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New biocatalytic route for the production of enantioenriched β-alanine derivatives starting from 5- and 6-monosubstituted dihydrouracils

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ABSTRACT

Taking advantage of the catalytic promiscuity of pyrimidine-catabolism enzymes (dihydropyrimidinase (E.C. 3.5.2.2), *N*-carbamoyl-β-alanine amidohydrolase (E.C. 3.5.1.6)), the production of different β-alanine derivatives starting from 5- and 6-monosubstituted dihydrouracils has been evaluated using a mimesis approach. In this work, the *S*-enantioselective character of dihydropyrimidinase from *Sinorizhobium meliloti* toward 6-monosubstituted dihydrouracil derivatives has been shown. An inverted *R*-/*S*-enantioselectivity of *N*-carbamoyl-β-alanine amidohydrolase from *Agrobacterium tumefaciens* toward two different *N*-carbamoyl-β-amino acids has been proved. Our results have shown for the first time that this mimetic tandem constitutes an interesting biotechnological tool for the preparation of different β-alanine derivatives in an environmentally friendly way, allowing the production of enantioenriched (*R*)-α-phenyl-β-alanine (e.e. > 95%) and (*R*)-α-methyl-β-alanine (e.e. > 90%).

1. Introduction

β-Amino acids have gained attention over the last decade partly due to their involvement in several natural products such as taxol, dolastatins, jasplakinolide, theopalauammide, and many others [1 and references therein]. β-Amino acids are key structural elements of peptides, peptidomimetics and many other physiologically active compounds [2]. Furthermore, a number of β-amino acids show interesting pharmacological properties in their free form [3], or as their cyclized (β-lactam) derivates [4]. β-Alanine supplementation is becoming popular in the sports field, as it has been shown to increase muscle buffer capacity in humans, with the potential to elicit enhanced physical performance during high-intensity exercise, and also to delay the onset of neuromuscular fatigue [5,6]. The racemic form of β-homoalanine (3-aminobutyric acid, 3-ABA) protects numerous plants against various pathogens [7 and references therein], by a natural defense machinery known as "induced resistance". This strategy has been proposed as an alternative environment-friendly approach for plant protection which would contribute to the development of sustainable agriculture [8]. α-Methyl-β-alanine (3-AiBA) has recently been described to reduce body weight in mice, suggesting it as an attractive pharmacological strategy in order to prevent (and/or treat) obesity in some individuals [9,10].

Chemical synthesis of β-amino acids has attracted the attention of many scientists over the last decade, as is evidenced by the number of reviews on asymmetric synthesis strategies found in the literature [1–3,11–17]. Several successful enzymatic strategies have also been investigated [2,3,18–20]. However, as stated previously, the resolution of β^2-amino acids have not been studied to the same extent as their β^3- and $\beta^{2,3}$-counterparts,[1] possibly due to the more laborious synthesis of the former compounds [18].

β-Alanine and 3-AiBA are produced naturally through the reductive catabolism of pyrimidines [21]. Among the enzymes involved in this metabolic pathway, dihydropyrimidinase (hydantoinase, E.C. 3.5.2.2) and *N*-carbamoyl-β-alanine amidohydrolase (β-alanine synthase, ureidopropionase, E.C. 3.5.1.6) are

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[1] Substituted β-amino acids are denominated β^2, β^3, and $\beta^{2,3}$, depending on the position of the side chain(s) (R) at the 3-aminopropionic acid core [29]. For more information, see Table 1, SD.

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responsible for the breakdown of the cyclic amide ring to the corresponding β-amino acid, CO_2 and NH_3. Although dihydropyrimidinases/hydantoinases[2] are mainly known/studied in the context of the "hydantoinase process" [22–31], enzymes hydrolyzing different 5- and 6-monosubstituted dihydrouracils have been reported [32–34]. This extended substrate promiscuity allowed Liljeblad and Kanerva to hypothesize that hydantoinases might "open up a new kinetic resolution route to enantiopure β-amino acids" [18]. This inference has been confirmed during the development of this manuscript by two independent groups [35,36], proving that different isolated and commercially available hydantoinases hydrolyze enantioselectively several 6-monosubstituted dihydrouracils, and demonstrating their potential for the biosynthesis of enantioenriched β^3-amino acids (Fig. 1, SD). Our group also proved that the β-alanine synthase from *Agrobacterium tumefaciens* C58 (Atβcar) is able to hydrolyze compounds other than those that are naturally degraded [37,38], making it the only β-alanine synthase to date that is known to do so. This enzyme proved to be enantiospecific for *N*-carbamoyl-L-α-amino acid hydrolysis [37]. On the other hand, it proved to be enantioselective toward *N*-carbamoyl-3-AiBA and *N*-carbamoyl-GABOB (β- and γ-amino acid precursors, respectively [37]). Taking advantage of the substrate promiscuity of dihydropyrimidinase from *Sinorizhobium meliloti* (SmelDhp) and Atβcar [33,37,38], we have evaluated the production of different β-alanine derivatives starting from dihydrouracil derivatives using a mimesis approach (Fig. 1, SD). According to the literature, at least some dihydrouracils derivatives can be synthesized from cheap materials such as urea and the corresponding α,β-unsaturated acid (acrylic, methacrylic and crotonic acids [41]), and thus, our results provide additional evidence that this enzymatic tandem might become an interesting biotechnological tool for the preparation of different β^2-amino acids in an environment-friendly way.

2. Materials and methods

2.1. General protocols and reagents

Standard methods were used for the cloning and expression of the different genes [42,43]. Restriction enzymes and T4 DNA ligase were purchased from Roche Diagnostic S.L. (Barcelona, Spain), the primers for PCR from IDT (Biomol S.L., Sevilla, Spain) and the thermostable KAPA HiFi polymerase from Kapa Biosystems (Cultek, Madrid, Spain). Talon metal-affinity resin was purchased from Clontech Laboratories, Inc. (Biomol S.L., Sevilla, Spain).(*R*,*S*)-3-Aminobutyric acid[3] (3-ABA), (*S*)-3-ABA, 1,2,4-thiadiazinan-3-one-1,1-dioxide (SULDHU), taurine, (*R*,*S*)- and (*S*)-3-amino-5-methylhexanoic acid, (*R*,*S*)- and (*S*)-3-amino-4-phenylbutyric acid and (*R*)-3-amino-2-phenylpropionic acid ((*R*)-α-phenyl-β-alanine) were acquired from Sigma–Aldrich Quimica (Madrid, Spain). β-Alanine, (*R*,*S*)-3-aminoisobutyric acid (3-AiBA) and (*R*,*S*)-3-amino-3-phenylpropionic acid were purchased from Acros (Scharlab, Barcelona, Spain). (*R*)-3-Amino-3-phenylpropionic acid was obtained from Alfa Aesar (VWR, Barcelona, Spain). (*R*,*S*)-α-Phenyl-β-alanine was previously obtained [38].

Dihydrouracil (DHU), the other monosubstituted dihydrouracils (5- and 6-methyl-dihydrouracil (METDHU), 6-phenyldihydrouracil (PHEDHU), 6-benzyldihydrouracil (BzDHU), 6-isobutyldihydrouracil (iBUTDHU)), and the corresponding *N*-carbamoyl-β-amino acids used in this work were synthesized using the same method used for α-amino acids described in the literature [44,45]. Briefly, the corresponding amino acids were refluxed in the presence of potassium cyanate in water solution, resulting in the formation of *N*-carbamoyl-β-amino acids. The subsequent formation of monosubstituted dihydrouracils was accomplished by refluxing the corresponding *N*-carbamoyl-β-amino acids in dilute aqueous HCl (0.3 M). All chemicals were analytical grade and were used without further purification.

2.2. Plasmids and microbes

Plasmids pSER38 and pAMG4 containing dihydropyrimidinase gene from *S. meliloti* CECT4114 (*SmelDhp*; 1455 bp; Genbank accession no. DQ779921) and *N*-carbamoyl-β-alanine amidohydrolase gene from *A. tumefaciens* C58 (*Atβcar*; 1248 bp; Genbank accession no. EF507843;), respectively, were used as DNA source for PCR amplifications [38,46]. Plasmid pJOE4036.1 (based on the *rhaBAD* promoter [47,48]) was used to reclone both genes. *Escherichia coli* DH5α (Stratagene, Agilent, Barcelona, Spain) was used for the cloning of *SmelDhp* and *Atβcar* genes, and *E. coli* BL21 Gold (Stratagene, Agilent, Barcelona, Spain) were used to overexpress them. Plasmids were purified with QIAprep spin Miniprep kit (Qiagen, Izasa, Barcelona, Spain), from overnight cultures of *E. coli* grown in Luria-Bertani medium (LB) (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.2) supplemented with 100 μg ml^{-1} of ampicillin, incubated at 37 °C with shaking.

2.3. Cloning of Atβcar and SmelDhp genes in pJOE4036.1

pAMG4 and pSER38 plasmids were used as templates for PCR amplification of *Atβcar* and *SmelDhp* genes [38,46]. The primers used were Atβcar5 (GGAATTCCATATGAC-GGCGGGTAAAAACTTGACGGTC) and Atβcar32 (TTAAGCTTTCAATGATGAT-GATGATGATGTTGCACGATCTCCGCAGTC) for the former, and SmelDhp52 (AAAAAAACATATGAGCACTGTCATCAAGGG) and SmelDhp32 (TTAAGCTTT-TAATGATGATGATGATGATGGACGCCGCTTGCGGGAATGCCGC) for the latter. The PCR products and the vector pJOE4036.1 were digested with *Nde*I and *Hind*III enzymes,[4] gel-purified and ligated, to create the plasmids pAMG4rha (containing *Atβcar* gene) and pSER38rha (containing *SmelDhp* gen). Verification of the sequence was carried out using the dye dideoxy nucleotide sequencing method in an ABI 377 DNA Sequencer (Applied Biosystems).

2.4. Expression of the different genetic constructions

E. coli BL21 Gold harboring pAMG4rha and pSER38rha plasmids were grown in LB medium supplemented with 100 μg ml^{-1} of ampicillin. A single colony was transferred into 10 ml of LB medium with ampicillin at the above-mentioned concentration. This culture was incubated overnight at 37 °C with shaking. 1000 ml of LB medium with the appropriate concentration of ampicillin was inoculated with 10 ml of the overnight culture. After 3–4 h of incubation at 37 °C with vigorous shaking, the OD_{600} of the resulting culture was 0.3–0.5. For expression induction of the *Atβcar* and *SmelDhp* genes in pAMG4rha and pSER38rha, L-rhamnose was added to a final concentration of 0.2% (w/v) and the culture was incubated at 32 °C overnight. The cells were collected by centrifugation (Beckman JA2-21, 7000 *g*, 4 °C and 15 min) and stored at −20 °C until use.

2.5. Purification of Atβcar and SmelDhp enzymes

Procedures for the purification and preparation of the enzymes were the same as those previously described using cobalt affinity chromatography [38,46]. An additional gel filtration chromatography step was carried out using a Superdex 200 gel-filtration column (GE Healthcare, Madrid, Spain) in a BioLogic DuoFlow fast-performance liquid chromatography (FPLC) system (Bio-Rad, Madrid, Spain) to prevent any DNA or protein co-eluting with the protein of interest. The purified enzymes were concentrated using the vivaspin concentrators (Sartorius, Dicsa, Almería, Spain), dialyzed against 0.1 M sodium phosphate buffer pH 8.0, and stored at 4 °C until use. Protein concentrations were determined using the Lowry method.

2.6. Bienzymatic system characterization

To analyze the effect of Ni^{2+} on enzymatic activity, samples of purified Atβcar and SmelDhp enzymes were incubated together in the presence of different concentrations of $NiCl_2$ at 4 °C for several days. Standard enzymatic reaction was carried out with (*R*,*S*)-5-METDHU as substrate (10 mM) in 100 mM sodium phosphate buffer (pH 8.0), at 30 °C in 1 ml reaction volume. Aliquots of 100 μl were taken and stopped by addition of 900 μl 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, Madrid, Spain) was used to determine the concentrations of 5-METDHU, *N*-carbamoyl-3-AiBA, and 3-AiBA. The mobile phase used in the analysis was 95% phosphoric acid (20 mM, pH 3.2) and 5% methanol, pumped at a flow rate of 1 ml min^{-1}. The UV detector was fixed at 200 nm. All reactions conducted for the bienzymatic system characterization were carried out in triplicate.

Optimal temperature of the bienzymatic system was evaluated from 25 to 60 °C. Thermal stability was measured after 24 h of preincubation at temperatures from 4 to 50 °C. A pH range of 6.0–9.0 was assayed (sodium phosphate

[2] For ease of comprehension the present work does not distinguish between dihydropyrimidinases and hydantoinases. For further knowledge on this topic, see [39,40].

[3] Due to the different nomenclature found in the literature for the β-amino acids used in this work, a list of synonyms has been included in Supplementary data (Table 1) for better understanding of the reader.

[4] Although this plasmid directly allows insertion of any gene fused to a His-tag by direct insertion in its *Nde*I-*BamH*I site, we could not use the latter enzyme as it cut both *Atβcar* and *SmelDhp* genes. Thus, we had to include a His-tag sequence in the reverse PCR primers.

Table 1
HPLC measurement conditions for each of the compounds.

Substrate/intermediate/product	Mobile phase[a]	Flow (ml min^{-1})
Reverse-phase HPLC separations		
DHU/*N*-carb-β-alanine/β-alanine	100% C	0.50
SULDHU/*N*-carb-taurine/taurine	100% C	0.50
5-METDHU/*N*-carb-3-AiBA/3-AiBA	5% A:95% B	1.00
6-METDHU/*N*-carb-3-ABA/3-ABA	5% A:95% B	1.00
5-PHEDHU/*N*-carb-α-phenyl-β-alanine/α-phenyl-β-alanine[b]	30% A:70% B	0.75
Compound	**Mobile phase[a]**	**Flow (ml min^{-1})**
Chiral HPLC separations		
3-AiBA[c]	100% A	0.50
3-ABA[c]	100% A	0.50
α-Phenyl-β-alanine	50% D:50% E	0.60
6-PHEDHU	90% E:10% A	0.60
6-BzDHU	90% E:10% A	0.60
6-iBUTDHU	90% E:10% A	0.60

[a] A, methanol; B, H_3PO_4 20 mM pH 3.2; C, NaH_2PO_4 50 mM pH 4.5; D, ethanol; E, H_2O.
[b] Although the order of elution for this kind of compounds is usually product < intermediate < substrate, for this amino acid and its derivatives it occurs as: product < substrate < intermediate.
[c] Assignment of the enantiomers is that reported previously [55].

and Tris/HCl buffers), at a concentration of 100 mM. Standard enzymatic reaction was then carried out with the Ni-amended Atβcar (1 μM) and SmelDhp (1 μM) enzymes. The reaction progress was monitored by HPLC analysis as described above.

Substrate specificity studies were performed with each different dihydrouracil derivative dissolved in 100 mM sodium phosphate buffer (pH 8.0) together with the purified enzymes (in triplicate). The enzyme ratio for each substrate was calculated with Ni-amended Atβcar and SmelDhp enzymes (concentrations ranging from 1 to 1000 μM for the former and 1 to 10 μM for the latter, depending on the substrate used). Reactions were carried out at 35 °C and pH 8.0, and stopped by addition of 1% H_3PO_4. The mobile phases used for each substrate and its corresponding *N*-carbamoyl-β-amino acid and β-amino acid are summarized in Table 1. Compounds were detected with a UV detector at a wavelength of 200–210 nm.

Larger-scale reactions were carried out with (*R*,*S*)-5-METDHU at 0.1 and 1 M (10 ml reaction volume), using Ni-amended Atβcar (10 and 100 μM) and SmelDhp (10 and 100 μM) enzymes, at pH 8.0 and 35 °C. We also considered the strategy known as "membrane-enclosed enzymatic catalysis", often called "tea-bags" [49,50]. For this approach, we used Spectra/Por® Float-A-Lyzer® G2 devices (8–10 kD, 1 ml, Sigma–Aldrich Quimica, Madrid, Spain).

2.7. Enatioselectivity of Atβcar and SmelDhp enzymes

Substrate specificity studies for Atβcar and SmelDhp were performed in 100 mM sodium phosphate buffer (pH 8.0) together with the Ni-amended enzymes (in triplicate, concentrations ranging from 0.5 to 20 μM, depending on the substrate used). Reactions were carried out at 35 °C. The reaction was stopped by retrieval of 100 μl aliquots and addition of the same volume of 1% H_3PO_4. 800 μl of the corresponding mobile phase were added to each sample (retention times depended greatly on the pH and the matrix effect). The mobile phases used for each compound are summarized in Table 1. Compounds were detected with a UV detector at a wavelength of 200–210 nm. To assess if spontaneous racemization of 6-monosubstituted dihydrouracils occurs (*S*)-6-BzDHU and (*S*)-6-IBDHU (2 mM) were incubated in phosphate buffer pH 8.0 at 35° for 48 h (1 ml). 100 μl aliquots were diluted with 100 μl of 1% H_3PO_4 and 800 μl of the corresponding mobile phase, and chromatographically detected as stated above.

3. Results and discussion

3.1. Effect of nickel cation on the bienzymatic system

Previous works have demonstrated that Atβcar and SmelDhp are metalloenzymes [33,38]. The best cations for Atβcar and SmelDhp were Ni^{2+} and Zn^{2+}, respectively. However, the latter inhibited Atβcar activity [38]. As Ni^{2+} also greatly enhanced SmelDhp activity [33], all experiments were conducted using this cofactor. The bienzymatic system activity was tested at different incubation times and different concentrations of $NiCl_2$ using the standard reaction. The optimum activity was achieved with over 1 mM of cation and at least 24 h of incubation (Fig. 2, SD).

3.2. Enantioselectivity of Atβcar and SmelDhp

The preference of Atβcar for the *R*-enantiomer of *N*-carbamoyl-3-AiBA was previously shown [37]. In this study, Atβcar has shown high enantioselectivity toward *N*-carbamoyl-(*R*)-α-phenyl-β-alanine, where an enantiomeric excess (e.e.) of over 95% was found for the production of (*R*)-α-phenyl-β-alanine (Fig. 1A). The contrary was found toward the only *N*-carb-β^3-amino acid derivative that Atβcar was able to hydrolyze (*N*-carb-(*R*,*S*)-3-ABA), where a preference toward the *S*-enantiomer could be observed (Fig. 3, SD). Although the previous results with this enzyme suggested that Atβcar was *R*-enantioselective toward *N*-carbamoylated-β^2-alanine derivatives, the *S*-enantiomer reacts with the β^3-alanine derivative.

The enantiopreference of SmelDhp toward different enantiomers of 5- and 6-monosubstituted dihydrouracils has not been previously studied. As chemical racemization of 5-monosubstituted hydantoins is known to occur [51], we wanted to know whether dihydrouacil derivatives have been studied in this sense. Through literature revision, we only found a work related with the racemization of dihydrouracil-derivatives [52]. In this paper, the study on the interchange of protons in the carbon 5 (C5) was proved, indicating that 5-monosubstituted dihydrouracils also racemize spontaneously, as expected for an acidic proton at the adjacent carbon to a carbonyl group. From the same work, it also seemed clear that dihydroorotate (6-carboxy-dihydrouracil) did not racemize. This has been further confirmed in this work: no racemization was observed for (*S*)-6-BzDHU and (*S*)-6-IBDHU in phosphate buffer pH 8.0 at 35° in 48 h. SmelDhp showed a marked preference toward the *S*-enantiomer of 6-BzDHU, 6-iBUTDHU (Fig. 1B) and 6-PHEDHU, with relative S/R rates of 7, 3 and 2, respectively. On the other hand, this enzyme is *R*-specific for 5-monosubstituted hydantoins [33]. We could not determine the enantioselectivity toward 5-METDHU, 6-METDHU and 5-PHEDHU by chiral HPLC, as we were not able to separate the enantiomers using different mobile phases. However, from the progression curves of these compounds (see Fig. 4, SD), a preference toward one of the enantiomers for 6-METDHU and 5-PHEDHU could be inferred. Our results reaffirm the findings of O'Neill et al. [36] and Engel et al. [35] during the development of this work: the

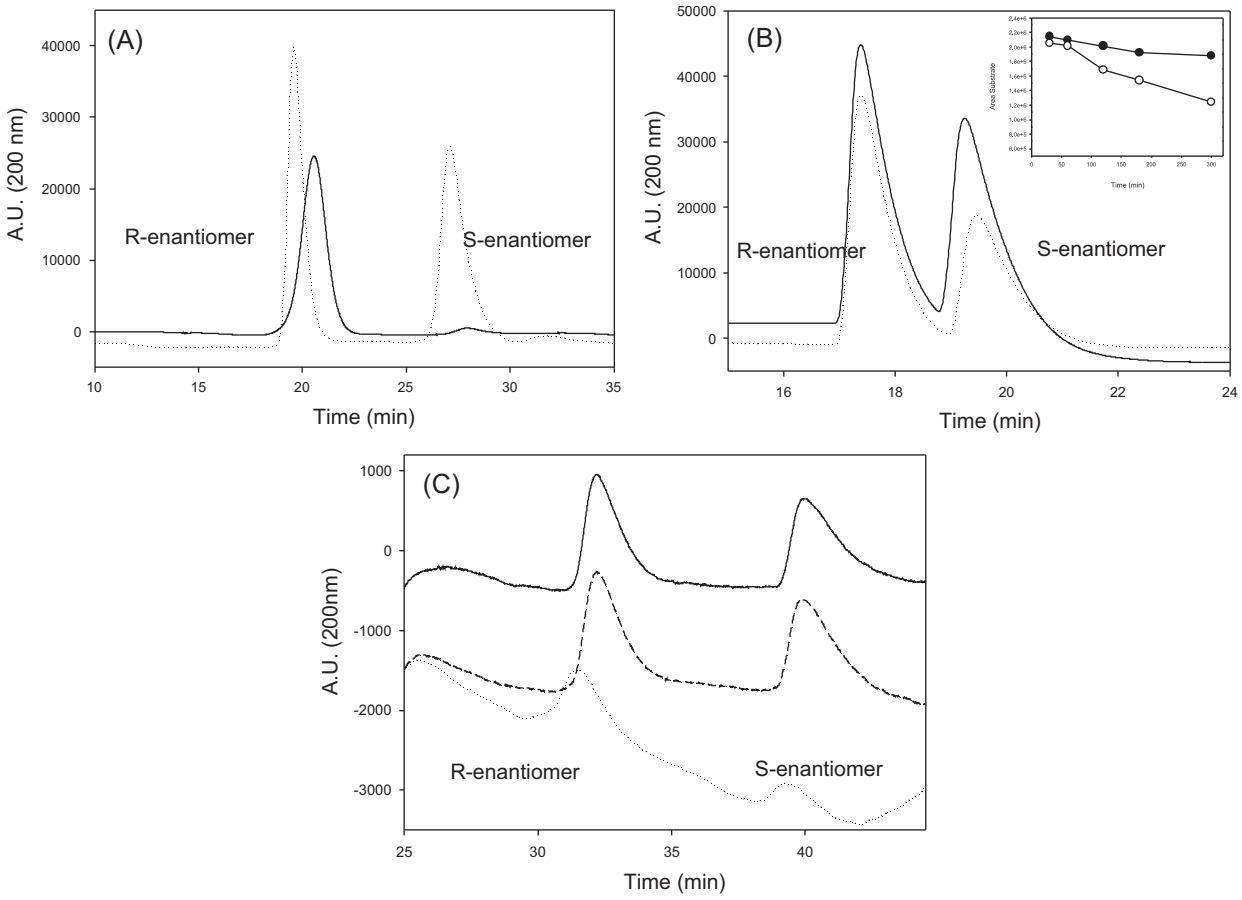

Fig. 1. Examples of the chiral separations carried out to asses the enantioselectivity of Atβcar (A) and SmelDhp (B), and to determine the enantiomeric excess of 3-AiBA produced using the bienzymatic system and (*R*,*S*)-5-METDHU as substrate (C). (A) Dotted line (*R*,*S*)-α-phenyl-β-alanine, solid line, reaction result using Atβcar with racemic (*R*,*S*)-carb-α-phenyl-β-alanine as substrate, showing appearance of (*R*)-α-phenyl-β-alanine. (B) Solid line (*R*,*S*)-6-iBUTDHU, dotted line, reaction course of SmelDhp using (*R*,*S*)-6-iBUTDHU as substrate, showing faster consumption of the *S*-enantiomer. The inset represents the decreasing areas of both (*R*)- (●) and (*S*)-iBUTDHU (○) during the reaction course, showing the *S*-stereoselectivity of SmelDhp toward this substrate. (C) Solid line, (*R*,*S*)-3-AiBA; dashed and dotted lines, reaction course of bienzymatic system at the end of the reaction and at approximately 50% consumption of (*R*,*S*)-5-METDHU, respectively (this graph corresponds to the same experiment shown in Fig. 4B). (*R*)-3-AiBA is preferentially produced at the beginning of the reaction [55].

commercially available hydantoinase from *Vigna angularis* and several hydantoinases from bacterial sources are able to hydrolyze enantioselectively several β^3-amino acid derivatives.

3.3. Effect of pH and temperature

The bienzymatic system showed maximum activity in Tris and phosphate buffers at pH 8.0 when it was examined in 100 mM sodium phosphate buffer (pH 6.0–8.0) and 100 mM Tris–HCl buffer (pH 8.0–9.0) (Fig. 2A). The optimum temperature for production of 3-AiBA was 45 °C (Fig. 2B), intermediate between the optimal temperatures of the isolated enzymes [33,38]. Thermal stability of the system was studied by preincubation of the system for 1 and 24 h in 100 mM sodium phosphate buffer, pH 8.0, at different temperatures. Although the melting temperatures of both enzymes were proved to be higher than 45 °C [37,53], the stability of the system proved to be highly dependent on the incubation time (Fig. 2B). Activity decreased drastically when the enzymes were incubated at temperatures of over 35 °C for 24 h, and was lost completely at over 45 °C (Fig. 2B). Thus, we can conclude that the results obtained in this work are due to the prolonged incubation time, and more specifically affect the second enzyme in the system. In view of the above data, it would not be advisable to carry out reactions of over 24 h or over 35 °C.

3.4. Bienzymatic production of non-chiral and enantioenriched β-amino acids

The opposite enantioselectivity of both enzymes is in principle a drawback for the application of a bienzymatic system. However, based on the substrate promiscuity of both enzymes and in the reverse reaction known to occur for dihydropyrimidinase, we decided to evaluate the possible application of the Atβcar/SmelDhp tandem for the production of different β-amino acids starting from 5- and 6-monosubstituted dihydrouracils, imitating the degradation of pyrimidines that occurs naturally in the metabolism [21]. Reactions were carried out with 10 mM of different substrates in sodium phosphate buffer (pH 8.0) at 35 °C after preincubation of the system with $NiCl_2$ as described in materials and methods. Different concentrations of the enzymes were assayed based on (a) the molar catalytic activity of both enzymes at the concentration used in the reaction for the different substrates [33,37,38], and (b) using an excess of Atβcar, as it is the limiting step of the enantioenrichment reaction. Using 1 μM of each enzyme, the natural substrates DHU and 5-METDHU were totally converted into β-alanine and 3-AiBA in 18 and 90 min, respectively (Fig. 3A and B). In this sense, an e.e. > 90% of (*R*)-3-AiBA was monitored during the reaction course until half of (*R*,*S*)-5-METDHU was consumed (Figs. 3B and 1C). Total conversion of 6-METDHU into 3-ABA was accomplished in

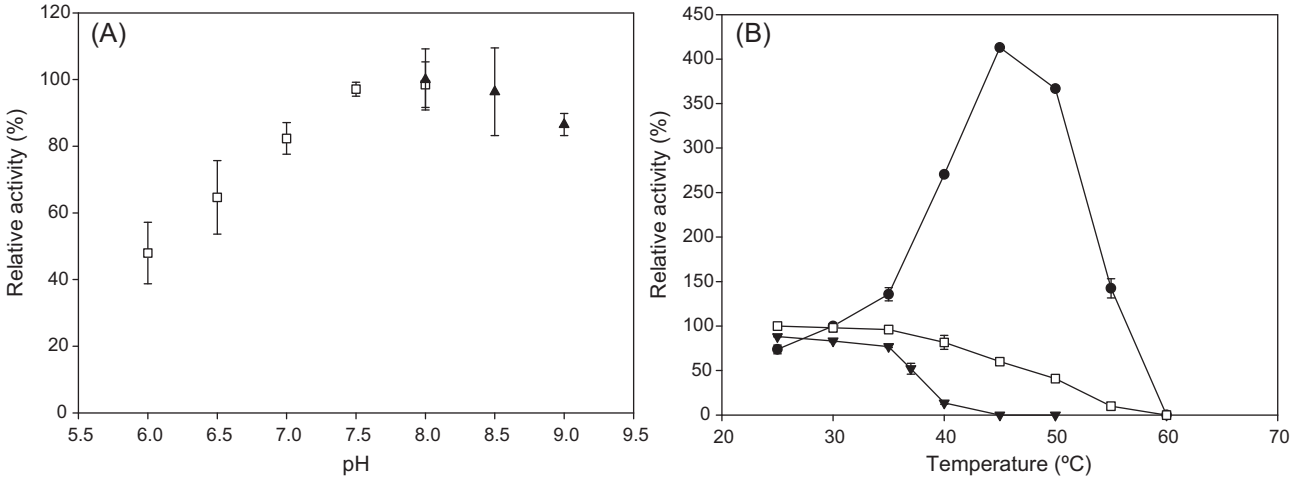

Fig. 2. (A) Optimal pH for 3-AiBA production using phosphate (□) and Tris (▲) buffers. (B) Optimum temperature for 3-AiBA production (●), and remaining relative activity of the bienzymatic system after 1-h (□) and 24-h (▼) preincubation at 35 °C. The results are the means of three experiments, and the error bars indicate the standard deviations of the means.

9.5 h, with an e.e. ≈ 50% for (*S*)-3-ABA before half consumption of (*R*,*S*)-6-METDHU (2.5 μM SmelDhp and 100 μM Atβcar, Fig. 3C). Taurine production was not possible because SmelDhp was not able to hydrolyze the substrate SULDHU in the conditions tested, although Atβcar was shown to hydrolyze the intermediate of the reaction [36]. (*R*)-α-phenyl-β-alanine with a high enantiomeric purity (e.e. > 95%) was obtained after 5 h of reaction, using 10 μM of SmelDhp and 1 mM of Atβcar (Fig. 3D). This occurs due to the high enantioselectivity of Atβcar toward the *R*-enantiomer of the intermediate of the reaction (Fig. 1A). In comparative terms, the

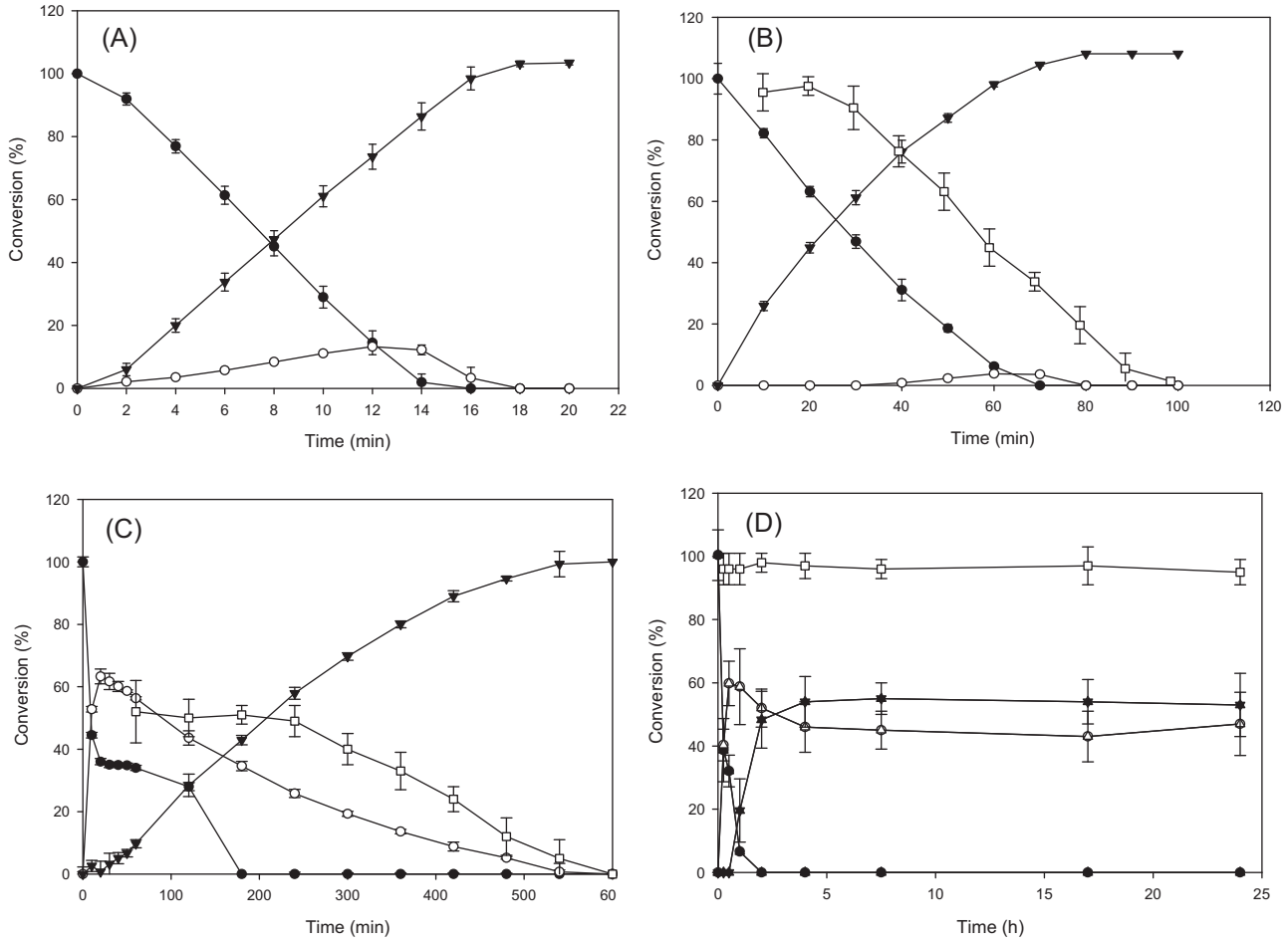

Fig. 3. Profiles of β-alanine (A), 3-AiBA (B), 3-ABA (C) and α-phenyl-β-alanine (D) production using the bienzymatic system. The symbols represent the substrate (●), the intermediate (○) and the product (▼) of the reaction. The squares (□) in (B), (C) and (D) represent the e.e. of the amino acid during the reaction. Reactions and measurements were carried out in triplicate as described in Section 2.

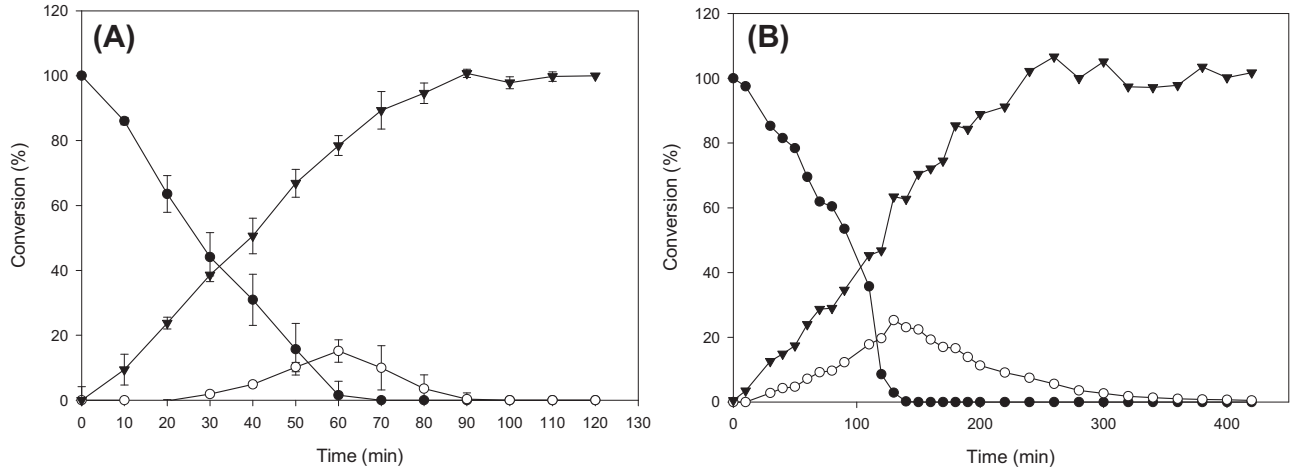

Fig. 4. Profiles of conversion of 0.1 M (A) and 1 M (B) by 10 μM and 100 μM of each enzyme respectively (●, 5-METDHU; ○, *N*-carb-3-AiBA; ▼, 3-AiBA). Reactions and measurements were carried out in triplicate as described in Section 2.

conversion efficiency of the tandem for 6-METDHU and 5-PHEDHU was three orders of magnitude lower than for the natural substrates 5-METDHU and DHU.

Following our hypothesis on the use of a β-alanine synthase for this approach [38], O'Neill et al. also tried to hydrolyze the *N*-carbamoyl-β-derivative produced by the hydantoinase of *V. angularis* using a commercially available carbamoylase [36]. They found that this was not possible. This may be explained by their using a D-carbamoylase (E.C. 3.5.1.77) instead of a β-alanine synthase (E.C. 3.5.1.6), which to the best of our knowledge has not been yet reported to hydrolyze *N*-carbamoyl-β-D-amino acids [40]. On the other hand, our previous results showed that Atβcar would not be a good candidate for the hypothetical production of β^3-amino acids, as it was only able to hydrolyze the precursor of 3-ABA, but not carbamoyl derivatives of larger substituents [38]. Thus, enriched β^3-amino acids such as 3-amino-3-phenylpropionic acid, β-homoleucine and β-homophenylalanine could not be produced by the bienzymatic approach. On the other hand, enriched (*S*)-β^3-amino acids could be produced using SmelDhp alone, with subsequent treatment of the reaction intermediate with $NaNO_2$, as proposed by the groups of Turner and Syldatk [35,36] (Fig. 1, SD).

The behavior of any enzymatic system at high substrate and product concentrations is of considerable interest, as its subsequent scale-up depends on several factors, such as whether high concentrations of substrate/products inhibit the enzymatic reaction. For this reason, we have analyzed the bienzymatic system for 3-AiBA production using 0.1 and 1 M (*R,S*)-5-METDHU in phosphate buffer (pH 8.0) at 35 °C in a reaction volume of 10 ml. Starting from 0.1 M substrate, total conversion was achieved in 90 min (Fig. 4A), similar to the time observed with in the small-scale experiment (Fig. 3B). Using 1 M 5-METDHU (the substrate was not completely dissolved), total conversion was achieved after 300 min (Fig. 4B). The higher time found for 1 M 5-METDHU seems to be due to a combination of the lower ratio of Ni^{2+} cation/enzyme in the reaction (cation precipitates over 1 mM at pH 8.0) and the solubility process of the non-dissolved substrate. The same effect observed in small-scale reactions with (*R,S*)-5-METDHU occurred in this case, with an e.e. > 90% of (*R*)-3-AiBA before half consumption of 5-METDHU (data not shown). We also evaluated the use of the "tea-bag" strategy [49,50], as it allows the reuse of any enzymatic system, thus decreasing production costs. Under the conditions assayed, total conversion was achieved after 5 days (Fig. 5, SD), meaning that it proved less effective than the system with the free enzymes. The evidence increasingly supports diffusional limitations, as has been observed with other "tea-bag" systems [54].

4. Conclusions

In brief, our results show that the mimetic tandem dihydropyrimidinase/β-carbamoylase is an interesting biotechnological tool for the preparation of different β-alanine derivatives in an environmentally friendly way. An analytical scale production of enantiomerically enriched (*R*)-α-phenyl-β-alanine (e.e. > 95%) and (*R*)-3-AiBA (e.e. > 90%) has been achieved. As the enzymes stand high concentrations of substrate and product, they might show high potential in preparative scale as well. On the other hand, further studies should be carried out to improve the economy of the system, as for example, in this work the substrates were prepared from β-amino acids as to show the potential of the enzymatic tandem. The necessity for new enzymes with different substrate spectrum and enhanced enantioselectivity is mandatory to increase the versatility of this bienzymatic system in the future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.procbio.2012.07.026.

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