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Journal of Biotechnology 84 (2000) 63–66

JOURNAL OF Biotechnology

www.elsevier.com/locate/jbiotec

Short communication

Hydrolysis of lysergamide to lysergic acid by *Rhodococcus equi* A4

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Received 13 January 2000; received in revised form 13 June 2000; accepted 12 July 2000

Abstract

From a mixture of lysergamide and its epimer isolysergamide, *Rhodococcus equi* A4 containing amidase preferentially hydrolyzed lysergamide into lysergic acid.

Keywords: *Rhodococcus equi*; Amidase; Lysergamide; Isolysergamide; Lysergic acid; Isolysergic acid

1. Introduction

Selective (bio)transformations of ergot alkaloids under mild conditions attracted many researchers (for review see Křen, 1999) because of the molecule intricacy and sensitivity to harsh reaction conditions. Despite of high industrial and commercial importance, enzymatic hydrolysis of lysergamide ((5*R*, 8*R*)-9,10-didehydro-6-methylergoline-8β-carboxamide, 1a, Fig. 1) by a bacterial amidase was not reported to our knowledge. Hydrolysis of lysergamide by the fungus *Claviceps purpurea* was described in a patent (Amici et al., 1964) but the method is hardly reproducible as this strain is no more available and other *Claviceps* strains have not this activity. Moreover, the productivity of the conversion catalyzed by the fungal cells was extremely low (less than 2 mmol l^{-1} within 7 days).

Here, we describe the hydrolysis of lysergamide by *Rhodococcus equi* A4. This enzymatic reaction proceeds with a high C-8 stereoselectivity (Fig. 1) and provides a convenient route to lysergic acid ((5*R*, 8*R*)-9,10-didehydro-6-methylergoline-8β-carboxylic acid, 1b, Fig. 1) as a crucial intermediate for the semisynthetic alkaloid production (Cvak, 1999). Most lysergic acid is produced by alkaline hydrolysis of peptide ergot alkaloids (e.g. ergotamine) from parasitic production by *C. pur-*

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0168-1656/00/$ - see front matter
PII: S0168-1656(00)00332-1

purea (Németh, 1999). The bottleneck of this procedure is the lysergamide hydrolysis that needs drastic conditions leading to material losses and lysergic acid–isolysergic acid isomerization (balance mixture of both isomers is obtained). Isomerization at C-5 was also observed to some extent (1–3%). On contrary, partial hydrolysis of the peptide alkaloids into a mixture of lysergamide/isolysergamide proceeds (2a Fig. 1) rapidly under milder conditions and gives very good yields. Such a mixture can be also easily obtained by hydrolysis of the mixture of lysergic acid α-hydroxyethylamides and ergometrine produced by submerged cultivation of *C. paspali* (Malinka, 1999).

2. Materials and methods

2.1. Chemicals

Lysergamide, isolysergamide, lysergic acid, isolysergic acid and ergotamine were gifts of Galena Pharmaceuticals Ltd. (Czech Republic). All other chemicals were of analytical grade purity and supplied from standard commercial sources.

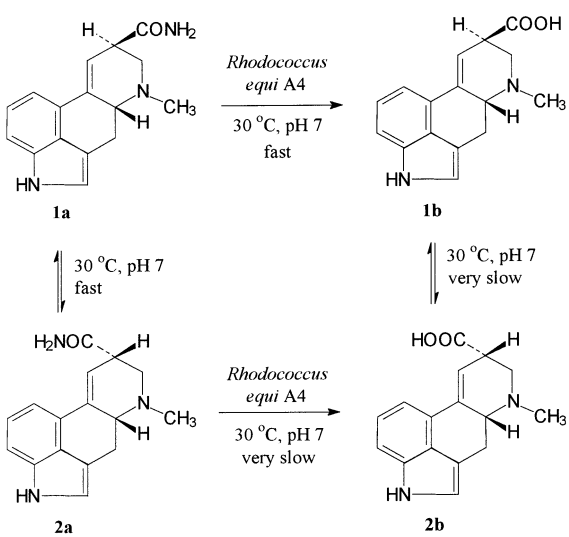

Fig. 1. Structural formulas of lysergamide (1a), lysergic acid (1b), isolysergamide (2a), and isolysergic acid (2b).

2.2. Ergotamine hydrolysis

Ergotamine (30 g, 0.0515 mol) was hydrolyzed using 2 h reflux with KOH (4 g) in 120 ml of water:ethanol (1:3). The resulting mixture contained lysergamide (39.1 mol.%), isolysergamide (35.9 mol.%), lysergic acid (8.9 mol.%) and isolysergic acid (3.0 mol.%).

2.3. Microorganism and biotransformation

The soil isolate *R. equi* A4 (Martínková et al., 1995) which is deposited in the culture collection of the Institute of Microbiology, Prague, was cultured in a basal medium (10 g glucose, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7\ H_2O$, 0.01 g $FeSO_4 \cdot 7\ H_2O$, in 1 l of tap water, pH 7.5 (Watanabe et al., 1987) modified by addition of 2 g yeast extract l^{-1}) at 30°C under shaking (220 rpm, amplitude 23 mm, 100 ml medium/500-l Erlenmeyer flask) for 1 day. The cells were harvested by centrifugation (6000 rpm, 30 min, 4°C) and washed with Na_2HPO_4/KH_2PO_4 buffer (54 mM, pH 7.0). Cell extract was prepared by ultrasonication (Cole-Parmer, USA, 4710 sonifier) of the cells in Tris/HCl buffer (50 mM, pH 7.5) containing dithiothreitol and ethylene diamine tetraacetate (EDTA, 1 mM each). Lysergamide/isolysergamide (1 mM, from 25 mM stock solutions in methanol with 0.025 ml of 1 M HCl per ml, or the same concentration from ergotamine hydrolysate) were shaken with the whole cells (4.6 mg dry cell weight per ml) or the cell extract (1.5 mg protein per ml) at 30°C (850 rpm, Thermomixer Compact, Eppendorf). Aliquots of 0.2 ml were withdrawn and mixed with 0.2 ml methanol and 0.02 ml 1 M HCl. The cells or the precipitated protein were removed by centrifugation. The reactions were monitored by high performance liquid chromatography (HPLC) as described below or by thin layer chromatography (TLC) on silica gel plates (Merck) developed with chloroform:methanol:ethanol:water, 65:26:5:4.

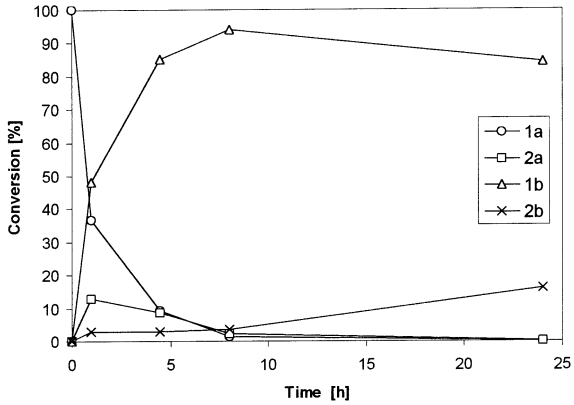

Fig. 2. Biotransformation of lysergamide (1a, 1 mmol l^{-1}) by *R. equi* A4 at pH 7 and 30°C (see Section 2 for experimental details and Fig. 1 for substrates and products).

2.4. Analytical HPLC

The HPLC system (Millenium Chromatography Manager 2.0; solvent delivery system 600, photo diode array detector 996, Waters Associates, Milford, MA, USA) was equipped with a RP-C18 column (250 × 4 mm, 7 μm; Tessek Prague, Czech Republic). Compounds 1a, 1b, 2a and 2b eluted at 2.8, 3.2, 6.1 and 8.8 min, respectively, with mobile phase containing methanol:potassium phosphate buffer (5 mM, pH 6.9), 40:60, at 1.0 ml min^{-1} and 35°C. Integration was carried out at 310 nm.

3. Results and discussion

Whole cells of *R. equi* A4 hydrolyzed lysergamide 1a (1 mM) into lysergic acid 1b (Figs. 1 and 2). 8-Epimerization of lysergamide into isolysergamide 2a (and vice versa) proceeding in the reaction mixture was not catalyzed enzymatically as it occurred under the same conditions in the absence of the biocatalyst. Isolysergamide was hydrolyzed by the microorganism only to a limited extent. The 8-epimerization of lysergic acid into isolysergic acid 2b proceeded at a significantly lower rate in comparison to lysergamide/isolysergamide epimerization. As a result, the diastereomeric excess (d.e.) of lysergic acid (5*R*, 8*R*) over isolysergic acid (5*R*, 8*S*) was 93% at 94% conversion. Stability of the configuration at C-8 was not absolute, as the d.e. of lysergic acid decreased to 80% after 24 h reaction. No other products were detected by HPLC.

When using hydrolysate of ergotamine as substrate, the reaction mixture contained 0.83 mol.% lysergic acid, 0.11 mol.% isolysergic acid, 0.02 mol.% lysergamide and 0.03 mol.% isolysergamide after 8 h incubation. The presence of isolysergic acid in the original hydrolysate caused some decrease of the diastereomeric purity of lysergic acid (d.e. 76.6%) compared with the biotransformation of pure lysergamide.

Stereoselectivity of lysergamide hydrolysis was given by the properties of the amidase and not by the cell-transport phenomena, as it was also observed with the cell extract. After 4 h reaction, d.e. of lysergic acid was $>95\%$ at 31% conversion.

The amidase activity in *R. equi* A4 could be either produced constitutively or induced by some amide-like compounds in the medium. When the microorganism was grown on acetonitrile as the sole source of nitrogen (Martínková et al., 1995), lysergamide-hydrolyzing activity was also present in the cells together with amidase activity for low-molecular-weight amides (Martínková et al., 1998). Similarly to other 'enantioselective amidases' (for a brief review see Hirrlinger et al., 1996), the amidase of *R. equi* A4 was highly enantioselective for some 2-arylpropionamides (Martínková et al., 1996). Further studies are needed to clarify if the lysergamide amidase belongs to 'enantioselective amidases' or if two amidases with different substrate specificity are produced by *R. equi* A4.

C. purpurea (Amici et al., 1964) produced lysergic acid from lysergamide as well as from isolysergamide under conditions supporting lysergamide isomerization at C-8. This suggests that the enzyme of this fungus selectively hydrolyzed the amide with 8*R*-configuration as the amidase from *R. equi* A4. Evolutionary relation between these two enzymes is possible because amidases from bacteria ('enantioselective amidases', Mayaux et al., 1991) and the fungi *Saccharomyces cerevisiae* (Chang and Abelson, 1990) and *Aspergillus nidulans* (Corrick et al., 1987) were partially homologous.

Stereoselective bacterial amidases were found to be useful for the preparation of optically active hydroxy acid amino acids and 2-aryl(oxy)propionic acids (for review see Sugai et al., 1997). In the present work, application of an amidase from *R. equi* for another pharmaceutical production, the preparation of lysergic acid of a high diastereomeric purity, was proposed.

Acknowledgements

Financial support of this work from the Grant Agency of the Academy of Sciences of the Czech Republic (Projects A4020802/1998 and A4020901/1999) is gratefully acknowledged. We also wish to thank Magdalena Balvínová for her excellent technical assistance.

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