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L-Amino Acid Production by a Immobilized Double-Racemase Hydantoinase Process: Improvement and Comparison with a Free Protein System

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Abstract: Protein immobilization is proving to be an environmentally friendly strategy for manufacturing biochemicals at high yields and low production costs. This work describes the optimization of the so-called "double-racemase hydantoinase process," a system of four enzymes used to produce optically pure L-amino acids from a racemic mixture of hydantoins. The four proteins were immobilized separately, and, based on their specific activity, the optimal whole relation was determined. The first enzyme, D,L-hydantoinase, preferably hydrolyzes D-hydantoins from D,L-hydantoins to *N*-carbamoyl-D-amino acids. The remaining L-hydantoins are racemized by the second enzyme, hydantoin racemase, and continue supplying substrate D-hydantoins to the first enzyme. *N*-carbamoyl-D-amino acid is racemized in turn to *N*-carbamoyl-L-amino acid by the third enzyme, carbamoyl racemase. Finally, the *N*-carbamoyl-L-amino acid is transformed to L-amino acid by the fourth enzyme, L-carbamoylase. Therefore, the product of one enzyme is the substrate of another. Perfect coordination of the four activities is necessary to avoid the accumulation of reaction intermediates and to achieve an adequate rate for commercial purposes. The system has shown a broad pH optimum of 7–9, with a maximum activity at 8 and an optimal temperature of 60 °C. Comparison of the immobilized system with the free protein system showed that the reaction velocity increased for the production of norvaline, norleucine, ABA, and homophenylalanine, while it decreased for L-valine and remained unchanged for L-methionine.

Keywords: protein immobilization; enzymatic cascade; L-amino acids

1. Introduction

Amino acids are precursors of drugs, cosmetics, pesticides, and sweeteners as well as compounds for direct use in plant and animal nutrition [1]. Some of them contain a chiral carbon that has been shown to be the key to their applicability, since both isomers present different utility according to their chirality. For example, D-valine is a precursor of fluvalinate, a synthetic pyrethroid used in the control of varroa mites, but L-valine is used in pharmacology to form an ester with acyclovir to increase the bioavailability of the antiviral. D-Phenylalanine is one of the compounds of the natural antibiotic polyxymid, and the L-enantiomer is one of the three compounds of the sweetener aspartame. The manufacture of enantiomeric racemic mixtures lacks industrial interest, and optically pure production is crucial. Several methods have been used for L-amino acid production: protein

hydrolysis, microbial fermentation, chemical synthesis, and enzymatic catalysis [2]. Protein hydrolysis and microbial fermentation are limited to obtaining proteinogenic amino acids of L-symmetry. Non-enzymatic synthesis procedures can be used to synthesize a broad spectrum of amino acids, but also a mixture of D- and L-stereoisomers that requires the additional process of separation. Finally, enzymatic biosynthesis has been shown to be an advantageous method of obtaining natural and non-natural amino acids that are enantiomerically enriched (>99%) in one of the isomers.

Some examples for the specific production of L or D amino acids are as follows: hydrolysis by D- or L-acylase to obtain optically pure amino acids from D,L-acyl-amino acids, the conversion of D,L-methionine by the enzymes D-amino acid oxidase and leucine dehydrogenase [3], and the addition of ammonia to fumaric acid to obtain L-aspartate catalyzed by aspartase [1] or to obtain L-cysteine by the hydrolysis of D,L-2-amino-2-thiazoline-4-carboxylic acid by three enzymes [3]. All of these methods are examples of systems that produce one specific amino acid. However, the so-called "hydantoinase process" is one of the most efficient methods able to produce a broad spectrum of both proteinogenic and non-proteinogenic amino acids that are enantiomerically pure [1]. The starting materials for this process are 5-monosubstituited hydantoins, which are low-price compounds that can be easily obtained by different routes [4]. The system has mainly been developed for D-amino acid because two of the enzymes are D-enantiospecific. The process combines the activity of three enzymes: D,L-hydantoinase, hydantoin racemase, and L- or D-carbamoylase. The D,L-hydantoinases described to date show D- or L- selectivity depending on the substrate or the source from which they are isolated, but tend to be more D-enantioselective (95:5). Hydantoinase cleaves carbon–nitrogen bonds of 5- or 6- hydantoins or dihydrouracils substituted in the 5′ position, producing the corresponding *N*-carbamoyl-amino acid. Two exceptions are the hydantoinase from *Arthrobacter aurescens* DSM3745, which showed substrate-dependent enantioselectivity, with L-selectivity for D,L-5-indolylmethylhydantoin, preference for D-enantiomer for D,L-methylthioethylhydantoin and D-selective for D,L-methylhydantoin [5], and D,L-hydantoinase from *Brevibacillus agrii* NCHU1002 which cleaved both isomers of D,L-homophenylalanylhydantoin [6]. Consequently, the hydantoinase process has mainly been applied to the industrial production of D-amino acids. Hydantoin racemase shows activity for L- and D- enantiomers, racemizing the D- or L-substrate not hydrolyzed by the D,L-hydantoinase, supplying continuous substrate to the hydantoinase, and allowing total conversion from D,L-hydantoin to L-amino acids. The third enzyme, *N*-carbamoylase, has been isolated from different sources with both D- and L-enantioselectivity [7,8] with an effective yield and velocity in both cases. For D-amino acid production, the enzymatic cascade with these three enzymes works efficiently because the higher D- selectivity of the D,L-hydantoinase favors the production of this racemate. However, for L-amino acid production, it has been necessary to add a fourth enzyme able to racemize *N*-carbamoyl-D-amino acid to *N*-carbamoyl-L-amino acid. A highly effective enzymatic cascade, namely the "double-racemase hydantoinase process," has been developed including an *N*-amino acyl racemase as carbamoyl racemase and using an L-carbamoylase with a broad substrate spectrum [9] (Figure 1).

The double-racemase hydantoinase process consists of the four enzymes D,L-hydantoinase, hydantoin racemase, carbamoyl racemase, and L-carbamoylase, all of which show different physical-chemical properties, cofactor requirements, and optimal conditions of activity. D-Hydantoinase from *Agrobacterium tumefaciens* BQL9 (AtDHyd) has been described as a metal-dependent enzyme with Mn^{2+} as a cofactor in expression induction. Its maximum activity is at pH 8.7–9.7 and 30 °C, and it shows low thermal stability [10]. The enzyme opens rings of 5 and 6 carbons, and its activity is enhanced by the presence of an aliphatic or aromatic group in carbon 5. Hydantoin racemase from *Agrobaterium tumefaciens* C58 (AthyuA1) shows high thermal stability, remaining active above 55 °C for 30 min and showing maximum activity from 55 to 60 °C in standard reaction conditions. The enzyme presents an optimum pH at 7.5 and is more active when the hydantoins contain aliphatic substituents [11]. L-*N*-Carbamoyl amino acid amidohydrolase from *Geobacillus stearothermophilus* CECT43 (BsLcar) is a stereospecific enzyme with the capacity to hydrolyze *N*-acetyl

and *N*-formyl-L-amino acids as well as *N*-carbamoyl-L-amino acids, showing the best results for *N*-fomyl-L-amino acids. Optimal conditions are 65 °C and pH 7.5, and its activity increases with Co^{2+} addition [12]. It is a stable enzyme that maintains 60% activity after incubation at 70 °C for one hour. To obtain 100% conversion, it is necessary to incorporate the fourth enzyme, an *N*-succinyl amino acid racemase (NSAAR) with *N*-carbamoyl amino acid racemase activity that allows the transformation of D-carbamoyl to L-carbamoyl, which is the substrate of the L-*N*-carbamoylase. The NSAAR enzyme from *Geobacillus kaustophilus* (GkNSAAR) has been characterized as metal-dependent, with optimal reaction conditions at pH 8 and 55 °C with the addition of Co^{2+}. GkNSAAR shows activity for aliphatic and aromatic substrates and is active up to 60 °C, above which activity falls sharply. It is able to hydrolyze a broad spectrum of substrates, such as *N*-acetyl, *N*-carbamoyl, and *N*-succinyl amino acids, but not *N*-formyl-amino acids [13]. The enzymatic cascade of these four enzymes, termed the "double-racemase hydantoinase process," has been developed to produce a broad spectrum of L-amino acids from racemic mixtures of D,L-hydantoins. The optimal enzymatic ratio has been found to be 1:2.5:26:69 for AtDhyd:BsLcar:AthyuA1:GkNSAAR, respectively. The optimum pH of the system is 7.5 in a potassium phosphate buffer at 55 °C. The system has shown thermal stability for 5 h up to 60 °C, but activity falls to 70% at 50 °C when the protein is incubated for 14 h. The best rates have been obtained for the production of L-norleucine, L-norvaline, L-methionine, and L-aminobutyric acid [9].

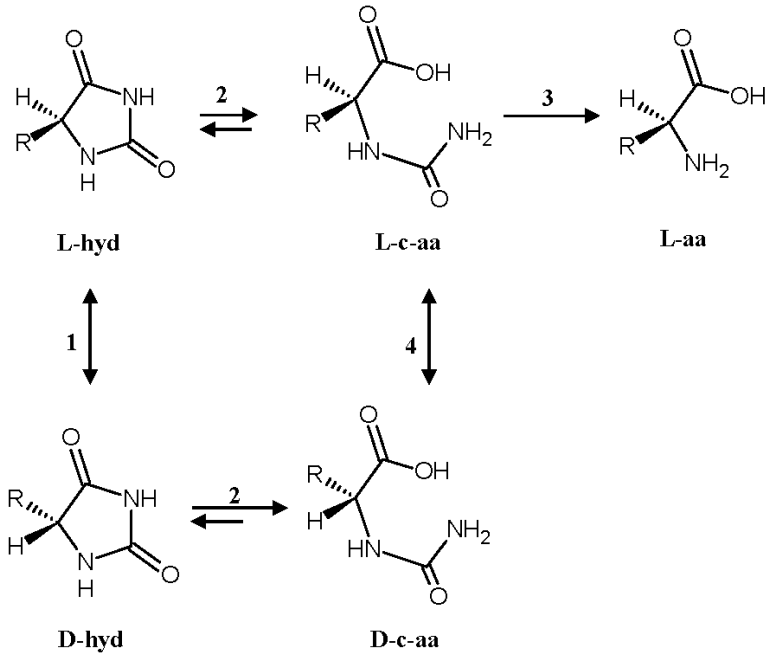

Figure 1. Reaction scheme of the so-called "double-racemase hydantoinase process" for optically pure L-amino acid production. (1) Hydantoin racemase. (2) D,L-Hydantoinase. (3) L-Carbamoylase. (4) *N*-Succinyl-amino acid racemase as carbamoyl racemase.

The pharmaceutical industry demands pure products at high yield and at low production costs. Protein immobilization leads to continuous production without purification steps, and it is environmentally friendly [14]. It allows control of the reaction, avoids contamination, and permits reusage of the biocatalyst over many cycles. On the other hand, in a multistep enzymatic system, the specific activity of one of the proteins may not be compatible with the overall system. Working with immobilized proteins, the quantity of each catalyst is selected according to its specific activity, providing a combination in the appropriate proportions to avoid intermediate accumulation. A wide range of methods can be employed for protein immobilization. Covalent binding by epoxy groups is easy and compatible with the rational immobilization of proteins. It is non-invasive and maintains the quaternary nature of the protein. The epoxy groups of the support bind to carboxy, thiol, phenolic,

or amino groups of the proteins depending on the pH. The location of these reactive groups determines the protein accessibility to the supports and gives stable and active immobilized proteins. The material of the supports must be inert, not swell in water, present low chemical reaction with proteins, and be reusable over several cycles [14].

Several supports from two companies have been tested to immobilize the proteins of the double-racemase hydantoinase process [15], and in the present study we have optimized the enzymatic cascade of four enzymes to produce a broad spectrum of optically pure L-amino acids. Protein saturation of all binding sites in the support does not always result in greater effectiveness due to crowding and substrate diffusion limitations [16]. It is necessary to calculate the minimum amount of protein necessary to saturate the attachment points of covalent binding to obtain the maximum activity and avoid protein waste. Finally, we studied pH, temperature, and stability of the four enzymes, monitoring the sequential activity and establishing the number of cycles over which the system maintains at least 80% activity.

2. Results and Discussion

2.1. Protein Loading

The production of L-amino acids by the double-racemase hydantoinase process requires that the activity of four enzymes obtain 100% substrate conversion. Each of the four enzymes has specific requirements and different physical and chemical characteristics. In a previous work, they were immobilized in several supports, thus determining the most adequate for each one. When the four immobilized enzymes were mixed, the different density and nature of the supports impeded the matter transfer and thus led to the accumulation of intermediate reaction products. Finally, the four proteins were immobilized in Sepabeads IB350, containing an epoxy group for covalent binding [15]. The rate of production and number of cycles over which the system could be reused increased at greater quantities of protein per mg of beads. We have varied the quantity of protein (Table 1) from 0.8 to 5 mg versus 400 mg of beads. AtDhyd, GkNSAAR, and BsLcar were concentrated to 6.2 mg/mL, but AthyuA1 could not be concentrated to more than 1.3 mg/mL. The conditions of immobilization were as follows: constant mixing of the beads in the presence of the different volumes of each protein individually, for 12 h at room temperature in 100 mM borate–HCl buffer pH 8. Table 1 shows how an increase in the amount of the proteins AtDhyd, BsLcar, and GkNSAAR does not result in better specific activity or a greater percentage of immobilization. The optimal quantities were 0.84 mg for AtDHyd, 2.60 mg for AthyuA1, 0.83 mg for BsLcar, and 0.84 mg for GkNSAAR versus 400 mg of support.

There is a maximum quantity of protein able to bind to the functional groups in the supports. For AtDHyd and AthyuA1, there was a decrease of immobilized protein; for BsLcar and GkNSAAR, the quantity remained constant even at higher concentrations. Wilchek and Miron indicated that an increase in the amounts of protein used for immobilization causes a competition for active groups in the solid phase and the proteins bind via fewer sites [17]. Another parameter that determines the effectivity of the binding is the specific activity of the immobilized protein. For AthyuA1, this effect was critical; however, for AtDhyd, BsLcar, and GkNSAAR, raising the quantity of protein did not result in an increase in specific activity, so for these enzymes less quantity is more effective. In these cases, the immobilization was constant, but accessibility to the substrate was impeded and the activity decreased. For BsLcar, increasing the protein from 0.83 to 2.49 mg produced only a slight increase in specific activity, from 0.73 to 0.78 mg/mL per mg of immobilized protein, respectively. On the other hand, for hydantoin racemase A1 (AthyuA1), the best results were obtained using 2.6 mg of protein. This effect has been observed when hydantoinase and L-*N*-carbamoylase from *Arthrobacter aurescens* were immobilized in Eupergit C250 and EAH Sepharose 4B; the increase in the quantity of protein did not result in an increase of loading or specific activities [18]. Optimal enzyme loading to the support is empirical and specific for each protein because the accessibility of the substrate to the active center depends on the disposition of the macromolecules on the support and the steric impediment.

Table 1. Optimization of the quantity of protein necessary to saturate the attachment point of covalent binding to 400 mg of the support IB-350. Activity was measured using 10 mM D-ethylhydantoin for AtDhyd activity; 10 mM L-ethylhydantoin for hydantoin racemase from *Agrobacterium tumefaciens* C58 (AthyuA1) activity coupling with D-hydantoinase from *Agrobacterium tumefaciens* BQL9 (AtDHyd); 10 mM D-carbamoyl methionine for *N*-succinylamino acid racemase from *Geobacillus kaustophilus* (GkNSAAR) activity coupling with L-*N*-carbamoyl amino acid amidohydrolase from *Geobacillus stearothermophilus* CECT43 (BsLcar); 10 mM L-carbamoyl methionine for BsLcar activity. All reactions were carried out in the presence of 1 mM $CoCl_2$, except for AthyuA1, 100 mM borate buffer pH 8, 60 °C for 30 min with constant stirring. The compounds were separated by HPLC water with a Zorbax C_{18} column, mobile phase of 80:20 20 mM phosphoric acid, pH 3.2, and methanol, 0.4 mL/min, and measurement at λ = 203 nm.

AtDHyd			AthyuA1			BsLcar			GkNSAAR		
Prot *	**% I ***	**Act ***	**Prot ***	**% I ***	**Act ***	**Prot ***	**% I ***	**Act ***	**Prot ***	**% I ***	**Act ***
0.84	98	6.80 ± 0.71	0.52	88	0.47 ± 0.03	0.83	99	0.73 ± 0.06	0.84	100	0.12 ± 0.01
1.70	85	4.02 ± 0.37	1.04	66	0.67 ± 0.07	1.66	98	0.64 ± 0.05	1.68	99	0.09 ± 0.01
2.50	76	2.86 ± 0.24	2.60	48	0.73 ± 0.08	2.49	97	0.78 ± 0.07	2.63	99	0.12 ± 0.01
5.00	69	1.77 ± 0.11	5.20	48	0.49 ± 0.03	3.57	98	0.37 ± 0.04		-	-

* Prot: mg of protein; % I: percentage of immobilized protein; Act: activity as μmol/min mg immobilized protein.

2.2. Optimal Reaction Conditions

Optimum reaction parameters were studied for the production of L-amino acids by varying pH, temperature, and the ratio of immobilized enzymes. pH was changed from 6 to 9.5 using 10 mM cacodylate with a pH from 6 to 7, 10 mM triethanolamine with a pH from 7 to 8, 10 mM borate with a pH from 8 to 9, and 10 mM glycine with a pH from 9 to 10 (Figure 2). The system showed a broad spectrum of pH from 7 to 9, with maximum activity at 8 in 10 mM borate–Na buffer. In contrast, with protein in solution, the spectrum was narrower, as the enzymatic cascade showed a 40% loss of activity at pH 7 and 9 [9]. The temperature of the system was tested from 50 to 70 °C, maintaining more than 80% activity up to 70 °C (Figure 3). When the system was incubated for 4 h at different temperatures from 30 to 80 °C, it maintained 100% activity at 60 °C, 60% at 70 °C, and 20% at 80 °C (Figure 4).

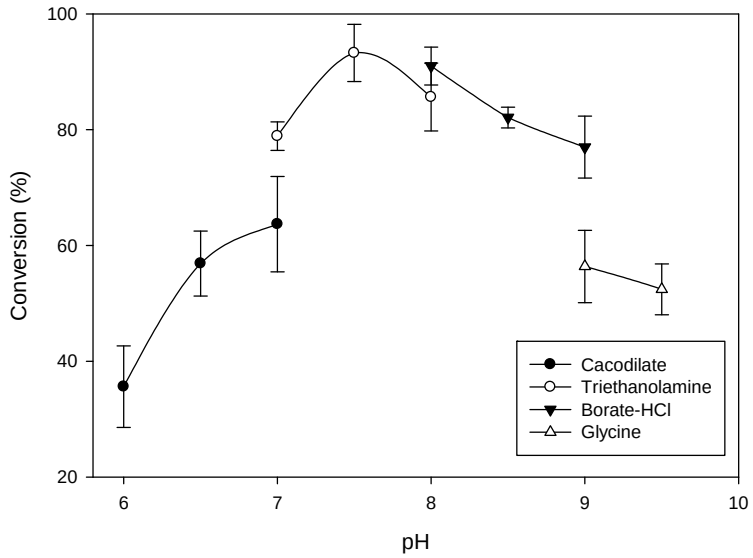

Figure 2. pH optimization using 10 mM D,L-methylthioethylhydantoin as substrate, 100 mM of each buffer (cacodylate, pH 6–7; triethanolamine, pH 7–8; borate, pH 8–9; glycine, pH 9–10) supplemented with 1 mM $CoCl_2$, for 4 h at 60 °C with constant stirring. Compounds of the multi-step reaction were separated in HPLC Water with a Zorbax C_{18} column, mobile phase of 80:20 20 mM phosphoric acid, pH 3.2, and methanol; 0.4 mL/min, and measurement at λ = 203 nm.

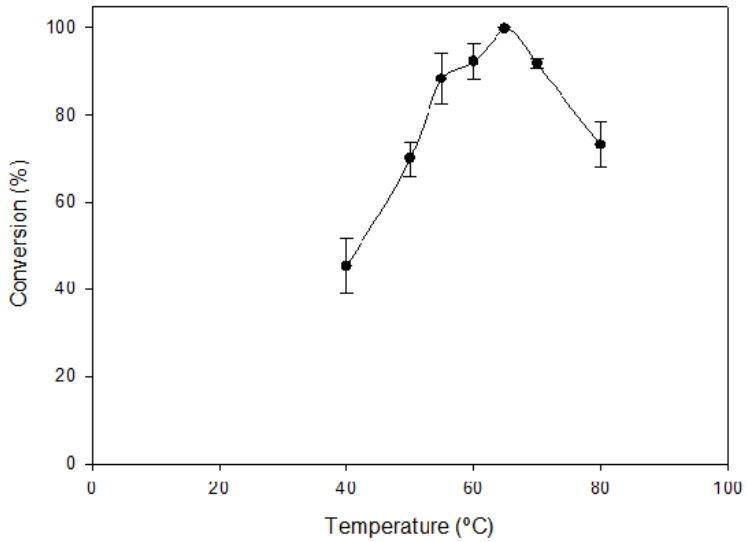

Figure 3. Optimal reaction temperature of the system using 10 mM D,L-methylthioethylhydantoin as substrate, 100 mM borate buffer pH 8 supplemented with 1 mM $CoCl_2$, for 4 h at 50–70 °C with constant stirring. Compounds of the multi-step reaction were separated in HPLC water with a Zorbax C_{18} column, mobile phase of 80:20 20 mM phosphoric acid, pH 3.2, and methanol, 0.4 mL/min, and measurement at λ = 203 nm.

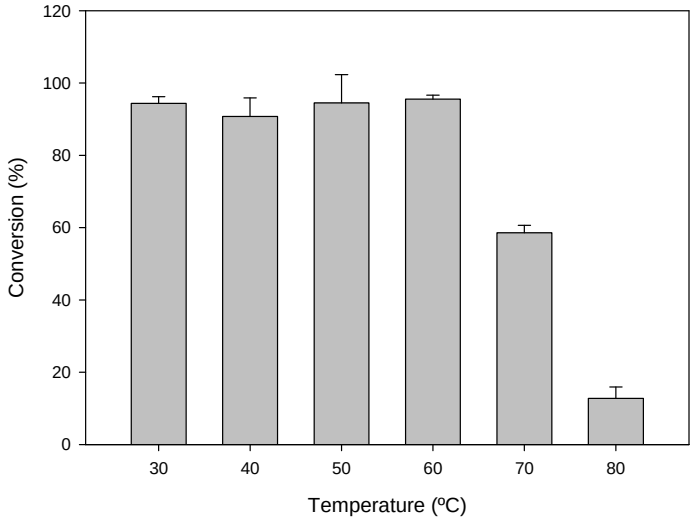

Figure 4. The immobilized four-enzyme system was incubated for 4 h at each temperature followed by a standard assay at 60 °C. The results are the mean of five experiments.

In previous studies of these enzymes, only BsLcar showed thermostability at 70 °C, whereas AtDHyd, AthyuA1, and GkNSAAR showed a drastic loss of activity, decreasing to 25%, 60%, and 40% of activity, respectively, after 30 min [10–13]. At high temperatures, free enzymes suffer a slight unfolding, so hydrophobic interphases can interact and the enzymes are inactivated by aggregation. When the proteins are covalently immobilized, they do not undergo this inactivation [19]. This increase in the optimum temperature is an advantage when the substrates are poorly soluble and a slight increase in temperature is necessary to facilitate their dissolution. L-*N*-carbamoylase and hydantoinase from *Arthrobacter aurescens* showed similar behavior when immobilized in EAH-Sepharose 4B, presenting a broad spectrum of pH and remaining active at higher temperatures, from 25 to 60 °C and 25 to 45 °C, respectively [20].

2.3. Efficiency of the System: Substrate Spectra and Reaction Cycles

The system has been tested for the production of 11 coded and non-coded amino acids. The following optically pure amino acids have been obtained from the corresponding racemic mixture of hydantoins in the reaction times indicated as follows: L-methionine in 150 min, L-norvaline in 200 min, L-norleucine in 200 min, L-ABA (amino butyric acid) in 50 min, L-homophenylalanine in 25 min, and L-valine in 40 min. The enzymatic cascade showed a slight accumulation of carbamoyl, as we can see in the production of L-methionine in Figure 5. For the L-amino acids, L-phenylglycine, L-phenylalanine, L-para-hydroxy-phenylglycine, L-tryptophan, and L-*tert*-leucine the system did not work efficiently (data not shown). The hydantoins D,L-p-hydroxyphenylhydantoin, D,L-phenylhydantoin, D,L-benzylhydantoin, and D,L-indolyl-methylhydantoin were hydrolyzed to the corresponding carbamoyl but not to the corresponding L-amino acid. The system has been tested over several reaction cycles (Figure 6), maintaining 80% activity for over 15 cycles and complete stability of the proteins. The immobilization has proven to be stable and correctly arranged in the porous matrix.

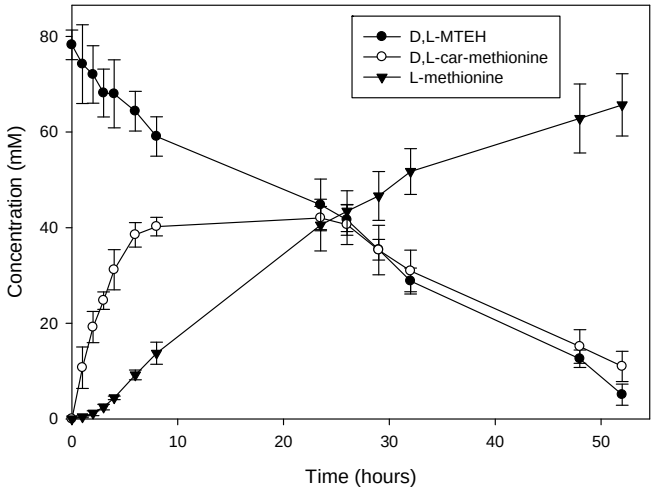

Figure 5. Conversion curves of D,L-5-methylthioethylhydantoin into L-methionine by immobilized biocatalyst on IB-350 (Cycle 1). (●) D,L-5-methylthioethylhydantoin, (○) *N*-carbamoyl-D-methionine, and (▼) L-methionine.

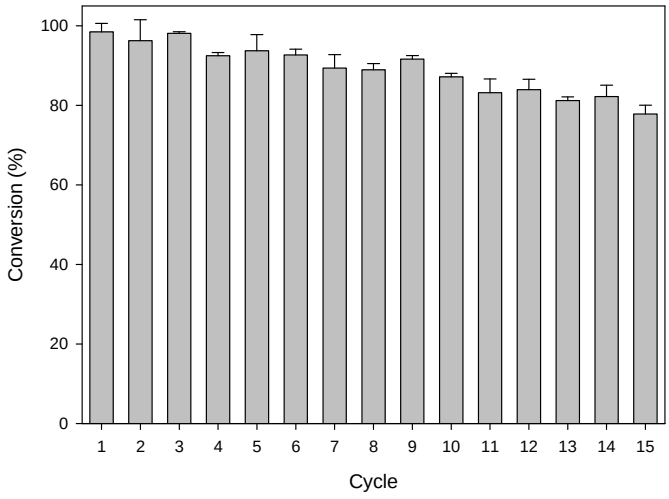

Figure 6. Number of cycles of activity of the system following the standard reaction with D,L-5-methylthioethylhydantoin as substrate and the percentage of amino acid produced. The beads were gently washed with water between cycles.

The immobilized system has shown the same substrate spectra as free protein: norvaline, norleucine, ABA, homophenylalanine, valine, and methionine. Comparison of the quantity of protein used over 15 reactions as free or immobilized protein reveals that immobilization requires five times less quantity of AthyuA1, 13 times less GkNSAAR, but 3 times more of both AtDHyd and BsLcar [9]. This indicates that AtDHyd and BsLcar present more efficiency as free protein. Even taking into account this loss of efficiency, immobilization proves cost-effective as the proteins are reusable over several cycles, control of the reaction is easier, and contamination of the final products is avoided [14].

Few multi-enzymatic systems have been immobilized, probably due to the complications involved in combining the specific activity of several enzymes and the difficulty of finding a suitable support for all of them simultaneously. A tri-enzyme system for selective oxidation of glycerol was improved, but in this case it was necessary to combine protein mutagenesis and immobilization techniques. The three enzymes were co-immobilized sequentially in the same porous carrier and co-crosslinked [21]. A bi-enzymatic system consisting of L-*N*-carbamoylase from *Geobacillus stearothermophilus* CECT43 (BsLcar) and *N*-succinyl-amino acid racemase from *Geobacillus kaustophilus* CECT 4264 (GkNSAAR) as carbamoyl racemase was immobilized in Immobeads IB-161, showing 75% activity after 10 reaction cycles. For both enzymes the specific activity decreased when they were immobilized, but even with this setback, the balance was positive, due to the greater stability and pH range, the greater number of substrates able to hydrolyze, and the increased reusage capacity of the system [20].

3. Materials and Methods

3.1. Protein Purification

The four enzymes were expressed in *Escherichia coli* BL21 containing one of these plasmids, pJMC44 for AtDhyd, pSER12 for AthyuA1, pJPD25 for GkNSAAR, and pJAVI80 for BsLcar. The bacteria were grown in LB medium with 100 µg/mL ampicillin overnight at 37 °C and continuous shaking at 150 rpm; a 2 L flask with 800 mL of LB media supplemented with ampicillin was then inoculated with 10% of overnight culture. The cultures were incubated at 37 °C with vigorous shaking at 180 rpm to an OD_{600} of 0.3–0.5. At this point, the cultures were induced with 0.2% (*w*/*v*) rhamnose and additional shaking for 6 h at 32 °C. For the expression of AtDhyd, it was necessary to add 0.5 mM $CoCl_2$ and maintain incubation at 30 °C overnight. The cellular debris was precipitated by centrifugation at 6300 *g* for 25 min at 4 °C (Beckman Avanti J-26XPi, Brea, CA, USA), the supernatant was discarded, and the precipitated cells frozen. The pellets were resuspended in a wash buffer (300 mM NaCl, 50 mM sodium phosphate pH 7.0), and the cells were disrupted by sonication (UP200S Ultrasonic Processor, Dr. Hielscher GmbH, Germany). The cellular remains were precipitated by centrifugation at 20,400 *g* at 4 °C (Beckman JA-21, Brea, CA, USA), and the supernatant was applied to a Tallon™ resin (Clontech Laboratories, Inc., Nucliber, Madrid, Spain). After washing the columns twice with water, the proteins were eluted with elution buffer (2 mM Tris buffer pH 8, 100 mM NaCl, 150 mM Imidazole) measuring the quantity of eluted protein at Abs_{280}.

3.2. Immobilization

Proteins were dialyzed versus 100 mM borate/HCl buffer pH 8 and concentrated to 2 mg/mL for AtDhyd, BsLcar, and GkNSAAR, as opposed to 1.3 mg/mL for AthyuA1. Each enzyme was then immobilized separately in IB-350 Sepabeads (ChiralVision, Leiden, Netherland) mixing 100 mg of matrix with 0.4, 0.8, or 1.2 mL of AtDhyd, BsLcar, or GkNSAAR (2 mg/mL) and 0.4, 0.8, 1.2, 1.5, 2.3, or 4 mL of 1.3 mg/mL AthyA1 in 100 mM sodium borate/HCl at pH 8 for 12 h at RT [9]. The non-immobilized protein was quantified by the Lowry method, concentrated, and visualized by SDS-PAGE.

Based on the specific activity measurements of the individual immobilized enzymes, three combinations were studied in order to obtain the best rates and avoid intermediate accumulation: SysA 1:1:1:1, SysB 1:1:1:2, SysC 1:1:1:4, AtDhyd:AthyA1:BsLcar:GkNSAAR. The optimal reaction temperature

was tested from 50 to 70 °C at pH 8. The pH was varied from 7 to 9, in 100 mM triethanolamine buffer for pH 7 to 7.5, and in 100 mM borate–HCl buffer for pH 8 to 9, at 60 °C and 700 rpm for 4 h with 10 mM D,L-methylthioethylhydantoin as substrate. To determine the thermostability of the immobilized proteins, each immobilized enzyme was incubated at 30, 40, 50, 60, 70, and 80 °C for 4 h followed by the standard assay at 60 °C. Finally, the system was evaluated to determine the number of cycles over which it maintained 80% activity.

3.3. System Characterization

Synthesis of the hydantoins was carried out as described in [22], while that of the carbamoyls is described in [23]. The amino acids were purchased from Sigma-Adrich Química S.A. (Madrid, Spain). The standard enzymatic reaction was carried out in 100 mM borate buffer at pH 8 supplemented with 1 mM $CoCl_2$, 10 mM racemic mixture of hydantoins, for 4 h at pH 8 and 60 °C. Activity was monitored taking aliquots of 50 µL, adding 450 µL of 1% H_3PO_4, and centrifuging at 12,000 *g*. The supernatants were analyzed by HPLC (Breeze, Waters, Madrid, Spain) with separation in a C_{18} Zorbax column (3 mm × 250 mm, Agilent Techology) using several mobile phases depending on the substrate and measurement at 203 nm in a UV detector.

For all the compounds, the mobile phase was a combination of methanol and phosphoric acid (20 mM; pH 3.2), and the products obtained from each substrate and the corresponding retention times varied. They are listed as follows: for the substrate D,L-ethylhydantoin the intermediate was *N*-carbamoyl-D-ABA, and the amino acid was aminobutyric acid (ABA), the mobile phase was 90:10 at 0.4 mL/min and the detection was at λ: 192/203 nm. Substrate D,L-propilhydantoin, *N*-carbamoyl-D-norvaline, and norvaline; mobile phase of 90:10 at 0.6 mL/min, λ: 203 nm. Substrate D,L-5-butylhydantoin, *N*-carbamoyl-D-norleucine, and norleucine; mobile phase of 90:10 at 0.6 mL/min, λ: 203 nm. Substrate D,L-homophenylalanine hydantoin, *N*-carbamoyl-D-homophenylalanine, and homophenylalanine; mobile phase of 82:18 at 0.4 mL/min, λ: 203 nm. Substrate D,L-isopropylhydantoin, *N*-carbamoyl-D-valine, and L-valine; mobile phase of 90:10 at 0.5 mL/min, λ: 203 nm. Substrate D,L-p-hydroxyphenylhydantoin, *N*-carbamoyl-D-p-hydroxyphenylglycine, and L-p-hydroxyphenylglycine; mobile phase of 90:10 at 0.4 mL/min, λ: 203 nm. Substrate D,L-phenylhydantoin, *N*-carbamoyl-D-phenyl glycine, and L-phenylglicine; mobile phase of 85:15 at 0.4 mL/min, λ: 203 nm. Substrate D,L-5- benzylhydantoin, *N*-carbamoyl-D-phenylalanine, and L-phenylalanine; mobile phase of 70:30 at 0.4 mL/min, λ: 203 nm. Substrate D,L-5-indolylmethylhydantoin, *N*-carbamoyl-D-tryptophan, and L-tryptophan; mobile phase of 70:30 at 0.6 mL/min, λ: 203 nm. Substrate *N*-carbamoyl-D-tert-leucine and L-tert-leucine; mobile phase of 70:30 at 0.6 mL/min, λ: 203 nm.

4. Conclusions

Immobilization of a four-enzyme system presents a high degree of difficulty as it involves the combination of several factors. Optimum pH and temperature conditions, which allow maximum activity of the mixture of proteins, must first be established. Subsequently, the quantity of each protein must be varied according to their specific activities and velocity in order to avoid reaction intermediates [15]. However, it should be noted that, although the fine tuning of the system requires a great deal of effort, once it is achieved, the cascade of activity has been stable for more than one year, opening future lines of work.

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