erythro*-D-NCMphe and the remaining *erythro*-L-NCMphe was chemically hydrolyzed to *erythro*-L-Mphe.

Keywords: α,β-Diastereomeric amino acid; β-Methylphenylalanine; D-Carbamoylase; L-Carbamoylase

1. Introduction

Recently *N*-carbamoyl-D-α-amino acid amidohydrolase (D-carbamoylase) and *N*-carbamoyl-L-α-amino acid amidohydrolase (L-carbamoylase) were found to recognize the configuration of not only the α-carbon but also the β-carbon of α-amino acids [1,2]. These features are useful for the stereospecific synthesis of α,β-diastereomeric amino acids which have four stereoisomers and thus are difficult to synthesize selectively by means of conventional organic and enzymatic methods.

β-Methylphenylalanine (Mphe) is one such α,β-diastereomeric amino acid and is found among some microbial secondary metabolites. As summarized in Fig. 1 D-carbamoylase specifically hydrolyzes *threo*-D-*N*-carbamoyl-β-methylphenylalanine (NCMphe) with a little side activity toward *erythro*-D-NCMphe and L-carbamoylase specifically hydrolyzes *threo*-L-NCMphe in a mixture of the four stereoisomers of NCM-

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1381-1177/01/$ – see front matter
PII: S1381-1177(00)00205-8

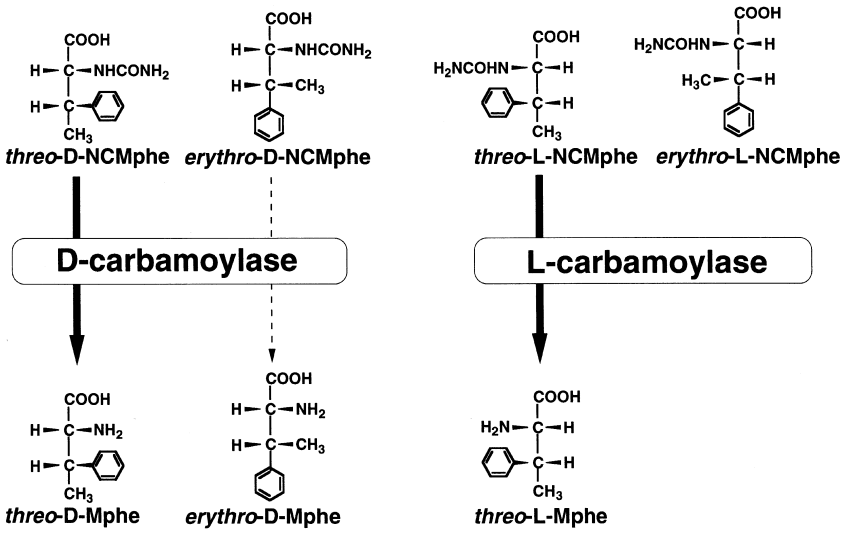

Fig. 1. Stereoselectivity of carbamoylases as to NCMphe stereoisomers.

phe. In this study the characteristics of these carbamoylases were applied to the practical preparation of each stereoisomer of Mphe from mixtures of the four stereosiomers. Fig. 2 summarizes the strategy for the separative preparation of Mphe isomers with the carbamoylases.The *threo*-D-Mphe can be obtained through the hydrolysis of a mixture of the four stereoisomers of NCMphe by D-carbamoylase. The *threo*-L-Mphe can be obtained through the hydrolysis of the same mixture by L-carbamoylase. The *erythro*-D-Mphe can be obtained with the side activity of D-carbamoylase toward *erythro*-D-NCMphe from *erythro*-DL-NCMphe which is obtained by separative crystallization of benzoyl Mphe and successive debenzoylation and carbamoylation. Chemical hydrolysis of the remaining *erythro*-L-NCMphe leads to *erythro*-L-Mphe.

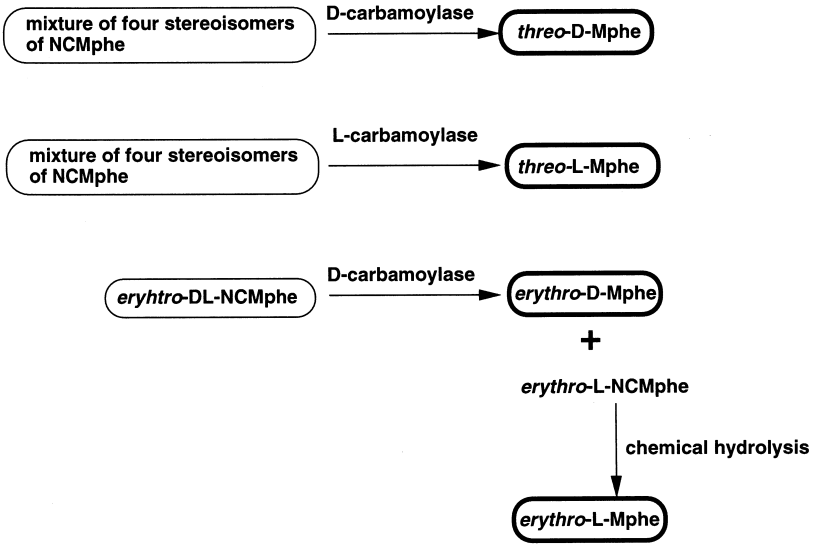

Fig. 2. Strategy for the separative preparation of Mphe stereoisomers with carbamoylases.

2. Materials and methods

2.1. Chemicals

A mixture of the four stereoisomers of Mphe was synthesized according to the method described previously [3].The *erythro*-DL-Mphe was obtained by separative crystalization of benzoyl Mphe and succesisive debenzoylation [3]. The molar ratios of the *erythro*-L, *threo*-L, *erythro*-D and *threo*-D isomers in Mphe and *erythro*-DL-Mphe were 27/23/27/23 and 48/2/48/2, respectively. *N*-Carbamoyl derivatives of amino acids were synthesized by the method of Nyc and Mitchell [5].

2.2. Microorganisms, culture conditions and enzyme preparation

The partially purified enzyme fraction from *Blastobacter* sp. A17p-4 AKU 990 [6] and washed cells of *Alcaligenes xylosoxidans* A35 AKU 110 [7] (AKU Culture Collection, Faculty of Agriculture, Kyoto University) were used as the sources of D-carbamoylase and L-carbamoylase, respectively. *Blastobacter* sp. was cultivated in medium comprising 0.1% (w/v) KH_2PO_4, 0.1% K_2HPO_4, 0.03% $MgSO_4{\cdot}7H_2O$, 0.03% $FeSO_4{\cdot}7H_2O$, 0.3% yeast extract, 0.3% meat extract, 1.0% glycerol, 0.2% peptone and 0.15% uracil (pH 7.0) at 28°C for 7 days as described previously [6]. D-Carbamoylase was partially purified from a cell-free extract of *Blastobacter* sp. through DEAE-Sephacel column chromatography as described previously [6,8]. *A. xylosoxidans* was cultivated in medium comprising 0.1% (w/v) KH_2PO_4, 0.1% K_2HPO_4, 0.03% $MgSO_4{\cdot}7H_2O$, 0.1% yeast extract, 1.0% glycerol, 1.0% NH_4Cl and 0.15% *N*-carbamoyl-L-leucine (pH 7.0) at 28°C for 1 day. Washed cells of *A. xylosoxidans* were prepared as described previously [2].

2.3. Reaction conditions for threo-D-Mphe production with D-carbamoylase from a mixture of the four stereoisomers of NCMphe

The reaction mixture comprised of 5 ml, 50 mg (45 mM) NCMphe (mixture of the four stereoisomers), 200 mM potassium phosphate buffer (pH 7.0) and partially purified D-carbamoylase (10 U). The reaction was carried out at 30°C. Aliquots of the reaction mixture were taken and used to monitor the reaction. After the reaction the Mphe produced was isolated with a Sep-Pak Plus C18 cartridge using 3% (v/v) methanol as the mobile phase and then used for analysis.

2.4. Reaction conditions for threo-L-Mphe production with L-carbamoylase from a mixture of the four stereoisomers of NCMphe

The reaction mixture comprised of 5 ml, 50 mg (45 mM) NCMphe (mixture of the four stereoisomers), 200 mM Tris–HCl (pH 7.4) and 500 mg washed cells of *A. xylosoxidans* (corresponding to 20 U L-carbamoylase). The reaction was carried out at 30°C. Aliquots of the reaction mixture were taken and used to monitor the reaction. After the reaction the reaction mixture was centrifuged (3500 rpm, 10 min) and the Mphe produced in the supernatant was isolated by Sep-Pak cartridge chromatography as described above and then used for analysis.

2.5. Reaction conditions for erythro-D-Mphe and erythro-L-Mphe production with D-carbamoylase and chemical hydrolysis from erythro-DL-NCMphe

The reaction mixture comprised of 5 ml, 50 mg (45 mM) *erythro*-DL-NCMphe, 200 mM potassium phosphate buffer (pH 7.0) and partially purified D-carbamoylase (100 U). The reaction was carried out at 30°C. Aliquots of the reaction mixture were taken and used to monitor the reaction. After the reaction was completed the Mphe produced and NCMphe remaining were isolated by Sep-Pak cartridge chromatography as described above. The isolated Mphe was directly used for analysis and NCMphe was further chemically hydrolyzed to *erythro*-L-Mphe by treatment with nitrous acid [9] and purified with a Sep-Pak cartridge and then used for analysis.

3. Analytical methods

Quantitative analysis of the four stereoisomers of Mphe was performed by the method of Buck and Krummen [10] after derivatization with *o*-phthalaldehyde and *N*-acetyl-L-cysteine. Analysis was performed

by high-performance liquid chromatography (HPLC) with a Shim-pack CLC-ODS(M) column, 4.6 × 250 mm (Shimadzu, Japan) and a fluorescence detector as described previously [1]. Enantiomeric purity was expressed as % enantiomer excess (*e.e.*) for both the α-carbon (Cα) and the β-carbon (Cβ). The *e.e.* (%) for Cα was expressed as the absolute value obtained with the formula $100 \times [(\text{D-L})/(\text{D} + \text{L})]$. The *e.e.* (%) for Cβ was expressed as the absolute value obtained with the formula $100 \times [(\textit{threo} - \textit{erythro})/(\textit{threo} + \textit{erythro})]$. Quantitative analysis of *threo*-DL- and *erythro*-DL-NCMphe was performed by HPLC with a Cosmosil 5C18-AR column (4.6 × 250 mm Nacalai, Tesque, Japan) and 35% methanol (pH 2.5) as the mobile phase, as described previously [2].

4. Results

4.1. *threo*-D-Mphe production with D-carbamoylase from a mixture of the four stereoisomers of NCMphe

NCMphe hydrolysis by the *Blastobacter* D-carbamoylase is shown in Fig. 3. The enzyme preferentially hydrolyzed *threo*-D-NCMphe with very little activity toward *erythro*-D-NCMphe. After 50 h the reaction was stopped and *threo*-D-Mphe (9.7 mg) was isolated (molar yield, 19.4%; *e.e.* for Cα, >99%; *e.e.* for Cβ, 89.9%).

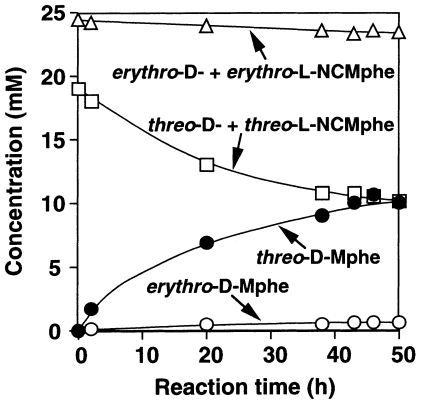

Fig. 3. NCMphe hydrolysis by the *Blastobacter* sp. D-carbamoylase. The reaction was carried out under the conditions given in Section 2. (●), *threo*-D-Mphe; (○), *erythro*-D-Mphe; (□), sum of *threo*-D- and *threo*-L-NCMphe; (△), sum of *erythro*-D- and *erythro*-L-NCMphe.

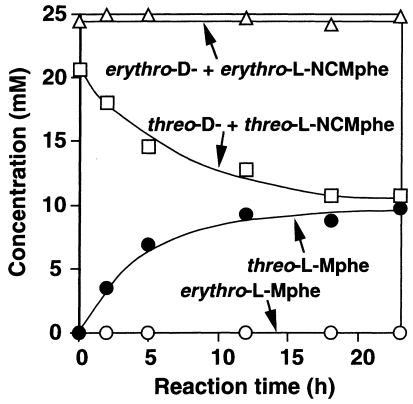

Fig. 4. NCMphe hydrolysis by *A. xylosoxidans* cells containing L-carbamoylase. The reaction was carried out under the conditions given in Section 2. (●), *threo*-L-Mphe; (○), *erythro*-L-Mphe; (□), sum of *threo*-D- and *threo*-L-NCMphe; (△), sum of *erythro*-D- and *erythro*-L-NCMphe.

4.2. *threo*-L-Mphe production with L-carbamoylase from a mixture of the four stereoisomers of NCMphe

NCMphe hydrolysis by *A. xylosoxidans* cells is shown in Fig. 4. The cells stereospecifically hydrolyzed *threo*-L-NCMphe. After 23 h the reaction was stopped and *threo*-L-Mphe (9.5 mg) was isolated (molar yield, 19.0%; *e.e.* for Cα, >99%; *e.e.* for Cβ, >99%).

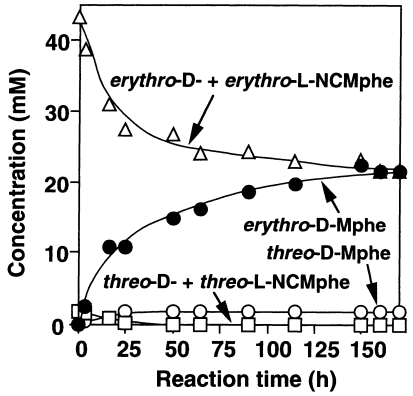

Fig. 5. Hydrolysis of *erythro*-DL-NCMphe by the *Blastobacter* sp. D-carbamoylase. The reaction was carried out under the conditions given in Section 2. (●), *erythro*-D-Mphe; (○), *threo*-D-Mphe; (□), sum of *threo*-D- and *threo*-L-NCMphe; (△), sum of *erythro*-D- and *erythro*-L-NCMphe.

4.3. *erythro*-D-*Mphe and erythro*-L-*Mphe production with* D-*carbamoylase and chemical hydrolysis from erythro*-DL-*NCMphe*

Hydrolysis of *erythro*-DL-NCMphe by the D-carbamoylase is shown in Fig. 5. After 170 h the reaction was stopped and *erythro*-D-Mphe (15.7 mg) was isolated (molar yield, 39.0%; *e.e.* for Cα, >99%; *e.e.* for Cβ, 91%). The *erythro*-L-NCMphe (23.2 mg, 46.4% yield) isolated from the reaction mixture was then treated with nitrous acid and *erythro*-L-Mphe (14.9 mg) was isolated (molar yield, 29.8%; *e.e.* for Cα, 88.6%; *e.e.* for Cβ, 92.4%).

5. Discussion

All stereoisomers of Mphe were obtained with considerably high enantiomeric purity and in practically high yields. These stereoisomers are practically useful for the synthesis of some microbial secondary metabolites. Bottromycin a peptide antibiotic discovered in the fermentation broth of *Streptomyces bottropensis* [3] and AK-toxin I a host-specific phytotoxic metabolite produced by the *Alternaria alternata* Japanese pear pathotype [4] contain Mphe as a component.

The present study is a good example of the use of enzymes for the stereoselective synthesis of α,β-diastereomeric amino acids. The other reported examples are the use of threonine aldolases for the stereospecific synthesis of α,β-diastereomeric amino acids. However, threonine aldolases are only useful for amino acids with a hydroxy-group at the β-carbon [11–13], i.e. not for those without a hydroxy-group like Mphe. The present results show the potential of carbamoylases which react with a wider range of substrates [14] for the stereoselective synthesis of a variety of α,β-diastereomeric amino acids.

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