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BIOCHEMISTRY, BIOPHYSICS, AND MOLECULAR BIOLOGY

Stereoselective Biotransformation of Phenylglycine Nitrile by Heterogeneous Biocatalyst Based on Immobilized Bacterial Cells and Enzyme Preparation

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Abstract—We studied the effect of a heterogeneous environment on the stereoselectivity of transformation of racemic phenylglycine nitrile. Immobilized biocatalysts were prepared by adhesion of *Pseudomonas fluorescens* C2 cells on carbon-containing supports and covalent crosslinking of nitrile hydratase and amidase of *Rhodococcus rhodochrous* 4–1 to activated chitosan as well as by the method of cross-linked aggregates. At a reaction duration of 20 h, the ratio of phenylglycine stereoisomers changes depending on the presence of support in medium. The highest optical purity of the product (enantiomeric excess of L-phenylglycine solution, 98%) is achieved when enzyme aggregates of nitrile hydratase and amidase cross-linked with 0.1% glutaraldehyde are used as a biocatalyst.

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L- and D-enantiomers of α-amino acids, which are used as chiral building blocks for asymmetric synthesis of a number of compounds (in particular, certain antibiotics, peptides, and peptidomimetics), can be synthesized and separated with the use of enzymes. D-phenylglycine is a side chain of cefalexin and ampicillin and an intermediate of viridenomycin, a new antitumor lactam antibiotic with a strong antifungal and antibacterial activity. L-Phenylglycine, although it has no direct commercial value, is the building block of neurokinin NK1 receptor antagonist and a component of thymidylate synthase inhibitors [1]. There are data [2] that L-phenylglycine can be used in synthesis as a side chain of some antibiotics and paclitaxel, the active component of the pharmaceutical agent taxol.

Phenylglycine enantiomers can be prepared enzymatically from racemic phenylglycine nitrile [3, 4]. It is known that enzymatic hydrolysis of nitriles is performed either in one state (by nitrilase to the corresponding carboxylic acid) or in two stages (by nitrile hydratase to amides followed by hydrolysis by amidase to the carboxylic acid) [5]. The main source of nitrile-hydrolyzing enzymes are microbial cells [6]. It is known that certain nitrilases and amidases exhibit stereoselectivity [7].

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Heterogeneous biocatalysis performed by immobilized enzymes or microbial cells has several advantages over homogeneous biocatalysis. The possibility of a continuous synthesis appears, the enzymatic activity is stabilized, and the biocatalyst can be easily separated from the reaction medium and reused. When developing a heterogeneous process of biocatalytic production of an enantiomerically pure substance, the question of the effect of immobilization of biocatalysts on the stereoselectivity of reaction arises. Data on the stereoselective hydrolysis of phenylglycine nitrile by immobilized biocatalysts are scanty. For example, the conversion of phenylglycine nitrile to D-phenylglycine by whole *Pseudomonas aeruginosa* 10145 cells entrapped in calcium alginate gel beads was performed at an enantiomeric excess of the D-isomer (98%) and a 20% yield [8]. An asymmetric synthesis of D-N-formylphenylglycine by cross-linked enzyme aggregates of nitrilase isolated from *Sphingomonas wittichii* RW1 was performed with a 95% yield and an enantiomeric excess of 97% [9]. However, any information about the transformation of this compound by adherent bacterial cells or by enzymes immobilized on a support was not found in the available literature.

The aim of this study was to investigate the stereoselective hydrolysis of L-, D-phenylglycine nitrile by *Pseudomonas fluorescens* C2 cells adhered to carbon-containing supports and by nitrile hydratase and amidase isolated from *Rhodococcus rhodochrous* 4-1 and immobilized in different ways.

P. fluorescens C2 and *R. rhodochrous* 4-1 strains, isolated and maintained in the Laboratory of Molecular Microbiology and Biotechnology, Institute of

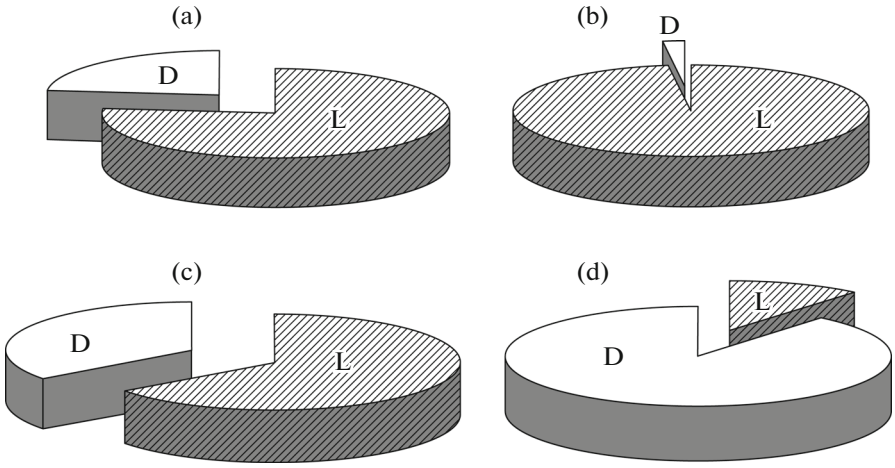

Fig. 1. The ratio of D- and L-isomers at (a, c) 1-h and (b, d) 20-h hydrolysis of phenylglycine nitrile by *P. fluorescens* C2 cells adhered to (a, b) Sibunit and (c, d) Sapropel.

Ecology and Genetics of Microorganisms, Ural Branch, Russian Academy of Sciences, were grown at 30°C on a synthetic mineral medium containing glucose as a carbon source and acetonitrile as a nitrogen source. *P. fluorescens* C2 cells were separated by centrifugation at 10 500 *g*, washed once with 0.01 M potassium phosphate buffer (pH 7.2 ± 0.2), and recentrifuged. The pellet was resuspended in the potassium phosphate buffer. The cells were adsorbed on carbon-containing substrates Sibunit (Boreskov Institute of Catalysis, Siberian Branch, Russian Academy of Sciences) and Sapropel (Institute of Hydrocarbon Processing Problems, Siberian Branch, Russian Academy of Sciences) at 22°C for 2 h with shaking on a shaker at 120 rpm. The substrates were washed once from the unbound cells with potassium phosphate buffer, and the amount of adherent cells on the sorbent was determined as described previously [10].

The enzyme preparation containing nitrile hydratase and amidase from *R. rhodochrous* 4-1 was obtained and immobilized on chitosan beads activated with 0.1% benzoquinone as described in our previous work [11]. The protein content in the sample was determined by the method of Bradford [12]. Amidase activity was assessed spectrophotometrically at 230 nm by the increase in the optical density of the solution for 1 min after the addition of substrate acrylamide.

The cross-linked enzyme aggregates (CLEAs) of the sample containing nitrile hydratase and amidase were prepared by simultaneous salting out a cell-free extract with ammonium sulfate to a concentration of 60% and a covalent cross-linking of proteins with 0.1% glutaraldehyde or benzoquinone.

The hydrolysis of 10 mM phenylglycine nitrile was performed at 30°C in 0.01 M potassium phosphate buffer (pH 7.2 ± 0.2). Samples were taken 1 and 20 h after the start of hydrolysis, after which the reaction was stopped by fast freezing.

The concentration of phenylglycine nitrile and D-, L-phenylglycine was determined by HPLC on a LC-10 chromatograph (Shimadzu, Japan) in a CROWNPAK® CR (-)column (Daicel Corporation, Japan). Elution was performed with a $HClO_4$ solution (pH 2.0) and 10% methanol. The flow rate was 0.300 mL/min at 30°C. Detection was performed at 210 nm.

We have found that, after 1-h conversion of phenylglycine nitrile by the cells immobilized on Sibunit, the enantiomeric excess (*ee*) of the L-isomer was 54%, whereas at a reaction time of 20 h *ee* increased to 96% (Fig. 1). When using *Pseudomonas* cells adhered to Sapropel, *ee* of the L-isomer after 1-h conversion was 30%. However, when the reaction time increased to 20 h, a shift of the reaction towards the D-isomer yield was observed (by this time, *ee* reached 78%). The reaction that was performed with using suspended cells served as a control. In this case, the stereoselectivity of the reaction only slightly changed over time, and *ee* of L-phenylglycine was 50–68%. The nitrilase activity of suspended cells and cells adhered to Sibunit and Sapropel at conversion of this substrate was 1.57 ± 0.42, 0.86 ± 0.55, and 3.14 ± 1.82 mmol/(g/h), respectively.

Next, we performed a biocatalytic transformation of D,L-phenylglycine nitrile by nonselective nitrile hydratase and L-specific amidase, which were coimmobilized by covalent crosslinking and CLEAs method (table). We have found that, in the case of covalent linking with the activated chitosan, the stereoselectivity of amidase increased. The highest L-stereoselectivity was exhibited by the enzyme aggregates that were obtained by crosslinking with glutaraldehyde (0.1% solution). When amidase molecules were crosslinked with benzoquinone, the stereoselectivity of the reaction was lower than that of the free enzyme.

Thus, we have shown that the immobilization method and supports affect the stereoselectivity of

Catalytic and stereoselective properties of the enzyme preparation containing nitrile hydratase and amidase of *R. rhodochrous* 4-1, immobilized by different methods

Amidase		Activity against L-isomer, mmol/g/h	Ratio of phenylglycine enantiomers L : D, %	*ee*, %
In solution		48.0 ± 0.9	87 : 13	74
Covalently attached to chitosan		9.0 ± 5.4	92 : 8	84
CLEAs	glutaraldehyde	24.0 ± 0.7	99 : 1	98
	benzoquinone	42.0 ± 27.0	74 : 26	48

Data are represented as $M \pm m$, $n = 3$.

enzymatic hydrolysis of phenylglycine nitrile. A heterogeneous medium may affect the optical purity of the product probably due to changes in the pH of the microenvironment of the immobilized biocatalyst.

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