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Amidohydrolase Process: Expanding the use of L-*N*-carbamoylase/*N*-succinyl-amino acid racemase tandem for the production of different optically pure L-amino acids

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ABSTRACT

A bienzymatic system comprising an *N*-succinylamino acid racemase from *Geobacillus kaustophilus* CECT4264 (GkNSAAR) and an enantiospecific L-*N*-carbamoylase from *Geobacillus stearothermophilus* CECT43 (BsLcar) has been developed. This biocatalyst has been able to produce optically pure natural and non-natural L-amino acids starting from racemic mixtures of *N*-acetyl-, *N*-formyl- and *N*-carbamoyl-amino acids by dynamic kinetic resolution. The fastest conversion rate was found with *N*-formyl-amino acids, followed by *N*-carbamoyl- and *N*-acetyl-amino acids, and GkNSAAR proved to be the limiting step of the system due to its lower specific activity. Metal ion cobalt was essential for the activity of the biocatalyst and the system was optimally active when Co^{2+} was added directly to the reaction mixture. The optimum pH for the biocatalyst proved to be 8.0, for both *N*-formyl- and *N*-carbamoyl-amino acid substrates, whereas optimum temperature ranges were 45–55 °C for *N*-formyl-amino acids and 55–70 °C for *N*-carbamoyl-derivatives. The bienzymatic system was equally efficient in converting aromatic and aliphatic substrates. Total conversion was also achieved using high substrate concentrations (100 and 500 mM) with no noticeable inhibition. This "Amidohydrolase Process" enables the production of both natural and non-natural L-amino acids from a broad substrate spectrum with yields of over 95%.

1. Introduction

Optically pure natural and non-natural L-amino acids are of considerable economic importance because of the broad spectrum of their industrial applications. Proteinogenic amino acids are the building blocks of life, used in human nutrition and health, or as additives, flavor enhancers and sweeteners [1]. Additionally, non-natural L-amino acids are in increasing demand as valuable intermediates in the pharmaceutical industry. By way of example, L-homophenylalanine is a precursor for the preparation of angiotensin-converting enzyme (ACE) and renin inhibitors, such as enalapril, lisinopril, quinapril, ramipril, trandolapril and benazepril, among others [2]. L-α-Aminobutyric acid (L-ABA) is an intermediate of ophthalmate, a sensitive indicator of hepatic glutathione (GSH) depletion, and is used as a biomarker for oxidative stress [3]. This amino acid, also named L-homoalanine, is a key chiral intermediate for the synthesis of several important drugs, such as levetiracetam or brivaracetam (antiepileptic drugs) and ethambutol (antituberculosis drug) [4].

Biocatalytic methods based on chemoenzymatic processes have been described for optically pure amino acid production, such as the hydantoinase [5], amidase [6] and acylase [7] processes. These methods take advantage of the enantiospecificity or enantioselectivity of (at least) one enzyme to achieve enantiopure (or highly enantiomeric enriched) D- or L-amino acids. In the

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hydantoinase and acylase processes, the use of in situ racemization of a non-hydrolyzed substrate turns these methods into "dynamic kinetic resolution" (DKR) methods, allowing total conversion of the racemic substrates used in each case. The "Hydantoinase Process" is an inexpensive and environment-friendly enzymatic method [8] based on the DKR of D,L-5-monosubstituted hydantoins. The enantiomeric purity of the amino acid obtained by this method depends on the stereospecificity of the last enzyme in the reaction cascade (*N*-carbamoyl-D- or L-amino-acid amidohydrolase, also known as D- or L-*N*-carbamoylase). In the case of L-amino acid production, an L-*N*-carbamoylase is responsible for the enantiospecificity of the reaction product, allowing 100% e.e. of the L-amino acid produced, due to its strict enantiospecifity toward the L-enantiomer of the carbamoyl-intermediate of the three-step process [9, and references therein]. The racemization step of this process can be produced chemically (at extreme pHs), or enzymatically by a hydantoin racemase [5,8]. Similarly, in the "Acylase process" an *N*-acylamino acid racemase [10] (NAAAR; recently re-assigned as *N*-succinylamino acid racemase, NSAAR [11]) is coupled with a D- or L-aminoacylase to produce D- or L-amino acids, starting from racemic mixtures of *N*-acetyl-amino acids (>98% purity) [12,13].

Our group has recently demonstrated the substrate promiscuity of the recombinant *N*-succinylamino acid racemase from *Geobacillus kaustophilus* CECT4264 (GkNSAAR), which was able to racemize both isomers of *N*-acetyl-, *N*-succinyl- and *N*-carbamoyl-amino acids [14]. We have also proved the substrate promiscuity of L-*N*-carbamoylase from *Geobacillus stearothermophilus* CECT43 (BsLcar), which hydrolyzed enantiospecifically different *N*-acetyl-, *N*-formyl- and *N*-carbamoyl-L-amino acids [15]. Taking the "Acylase process" [7] as reference, the aim of this work is to develop a biocatalyst joining the GkNSAAR enzyme together with the enantiospecific BsLcar, in order to convert racemic mixtures of several *N*-derivative-amino acids into natural and non-natural L-amino acids by a DKR approach. As has been shown previously, the use of BsLcar guarantees the enantiomeric purity of the L-amino acid obtained, as only the L-*N*-substituted-amino acid can be recognized by the enzyme [9,15 and references therein]. At the same time as the L-*N*-derivative amino acid of the racemic mixture is hydrolyzed by BsLcar, the remaining non-hydrolyzed D-*N*-derivative-amino acid is racemized by GkNSAAR (Fig. 1).

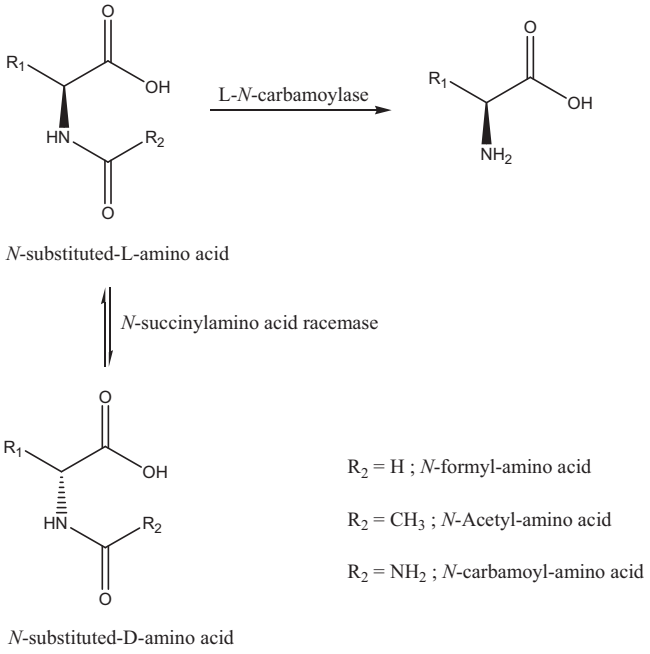

Fig. 1. Reaction scheme for optically pure L-amino acid production from racemic mixtures of *N*-acetyl-, *N*-carbamoyl- and *N*-formyl-amino acids by the "Amidohydrolase Process". R_1, lateral chain of the corresponding amino acid. R_2, *N*-substituent.

2. Materials and methods

2.1. General protocols and reagents

Standard methods were used for the cloning and expression of the different genes [16,17]. Restriction enzymes, T4 DNA ligase and the thermostable *Pwo* polymerase together with primers for PCR were purchased from Roche Diagnostic S.L. (Barcelona, Spain). Racemic mixtures and optically pure L-amino acids were purchased from Sigma Aldrich Quimica S.A. (Madrid, Spain). *N*-Acetyl-methionine was purchased from Sigma–Aldrich (Madrid, Spain). The *N*-carbamoyl- and *N*-formyl-amino acids were synthesized according to previous works, with slight modifications [18,19]. Briefly, *N*-carbamoyl-amino acids were obtained by dissolving 10 mmol of the corresponding amino acid into water. After addition of 25 mmol of KOCN, the solution was refluxed for 1 h at 90 °C. It was then ice-cooled, and acidified with concentrated HCl (pH = 2–3). This acidified solution was kept on ice for up to 2–3 days, and crystals of *N*-carbamoyl-amino acid were recovered by filtration. *N*-Formyl-amino acids were obtained by dissolving the corresponding amino acid into formamide (5 eq.) and heating at 100 °C under inert atmosphere. When the solution became totally transparent, formamide was subjected to evaporation into a rotavapor under vacuum (60–90 °C). 2.5 water eq. were then added to the flask, ice-cooled, and acidified with concentrated HCl (pH = 2). This acidified solution was kept on ice for up to 2–3 days, and crystals of *N*-formyl-amino acid were recovered by filtration.

2.2. Plasmids and culture conditions

L-*N*-Carbamoylase from *G. stearothermophilus* CECT43 (BsLcar) was overexpressed and purified from BL21 cells harboring pJAVI80Rha plasmid as previously described [20]. *N*-Succinylamino acid racemase gene (*nsaar*) from *G. kaustophilus* CECT4264 (*Gknsaar*) was cloned into a rhamnose-inducible expression vector including a C-terminal His6-tag (pJOE4036.1 [21]; Altenbuchner, pers. communication), PCR amplification of *the Gknsaar* gene (1128 bp; GenBank accession no. EU427322) was carried out using the recombinant plasmid pJPD25 as template [14], which already contained the corresponding gene. The PCR primers were 5′-AGAAAGGGGAGAGCTCATGGCGATCAACA-3′ (*inlcuding a Sac*I site, in italics) and 5′-GGATCCTGCCGTCGCCGTACGATGAAACA-3′ (*including a BamH*I site, in italics). The PCR product was purified from an agarose gel using E.Z.N.A. Gel Extraction Kit (Omega Bio-Tek, Inc., USA), treated with SacI and BamHI enzymes, and then ligated into pJOE4036.1 plasmid which was cut with the same enzymes to create plasmid pJPD25rha. Its sequence was analyzed at least twice using the dye dideoxy nucleotide sequencing method in an ABI 377 DNA Sequencer (Applied Biosystems).

2.3. Expression of Bslcar and Gknsaar genes

The transformants in BL21 strain (BL21pJAVI80rha and BL21pJPD25rha) were grown in LB medium supplemented with 100 μg ml^{-1} of ampicillin. The expression protocol was the same for both transformants. A single colony was transferred into 10 ml of LB medium with ampicillin at the above-mentioned concentration in a 100 ml flask. This culture was incubated overnight at 37 °C with shaking (150 rpm). In a 2 l flask, 500 ml of LB supplemented with 100 μg ml^{-1} of ampicillin was inoculated with 5 ml of the overnight culture. After 2 h of incubation at 37 °C with vigorous shaking (180 rpm), the OD_{600} of the resulting culture was

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