Amidohydrolase Process, a cascade chemoenzymatic process for multisubstrate conversion to optically pure L-amino acids by dynamic kinetic resolution

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ABSTRACT

A bienzymatic system containing a *N*-succinylamino acid racemase from *Geobacillus kaustophilus* CECT4264 (GkNSAAR) and of L-*N*-carbamoylase from *Geobacillus stearothermophilus* CECT43 (BsLcar) has been developed. This biocatalyst was able to produced optically pure natural and non-natural L-amino acids form racemic mixtures of *N*-acetyl, *N*-formyl- and *N*-carbamoyl-amino acids by dynamic kinetic resolution, showing the fastest conversion on the *N*-formyl-amino acids followed by *N*-carbamoyl and *N*-acetyl ones. Metal ion cobalt was essential for the activity of the biocatalyst. Co^{2+} was added during protein induction or preincubated with the recombinant enzymes before reaction or directly added into this, and the system was optimally active when Co^{2+} was added directly to the reaction. Optimum reaction conditions for the biocatalyst were pH 8 and 45 and 65ºC for *N*-formyl- and *N*-carbamoyl-amino acids, respectively. The bienzymatic system was equally efficient to convert aromatic and aliphatic substituents but always faster *N*-formyl than *N*-carbamoyl-amino acids. This new Amidohydrolase Process enables the natural and unnatural L-amino acids production from broad substrate spectrum.

INTRODUCTION

Optically pure natural and non-natural L-amino acids are of considerable economic importance because of the broad spectrum of their industrial applications. The former, proteinogenic amino acids are building block of life, used in human nutrition and health or as additives, flavour enhancers and sweeteners (5). Additionally, non-natural L-amino acids are in increasing demand as valuable intermediates in the pharmaceutical industry. By way of example, L-homophenylalanine is a precursor for the preparation of agniotensin-converting enzyme (ACE) and rennin inhibitors, such as enalapril, lisinopril, quinapril, ramipril, trandolapril and benazepril, among others (10). L-α-aminobutyric acid (L-ABA) is an intermediate of ophthalmate, a sensitive indicator of hepatic glutathione (GSH) depletion, and designed as new biomarker for oxidative stress (18).

Biocatalytic methods based on chemo-enzymatic processes have been described for optically pure amino acid production. Some of these methods are the hydantoinase (6), amidase (2) and acylase (14) processes. The "Hydantoinase Process" is based on the dynamic kinetic resolution of D,L-5-monosubstituted hydantoins using an inexpensive and environmental friendly enzymatic method (1). The chirality of the amino acid obtained depends on the stereospecificity of the last enzyme in the reaction cascade (*N*-carbamoyl-L-amino-acid amidohydrolases, also known as L-*N*-carbamoylases) (13). In the "Acylase process" a *N*-acylamino acid racemase (NAAAR) together with a L-aminoacylase can produce a final yield of 99% optically pure amino acid in one step from racemic mixtures of *N*-acetylamino acids, by enzymatic racemization of the non-hydrolysed *N*-acetyl-L-amino acid (21).

Our group has recently demonstrated the substrate promiscuity of a recombinant *N*-succinylamino acid racemase from *Geobacillus kaustophilus* CECT4264 (GkNSAAR) (15) and of L-*N*-carbamoylase from *Geobacillus stearothermophilus* CECT43 (BsLcar) (16). Both enzymes allowed the racemization or hydrolysis of *N*-acetyl-, *N*-formyl- and *N*-carbamoyl-amino acids. The aim of this work is to develop a biocatalyst joining both enzymes as a bienzymatic system for natural and non-natural optically pure L-amino acids production. This Amidohydrolase Process will be able to convert racemic mixtures of *N*-acetyl-, *N*-carbamoyl- and *N*-formyl-amino acids into L-amino acids, with 100% of yield, by dynamic kinetic resolution.

MATERIALS AND METHODS

General protocols and reagents.

Standard methods were used for the cloning and expression of the different genes (3,17). Restriction enzymes, T4 DNA ligase and the thermostable *Pwo* polymerase together with primers for PCR were purchased from Roche Diagnostic S.L. (Barcelona, Spain). Racemic mixtures and optically pure D- and L-amino acids were purchased from Sigma Aldrich Quimica S.A. (Madrid, Spain). *N*-acetyl D- and L-methionine were purchased from Sigma-Aldrich (Madrid, Spain). The *N*-carbamoyl- and *N*-formyl-amino acids were synthesized according to previous works (4,9).

Plasmids and culture conditions.

Two recombinant *Escherichia coli* BL21 strains were grown in Luria-Bertani medium (LB) (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.2) as previously described (15,11). One harboured pJAVI80 containing L-*N*-carbamoylase gene from *Geobacillus stearothermophilus* CECT43 (*Bslcar*) and the other harboured pJPD25 containing *N*-succinylamino acid racemase gene (*nsaar*) from *Geobacillus kaustophilus* CECT4264 (*Gknsaar*). Both genes were taken from the above mentioned plasmids and cloned in the rhamnose-inducible expression with His-tag pJOE4036.1 (20); Altenbuchner, pers. communication), to increase the overexpression of the genes in *E. coli* BL21 strains (19)**.** The development of the plasmid pJAVI80rha is described in (12).

The pJPD25rha construction was made by PCR amplification of *Gknsaar* gene (1128 bp; Genbank accession no. EU427322) from the recombinant plasmid pJPD25 that was used as template (16). The PCR primers were 5´-AGAAAGGGGA*GAGCTC*ATGGCGATCAACA-3´ (the *Sac*I site in italics) and 5´-

GGATCCTGCCGTCGCCGTACGATGAAACA-3´ (the *BamH*I site in italics). Amplifications were performed in a Applied Biosystems thermal cycler 2720 programmed as follows: initial denaturation at 94ºC for 5 min; 30 sec at 94ºC for denaturing, 30 sec at 57ºC for annealing, 1 min at 72ºC for synthesis, repeated 35 cycles; and a final extension step of 5 min at 72ºC. The PCR products were purified from agarose gel using E.Z.N.A. Gel Extraction Kit (Omega Bio-Tek, Inc., USA), treated with the corresponding enzymes and then ligated into pJOE4036.1 which was cut with the same enzymes to create the plasmid pJPD25rha.

After cloning, the plasmid was transferred to *E. coli* DH5α to verify the presence of the insert. The sequence was analyzed at least twice using standard T3 and T7 primers. Sequencing analysis was carried out using the dye dideoxy nucleotide sequencing method in an ABI 377 DNA Sequencer (Applied Biosystems).

Expression of *Bslcar* and *Gknsaar* genes.

The transformants in BL21 strain (BL21pJAVI80rha and BL21pJPD25rha) were grown in LB medium supplemented with 100 μg ml^{-1} of ampicillin. The expression protocol was the same both transformants. A single colony was transferred into 10 ml of LB medium with ampicillin at the above-mentioned concentration in a 100 ml flask. This culture was incubated overnight at 37°C with shaking. In a 2 l flask 500 ml of LB with the appropriate concentration of ampicillin was inoculated with 5 ml of the overnight culture. After 2 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 *lcar* and *nsaar* genes, L-rhamnose (Prolabo, VWR) was added to a final concentration of 0.2 % (w/v) and the culture was incubated at 32°C for an additional 6 hours. The cells were collected by centrifugation (Beckman JA2-21, 7,000 *g*, 4°C, 10 min) and stored at -20ºC. The

151 freezed pellet was washed twice and resuspended in 50 ml wash buffer (300 mM NaCl,
152 20 mM imidazole, 50 mM sodium phosphate; pH 7.0). The cell walls were disrupted in
153 ice by sonication using a UP 200 S Ultrasonic Processor (Dr. Hielscher GmbH,
154 Germany) for 6 periods of 60 s, pulse mode 0.5 and sonic power 60%. The pellet was
155 precipitated by centrifugation (Beckman JA2-21, 10,000 g, 4°C, 20 min) and discarded.
156 The supernatant was applied to a column with TALON^{TM} metal affinity resin
157 (CLONTECH Laboratories, Inc., Nucliber, Madrid, Spain) and then washed three or
158 four times with wash buffer. After washing, BsLcar and GkNSAAR were eluted with
159 elution buffer (100 mM NaCl, 150 mM imidazole, 2 mM Tris, pH 8.0). The purified
160 enzymes were dialysed against 0.1 M borate/HCl pH 8.0 and stored at -20ºC until use.
161
162 **Conversion assay.**
163 Standard enzymatic reaction was carried out with the purified BsLcar and
164 GkNSAAR enzymes (at a final concentration of 3.1 µM and 8.1 µM, respectively) in
165 presence of $CoCl_2$ (final concentration of 1 mM) together with *N*-derivate of D,L-
166 methionine as substrate (15 mM) dissolved in 100 mM borate/HCl (pH 8.0) in 390 µl
167 reaction volume. The reaction mixture was incubated at 65ºC and 60 min for *N*-
168 carbamoyl-D,L-methionine conversion and 15 min at 45ºC for *N*-formyl-D,L-
169 methionine one. For *N*-acetyl-D,L-methionine conversion final concentration of BsLcar
170 and GkNSAAR enzymes was 35.64 µM and 73.49 µM, respectively, with a reaction
171 time of 240 min at 65ºC. The reactions were stopped by addition of 10 times the
172 reaction volume of 1% H_3PO_4.
173 After centrifuging, the resulting supernatants were analysed by high
174 performance liquid chromatography (HPLC). The HPLC system (LC2000Plus HPLC
175 System, Jasco, Madrid, Spain) equipped with a Luna C_{18} column (4.6 × 250 mm,

Phenomenex) was used to detect *N*-acetyl, *N*-formyl and *N*-carbamoyl-D,L-methionine and L-methionine. The mobile phase was methanol/phosphoric acid (20 mM, pH 3.2) (vol/vol, 20:80), pumped at a flow rate of 0.80 ml min^{-1} and measured at 200 nm.

Substrate specificity studies were performed with each different *N*-formyl and *N*-carbamoyl-amino acid dissolved in 100 mM borate/HCl (pH 8.0) together with the purified enzyme at the same concentration described above in presence of 1 mM $CoCl_2$. Reactions were carried out at 45 ºC and 65ºC, and stopped by addition of 1% H_3PO_4. The mobile phase of the different substrates and their corresponding L-amino acids was methanol-phosphoric acid (20 mM, pH 3.2) (5:95 to 50:50 vol/vol, depending on the compound), pumped at a flow rate of 0.50 ml min^{-1}. Compounds were detected with a UV detector at a wavelength of 200 nm.

System characterization.

Optimal temperature was evaluated from 30 to 80 ºC in 100 mM borate/HCl (pH 8.0). The thermal stability of the cascade enzymatic process was measured after 0, 2, 4, 6, 8, 10 and 24 hours of preincubation at temperatures from 40 to 70 ºC in 100 mM sodium borate/HCl buffer pH 8. Studies of pH were assayed in several buffers at pH 5.5 to 10.5 (sodium citrate, sodium cacodylate, borate/HCl and borate/NaOH) at a concentration of 100 mM. Enzyme assays, for optimal temperature and pH studies, were then carried out at 45 ºC for 30 min with *N*-formyl-D,L-methionine and 65ºC for 60 min with *N*-carbamoyl-D,L-methionine.

RESULTS AND DISCUSSION

Multisubstrate hydrolysis

Recombinant BsLcar has demonstrated to hydrolyse *N*-acetyl and *N*-formyl-amino acids in addition to *N*-carbamoyl-amino acids (16). Aditionally, GkNSAAR has shown the ability to racemize different *N*-acetyl and *N*-carbamoyl-amino acids (15). Evidenced this substrate promiscuity of both enzymes, BsLcar and GkNSAAR were combined to transform *N*-acetyl, *N*-formyl- and *N*-carbamoyl-amino acids into optically pure L-amino acids. The bienzymatic system was able to hydrolyse all three racemic substates into L-methionine, showing the fastest conversion on the *N*-formyl-amino acid followed by *N*-carbamoyl and *N*-acetyl ones (Fig. 1), for this we have named it as "Amidohydrolase Process". These results are in accordance with those obtained for BsLcar, where the highest conversion was for *N*-formyl-amino acids followed by *N*-carbamoyl- and *N*-acetylamino acids (16). Previous studies with GkNSAAR demonstrated that catalytic efficiency (k_{cat}/K_m) to racemize both isomers of *N*-acetyl- and *N*-carbamoyl-methionine were very similar (15), and there is no data about racemization of *N*-formyl-amino acids with GkNSAAR. From the present studies with de bienzymatic system and those with BsLcar and GkNSAAR separately, we can assume that BsLcar is the reponsable that *N*-formyl amino acids are fasted hydrolysed compared with the *N*-carbamoyl- and *N*-acetyl- ones. At last, when *N*-succinyl-amino acids, the natural substrates of GkNSAAR, were used as substrates for BsLcar we did not detect any activity (data not shown).

Effect of enzyme ratios in the bienzymatic system

Enzyme ratio plays a critical role in driving the overall reaction. Optimize the right proportion of each enzyme allows to reduce both time reaction and protein consumption. BsLcar has shown higher catalytic efficiency for the hydrolysis of these types of substrates than GkNSAAR to racemizate them (15,16). Five different BsLcar/GkNSAAR ratios, with constant presence of BsLcar and always increasing the GkNSAAR proportion in the mixture (1:1, 1:3, 1:6, 1:12 and 1:18), were assayed to achieve maximum conversion efficiencies for L-methionine from *N*-formyl-D,L-methionine (Figure 2). When the proportion of both enzymes was the same (ratio 1:1), the conversion was only 50% after 350 min of reaction. After increased 6, 12 and 18 times GkNSAAR proportion respect BsLcar, conversion efficiencies approached nearly 100%. In order to reduce GkNSAAR consumption 1:6 ratio was selected as the best for biocatalytic transformation (Fig. 2).

Cobalt effect in the bienzymatic system

BsLcar and GkNSAAR have been described as metalloenzymes and for both enzymes Co^{++} as the best cofactor (15,16). For GkNSAAR it is not necessary a preincubation with the metal ion for hours and the Co^{++} is adding directly to the reaction as a component (15). However, our previous studies for BsLcar were made with preincubation with Co^{++} at 4ºC (16). In order to balance the way to obtain the active protein by metal ion, two strategies were developed: a) compare the BsLcar activity after preincubation with Co^{++} after adding the metal ion directly in the reaction, in order to avoid the preincubation, and b) include Co^{++} in the grow medium to obtain recombinant enzymes including active avoiding to add the metal ion in the reaction.

To evaluate the first strategy, BsLcar was incubated at 4ºC for 60 min with 1 mM Co^{++} to enhance the activity. However, there was no activity difference between preincubated BsLcar and when the metal ion was added directly to the reaction (data not shown). Thus, to activate the enzymes is enough adding Co^{++} in the reaction directly, avoiding time consuming in enzyme preincubation.

The second strategy tried to avoid adding the cobalt in the reaction by including this in the induction step. For this, BL21 pJAVI80rha and BL21 pJPD25rha were induced as described in materials and methods, but including Cl_2Co 0.2 mM at the end concentration. BsLcar activity was the same for both BsLcar overexpresed in a medium supplemented with Co^{++} and that one induced without metal ion but added directly to the reaction (Fig. 3A). In opposite, GkNSAAR was not active at all when the enzyme was overexpresed in a medium supplemented with Co^{++} (Fig. 3B), restoring the activity after adding directly Cl_2Co 1mM to the reaction mixture. Additionally, two effects were detected when Co^{++} was included in the cells grow medium: a) a toxic effect in bacterial cells decreasing the number recombinant cells produced during the induction, consequently the purified protein yield drastically dropred, and b) substantial insolubility of the recombinant enzymes produced by cells that accumulate the Co^{++} during the grow and induction. Thus, BsLcar could be concentrate up to 20 mg/ml if the cells were grown without Co^{++} and 7.7 mg/ml in presence of the metal ion. Similar phenomenon was detected for GkNSAAR, where the solubility down from 17 to 9.5 mg/ml, without and with metal ion, respectively. All these studies ended in directly addition of Co^{++} in the reaction mixture.

For determinate the optimum Co^{++} concentration in the reaction, Cl_2Co from 0 to 6mM was added to the reaction (Fig. 4). In absence of cobalt the bienzymatic system only transformed 50% of the substrate in optically pure L-amino acid. This result agrees

with those that demonstrated BsLcar activity with no cobalt in the reaction (16), and in the bienzymatic system this activity let the hydrolysis of the L-isome of the racemic formyl-methionine used as substrate. However, GkNSAAR was previously described as inactive without cobalt in reaction (15), and thus in the bienzymatic system the enzyme can not racemize the non-hydrolysed by BsLcar D-isomer. When the metal ion was presented in the reaction the conversion was 100% in the same time (Fig. 4) with no noticeable inhibition at higher cobalt concentrations (6 mM). Optimal conversion by bienzimatic system was detected at 0.25 mM of Co2+, and from now this is metak ion concentration used in standard reaction.

pH and temperature effect in the bienzymatic system

As we have described above the bienzymatic system transformed *N*-formyl, *N*-carbamoyl- and *N*-acetyl-amino acids into optically pure L-amino acids, but those latter very much slowly. For this reason the conversion reaction was optimized for the two former. Thus, L-methionine production from *N*-formyl and *N*-carbamoyl-methionine was evaluated at different pHs and temperatures. Optimal pH to obtain the maximum conversion efficiency of the system for both substrates was 8 (Fig. 5). This pH value is the same that obtained for GkNSAAR (15) and slightly higher than for BsLcar (16) with pH 7.5. BsLcar showed 95% of activity at pH 8, but GkNSAAR activity decreased until 80% at pH 7.5 (15,16), confirming pH 8 as the optimal for the bienzymatic system. The temperature activity profile of the bienzymatic system for the two different substrates was evaluated at different temperatures (Fig. 6). Maximum conversion activity was different for *N*-carbamoyl- and *N*-formyl-methionine with 65 and 45ºC, respectively.

The thermal stability of the cascade enzymatic process was measured after 0, 2, 4, 6, 8, 10 and 24 hours of preincubation at temperatures from 40 to 70 ºC in 100 mM

sodium borate/HCl buffer pH 8. The bienzymatic system showed 100% of activity after 24 hours of preincubation at 45ºC, down until 70 and 60% after 24 hours at 50 and 60ºC, respectively (Fig. 7).

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

After optimized the bienzymatic system conversion reaction from *N*-formyl and *N*-carbamoyl-amino acid to optically pure L-amino acid, this was evaluated for different substrates (Fig. 8). A previous work demonstrated the ability to produce L-homophenylalanine from racemic mixtures of *N*-carbamoyl- D,L-homophenylalanine by using a NSAAR from *Deinococcus radiodurans* and L-*N*-carbamoylase from *Bacillus kaustophilus* in a recombinant *E. coli* whole cell system, but not other L-amino acids (8). However, and to our knowledge, this is the first work to report a system able to convert, not only racemic mixtures of *N*-carbamoyl-amino acids, but also *N*-formyl-amino acids into optically pure L-amino acids. Following the results, the biocatalytic process converted more efficiently *N*-formyl than *N*-carbamoyl-amino acids into natural and unnatural L-amino acids (Fig. 8). The bienzymatic system was equally efficient to convert aromatic and aliphatic substituents. The highest initial reaction rate has been for the unique natural L-amino acid used as control in this work, L-methionine, with 0.6377 and 0.1581 U/mg protein from *N*-formyl and *N*-carbamoyl-methionine, respectively. The substrates hydrolyzed most slowly were L-aminobutyric acid (L-ABA) and L-phenylglycine, with aliphatic and aromatic substituents, respectively. However, this enzymatic method using racemic substrates is more efficient than the previously described for L-ABA production from optically pure L-threonine and L-aspartic acid in a whole cell biotransformation using recombinant *E. coli* cells expressing cloned genes for threonine deaminase, aromatic aminotransferase and acetolactate synthase (7).

The behaviour of the bienzymatic system in presence of high substrate concentration in reaction is a crucial point of interest. For this reason, we have analyzed the production of optically pure L-homophenylalanine from 100 mM *N*-formyl-homophenylalanine (Fig. 9). In spite of the low solubility of the L-amino acid, upper concentrations of 50 mM were not measurable, the total hydrolysis of the precursor (Fig. 9) and the only presence of the L-amino acid in the powder at the end of reaction by nuclear magnetic resonance (NMR) analysis (data no shown), have demonstrated the 100% conversion of the substrate. The total conversion was achieved in only 24 hours using the same enzyme concentrations that at the small-scale without noticeable inhibition effect for high substrate concentration.

This bienzymatic system has shown to be able to convert different racemic mixtures of substrates (*N*-acetyl, *N*-formyl- and *N*-carbamoyl-amino acids) in optically pure L-amino acids by dynamic kinetic resolution. This biocatalyst is a real alternative for supply non-natural L-amino acids to the Pharmaceutical Industry.

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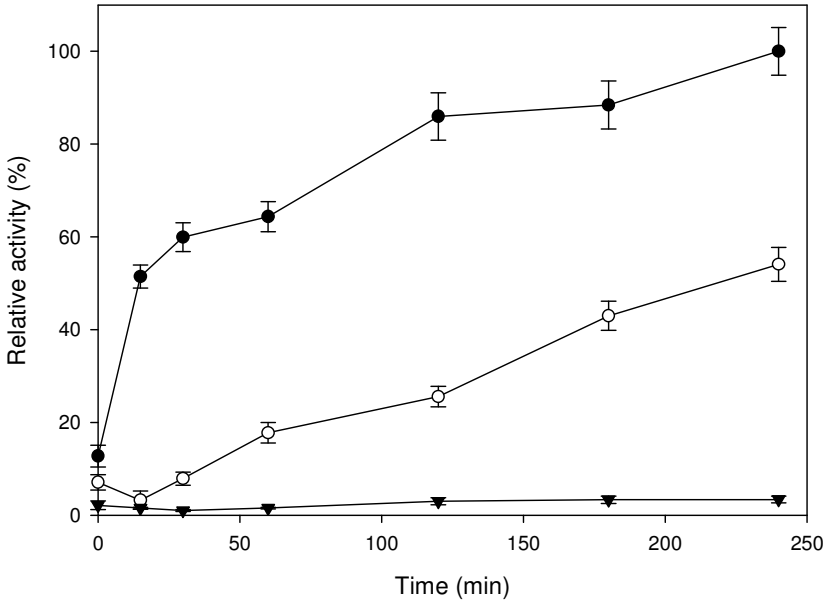

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437 Figure 1.- Optically pure L-methionine formation from *N*-formyl- (●), *N*-carbamoyl- (○)
438 and *N*-acetyl-methionine (▼) by using the Bienzymatic System formed by BsLcar and
439 GkNSAAR. Activity measures were performed using standard enzyme assay and in
440 triplicate (see Material and Methods).

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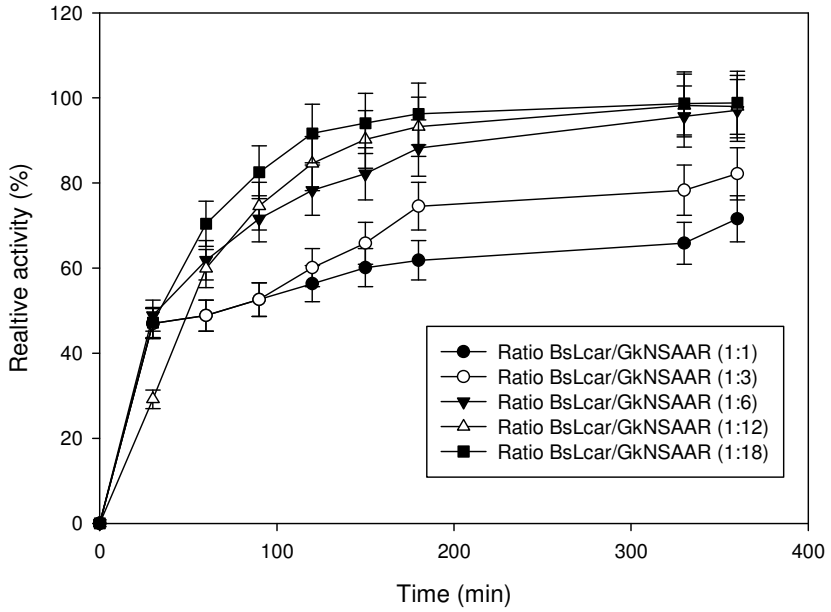


Figure 2.- Optically pure L-methionine formation from *N*-formyl-methionine by using the Bienzymatic System with different BsLcar and GkNAAAR ratios. Activity measures were performed using standard enzyme assay and in triplicate (see Material and Methods).

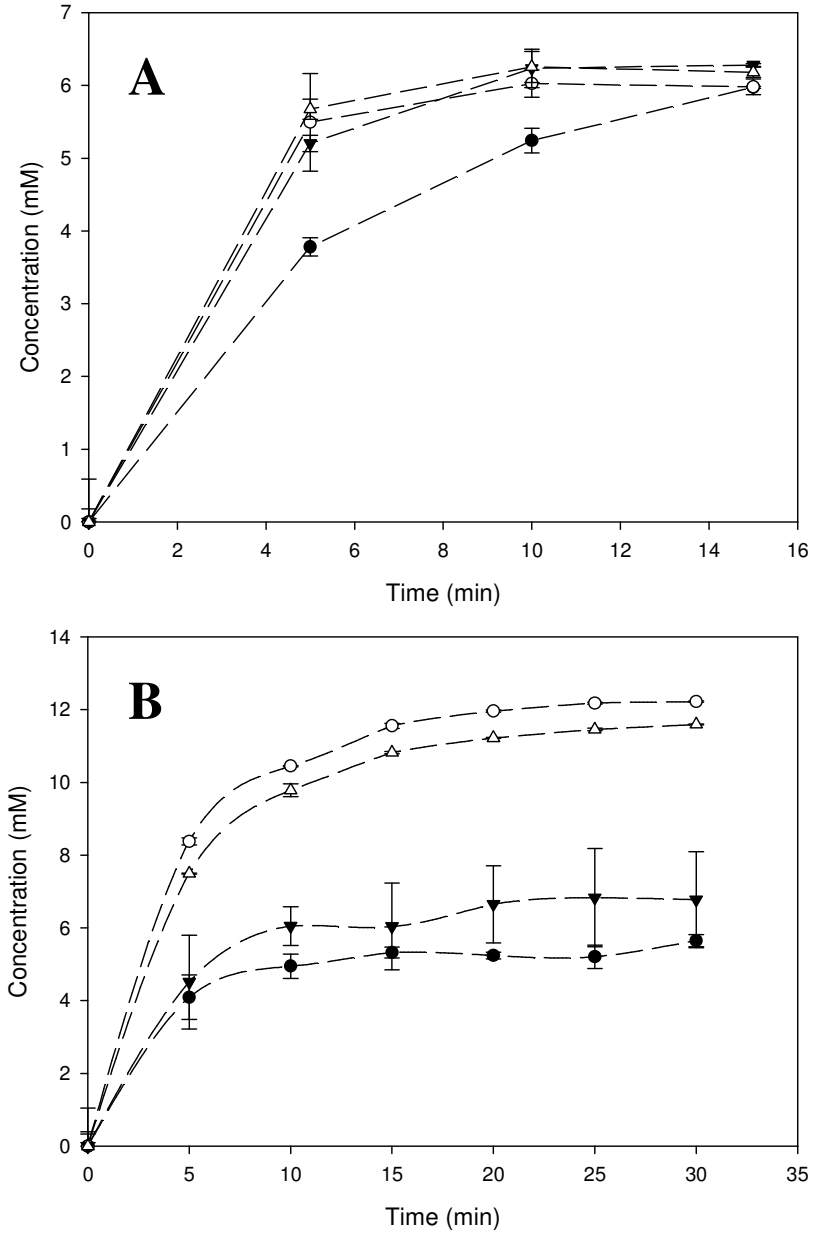
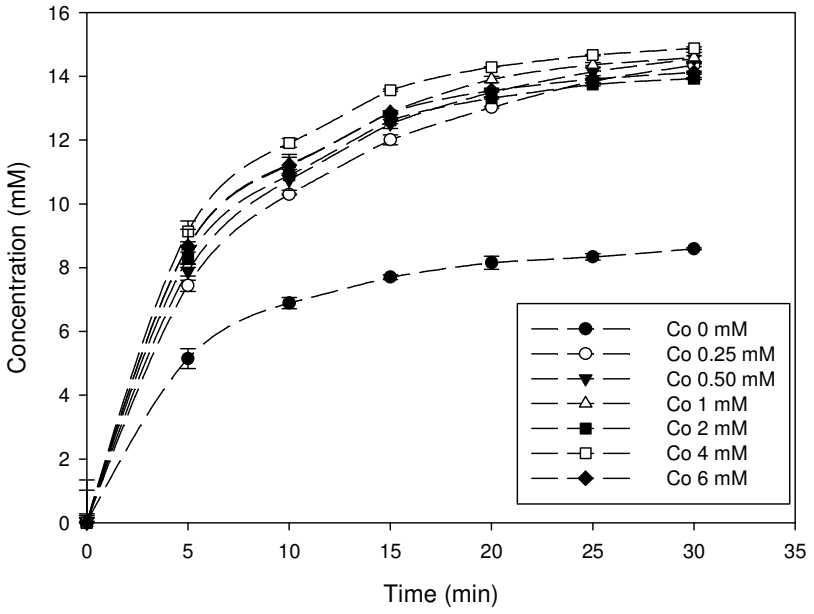


Figure 3.- Cobalt effect on BsLcar (A) and GkNSAAR (B). BsLcar activity was measured using *N*-formyl-D,L-methionine as substrate and the assays were performed in triplicate as described in (Pozo-Dengra, 2010). GkNSAAR activity was measured as accomplished reaction together BsLcar, using *N*-formyl-D,L-methionine as substrate and the assays were performed in triplicate as described in Material and Methods in presence of five times higher concentration of BsLcar. (●) Recombinant enzyme induced without Co^{++} and reaction assay without Co^{++}, (○) recombinant enzyme induced without Co^{++} and reaction assay with Co^{++}, (▼) recombinant enzyme induced with Co^{++} and reaction assay without Co^{++} and (Δ) recombinant enzyme induced with Co^{++} and reaction assay with Co^{++}.



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555 **Figure 4.-** Cobalt effect on Bienzymatic System after adding several concentrations of
556 metal ion in the reaction mixture. Reactions were made using *N*-formyl-D,L-methionine
557 as substrate and the assays were performed in triplicate as described in Material and
558 Methods with a protein concentration of 13.81 µM for GkNSAAR and 2.21 µM for
559 BsLcar.

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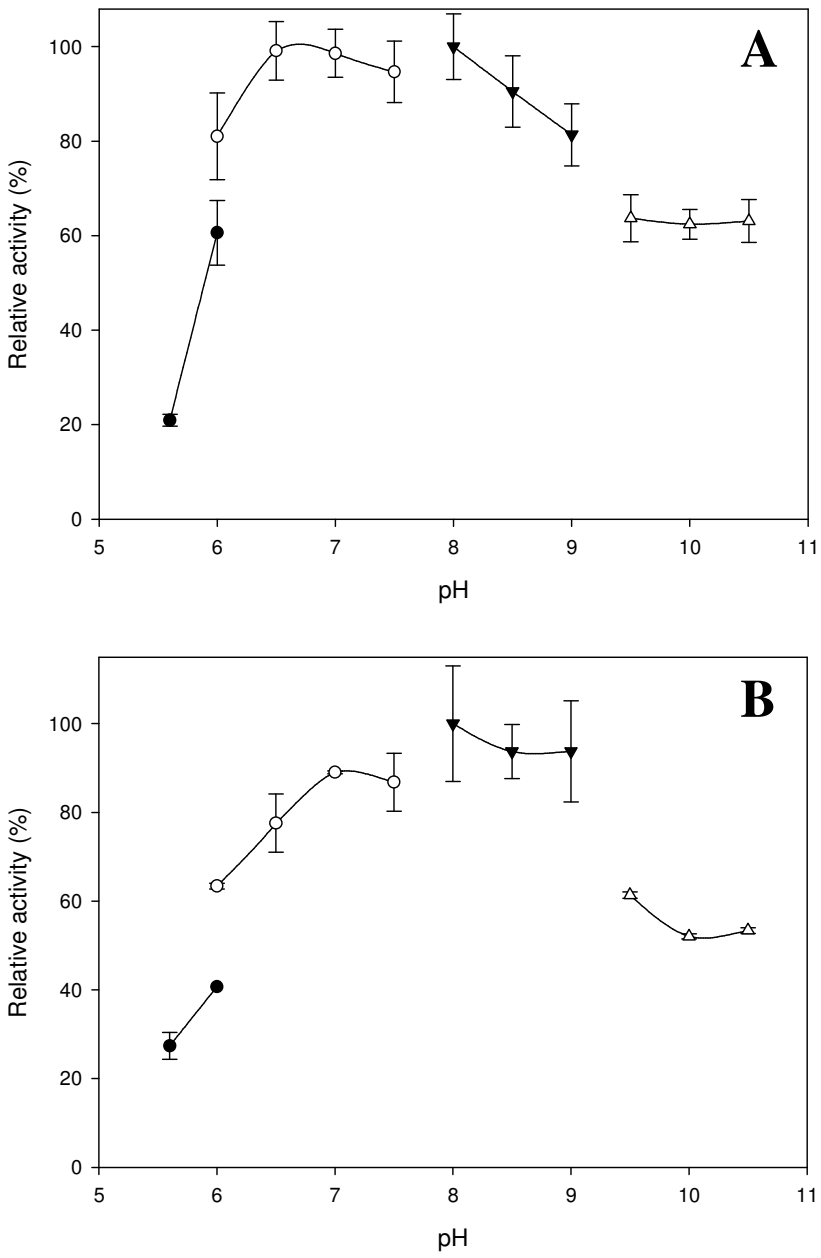


Figure 5. Effect of pH on *N*-carbamoyl- (A) and N-formyl-D,L-methionine (B) conversion into L-methionine. Studies of pH were assayed in several buffers at pH 5.5 to 10.5 (sodium citrate (●), sodium cacodylate (○), borate/HCl (▼) and borate/NaOH (Δ)) at a concentration of 100 mM. Enzyme assays were performed in triplicate as described in Material and Methods.

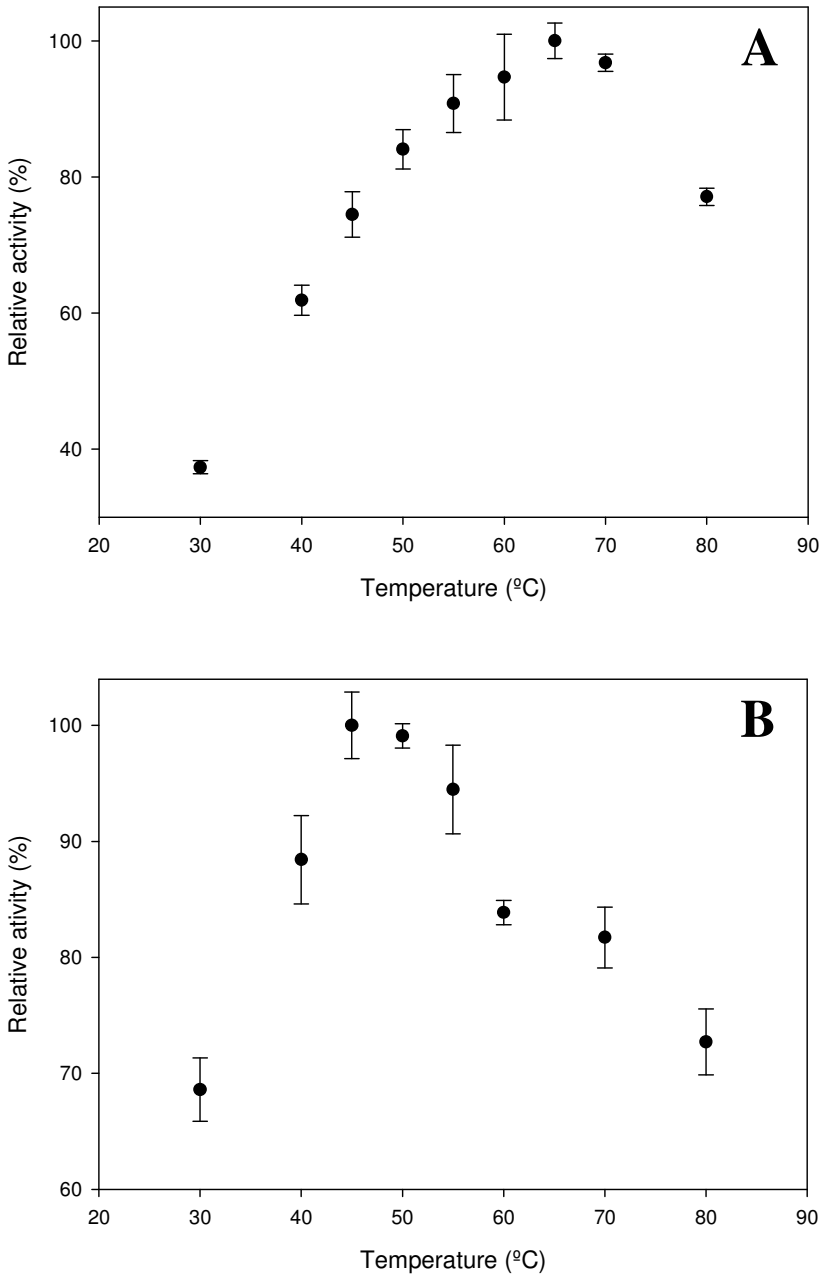


Figure 6. Effect of temperature on *N*-carbamoyl- (A) and N-formyl-D,L-methionine (B) conversion into L-methionine. Optimal temperature was evaluated from 30 to 80 ºC in 100 mM borate/HCl (pH 8.0). Enzyme assays were performed in triplicate as described in Material and Methods.

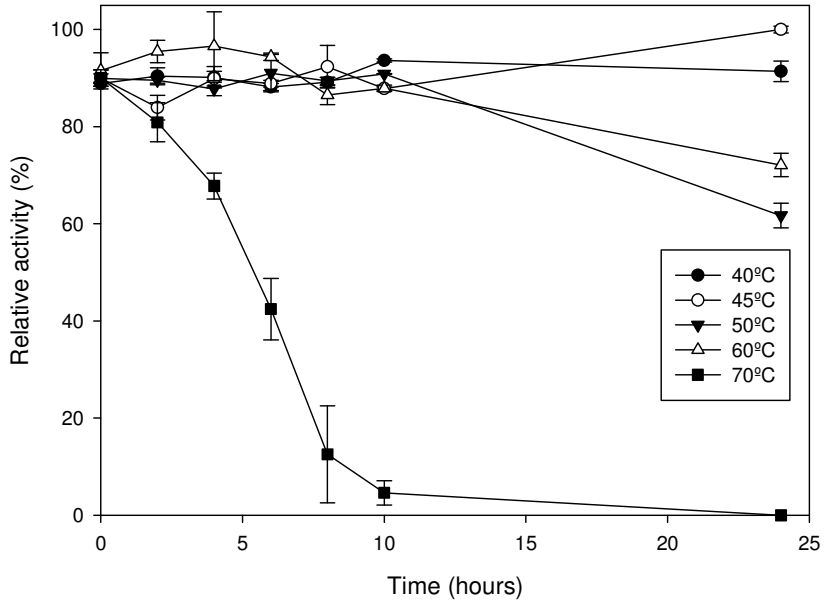
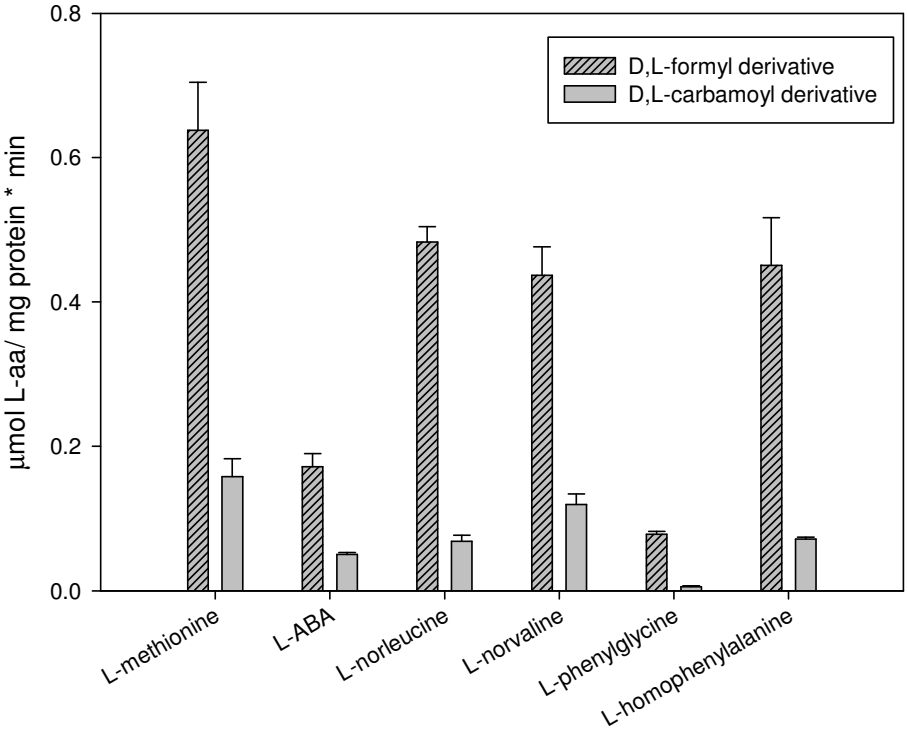


Figure 7. Thermal stability effect on L-methionine production from D,L-formyl methionine after different hours of preincubation at temperatures from 40 to 70 ºC in 100 mM sodium borate/HCl buffer pH 8. Thermal stability was evaluated with standard assays performed in triplicate as described in Material and Methods.



725 **Figure 8.** Initial reaction rates for the production of different optically pure L-amino
726 acids from D,L-formyl- and D,L-carbamoyl-amino acids. Enzyme assays were
727 performed in triplicate as described in Material and Methods.

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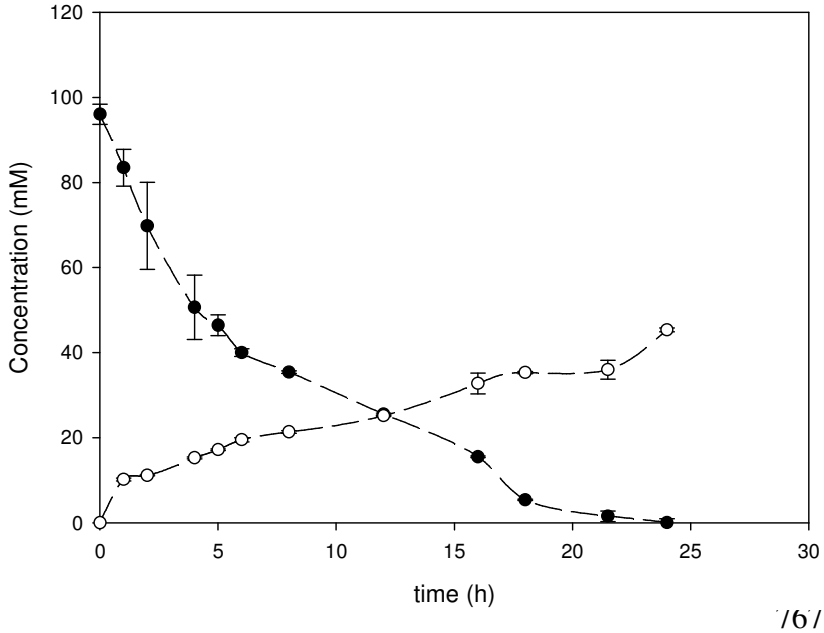

Figure 9. Profile of L-homophenylalanine production from 100 mM *N*-formyl-homophenylalanine. L-amino acid at higher concentration of 50 mM is insoluble, but after 24h of reaction the collected powder is only L-homophenylalanine (data checked by NMR). Enzyme assays were performed in triplicate as described in Material and Methods.