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Mild hydrolysis of nitriles by the immobilized nitrilase from *Aspergillus niger* K10

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Abstract

The cell free extract from the nitrile-hydrolyzing strain *Aspergillus niger* K10 (0.25 mg of protein) was adsorped onto a 1 mL HiTrap Butyl Sepharose column. The benzonitrile-hydrolyzing activity of the immobilized enzyme (about 1.6 U/mg of protein) was stable at pH 8 and 35 °C within the examined period (4 h). The enzyme load on the above column was increased 18 times in order to achieve high nitrile conversion. This enzyme preparation was used for the conversion of 3-cyanopyridine and 4-cyanopyridine under the above conditions. The initial substrate conversion was nearly quantitative. The activity was fairly stable; the conversion of 3-cyanopyridine decreased to 70% after 15 h, while the conversion of 4-cyanopyridine was 60% of the initial value after 39 h. The former substrate was converted into nicotinic acid and nicotinamide (molar ratio approximately 16:1) and the latter one into isonicotinic acid and isonicotinamide (molar ratio approximately 3:1).
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1. Introduction

Recently, there is considerable interest in the immobilization of nitrile-converting biocatalysts. Most of the reported methods consisted in the entrapment of whole cells in various hydrogels such as alginate, pectate, carrageenan, polyvinyl alcohol and polyacrylamide (see Ref. [1] for a review). The whole cells were also immobilized by adsorption onto Dowex 1 [2], by entrapment into porous dimethyl silicone rings [3] and sol–gel silica hybrids [4] or by chemical binding on polysulphone membranes [4].

On the other hand, the immobilized nitrile-transforming biocatalysts were rarely based on cell extracts or (partially) purified enzymes. The immobilization of nitrile hydratases seems to be difficult due to the instability of these enzymes [1]. The immobilization of nitrilases appears to be more promising. Recently, two different nitrilases (a cell free extract from *Pseudomonas fluorescens* and Nitrilase 1004 from Biocatalytics) were immobilized using precipitation with dimethoxyethane and subsequent cross-linking using a macromolecular cross-linker, dextran polyaldehyde [5].

Immobilization of enzymes by ionic and hydrophobic adsorption or by covalent binding onto solid carriers find a broad use in biocatalysis. For example, all these methods were examined for the immobilization of epoxide hydrolases. Ionic adsorption on DEAE cellulose increased the specific activity and the stability of the immobilized enzyme, while other methods were unsuitable [6]. Hydrophobic adsorption on Octadecyl-Sepabeads was useful in the case of lipases, which were adsorped in the form of the “open structure”; the enzymes were highly active and preserved 100% of their activity after 200 h incubation [7].

Herein, we report the immobilization of a nitrilase from *Aspergillus niger* K10 by hydrophobic adsorption. The fungal enzyme has been selected because of its activity towards heteroaromatic nitriles of industrial interest (cyanopyridines) [8] as well as its good thermostability [9]. The extracts from cells induced by 2-cyanopyridine and valeronitrile exhibited a high specific nitrilase activity [10] and, therefore, represented a suitable starting material for the immobilization.

2. Experimental

2.1. Nitrilase preparation and immobilization

A. niger K10 [8] is deposited in the Culture Collection of Fungi (Charles University Prague, Czech Republic). The microorganism was cultivated in shaken flasks with a modified

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Czapek-Dox medium [8] in which sodium nitrate was replaced by 2-cyanopyridine (20 mM) and valeronitrile (7.5 mM) as nitrilase inducers [10]. The mycelium was ground to powder in a mortar, extracted with Tris–HCl buffer (50 mM, pH 8) and centrifuged at 13,000 × *g* and 4 °C for 30 min. The cell extract exhibiting nitrilase activity (0.25 mg of protein; approximately 0.4 U with benzonitrile at pH 8 and 35 °C) in Tris–HCl buffer (50 mM, pH 8) containing 0.8 M ammonium sulfate was applied onto a 1 mL HiTrap Butyl FF column (Amersham Biosciences) at a flow rate of 0.25 mL min^{-1}. Tris–HCl buffer (50 mM, pH 8) with 0.8 M ammonium sulfate was passed through the column at a flow rate of 0.5 mL min^{-1} for 20 min and the eluate was checked for nitrilase activity.

2.2. Assay of the nitrilase activity

The initial activity of the immobilized enzyme was determined at 35 °C. Tris–HCl buffer (50 mM, pH 8) with 0.8 M ammonium sulfate and 5 mM of benzonitrile was passed through the column with the immobilized nitrilase at a flow rate of 0.5 mL min^{-1}. After the dead volume had been eluted, 1 mL fractions were collected for 10 min. The concentration of benzonitrile, benzoic acid and benzamide in the eluate was determined by HPLC as described previously [8]. The total product was the sum of benzoic acid and benzamide. The specific activity was calculated according to Eq. (1).

$$\text{Specific activity (U/mg protein)} = \frac{[\text{product concentration (mM)} \times \text{flow rate (mL min}^{-1})]}{\text{immobilized protein (mg)}} \quad (1)$$

The activity of the free enzyme was determined batchwise under the same conditions at shaking. The substrates were benzonitrile, 3-cyanopyridine and 4-cyanopyridine.

2.3. Assay of the storage stability of the nitrilase

The immobilized nitrilase was stored at 4 °C. At intervals, the enzyme preparation was checked for its benzonitrile-hydrolyzing activity as described above.

2.4. Assay of the operational stability of the nitrilase

The conversion of benzonitrile by the immobilized nitrilase was performed at 20, 35 and 45 °C for 30 min. The operational stability at 35 °C was also examined within 4 h. Other conditions were as described for the nitrilase activity assay.

2.5. Hydrolysis of 3-cyanopyridine and 4-cyanopyridine by the immobilized nitrilase

The nitrilase was immobilized as described above but the enzyme load on the column was increased to 7.2 U (assayed with benzonitrile). The hydrolysis of 3-cyanopyridine and 4-cyanopyridine (10 mM each) was carried out at pH 8 and 35 °C for 15 and 39 h, respectively. The flow rate was 0.25 mL min^{-1}. Fractions (7.5 mL) were collected and analyzed by HPLC as described previously [8].

2.6. Protein assay

Protein was determined according to Bradford [11] using bovine serum albumin as the standard.

3. Results and discussion

3.1. Adsorption of nitrilase onto Butyl Sepharose

Nitrilases were reported to be prone to deactivation under the conditions of some immobilization procedures. No nitrilase activity was recovered in cross-linked enzyme aggregates prepared with glutaraldehyde. It was postulated that the nitrilase sensitivity was due to a reactive lysine residue in the active site. Using an alternative cross-linker, dextran polyaldehyde, that did not penetrate the enzyme active center, 50–60% of the activity was recovered [5].

In this work, hydrophobic adsorption was tested as a milder immobilization technique. The fungal nitrilases are hydrophobic proteins as obvious from the late elution of the enzyme from Phenyl Sepharose columns. This phenomenon was observed for the nitrilases from *Fusarium oxysporum* f. sp. *melonis* [12] and *A. niger* (unpublished data). Therefore an efficient binding of the protein on the hydrophobic carrier could be expected. Previously, such expectation was not proved by experimental data obtained for another lipophilic protein, an epoxide hydrolase [6]. In this case, the hydrophobic carriers had a low binding capacity or inactivated the enzyme. Contrary, hydrophobic adsorption proved to be viable for the immobilization of the nitrilase from *A. niger*. The nitrilase applied in the form of a cell extract remained active after binding onto Butyl Sepharose. The ratio of the activity recovered after immobilization was 90%. About 20% of the applied protein was not adsorped on the column, but this protein did not contain any active nitrilase. Partial purification was thus achieved. The immobilization procedure can be further improved. First, the enzyme load can be increased. The method can be also optimized by decreasing the concentration of ammonium sulfate in the elution buffer. In this way, more contaminating protein may be removed, so that the binding capacity of the column becomes available for the enzyme.

Examination of the suitability of other carriers for the nitrilase immobilization is ongoing. Preliminary data suggest that hydrophobic adsorption onto Phenyl Sepharose and Octyl Sepharose gives comparable results as immobilization on Butyl Sepharose. Ionic binding of the enzyme onto Q Sepharose proved to be feasible as well. The hydrophobic and ionic immobilization will be also applied to other nitrilases such as the enzymes of the genus *Fusarium* which are similar to the enzyme of *A. niger* K10 in terms of their inducibility and substrate specificity [9].

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