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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).

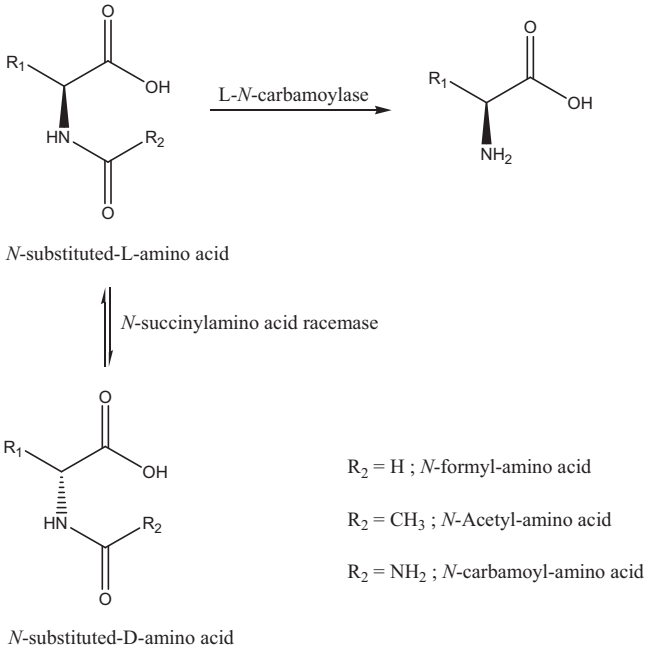

Fig. 1. Reaction scheme for optically pure L-amino acid production from racemic mixtures of *N*-acetyl-, *N*-carbamoyl- and *N*-formyl-amino acids by the "Amidohydrolase Process". R_1, lateral chain of the corresponding amino acid. R_2, *N*-substituent.

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

0.3–0.5. For expression induction of the *Bslcar* and *Gknsaar* genes, L-rhamnose (Prolabo, VWR) was added to a final concentration of 0.2% (w/v) and the culture was incubated at 32 °C for an additional 6 h. The cells were collected by centrifugation (Beckman JA2-21, 7000 × *g*, 4 °C, 20 min) and stored at −20 °C. The frozen pellet was washed twice and resuspended in 50 ml wash buffer (300 mM NaCl, 50 mM sodium phosphate pH 7.0). The cell walls were disrupted in ice by sonication using a UP 200 S Ultrasonic Processor (Dr. Hielscher GmbH, Germany) for 6 periods of 60 s, pulse mode 0.5 and sonic power 60%. The pellet was precipitated by centrifugation (Beckman JA2-21, 13,000 × *g*, 4 °C, 20 min) and discarded. The supernatant was applied to a column with TALON™ metal affinity resin (CLONTECH Laboratories, Inc., Nucliber, Madrid, Spain) and then washed three times with wash buffer. After washing, BsLcar and GkNSAAR were eluted with elution buffer (100 mM NaCl, 150 mM imidazole, 2 mM Tris, pH 8.0). The purified enzymes (over 95% purity) were dialyzed against 100 mM borate/HCl pH 8.0 and stored at 4 °C until use.

2.4. Standard conversion assay

Standard enzymatic reaction was carried out with purified BsLcar and GkNSAAR enzymes (at final concentrations of 1.50–2.0 μM and 10.0–12.0 μM, respectively) in presence of $CoCl_2$ (final concentration of 0.25 mM) using *N*-acetyl-, *N*-carbamoyl- or *N*-formyl-D,L-methionine as substrates (15–20 mM) dissolved in 100 mM borate/HCl pH 8.0. The reaction mixture (780 μl) was incubated at 65 °C for 30 min (*N*-carbamoyl-D,L-methionine), at 65 °C for 60 min (*N*-acetyl-D,L-methionine) and at 45 °C for 15 min (*N*-formyl-D,L-methionine). The reactions were stopped by addition of 10 times the reaction volume of 1% H_3PO_4.

After centrifuging, the resulting supernatants were analyzed by high performance liquid chromatography (HPLC). The HPLC system (LC2000Plus HPLC System, Jasco, Madrid, Spain) equipped with a Luna C_{18} column (4.6 × 250 mm, Phenomenex) was used to detect *N*-acetyl-, *N*-formyl- and *N*-carbamoyl-D,L-methionine and L-methionine. The mobile phase was methanol/phosphoric acid (20 mM, pH 3.2) (vol/vol, 20:80), pumped at a flow rate of 0.80 ml min^{-1} and measured at 203 nm.

2.5. Bienzymatic system characterization

N-Formyl- and *N*-carbamoyl-D,L-methionine (15–20 mM) were chosen for optimization of optimal pH and temperature reaction conditions due to their higher conversion rates, using the above-described standard enzymatic assay. All assays were carried out in triplicate. Optimal temperature was evaluated from 30 to 80 °C in 100 mM sodium borate/HCl buffer pH 8.0. pH studies were carried out in 100 mM buffer solutions ranging from pH 5.5 to 10.5 (sodium citrate, sodium cacodylate, sodium borate/HCl and sodium borate/NaOH). Thermal stability of the biocatalyst was measured after 0, 2, 4, 6, 8, 10 and 24 h of incubation at temperatures ranging from 40 to 70 °C in 100 mM sodium borate/HCl buffer pH 8.0 followed by the standard enzymatic assay.

Substrate specificity studies were performed with different *N*-formyl- and *N*-carbamoyl-amino acids (15–20 mM) dissolved in 100 mM sodium borate/HCl pH 8.0 together with the purified enzyme at the same concentration described in the standard enzymatic assay, in the presence of 0.25 mM $CoCl_2$. In these studies, the final reaction volume was 1.2 ml. Reactions were carried out in triplicate at 45 °C (*N*-formyl-amino acid) or 65 °C (*N*-carbamoyl-amino acid). One 75 μL-aliquot of the reaction sample was retrieved at different reaction times, and the reaction was stopped by addition of 10 volumes 1% H_3PO_4. The mobile phase of the different substrates and their corresponding L-amino acids was methanol-phosphoric acid (20 mM, pH 3.2) (5:95–50:50 vol/vol, depending on the compound), pumped at a flow rate of 0.50 ml min^{-1}. Compounds were detected with a UV detector at a wavelength of 203 nm.

2.6. Enantiomeric purity of the products of the reaction

The theoretical 100% e.e. of the amino acids produced by this system was confirmed by conversion assays of the *N*-formyl-D,L-derivatives of methionine, norleucine, norvaline, ABA, phenylglycine and homophenylalanine (15–20 mM) to the corresponding L-amino acids, carried out in the conditions described above for the substrate specificity assays. Samples were measured in the LC2000Plus HPLC System (Jasco, Madrid, Spain) equipped with a chirobiotic T column (Astec, Sigma–Aldrich). The mobile phase was 70:29.5:0.05 methanol:10 mM ammonium acetate:acetic acid, pumped at a flow rate of 0.3 ml min^{-1} and measured at 203 nm. Solid L-homophenylalanine produced "*in situ*" by the bienzymatic system was confirmed by NMR (Avance 300 DPX) in the Research Central Services of the University of Almería.

3. Results and discussion

3.1. Multisubstrate hydrolysis

The recombinant BsLcar used in this bienzymatic system, was previously shown to hydrolyze different *N*-acetyl-, *N*-formyl- and *N*-carbamoyl-amino acids [15]. On the other hand, GkNSAAR showed the ability to racemize different *N*-succinyl-, *N*-acetyl- and *N*-carbamoyl-amino acids [14]. Since the main aim of this work was to couple both enzymes to use the same type of substrate to convert it completely to the corresponding L-amino acid, we first checked whether (a) BsLcar presented activity toward *N*-succinyl-L-amino acids, and (b) GkNSAAR would racemize *N*-formyl-amino acid derivatives. BsLcar showed no hydrolytic activity toward *N*-succiniyl-L-methionine. On the other hand, GkNSAAR was able to racemize both *N*-formyl-D- and L-methionine. In view of these results, BsLcar and GkNSAAR were initially combined to evaluate their ability to transform racemic mixtures of *N*-acetyl-, *N*-formyl- and *N*-carbamoyl-amino acids into optically pure L-amino acids. The bienzymatic system was able to hydrolyze all three racemic substrates into optically pure L-methionine (Fig. 2),

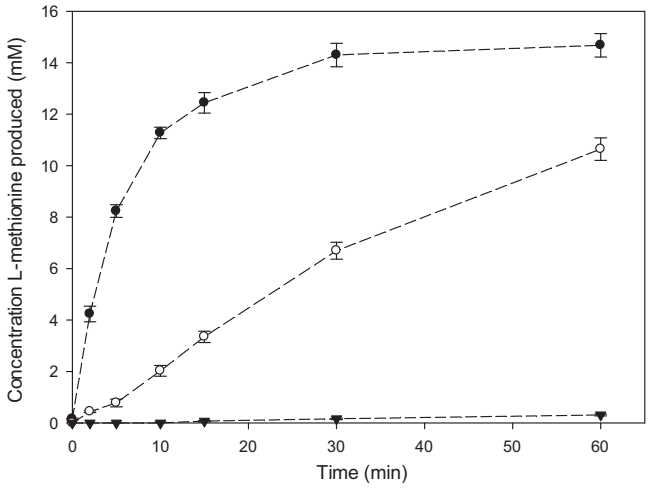

Fig. 2. Optically pure L-methionine formation from racemic mixtures of 15 mM *N*-formyl- (●), *N*-carbamoyl- (○) and *N*-acetyl-methionine (▼) using the bienzymatic BsLcar/GkNSAAR system. Conversion measurements were performed in triplicate using standard enzyme assay (see Section 2).

the fastest conversion being of *N*-formyl-amino acid, followed by *N*-carbamoyl- and *N*-acetyl-derivatives. Based on the substrate promiscuity of BsLcar and GkNSAAR, we previously hypothesized on the production of optically pure amino acids starting from different *N*-substituted amino acids, by coupling an L-*N*-carbamoylase and an NSAAR enzyme, imitating the "Acylase process" [9]. In fact, this hypothesis was initially confirmed by Hsu et al., who were able to produce L-homophenylalanine using *N*-carbamoyl-D,L-homophenylalanine as substrate [13]. On the other hand, this is the first time that the versatility of this tandem to use different substrates has been shown. Furthermore, since the slowest rate of conversion with this bienzymatic tandem was surprisingly found with *N*-acetyl-derivatives, we decided to adopt a more general term for this modified "Acylase process", namely "Amidohydrolase Process". Based on these initial results, we only characterized the bienzymatic tandem with *N*-formyl- and *N*-carbamoyl-amino acids, since they were the fastest converted substrates (Fig. 2).

The conversion rates found with the bienzymatic system are in accordance with previous results obtained for BsLcar, where the highest conversion rate was reached for *N*-formyl-amino acids, followed by *N*-carbamoyl- and *N*-acetyl-derivatives [15]. Based on the specific activities of GkNSAAR and BsLcar toward *N*-carbamoyl-methionine (3.2 and 30.3 nmol min^{-1} μg^{-1}, respectively) and *N*-formyl-methionine (16. 3 and 120.0 nmol min^{-1} μg^{-1}, respectively), we can conclude that GkNSAAR is the limiting step in the system due to its lower specific activity.

3.2. Effect of enzyme ratios on the bienzymatic system

Based on the lower specific activity of GkNSAAR, we carried out studies on the proportion of each enzyme in the biocatalyst to evaluate economy aspects of both reaction time and protein consumption. Different BsLcar/GkNSAAR ratios were assayed (1:1, 1:3, 1:6, 1:12 and 1:18), maintaining a constant concentration of BsLcar (1.5–2.0 μM) and increasing the concentration of GkNSAAR (Fig. S1 SD). The three highest BsLcar/GkNSAAR ratios produced over 95% yield in less than 6 h. With a view to maintaining the minimum consumption of GkNSAAR, the 1:6 ratio (1.5–2.0 μM BsLcar and 10.0–12.0 μM GkNSAAR) was selected as optimum for biocatalytic transformation, since higher proportions of GkNSAAR did not result in a significant decrease in consumption time compared to the amount of enzyme used (Fig. S1 SD).

3.3. Cobalt effect on the bienzymatic system

Both BsLcar and GkNSAAR have been previously described as metalloenzymes, and Co^{2+} proved the best cofactor [14,15]. We therefore evaluated two strategies to provide the enzymes with this cofactor: (a) direct addition of 0.2 mM $CoCl_2$ in the growth medium to assess whether the enzymes were able to incorporate the cation before IMAC purification, and (b) addition of $CoCl_2$ in the reaction mixture. No difference in BsLcar activity was found between these two strategies (Fig S2 SD). On the other hand, GkNSAAR was not active at all when the enzyme was overexpressed in a medium supplemented with Co^{2+} (Fig. S3 SD), but it was active when $CoCl_2$ 1 mM was added to the reaction mixture. In addition, two effects were detected when Co^{2+} was included in the growth medium: (a) a toxic effect on bacterial cells, leading to a decrease in the number of recombinant cells produced during induction, and consequently, a drastic drop in the purified protein yield; (b) substantial insolubility of the recombinant enzymes produced by cells that accumulate Co^{2+} during growth and induction.

In view of these results, we decided to add the Co^{2+} cation directly into the reaction, determining the necessary cofactor concentration for optimal activity. Under the standard conditions (1.5–2.0 μM BsLcar and 10.0–12.0 μM GkNSAAR), total conversion of 15 mM *N*-formyl-methionine was achieved in 30 min (Fig. 3) with no noticeable inhibition at higher cobalt concentrations (up to 6 mM). Since concentrations over 0.25 mM of Co^{2+} did not significantly decrease the reaction time, we established this cofactor concentration for use in the standard reaction.

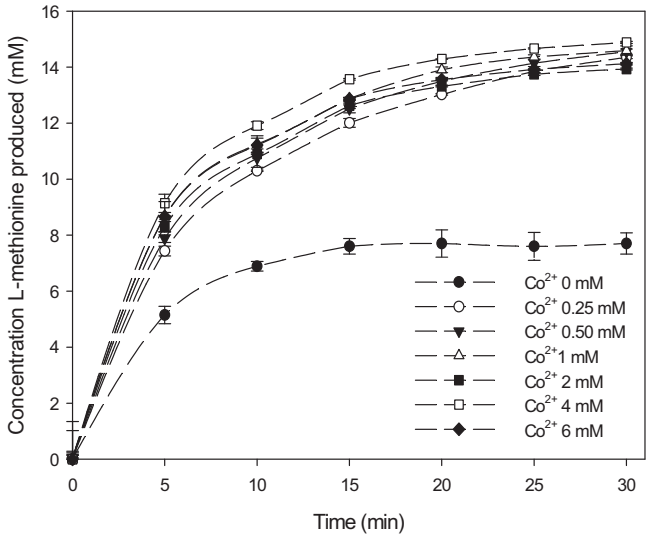

Fig. 3. Cobalt effect on the production of L-methionine by the bienzymatic system. Several concentrations of metal ion were added in the reaction mixture, and reactions were performed in triplicate using *N*-formyl-D,L-methionine as described in Section 2.

3.4. pH and temperature effect on the bienzymatic system

The study of parameters such as pH and temperature is of considerable importance when optimizing an enzymatic system. In order to find the optimal conditions for L-amino acids production, pH and temperature were studied starting from racemic mixtures of both *N*-formyl- and *N*-carbamoyl-methionine. The maximum conversion efficiency for both *N*-substituted substrates was detected in 100 mM borate buffer pH 8.0 (Fig. 4A). This pH value is the same as that obtained for GkNSAAR [14] and slightly higher than the value of pH 7.5 determined for BsLcar [15]. BsLcar showed 95% activity at pH 8.0, but GkNSAAR activity decreased to 80% at pH 7.5 [14,15], confirming pH 8.0 as the optimum for the bienzymatic system. Maximum conversion rates at this pH value was achieved in the range 55–70 °C (*N*-carbamoyl-methionine) and 45–55 °C (*N*-formyl-methionine) (Fig. 4B). These values are slightly different to those previously shown with BsLcar for *N*-formyl- and *N*-carbamoyl-amino acids (65 °C in both cases [15]). Since the optimal temperature of the isolated GkNSAAR had not been previously described for *N*-formyl or *N*-carbamoyl-amino acids [14], we carried out additional experiments to ascertain whether the optimal temperature found with the bienzymatic system was due to the GkNSAAR enzyme. The same optimal temperatures were found for GkNSAAR (45–55 °C for *N*-formyl-D,L-methionine; 55–70 °C for *N*-carbamoyl-D,L-methionine), thus showing that the obtained values are a result of the lower optimum temperatures of GkNSAAR. The substrate-dependent temperature found for GkNSAAR might reflect changes in enzyme structure (i.e. flexibility). Our results also underline the fact that the racemization step of the process constitutes the bottleneck of this bienzymatic system. Based on our results, we decided to carry out reactions at 45 °C for *N*-formyl-amino acids, and 65 °C for *N*-carbamoyl-amino acids, since higher temperatures did not result in a significant decrease in the conversion rates of the bienzymatic system.

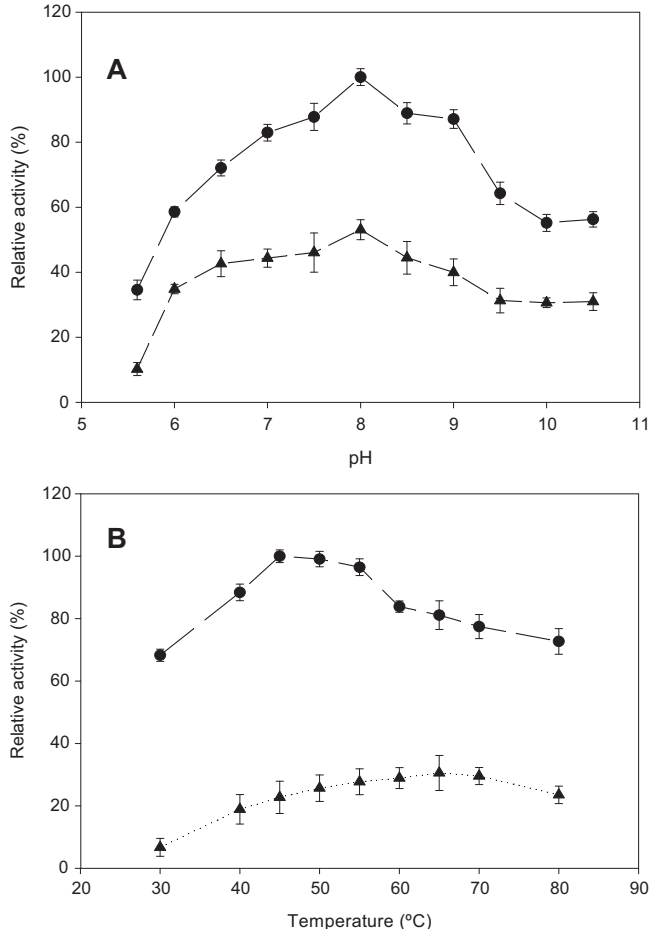

Fig. 4. Effect of pH (A) and temperature (B) on *N*-formyl- (●) and *N*-carbamoyl-D,L-methionine (▲) conversion into L-methionine. Studies of pH were assayed in several 100 mM buffers (sodium citrate (5.5–6.0), sodium cacodylate (6.5–7.5), sodium borate/HCl (8.0–9.0) and sodium borate/NaOH (9.5–10.5)). Temperature studies were carried out from 30 to 80 °C in 100 mM borate/HCl buffer pH 8.0. The figure shows the relative conversion rates of both *N*-formyl- and *N*-carbamoyl-methionine at different pH and temperature conditions. Enzyme assays were performed in triplicate as described in Section 2.

3.5. Thermal stability of the bienzymatic system

The thermal stability of the bienzymatic process was measured after 2, 4, 6, 8, 10 and 24 h of incubation, at temperatures ranging from 40 to 70 °C in 100 mM sodium borate/HCl buffer pH 8.0, followed by the standard enzyme assay. The bienzymatic system retained almost total operability after 24 h of incubation at 45 °C (Fig. S4 SD), thus showing that enzymatic reactions with this tandem can stretch to at least 24 h without loss of operability.

3.6. Substrate conversion to L-amino acid by the bienzymatic system

Once the reaction parameters resulting in lower conversion rates had been established, conversion reactions from different *N*-formyl- and *N*-carbamoyl-amino acids to the corresponding optically pure L-amino acids were evaluated (Table 1; Fig. S5 SD). As already mentioned, a previous work has shown L-homophenylalanine production, but no other L-amino acids, starting from racemic mixtures of *N*-carbamoyl-homophenylalanine, by using an *Escherichia coli* whole cell system comprising *Deinococcus radiodurans* NSAAR and *Bacillus kaustophilus* L-*N*-carbamoylase [13]. However, to the best of our knowledge, this is the first work to report a system able to convert not only racemic mixtures of *N*-carbamoyl-amino acids, but also *N*-formyl-amino acids into optically pure L-amino acids. The results show that the biocatalytic process converted *N*-formyl-amino acids more efficiently than *N*-carbamoyl-derivatives (Table 1), and that it was efficient in converting both aromatic and aliphatic substrates. In order to rule out the possibility that chemical hydrolysis of *N*-formyl- protecting group could be occurring during the reaction (*N*-carbamoyl-amino acids are known to be stable in solution), negative control reactions were carried out with all the substrates tested, incubating them in the same conditions as in the standard reaction assay (15 mM, 100 mM borate buffer pH 8.0, 45 °C) in the absence of enzymes. None of the *N*-formyl-amino acids showed spontaneous hydrolysis after 5 h. Since this incubation time is longer than the time required for total conversion of the majority of the *N*-formyl-substrates tested (Table 1), we can clearly conclude that no spontaneous hydrolysis occurs. No spontaneous hydrolysis was neither detected after incubation of 100 mM *N*-formyl-D,L-homophenylalanine in 100 mM borate buffer pH 8.0 for 24 h at 45 °C (in absence of enzymes).

Though the strict L-enantiospecificity of BsLcar and other L-*N*-carbamoylases has been previously demonstrated [9,15 and references therein], we checked again whether this enzyme hydrolyzed any of the following *N*-formyl-D- or *N*-carbamoyl-D-amino acids: methionine, ABA, norleucine, norvaline, ethionine, homophenylalanine and phenylglycine. None of these substrates were hydrolyzed under the conditions assayed. Furthermore, we calculated the enantiomeric excess of the L-amino acid produced by chiral-HPLC (Fig. 5), confirming that the e.e. of the amino acid obtained was 100% (Table 1). The highest initial reaction rate was found for L-methionine, with 0.64 and 0.16 U/mg protein from

Table 1
Optically pure L-amino acids produced by the bienzymatic tandem under the standard conditions assayed (15–20 mM *N*-formyl- or *N*-carbamoyl-amino acid). The corresponding times and the achieved yields are indicated.

Substrate	Product	Time (h)	% Conversion	e.e. (%)
N-Formyl-D,L-methionine	L-Methionine	0.5	>95	100
N-Carbamoyl-D,L-methionine	L-Methionine	2.5	>95	100
N-Formyl-D,L-norleucine	L-Norleucine	0.8	>95	100
N-Carbamoyl-D,L-norleucine	L-Norleucine	4	>95	100
N-Formyl-D,L-ABA	L-ABA	2	>95	100
N-Carbamoyl-D,L-ABA	L-ABA	10.8	>95	100
N-Formyl-D,L-homophenylalanine	L-Homophenylalanine	1.8	>95	100
N-Carbamoyl-D,L-homophenylalanine	L-Homophenylalanine	15	>95	100
N-Formyl-D,L-norvaline	L-Norvaline	0.8	>95	100
N-Carbamoyl-D,L-norvaline	L-Norvaline	4.5	>95	100
N-Formyl-D,L-phenylglycine	L-Phenylglycine	15	>95	100
N-Carbamoyl-D,L-phenylglycine	L-Phenylglycine	24	20	100
N-Formyl-D,L-ethionine	L-Ethionine	1	>95	100

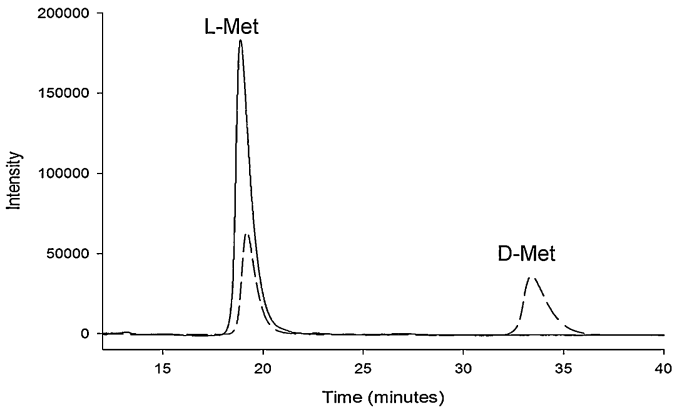

Fig. 5. Chiral-HPLC chromatograms showing commercial D,L-methionine (dashed line, 1 mM final concentration, that is 0.5 mM of each enantiomer) and a sample of a finished standard reaction of the bienzymatic system (continuous line, 1.5 mM final concentration), using racemic *N*-formyl-methionine as substrate. Optically pure L-methionine was produced by the enzymatic tandem (>95% yield, e.e. 100%).

N-formyl- and *N*-carbamoyl-methionine, respectively. The substrates hydrolyzed most slowly were L-aminobutyric acid (L-ABA) and L-phenylglycine, with aliphatic and aromatic substituents, respectively. Despite the lower conversion rate found for the production of L-ABA when compared to L-MET, the bienzymatic method presented in this work is more efficient than the alternative method for L-ABA production using threonine deaminase, aromatic aminotransferase and acetolactate synthase [22].

The behavior of the bienzymatic system at high substrate concentration is a crucial point to scale it to analytical or industrial scale. For this reason, we analyzed the conversion of racemic mixtures of *N*-formyl-phenylglycine (100 mM), *N*-formyl-homophenylalanine (100 mM) and *N*-formyl-L-ABA (500 mM), using the same amount of enzyme described for the standard small-scale assays. Conversion to optically pure 100 mM L-phenylglycine was achieved after 6 days, whereas total conversion to 500 mM L-ABA took 85 h, with no observed reaction inhibition. A striking effect occurred with the production of L-homophenylalanine at high substrate concentration; whereas the small-scale experiment showed a normal reaction profile (Fig. 6A), the experiment at high concentration seemed in principle to show inhibition, since the maximum concentration of L-homophenylalanine achieved was 30 mM (Fig. 6B). However, this reaction profile was not due to enzymatic inhibition, but to the low solubility of the reaction product. Whereas *N*-formyl-D,L-homophenylalanine is soluble at 100 mM, the corresponding product of the reaction is insoluble at concentrations over 30 mM. Thus, after approximately 30% of substrate conversion, a white precipitate started to appear in the solution, corresponding to the product of the reaction. The nature of the precipitated product obtained during the reaction was confirmed by NMR as L-homophenylalanine (Fig. S6 SD). The sum of both soluble and insoluble L-homophenylalanine was 98.2 ± 6.3 mM (Fig. 6B), resulting in a global yield over 95%. Under these particular circumstances, total conversion to 100 mM L-homophenylalanine was achieved after 24 h (Fig. 6B).

In conclusion, our results confirm that the bienzymatic BsLcar/GkNSAAR tandem is an interesting biotechnological tool for the preparation of different optically pure L-amino acids starting from different *N*-substituted amino acids by dynamic kinetic resolution. Analytical scale production of optically pure L-homophenylalanine, L-ABA and L-phenylglycine has been achieved. As the enzymes stand high concentrations of substrate and product, they might also show high industrial potential. Evaluation of economical aspects to ascertain whether this new biocatalyst is a real alternative for supplying non-natural L-amino acids to the pharmaceutical industry is currently being carried out. Furthermore, to improve the efficiency of this novel biocatalyst, our current research effort is focusing on the immobilization of these enzymes in order to enhance its stability and efficiency [23].

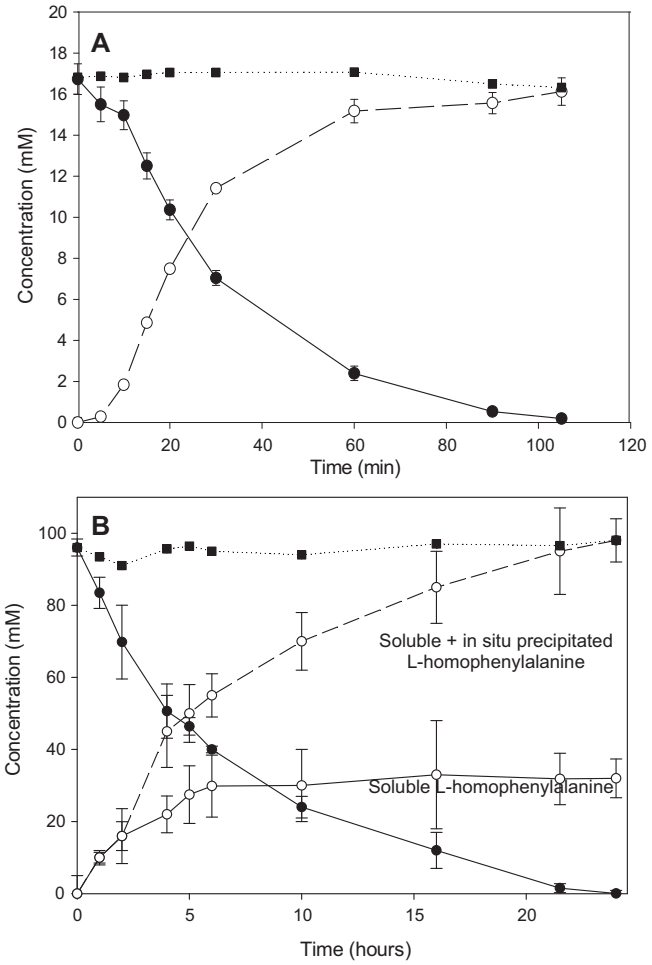

Fig. 6. Comparison of small-scale (17 mM, (A)) and large-scale (100 mM, (B)) experiments using *N*-formyl-D,L-homophenylalanine as substrate. Enzyme assays were performed in triplicate as described in Section 2. Plots show the profile of L-homophenylalanine production (○) starting from *N*-formyl-D,L-homophenylalanine (●), and the sum of the concentrations of both the product and the substrate of the reaction (■). In the large-scale experiment (B), two plots of L-homophenylalanine (○) are represented: one corresponding to the soluble homophenlyalanine produced during the reaction (continuous line), and other corresponding to the sum of the insoluble and soluble L-amino acid (dashed line), since L-homophenylalnine was soluble only up to 30 mM.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.procbio.2014.04.013.

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