CROATICA CHEMICA ACTA CCACAA **74** (4) 757–762 (2001)

ISSN-0011-1643
CCA-2764 *Original Scientific Paper*

Quantitative Acylation of Amino Compounds Catalysed by Penicillin G Acylase in Organic Solvent at Controlled Water Activity

Alessandra Basso, Stefania Biffi, Luigi De Martin, Lucia Gardossi, and Paolo Linda*

Dipartimento di Scienze Farmaceutiche, Università degli Studi di Trieste, Piazzale Europa 1, 34127 Trieste, Italy

Received December 12, 2000; revised April 27, 2001; accepted May 3, 2001

Covalently immobilised penicillin G acylase (PGA-450) accepts in toluene, at controlled water activity (a_w), a broad range of amino compounds as nucleophiles in kinetically controlled acylation. Hydrolytic reactions were prevented and complete conversions were achieved in short times even when working with an equimolar concentration of the substrates. The recovery of the products was facile, leading to high isolation yields. The obtained *N*-acylated derivatives of L-amino acids can be used in further reactions, since no purification steps are required in such conditions. This opens new perspectives to the application of PGA in selective protection of the amino function for peptide synthesis. All attempts to perform esterification and transesterification reactions with PGA in toluene, at the same a_w as used for the acylation of amino compounds, were unsuccesful.

Key words: penicillin G acylase, kinetically controlled synthesis, organic solvent, acylation, water activity, derivatised L-amino acids.

INTRODUCTION

Penicillin G acylase (EC 3.5.1.11) (PGA) is a commercially available enzyme with a wide substrate acceptance in water, since it is not only specific for penicillin G, but it also hydrolyses the amides and esters of phenylacetic

* Author to whom correspondence should be addressed. (E-mail: linda@univ.trieste.it)

acid,[1–5] showing a good flexibility as far as the amine/alcohol moiety is concerned.

Catalysis of the reverse reaction by PGA and its applicability to a wide range of substrates could favour the exploitation of this enzyme in the synthesis of biologically active peptides, for the protection/deprotection of the amino groups, since no hydrolytic activity towards peptide bonds for PGA has been shown.[6]

In general, the hydrolysis of phenylacetic acid esters, used as acylating agents, can be prevented by working in low-water media.

We have already demonstrated that PGA can be kept active in hydrophobic organic media only when the hydration of the enzyme is maintained sufficiently high by controlling the water activity (a_w) of the system ($0.50 < a_w < 0.85$) and we have developed several methods to use both native[7] and immobilised PGA in low-water media.[8–12] This paper presents a comprehensive study of the applicability of PGA in the protection of α-amino groups of derivatised L-amino acids in toluene.

EXPERIMENTAL

PGA-450 (PGA from *E. coli,* immobilised on a polymer the nature of which is not disclosed by the manufacturer, enzymatic activity = 401 U g^{-1} dry preparation) and 7-aminocephalosporanic acid (7-ACA) were a generous gift of Boehringer Mannheim. Celite R-640® rods were purchased from Fluka. L-Ala-OMe, L-Phe-OMe, Gly-OEt, L-PhGly-OMe, L-Trp-OMe and L-Leu-NH_2 were obtained from their respective hydrochlorides by adding an equimolar amount of $Na_2CO_3 \cdot 10H_2O$, as previously described.[12] All the substrates, L-Tyr-OEt and L-Tyr-OMe were obtained from Sigma. All solvents were dried over molecular sieves (4Å) prior to use. Ultrapure water was used in all experiments.

7-ACA-OMe was chemically synthesised from 7-ACA following a procedure previously reported for 6-aminopenicillanic acid (6-APA).[13]

Water activity was measured using a hygrometer (Novasina MS 1) equipped with a humidity-temperature sensor (enCR-3). The sensor was calibrated at 25 °C at five different a_w values (0.12; 0.33; 0.52; 0.75; 0.90) using standard salt solutions. After equilibration, measurements were carried out by sealing the sensor into the open end of 5 mL glass vials, thermostatted, until constant reading.

PGA-450 was partially dehydrated, prior to use in toluene, following a previously reported procedure.[10,12]

Enzymatic reactions were carried out by adding the substrates as powders to 1 mL of solvent, previously equilibrated with the catalyst for 24 hours at 30 °C in an orbital thermostatted shaker.

Initial rates were determined by following the first 10% of conversion of the reaction by RP-HPLC (Pharmacia) according to a previously reported procedure.[9]

Enzymatic activity of PGA-450 was assayed in phosphate buffer by automated titration (TTT80 Radiometer, Denmark) of the phenylacetic acid formed during the hydrolysis of benzylpenicillin (Aldrich). One enzymatic unit corresponds to the amount of enzyme that hydrolyses 1 μmol of benzylpenicillin in 1 minute at pH 8.0 at 37 °C.

Conversions and verification of the formed amides were obtained by means of RP-HPLC and ^{1}H NMR, respectively.

RESULTS AND DISCUSSION

In order to select the best acyl donor, PGA-450 was used to catalyse the acylation of L-PhGly-OMe with three different derivatives of phenylacetic acid in toluene (Table I).

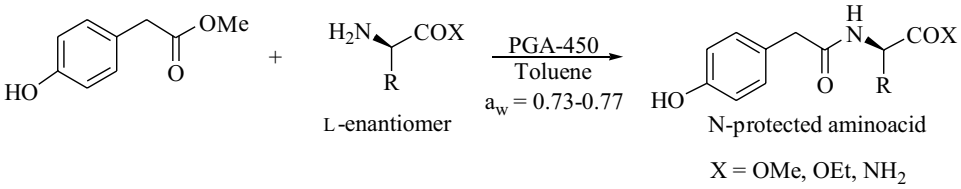
TABLE I

Acylation of L-phenylglycine methyl ester using different acyl donors catalysed by PGA-450 in toluene at controlled a_w[a]

Acyl donor	v_0 / mmol h^{-1}
Methyl 4-hydroxyphenylacetate	0.83
Methyl phenylacetate	0.43
Methyl phenoxyacetate	0.34

[a] Experimental conditions: reaction volume = 1 mL of dry toluene, T = 30 °C, a_w = 0.73–0.77, 62.5 mg of PGA-450 (18 U), 0.1 mmol of L-phenylglycine methyl ester, 0.1 mmol of acyl donor.

The highest initial rate (v_0) was observed when methyl 4-hydroxyphenylacetate was used as acyl donor. The coversion was >98% after 24 h in all reactions. Thus, methyl 4-hydroxyphenylacetate was chosen to perform the acylation of 7-aminocephalosporanic acid methyl ester (7-ACA-OMe) and of derivatised L-amino acids according to Scheme 1.



Scheme 1. Acylation of different derivatised L-amino acids and 7-ACA with methyl 4-hydroxyphenylacetate catalysed by PGA-450 in toluene.

Results are reported in Table II.

As reported in Table II, PGA accepts in toluene both aromatic (L-Tyr-OEt, L-Phe-OEt, L-Trp-OMe) and aliphatic (Gly-OEt, L-Ala-OMe, L-Leu-NH_2) derivatised L-amino acids, though the reaction rates differ considerably. PGA catalysed the acylation of all the examined amino acids, through a kinetically controlled synthesis, with high conversion (all above 98%) and isolation yields (all above 90%) in a reasonable reaction time (maximum 29 hours). The acylation of derivatised 7-ACA was also accomplished in toluene in about 24 hours. Moreover, since hydrolytic reactions were prevented by working at low a_w, it was possible to perform all the acylations using equimolar concentrations of the substrates.

TABLE II

Acylation of different derivatised L-amino acids and 7-ACA with methyl 4-hydroxyphenylacetate catalysed by PGA-450 in toluene[a]

Nucleophile	Reaction time[b] / h
L-Ala-OMe	0.5
L-Tyr-OEt	0.5
L-Tyr-OMe	3
L-PhGly-OMe	3
Gly-OEt	5
L-Leu-NH_2	5
L-Phe-OMe	10
L-Trp-OMe	29
7-ACA-OMe	24

[a] Experimental conditions: reaction volume = 1 mL of dry toluene, T = 30 °C, a_w = 0.73–0.77, 62.5 mg of PGA-450 (18 U), 0.1 mmol of nucleophile, 0.1 mmol of methyl 4-hydroxyphenylacetate.

[b] Reaction time needed for conversion above 98%.

In all syntheses, the formation of the products led to a precipitate. The products were easily recovered by washing the immobilised enzyme with ethyl acetate at the end of the reaction.

All the amino compounds considered were used in their esterified form. The zwitterionic form of the unprotected substrates reported in Table II was insoluble in organic solvent, and all attempts to perform the acylation of the free amino acids and 7-ACA in toluene were unsuccessful.

Results show that, in organic solvent, PGA accepts different esters of the amino acids considered, since both L-Tyr-OMe and L-Tyr-OEt were quantitatively acylated by PGA with complete conversion.

This study was extended to esterification and transesterification reactions catalysed by PGA in toluene at the same a_w as above. 1-Phenyl-1-propanol, 1-phenyl-1-ethanol, cinnamic alcohol and methanol were incubated in the presence of methyl 4-hydroxyphenylacetate as acyl donor. Surprisingly, after one week of incubation, no reaction was achieved using any of the alcohols, not even when the alcohol itself, in the case of phenylacetic acid esterification with methanol, was used in large excess, namely as reaction medium. This lack of reactivity cannot be ascribed to PGA inhibition by alcohols. It was verified that L-Tyr-OEt was completely converted to the corresponding amide when added to the reaction system where PGA had been previously incubated in the presence of alcohols for 24 h. Thus, the examined alcohols seem to be very poor nucleophiles for PGA-catalysed esterification and transesterification in organic solvent.

Even though it has been claimed in literature that PGA is able to perform also esterification and transesterification reactions,[4,14] to the best of our knowledge no experimental data of transesterification using PGA have been reported until now, whereas the PGA-catalysed hydrolysis of phenylacetic esters of many alcohols, such as 1-phenyl-1-propanol, 1-phenyl-1-ethanol,[1] in aqueous media has been largely described.

The results obtained in the acylation of amines and amino acids show that the synthetic potential of PGA in organic solvent can be exploited avoiding the problems related to the use of aqueous media. Furthermore, for the first time PGA was shown to accept a wide variety of amines also in pure hydrophobic organic solvent. The broad versatility of PGA towards different amino compounds could lead to new perspectives, especially in the field of peptide synthesis. We are currently verifying the applicability of this technique to a totally enzymatic approach for the kinetically controlled synthesis of peptides of pharmaceutical interest.

Acknowledgements. – We are grateful to Boehringer Mannheim for the generous gift of PGA-450. Thanks are due to C.N.R. and M.U.R.S.T. (Roma) for financial support to P. L. and to Università degli Studi di Trieste for financial support to L. G.

REFERENCES

1. E. Baldaro, P. D'Arrigo, G. Pedrocchi-Fantoni, C. M. Rosell, S. Servi, A. Tagliani, and M. Terreni, *Tetrahedron: Asymmetry* **4** (1993) 1031–1034.
2. C. Fuganti, C. M. Rosell, S. Servi, A. Tagliani, and M. Terreni, *Tetrahedron: Asymmetry* **3** (1992) 383–386.
3. D. Rossi, A. Calcagni, and A. Romeo, *J. Org. Chem.* **44** (1979) 2222–2225.
4. T. Pohl and H. Waldmann, *Tetrahedron Lett.* **36** (1995) 2963–2966.
5. A. Guy, A. Dumant, and P. Sziraky, *Bioorg. Med. Chem. Lett.* **3** (1993) 1041–1044.
6. H. Waldmann and D. Sebastian, *Chem. Rev.* **94** (1994) 911–937.

7. C. Ebert, L. Gardossi, and P. Linda, *Tetrahedron Lett.* **37** (1996) 9377–9380.
8. C. Ebert, L. Gardossi, and P. Linda, *J. Mol. Catal. B: Enzymatic* **5** (1998) 241–244.
9. L. De Martin, C. Ebert, G. Garau, L. Gardossi, and P. Linda, *J. Mol. Catal. B: Enzymatic* **6** (1999) 437–445.
10. A. Basso, L. De Martin, C. Ebert, L. Gardossi, P. Linda, and V. Zlatev, *J. Mol. Catal. B: Enzymatic* **11** (2000) 851–855.
11. A. Basso, L. De Martin, C. Ebert, L. Gardossi, A. Tomat, M. Casarci, and O. Li Rosi, *Tetrahedron Lett.* **41** (2000) 8627–8630.
12. A. Basso, P. Braiuca, L. De Martin, C. Ebert, L. Gardossi, and P. Linda, *Tetrahedron: Asymmetry* **11** (2000) 1789–1796.
13. W. Dürckheimer and M. Schorr, *Liebigs Ann. Chem.* **702** (1967) 163–168.
14. M. Cole, *Biochem. J.* **115** (1969) 747–755.

SAŽETAK

Kvantitativna acilacija aminospojeva katalizom Penicilin-G-acilaze u organskim otapalima uz kontroliranu aktivnost vode

Alessandra Basso, Stefania Biffi, Luigi De Martin, Lucia Gardossi i Paolo Linda

Kovalentno imobilizirana Penicilin-G-acilaza (PGA-450) reagira u toluenu, pri kontroliranoj aktivnosti vode (a_w), s raznim aminospojevima (nukleofilima) u kinetički kontroliranim acilacijama. N-acilirani derivati mogu se koristiti u drugim reakcijama, jer nije potrebno pročišćavanje. To otvara nove mogućnosti u primjeni PGA kod selektivne zaštite aminoskupine potrebne za sintezu peptida.