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Rational re-design of the "double-racemase hydantoinase process" for optically pure production of natural and non-natural L-amino acids

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ABSTRACT

The "hydantoinase process" is a well-established method for the industrial production of optically pure D-amino acids. However, due to the strict D-enantioselectivity of most hydantoinase enzymes, the process is less efficient for L-amino acid production. We present a new chemo-enzymatic cascade reaction for natural and non-natural L-amino acid production from racemic mixtures of 5-monosubstituted hydantoins. This system comprised the following enzymes: D-hydantoinase from *Agrobacterium tumefaciens* BQL9, hydantoin racemase 1 from *A. tumefaciens* C58 and L-*N*-carbamoylase from *Geobacillus stearothermophilus* CECT43, together with *N*-succinyl-amino acid racemase from *G. kaustophilus* CECT4264. This latter presents catalytic promiscuity and racemizes *N*-carbamoyl-amino acids. This activity avoids the accumulation of *N*-carbamoyl-D-amino acid in the reaction due to the strict D-enantioselectivity of the hydantoinase. The optimum pH for the system proved to be 8.0, whereas optimum temperature range was 50–65 °C, with the maximum reaction rate at 60 °C. The metal ion cobalt was added directly to the reaction mixture (end concentration 1 mM), but in the case of D-hydantoinase, overexpression in presence of 0.5 mM Co^{2+} was also necessary. The enzymatic cascade reaction produced different optically pure L-amino acids by dynamic kinetic resolution, achieving 100% conversion even at high substrate concentrations (100 mM) with no noticeable inhibition. This total conversion demonstrates that the "double-racemase hydantoinase process" upgrades the classical "hydantoinase process" for natural and non-natural L-amino acid production.

1. Introduction

Optically pure L-amino acids are among the most important products in industrial biotechnology [1]. Proteinogenic amino acids are the basic building blocks for proteins and key nutritional compounds [2]. Additionally, non-natural amino acids are in demand for the synthesis of novel drugs, giving rise to a new discipline of study, as can be inferred from the appearance of a complete book on this topic [3]. L-Homophenylalanine is a non- natural amino acid used as an intermediate for the preparation of angiotensin-converting enzyme (ACE) inhibitors [4]. L-α-Aminobutyric acid (L-ABA, also known as L-homoalanine) is used as a biomarker for oxidative stress [5] and is a key chiral intermediate for the synthesis of several important antiepileptic and antituberculosis drugs [6]. L-Norleucine (2-aminohexanoic acid) has recently been shown to present cytotoxic activity against human tumor cell lines [7], and it is also a key chiral intermediate for the synthesis of several new compounds [8,9]. In recent years, the possibility of site-specific incorporation of non-natural amino acids into proteins has had a major impact on protein engineering, and has proven of great value to test protein function for medical and industrial applications [10,11].

The "hydantoinase process" is one of the most widely-used enzymatic methods for the production of optically pure α-amino acids [12–14]. In this cascade process, the chemically synthesized D,L-5-monosubstituted hydantoin ring is first hydrolyzed by an enantioselective hydantoinase. Further hydrolysis of the resulting enantiospecific *N*-carbamoyl-α-amino acid (intermediate) to the corresponding free D- or L-amino acid is catalyzed by highly enantiospecific *N*-carbamoyl-α-amino acid amidohydrolase (*N*-carbamoylase). At the same time as the hydantoinase hydrolyzes the enantiospecific 5-monosubstituted hydantoin in the first step, the remaining non-hydrolyzed substrate is

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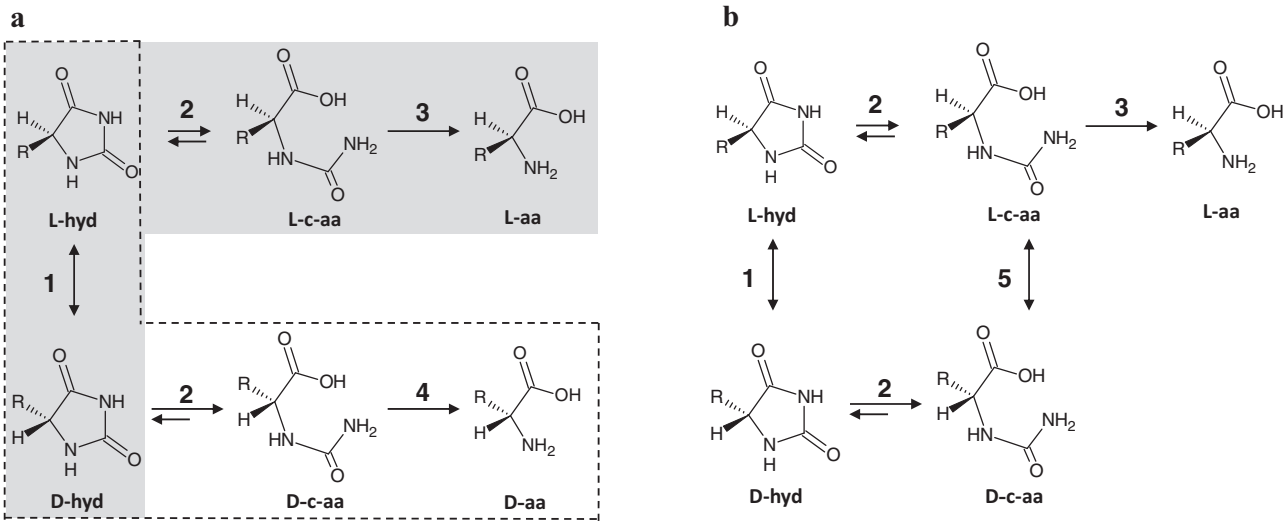

Fig. 1. (a) Reaction scheme for optically pure D-(dashed line) and L-(grey box) amino acid production from racemic mixtures of 5-monosubstituted hydantoins by the "hydantoinase process". (b) Reaction scheme for the proposed "double-racemase hydantoinase process" for optically pure L-amino acid production from the same substrates. (1) Hydantoin racemase, or chemical racemization; (2) D,L-hydantoinase; (3) L-carbamoylase; (4) D-carbamoylase; (5) *N*-succinyl-amino acid racemase.

racemized by the hydantoin racemase (Fig. 1a). This dynamic kinetic resolution process was initially used for the production of optically pure D-amino acids, D-phenylglycine and p-OH-phenylglycine, side chains for the β-lactam antibiotics ampicillin and amoxicillin [15]. Hydantoinases, as mentioned above, are enantioselective enzymes hydrolyzing both D- and L-5-monosubstituted hydantoins. However, from the practical point of view, hydantoinases have been classified into D-, L- and non-selective, depending on their enantioselectivity [16]. Due to the importance of D-amino acids as intermediates for the production of sweeteners, pesticides and other active compounds [17], and the fact that most reported hydantoinases were D-enantioselective [16], the "hydantoinase process" has mainly been employed for the production of D-amino acids [18]. Our group has developed biocatalytic systems overexpressing D-hydantoinase, D- carbamoylase and hydantoin racemase for optically pure D-amino acid production [19,20]. Hydantoinases do, on rare occasions, present L-enantioselectivity and applicability for natural and non- natural L-amino acid production by the "hydantoinase process" [18]. The hydantoinase from *Arthobacter aurescens* DSM3745 (AaHYD) showed substrate-dependent enantioselectivity, which was therefore predominantly L-enantiospecific for the cleavage of D,L-5-indolylmethylhydantoin (D,L-IMH), with hardly any cleavage for the D-isomer [21]. On the other hand, the same enzyme appeared to be D-enantiospecific for the hydrolysis of D,L-methylthioethylhydantoin (D,L-MTEH), and the activity for the D-enantiomer was 3-fold that for the L-enantiomer [21]. More recently, the D- hydantoinase from *Brevibacillus agri* NCHU1002 (BaDHP) has shown non-enantiospecificity for D,L- homophenylalanylhydantoin (D,L-HPAH), although it exhibited five times more substrate preference for the D-isomer (D- HPAH) [22]. However, despite this scant L-hydantoinase activity, together with an L-*N*-carbamoylase from *Bacillus kaustophilus* CCRC11223, a conversion yield of 49% for L-homophenylalanine (l-HPA) was reached in 16 h at pH 7.0 from 10 mM D,L-HPAH. Both hydantoinase enzymes have been improved by directed evolution (AaHYD) and by site-directed mutagenesis (BaDHP), and subsequently integrated in the "hydantoinase process" to produce L-methionine from D,L-MTEH [23] or L-HPA from D,L-HPAH [24], respectively. The evolved L-hydantoinase from AaHYD together with an L- *N*-carbamoylase and a hydantoin racemase produced 91 mM L-methionine from 100 mM D,L-MTEH in less than 2 h, whereas the whole-cell catalyst with the wild-type pathway produced about 66 mM of the L-amino acid in the same time [23] . Site-directed mutagenesis of BaDHP provided several variants with increased activity toward L-HPAH. One variant of BaDHP (L159 V) was used to convert D,L-HPAH to l-HPA using the "hydantoinase process". As compared with that of the wild-type enzyme, the conversion yield of l-HPA increased from 39% to 61% for this variant [24]. The l-selectivity of these designed enzymes was not impressive and left room for further improvements [23]. However, until that moment, these designed hydantoinases represented the only way to improve the productivity of whole cell catalysis for l-amino acid production by the "hydantoinase process" [25].

Our group has characterized several hydantoinases that have shown D-enantioselectivity for the hydrolysis of D,L-5-monosubstituted hydantoins [26,27]. D-hydantoinase and D-*N*-carbamoylase from *Agrobacterium tumefaciens* BQL9 were incorporated together with a hydantoin racemase from *A. tumefaciens* C58 for the production of optically pure D-amino acids [19,20]. Due to the D-enantioselectivity of this hydantoinase, the resulting enzymatic cascade reaction after combination with an L-*N*-carbamoylase from *Geobacillus stearothermophilus* CECT43 [28] and a hydantoin racemase is inoperative for L-amino acid production. In addition, the potential enhancement of this D-enantioselective hydantoinase by directed evolution or site-directed mutagenesis will be unsatisfactory, judging from the results described above for the non-enantiospecific hydantoinases.

An alternative method to produce optically pure amino acids is the "acylase process", where starting from racemic mixtures of *N*-acetyl-amino acids, an *N*-acetyl-amino acid racemase [29] (NAAAR; re-assigned as *N*- succinyl-amino acid racemase, NSAAR [30] is coupled with a D- or L-aminoacylase. In a previous work, we confirmed that a recombinant NSAAR from *G. kaustophilus* CECT4264 (GkNSAAR)), with substrate promiscuity, also racemized D- and L-*N*-carbamoyl-amino acids [31] . Thus, in the present work we aimed to develop a biocatalyst based on the "hydantoinase process" to produce optically pure L-amino acids from racemic mixtures of 5-monosubstituted hydantoins. Together with the enzymes D-hydantoinase from *A. tumefaciens* BQL9 [26], hydantoin racemase 1 from *A. tumefaciens* C58 [32] and l-*N*-carbamoylase from *G. stearothermophilus* CECT43 [28], GkNSAAR is also incorporated in the enzymatic cascade reaction (Fig. 1b). This NSAAR is able to racemize the accumulated *N*-carbamoyl-D-amino acid produced by the D-enantioselective hydantoinase, thus allowing the L-*N*-

carbamoylase activity to produce the corresponding free L-amino acid. This "double-racemase hydantoinase process" permits 100% conversion of racemic mixtures of hydantoins into optically pure L-amino acids using a D- enantioselective hydantoinase.

2. Material and methods

2.1. Materials and reagents

All chemicals were analytical grade and were used without further purification. Talon metal-affinity resin was purchased from Clontech Laboratories, Inc., Cloning enzymes and PCR primers were purchased from Roche Diagnostic S.L. (Barcelona, Spain) [34]. Racemic and optically pure D- and L-amino acids were purchased from Alfa Aesar GmbH & Co., K.G. (Barcelona, Spain). The monosubstituted hydantoins and the corresponding *N*-carbamoyl-amino acids used in this work were synthesized according to the potassium cyanate method for the carbamoylation and cyclation of amino acids, described elsewhere [33,34].

2.2. Cloning of the genes

L-*N*-carbamoylase from *Geobacillus stearothermophilus* CECT43 (*Bslcar*) and *N*-succinyl-amino acid racemase from *G. kaustophilus* CECT4264 (*Gknsaar*) were overexpressed and purified from *Escherichia coli* BL21 cells [35] harbouring the plasmids pJAVI80Rha [36] and pJPD25Rha [34], respectively, as previously described.

PCR amplification and subsequent cloning into the plasmid pJOE4036.1 [37] of the D-hydantoinase gene from *Agrobacterium tumefaciens* BQL9 (*Atdhyd*) and the hydantoin racemase 1 gene from *A. tumefaciens* C58 (*AthyuA1*) were performed as described in a previous work [34], with slight modifications. The PCR primers for *Atdhyd* gene amplification were 5′-AAAAAAA*CATATG*GATATCATCATCAAGAACGGAAC-3′ (including a *Nde*I site, in italics) and 5′-AA*CTGCAG***TTA**ATGATGATGATGATG ATGGGCGCAGGCGACGAGGGCTGG-3′ (including a *Hind*III site, in italics, stop codon in bold and six histidine tag underlined) using pBSH1 as template [26]. The resultant plasmid harbouring Atdhyd gene was named pJMC44rha. The same procedure was performed for *AthyuA1* gene. PCR primers 5′-AAAAAAA*CATATGCGTG*CGATGCATATTCGTTTGA-3′(including a *NdeI* site, in italics) and 5′-AACTGCAG**TTA**ATGATGATGATGATGATG GGCGCAGGCGACGAGGGCTGG-3′(including a *PstI* site, in italics, stop codon in bold and six histidine tag underlined) were used to amplify the gene from pSER12 [32] and cloned into pJOE4036.1 to produce pSER12Rha.

2.3. Expression of the different genes

The expression protocol for the four transformants (BL21pSER12rha, BL21pJAVI80rha, BL21pJPD25rha and BL21pJMC44rha) was as previously described in a recent work [34]. However, in the case of BL21pJMC44rha the growth and induction cultures were always supplemented by 0.5 mM of $CoCl_2$ (final concentration), which meant that the incubation time to reach an OD_{600} value of 0.3–0.5 increased to about 5 hours. Likewise, for *Atdhyd* expression overnight incubation (18–20 h) was required. The collection of induced cells, their disruption and enzyme purification by cobalt affinity chromatography are described in [34].

2.4. Purity and concentration of the recombinant enzymes

The purity of each recombinant enzyme was estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by the Laemmli method [38] using a low molecular weight marker kit (Amersham Biosciences, Barcelona, Spain). An additional gel filtration chromatography step was carried out by using a Superdex 200 gel filtration column (GE Healthcare) in a BioLogic DuoFlow FPLC system (BioRad) to eliminate any DNA co-eluting with the protein, with observation at 280 nm. The purified enzymes were dialyzed against 100 mM sodium borate/HCl pH 8.0 and stored at 4 °C until use. Protein concentrations were determined from the absorbance of coefficient extinction (ϵ) of tyrosine residues [39] . The ϵ values for the enzymes were 41,960 M^{-1} cm^{-1} for AtDhyd, 7680 M^{-1} cm^{-1} for AthyuA1, 43,430 M^{-1} cm^{-1} for BsLcar and 58,900 M^{-1} cm^{-1} for GkNSAAR, respectively.

2.5. Specific activity of studied enzymes

Specific activity of each enzyme was determined by enzymatic reaction in triplicate. For AtDhyd enzyme the reaction contained the protein at a final concentration of 5 μM using D-ethylhydantoin (D-EH) 10 mM as substrate. Activity of AthyuA1 (25 μM) was measured using 10 mM L-EH as substrate. BsLcar activity was evaluated in a reaction with 5 μM of enzyme using *N*-carbamoyl-L-2-aminobutyric acid (L-carABA) 10 mM as substrate. For GkNSAAR (25 μM), racemase activity was monitored using 10 mM D-carABA as substrate. All the reactions, with the exception of the AthyuA1 one, were carried out in presence of $CoCl_2$ (final concentration of 1 mM) dissolved in 100 mM sodium borate/HCl pH 8.0 at a final volume of 500 μL. The reactions were carried out at 50 °C for 30 min (Eppendorf Compact thermomixer at 800 rpm). Aliquots of 25 μL were stopped by addition of 475 μL of 1% H_3PO_4 (v/v) and centrifuged at 13,000 *g*. The resulting supernatants were analyzed by high performance liquid chromatography (HPLC).

To evaluate AtDhyd and BsLcar activities, an HPLC system (Breeze HPLC System, Waters, Madrid, Spain) with a UV detector and equipped with a Zorbax C_{18} column (4.6 × 250 mm, Agilent) at room temperature was used to detect D-EH, D- and L-carABA and L-2 -aminobutyric acid (L-ABA). The mobile phase was methanol:phosphoric acid (20 mM, pH 3.2) 10:90 (v/v), pumped at a flow rate of 0.40 mL min^{-1} and measured at 203 nm. Specific activity for each enzyme was defined as the μmoL substrate hydrolyzed (D-EH or L-carABA) per min and mg of protein.

For AthyuA1 and GkNSAAR enzymes, the racemase activity was monitored by chiral HPLC using an LC2000Plus HPLC System (Jasco, Madrid, Spain) with a UV detector and equipped with a chirobiotic T column (4.6 × 250 mm, Astec, Sigma–Aldrich) at room temperature, separating the D- and L- forms of EH and N- carABA, respectively. The mobile phase was water:methanol 60:40 (v/v), pumped at a flow rate of 0.3 mL min^{-1} and measured at 203 nm. The specific activity of AthyuA1 was defined as the amount of enzyme that catalyzed the formation of 1 μmol of D-EH from L-EH per min and mg of protein. The specific activity of GkNSAAR was defined as the amount of enzyme that catalyzed the formation of 1 μmol of L-carABA from D-carABA per min and mg of protein.

To analyze the effect of several metal ions on the AtDhyd enzyme, incubation and induction experiments were performed. For incubation studies, 5 μM of AtDhyd were incubated in presence of 1 mM of $MnCl_2$, $CoCl_2$ and $ZnCl_2$ in 100 mM sodium borate/HCl pH 8.0 (final volume 100 μL) at 4 °C for 1 and 24 h. To evaluate the effect of the metal ions on enzyme expression, each of the three cations was added to the growth and induction cultures to a final concentration of 0.5 mM. Specific activity of AtDhyd for each experiment was measured as described above using 5 μM of enzyme and D-EH (10 mM) as substrate.

2.6. Standard conversion assay for multi-enzymatic systems

The four purified enzymes (at final concentrations of 0.4 μM for AtDhyd, 1 μM for BsLcar, 10 μM for AthyuA1 and 28 μM for GkNSAAR, respectively) were mixed with 10 mM D, L-EH dissolved in 100 mM sodium borate/HCl pH 8.0, supplemented with 1 mM $CoCl_2$ (reaction volume 500 μL). During the reaction at 60 °C for 4 h (Eppendorf Compact thermomixer at 800 rpm), aliquots of 25 μL were retrieved and stopped by addition of 475 μL of 1% H_3PO_4 (v/v). The samples were centrifuged at 13,000 g and the resulting supernatants were analyzed by HPLC. The compounds were separated by reverse-phase column (Zorbax C_{18}) and UV detection was carried out at 203 nm, as described above. For the bienzymatic system and for the two triple systems, the enzyme concentrations were the same as for the double-racemase system with four enzymes.

2.7. Chemo-enzymatic cascade reaction characterization

The behavior of the multienzymatic cascade reaction at different pHs and temperatures was carried out following the standard reaction described above, using D, L-EH (10 mM) as substrate and in triplicate. For pH studies, different buffers were used at 100 mM (sodium citrate, MES (2-(*N*-morpholino) ethanesulfonic acid), triethanolamine hydrochloride, sodium borate/HCl and sodium borate/NaOH) ranging from pH 3.0 to 10.5. The effect of temperature on the conversion rate was studied from 20 to 80 °C. The thermal stability of the process was evaluated at different incubation times (0 to 24 h) at 50, 55 and 60 °C.

For substrate specificity studies the standard conversion assay was performed for racemic mixtures of different hydantoins (10 mM). To monitor the conversion to the corresponding L-amino acid, reverse phase HPLC was employed with detection at 203 nm in a mobile phase with methanol:phosphoric acid (20 mM, pH 3.2) at different ratios. The ratio 10:90 (v/v), pumped at a flow rate of 0.40 mL min^{-1} was used for L-ABA acid and L-valine, and the flow rate was slightly higher (0.60 mL min^{-1}) for L- norvaline and L-norleucine. The ratio 18:82 (v/v) was used for L-methionine and L-homophenylalanine (L-HPA), at a flow rate of 0.4 mL min^{-1}.

Larger-scale reactions were carried out at 60 °C (Eppendorf Compact thermomixer at 800 rpm) for D,L-EH and D,L-homophenylalanylhydantoin (D,L-HPAH) as substrates, both at 100 mM concentration, with the same amount of each enzyme as in the standard assay. The final reaction volume was 1 mL, buffered by 200 mM sodium borate/HCl pH 8.0. The different aliquots retrieved from the reaction were measured by HPLC as described above for small scale reactions.

2.8. Enantiomeric purity of the reaction products

Upon completion of the multienzymatic cascade reaction for the different racemic hydantoins, the theoretical 100% enantiomeric excess (e.e.) of each amino acid produced was evaluated by chiral HPLC, measuring the product as described above in racemase activity measurement (Section 2.5).

3. Results and discussion

3.1. Evaluation of "hydantoinase process" variations for L-amino acid production

Recombinant enzymes L-N-carbamoylase from Geobacillus stearothermophilus CECT43 (BsLcar) and *N*-succinyl-amino acid racemase from G. kaustophilus CECT4264 (GkNSAAR) were obtained as previously described by our group with over 95% purity [36,40] (Fig. 2). The expression of the hydantoin racemase

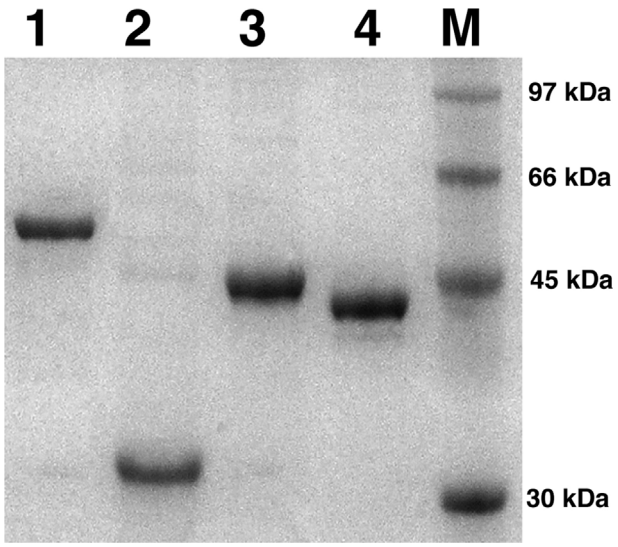

Fig. 2. SDS–PAGE analysis of the four purified enzymes. Lane 1, purified D-hydantoinase (AtDhyd). Lane 2, purified hydantoin racemase 1 (AthyuA1). Lane 3, purified L-*N*-carbamoylase (BsLcar). Lane 4, purified *N*-succinyl-amino acid racemase (GkNSAAR). Lane M, standard molecular-weight marker; phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa).

1 gene from Agrobacterium tumefaciens C58 (AthyuA1) and the D-hydantoinase gene from A. tumefaciens BQL9 (Atdhyd) has been reported by our group after cloning in pBluescript II SK (+) plasmid (pBSK) [32,41]. In the present study both genes have been recloned separately into the rhamnose-inducible expression vector pJOE4036.1 [37] that Prof. Altenbuchner donated to our group. The purity of the recombinant proteins AtDhyd and AthyuA1 was over 95% (Fig. 2), which is very similar to that obtained after overexpression of both enzymes with IPTG. The additional advantage of rhamnose induction with these enzymes is the higher yield and lower cost of induction as compared to IPTG.

Different variations of the "hydantoinase process" for L-2-aminobutyric acid (L-ABA) production were tested using as substrate D,L-ethylhydantoin (D,L-EH) (Fig. 3). A double system comprising AtDhyd and BsLcar was only able to convert the D-isomer of the substrate (D,L-EH) into *N*-carbamoyl-D-2-aminobutyric acid (D-carABA), but the BsLcar enzyme could not hydrolyze this D-carABA due to its enantioespecificity for L-carABA, and no amino acid (L-ABA) was produced. In this case, the non-hydrolyzed L-EH and the intermediate D- carABA each made up approximately 50% of the original racemic substrate (Fig. 3a). A triple system with AtDhyd, BsLcar and AthyuA1 enabled the hydrolysis of over 50% of D,L-EH into D- carABA. The third enzyme, AthyuA1, racemized the L-EH enantiomer into D,L-EH, and again the D-EH isomer was available to be hydrolyzed by AtDhyd. However, the intermediate D-carABA was accumulated and no amino acid (L-ABA) was produced (Fig. 3b). A second triple system, with AtDhyd, BsLcar and GkNSAAR (as *N*-carbamoyl-amino acid racemase) was able to produce optically pure L-ABA from a racemic mixture of D,L-EH as substrate (Fig. 3c). GkNSAAR allowed the racemization of D-carABA into D,L- carABA, and BsLcar only hydrolyzed the L-carABA isomer into L-ABA. After successive GkNSAAR and BsLcar reactions, all the D-carABA was transformed into L-ABA. However, L-ABA production was only 50% because only half of the racemic substrate (the D-EH isomer) was hydrolyzed by AtDhyd. Fig. 3c shows that 50% of non-hydrolyzed substrate (the L-EH isomer) is present at the end of the reaction together with 50% of the L-amino acid produced. Finally, Fig. 3d shows that a quadruple system with two racemases, AthyuA1 and GkNSAAR, together with AtDhyd and BsLcar, was able

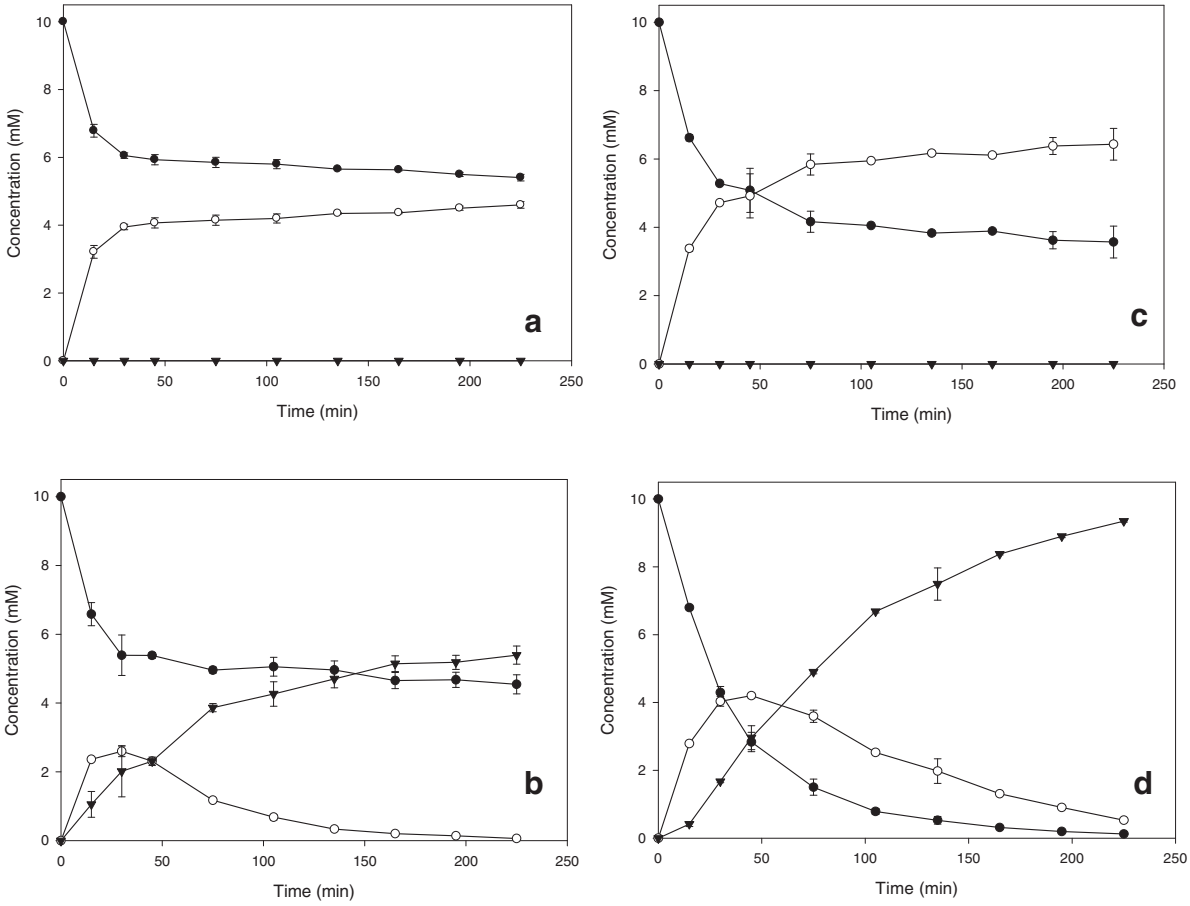

Fig. 3. Different variations of the "hydantoinase process" for L-ABA (▼) production using D,L-EH (●) with D-carABA (○) as intermediate. (a) Double system formed by AtDhyd and BsLcar enzymes. (b) Triple system formed by AtDhyd, BsLcar and AthyuA1 enzymes. (c) Triple system formed by AtDhyd, BsLcar and GkNSAAR enzymes. (d) Double-racemase system formed by AtDhyd, AthyuA1, BsLcar and GkNSAAR enzymes. Reactions were performed at 60 °C for 4 h in triplicate as described in material and methods and error bars represent the standard errors of the means.

to convert the racemic mixture of D,L-EH into optically pure L-ABA with a yield of 100%. In this enzymatic cascade reaction, AthyuA1 racemized the non-hydrolyzed isomer (L- EH), continuously providing the D-isomer to AtDhyd until the substrate was consumed. Likewise, GkNSAAR supplied L- carABA to BsLcar by racemization of the D-carABA isomer. This "double-racemase hydantoinase process" has been able to convert the racemic mixture of hydantoin into optically pure L-amino acid with 100% efficiency.

N-carbamoylase [22] and also with hydantoin racemase as a third enzyme [42]. Although most hydantoinases have been described as D-enantioselective [16], the above-mentioned systems were able to produce L-amino acids, as they included hydantoinases with unusual enantiomeric properties (see introduction section for details). Additionally, these two hydantoinases were improved by directed evolution (hydantoinase from *Arthobacter aurescens* DSM3745, AaHYD) and by site-directed mutagenesis (D-hydantoinase from *Brevibacillus agri* NCHU1002, BaDHP), increasing the L-amino acid productivity, but leaving scope for further enhancement of the process [23,24]. Our group has achieved D-amino acid production from racemic mixtures of 5-monosubstituted hydantoins by the "hydantoinase process" [19,20]. However, for L-amino acid production, from these cheap racemic precursors, only the combination of a D-enantionselective hydantoinase and an L-enantioespecific *N*- carbamoylase produce 50% conversion of the D-isomer of the substrate into *N*-carbamoyl-D-amino acid without any formation of L-amino acid (see as an example of L-ABA production Fig. 3a) or with relatively low yield [43]. The strict D-enantioselectivity of the majority of hydantoinases, including AtDhyd, led us to tackle novel strategies other than enzyme design by molecular engineering. Thus, we have developed a biocatalyst combining AtDhyd, AthyuA1, BsLcar and the promiscuous GkNSAAR [31] as *N*-carbamoyl-amino acid racemase, for the total conversion of racemic hydantoins into L-amino acids. This is the first chemo-enzymatic cascade reaction able to produce optically pure L- amino acids from these substrates using a strict D-enantioselective hydantoinase. We have proposed the name of "double-racemase hydantoin process" because in addition to the hydantoin racemase enzyme that contributes together with D-hydantoinase to the total hydrolysis of the racemic hydantoin, a second racemase, GkNSAAR, racemizes the D-carbamoyl-amino acid accumulated by the D-hydantoinase. The resultant L-carbamoyl-amino acid is converted to L- amino acid by the L-carbamoylase enzyme, until all the racemic substrate is consumed, producing the optically pure L- amino acid by dynamic kinetic resolution.

3.2. Metal ion effect on the "double-racemase hydantoinase process"

GkNSAAR and BsLcar have been previously described as metalloenzymes, and Co^{2+} proved the best cofactor [28,31]. Additionally, the maximum activity for these two enzymes was obtained when Co^{2+} was added to the reaction, without previous incubation with the cation [28,34]. The metalloenzyme AtDhyd had shown maximum activity, measured in E. coli recombinant cells, when Mn^{2+} was added to the induction culture [26]. However, in order to use

Table 1
Specific activity of D,L-hydantoinase from Agrobacterium tumefaciens BQL9 (AtDhyd) in presence of metal ions Co^{2+}, Zn^{2+} and Mn^{2+} after 24 h of incubation of the purified enzyme at 4 °C (1 mM of each cation) or supplemented during the induction (0.5 mM of each cation). Specific activity of incubated or induced AtDhyd was measured by standard reaction in presence (with) or absence (w/o) of cobalt. Enzyme assays were performed in triplicate using 10 mM D-ethylhydantoin as described in material and methods.

Metal ion	Specific activity (μmol/min mg prot)			
	Incubation (reaction w/o Co^{2+})	Incubation (reaction with Co^{2+})	Induction (reaction w/o Co^{2+})	Induction (reaction with Co^{2+})
None	0.122 ± 0.011	0.341 ± 0.031	0.126 ± 0.011	0.312 ± 0.228
Co^{2+}	0.585 ± 0.053	0.677 ± 0.061	8.976 ± 0.626	8.199 ± 0.656
Zn^{2+}	0.293 ± 0.026	0.337 ± 0.030	5.164 ± 0.464	4.123 ± 0.370
Mn^{2+}	0.326 ± 0.029	0.334 ± 0.026	2.573 ± 0.231	2.756 ± 0.248

the same cation for the three metalloenzymes of the system, AtDhyd activity was studied in presence of Co^{2+}. Enzyme activity was also measured in presence of $Mn^{2+}$and Zn^{2+} (Table 1), since the latter has been described as a cofactor for several hydantoinases [44]. When the recombinant enzyme AtDhyd was incubated in presence of 1 mM of the three different metal ions, the specific activity was slightly higher than without any metal ion incubation. On the contrary, when AtDhyd was overexpressed in presence of the three metal ions (end concentration 0.5 mM), the activity increased greatly. The maximum activity for the enzyme using D-EH as substrate was obtained after inducing the enzyme in presence of 0.5 mM Co^{2+}, whereas presence of Mn^{2+} produced the lowest activity (Table 1). Recently our group has evaluated the metal ion requirements of another cyclic amidohydrolase, the allantoinase enzyme from Bacillus licheniformis CECT 20T [45]. This enzyme showed more activity after induction of the chloride salts of cobalt compared to the non-amended enzyme, as did AtDhyd, suggesting that the incorporation of the cation into these enzymes occurs preferentially during protein folding. Additionally, since the BsLcar and GkNSAAR enzymes showed maximum activity when Co^{2+} was added to the reaction, AtDhyd activity was measured with Co^{2+} present in the reaction using both incubated and induced enzyme (Table 1). AtDhyd activity was very similar when Co^{2+} was added to the reaction, with no inhibition or rise in enzyme activity. Based on these results, the AtDhyd used for the multienzymatic system was overexpressed in presence of 0.5 mM Co^{2+}.

3.3. Enzyme ratio in the "double-racemase hydantoinase process"

Specific activity was studied for the four enzymes involved in the multienzymatic process in order to determine the ratio of each one. The enzyme with the highest activity was AtDhyd with 8.2 μmoL min^{-1} mg^{-1}, followed by BsLcar with 3.3 μmoL min^{-1} mg^{-1} and the two racemases, AthyuA1 and GkNSAAR, with 0.156 and 0.059 μmoL min^{-1} mg^{-1}, respectively. These specific activities correspond to a ratio of 1:2.5:52:139 for AtDhyd:BsLcar:AthyuA1:GkNSAAR, respectively. This enzyme ratio meant high consumption of both racemases, due to their low specific activity. In order to evaluate the reduction of the racemase proportion in the biocatalyst, the ratio of these two enzymes was reduced by half and compared with the conversion rate of L-ABA from D,L-EH (Fig. 4). When the AthyuA1 ratio was halved, the efficiency of the biocatalyst was very similar to that with the ideal ratio (approximately 99% conversion). Similarly, when the GkNSAAR ratio was halved the conversion was 90%. When both AthyuA1 and GkNSAAR ratios were halved the conversion was about 92%. This reduction in the ratio of both racemases allowed economy of protein with almost maximum efficiency in the biocatalyst, using a final ratio of 1:2.5:26:69 for AtDhyd:BsLcar:AthyuA1:GkNSAAR, respectively.

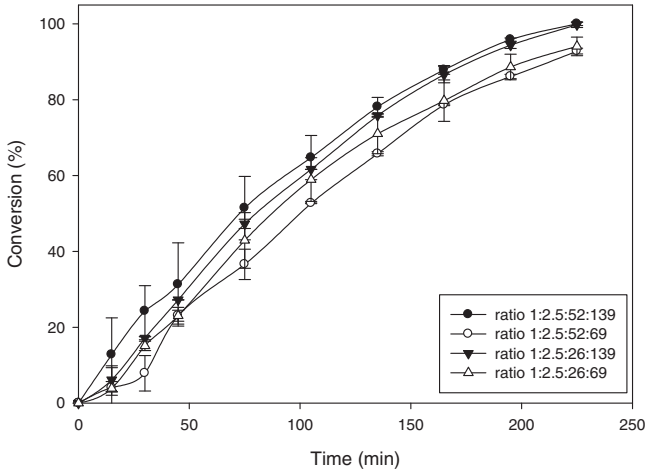

Fig. 4. Effect of the enzyme ratios in the multienzymatic system for L-ABA conversion using D,L-EH as substrate. The enzymes AtDhyd, BsLcar, AthyuA1, GkNSAAR were presented in the reaction in several ratios. A first system was formed by the ratio of each enzyme derived from their specific activity (●). The other three systems reduced the ratio of one racemase, GkNSAAR (○) and AthyuA1 (▼) or both racemases (Δ) by half. Measurements were performed at 60 °C for 4 h in triplicate as described in material and methods and error bars represent the standard errors of the means.

3.4. Effect of pH and temperature in the "double-racemase hydantoinase process"

mM sodium borate/HCl buffer pH 8.0 (Fig. 5). It should be noted that this pH value is roughly the same as those for the enzymes

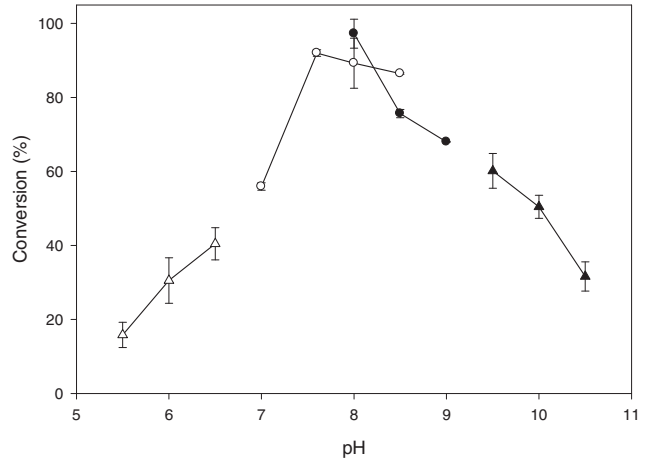

Fig. 5. Effect of pH on the multienzymatic system for D,L-EH conversion into L-ABA. Studies of pH were assayed in several 100 mM buffers, MES (5.5–6.5) (Δ), triethanolamine hydrochloride (7.0–8.5) (○), sodium borate/HCl (8.0–9.0) (●) and sodium borate/NaOH (9.5–10.5) (▲). Enzyme assays were performed at 60 °C for 4 h in triplicate as described in material and methods and error bars represent the standard errors of the means.

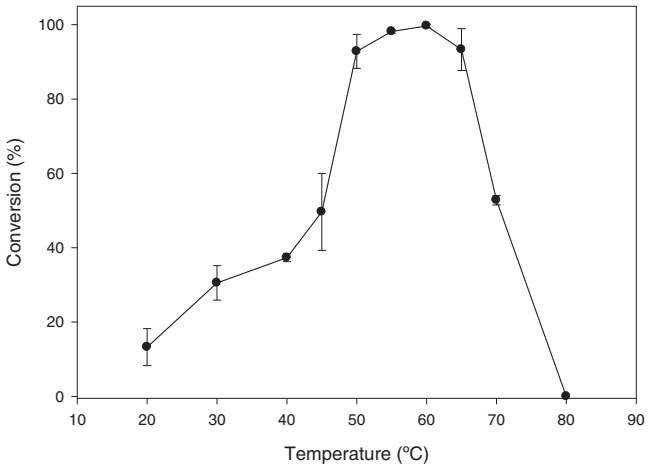

Fig. 6. Effect of temperature on the multienzymatic system for D,L-EH conversion into L-ABA. Temperature studies were carried out from 20 to 80 °C in 100 mM sodium borate/HCl buffer pH 8.0. The relative activity value at 60 °C was taken as 100%. Enzyme assays were performed for 4 h in triplicate as described in material and methods and error bars represent the standard errors of the means.

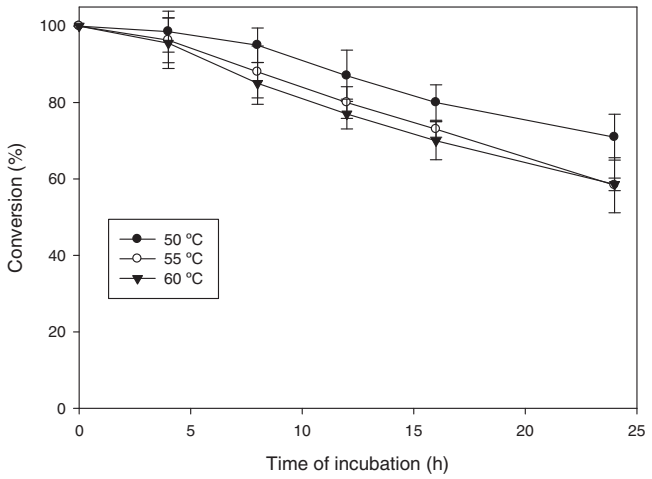

Fig. 7. Thermal stability of the multienzymatic system. Different incubation times (0–24 h) at temperatures of at 50 (●), 55 (○) and 60 °C (▼) in 100 mM sodium borate/HCl buffer pH 8.0 followed by the standard enzyme assay. Reactions were performed at 60 °C for 4 h in triplicate as described in material and methods and error bars represent the standard errors of the means.

purified separately. Optimal pH value for the purified BsLcar and AthyuA1 was 7.5 in 100 mM potassium phosphate buffer [28,32]. AtDhyd activity was measured in the recombinant pure enzyme by HPLC, colorimetric and isothermal thermal titration (ITC) assays at pH 7.5 in 100 mM sodium cacodylate buffer [41]. Likewise, GkNSAAR showed maximum activity at pH 8.0 in 100 mM sodium borate/HCl buffer but was not active in phosphate buffer [31]. Our group has recently proposed a tandem system for L-amino acid production, where BsLcar and GkNSAAR are involved, using sodium borate/HCl buffer pH 8.0 in the conversion reaction because the second enzyme is not active in phosphate buffer [34] .

°C, with the maximum reaction rate at 60 °C (Fig. 6). These temperatures are similar to those of the individual enzymes in the system, as both racemases GkNSAAR and AthyuA1 showed optimal activity at 55 °C [31,32] and BsLcar at 65 °C, due to its thermophilic origin [28]. Recombinant cells of *E. coli* with AtDhyd activity showed a linear decrease in activity as the temperature increased [26], and microcalorimetric assays on purified AtDhyd were carried out from 20 to 40 °C to provide the dependence of the catalytic rate constant on temperature [41]. However, a recombinant polycistronic system for D-amino acid production containing AtDhyd, AthyuA1 and a D-carbamoylase from *A. tumefaciens* BQL9 reached 100% conversion from D,L-MTEH to D-methionine at 55 °C [20]. Taken together, this previous result and those of the present work demonstrate that AtDhyd is active in this high temperature range, at least during the first conversion step where the substrate is hydrolyzed to the intermediate by AtDhyd. The thermal stability of the system was measured at several incubation times (0, 4, 8, 12, 16 and 24 h) at 50, 55 and 60 °C, followed by the standard enzyme assay (Fig. 7). The enzymatic cascade reaction was over 95% active after 4 hours of incubation at the three temperatures, but conversion fell to 70% at 50 °C after 24 h of incubation, and to 60% after the same time at both 55 and 60 °C.

3.5. Substrate conversion to L-amino acid by the "double-racemase hydantoinase process"

After establishing the reaction parameters of the "double-racemase hydantoinase process", its efficiency was evaluated in the conversion of different racemic hydantoins to the corresponding optically pure L-amino acids (Fig. 8). In all the studied reactions the enantiomeric excess (e.e.) of the L-amino acid produced was 100%. The L-amino acids obtained by this biocatalytic process were

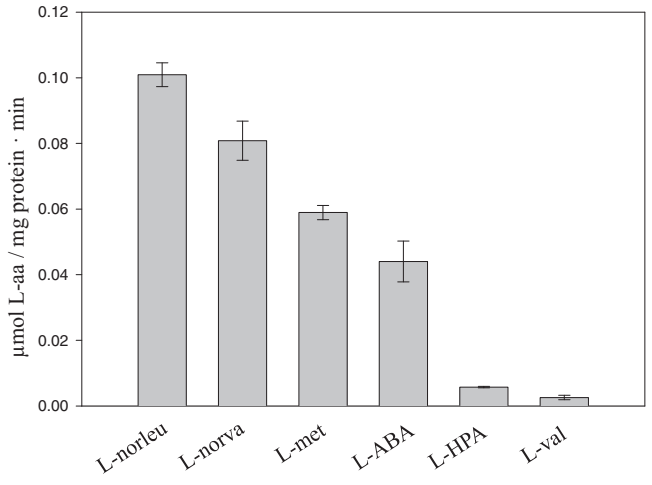

Fig. 8. Initial reaction rates for the production of different optically pure L-amino acids from 5-monosubstituted hydantoins using the "double-racemase hydantoinase process". The reactions were performed as described in material and methods at pH 8 and 60 °C, and error bars represent the standard errors of the means. L-norleu: L-norleucine; L-norva: L-norvaline; L-met: L-methionine; L-ABA: L-aminobutyric acid; L-HPA: L-homophenylalanine; L-val: L-valine; L-aa: L-amino acid.

non-natural, with the exception of L-methionine. The highest initial reaction rates were found for L-norleucine and L-norvaline, with 0.10 and 0.08 U/mg of protein, respectively. The L-amino acids produced most slowly were L-homophenylalanine (L-HPA) and L-valine, with 0.0057 and 0.0026 U/mg of protein, respectively. The enzyme promiscuity of GkNSAAR that has allowed the upgrade of the "hydantoinase process" for L-amino acid production from racemic mixtures of hydantoins has been used for other processes. Thus, our group has recently presented a wider use of this enzyme together with BsLcar for producing L-amino acid from *N*-acetyl- and *N*-carbamoyl-amino acids, and for the first time from *N*-formyl-amino acids [34]. This new process has been renamed "amidohydrolase process". In both systems, the substrate promiscuity has allowed the use of old enzymes for new targets, making possible the production of interesting compounds by new enzymatic cascade reactions.

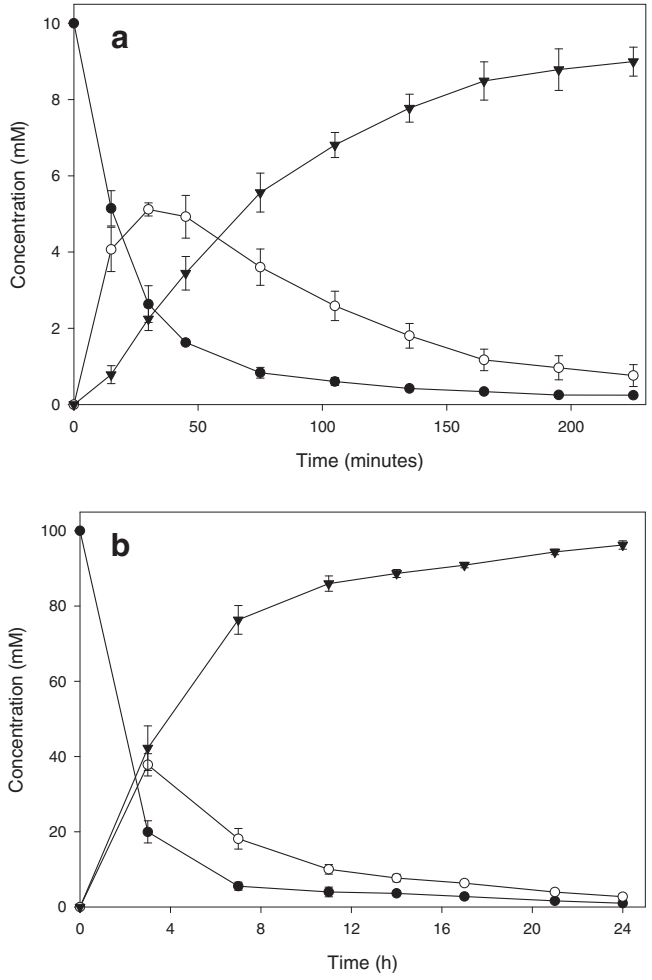

Fig. 9. Profiles of L-ABA production from 10 mM (a) and 100 mM (b) D,L-EH by using the "double-racemase hydantoinase process". D,L-EH (●), L-carABA (○), L-ABA (▼). Reactions were performed in triplicate as described in material and methods, and error bars represent the standard errors of the means.

Conversion of racemic mixtures of hydantoins at high concentration by the enzymatic cascade reaction was also studied. Conversion to optically pure L-ABA from 10 and 100 mM D,L-EH, respectively, was analyzed using the same amount of enzyme as described for standard assays (Fig. 9). The "double-racemase hydantoinase process" was able to convert 10 mM L-ABA in about 4 h, whereas total conversion to 100 mM took 24 h, with no observed reaction inhibition. The system was also studied for L-HPA production, which was achieved after 20 h from 10 mM of D,L-homophenylalanylhydantoin (D,L-HPAH) as substrate, and after about 60 h when the substrate concentration was increased tenfold. This enzymatic cascade reaction has been able to transform racemic hydantoins into optically pure natural or non-natural L-amino acids, even at high substrate concentration. Knowledge of the behavior of this multienzymatic cascade reaction in the presence of high substrate concentrations is a prerequisite to scale it up to an analytical or industrial scale. The system has shown higher yields than other methods used for L-HPA production from hydantoins [22]. For L-ABA production the biocatalyst has also proved simpler and with similar or even higher productivity compared to other methods using enzymatic cascade reactions, such as L-threonine deaminaseL-leucine dehydrogenase/formate dehydrogenase [46] or L-threonine deaminase/aromatic aminotransferase/acetolactate synthase/alanine racemase/D-amino acid oxidase [47].

4. Conclusion

N-succinyl-amino acid racemase as *N*-carbamoyl-amino acid racemase that allows the use of the majority of described hydantoinases, which are D-enantioselective [16], for L-amino acid production. Our group are currently looking the economic viability of the novel biocatalyst to provide non-natural L-amino acids to the pharmaceutical industry. Our main goals are to improve the yield of the process, increase the stability of the enzymes by immobilization [48] and also enhance their activity or selectivity by enzyme engineering strategies [25].

Acknowledgements

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