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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).

Keywords: Nitrilase; *Aspergillus niger*; Benzonitrile; Cyanopyridines; Immobilized enzyme

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].

3.2. Storage and operational stability of the immobilized nitrilase

The storage stability of the immobilized nitrilase was satisfactory, 80 and 70% of the initial activity being retained after 3 and 7 days at 4 °C. The immobilized nitrilase was operated at pH 8 – the activity optimum of the enzyme – at different temperatures for 30 min. At 20 and 35 °C, the activity was stable within this period. However, the lower temperature was not beneficial because the activity of the nitrilase was relatively low (about one-third of that at 35 °C). At 45 °C (activity optimum), the initial activity increased by approximately one-third compared to that at 35 °C but the activity drop within 30 min was about 15%. Therefore, the temperature of 35 °C was applied to the prolonged experiment (see Fig. 1) in which the nitrilase activity was monitored for 4 h. The concentration of benzonitrile eluted in the beginning of the run was lower than predicted from the substrate/product balance. This was probably caused by retardation of benzonitrile which was bound onto Butyl Sepharose through hydrophobic interaction.

The major product of the enzyme reaction was benzoic acid but minor amounts of benzamide were also detected. The concentrations of benzoic acid and benzamide in the eluate were stable within the examined period. Thus the ratio of benzoic acid and benzamide was also constant (about 11:1, i.e. similar as for the free enzyme). Production of both carboxylic acids and amides by nitrilases has been reported [12–14]. This ability of the nitrilase to produce amides as by-products is especially pronounced for the enzyme from *A. niger* K10 [8]. Though the production of amides from nitriles might support the assumption that the fungus produces a nitrile hydratase [4], other results contradict this hypothesis. These data involve the constant acid: amide ratio during the operation of the immobilized enzyme (see also below) and under different pH and temperatures (unpublished data).

3.3. Conversion of heterocyclic nitriles by the immobilized nitrilase

The immobilized nitrilase was used for the conversion of substrates with industrial impact, 3-cyanopyridine and 4-cyanopyridine (10 mM each). Previously, it was reported that 3-cyanopyridine was transformed by the whole cells of the fungus at a lower rate than benzonitrile [8]. This was confirmed by an experiment using a cell free extract whose specific activity for 3-cyanopyridine was about half of that for benzonitrile. The enzyme load on the column was increased approximately 18 times to achieve total conversion and to outweigh the difference between the specific activities towards 3-cyanopyridine and benzonitrile. At the same time, the elution rate was decreased.

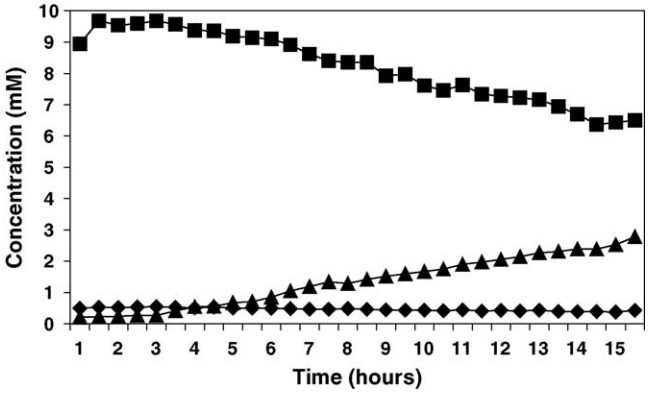

Fig. 2. Conversion of 10 mM 3-cyanopyridine (▲) into nicotinic acid (■) and nicotinamide (♦) by the immobilized nitrilase (7.2 U per 1 mL of Butyl Sepharose). The column was operated at 35 °C at a flow rate of 0.25 mL min^{-1}. See Section 2 for details.

Nicotinic acid was the major product of the reaction, while nicotinamide formed about 6% of the total product. Up to 3 h of the column operation, a nearly quantitative conversion of 3-cyanopyridine was achieved. At later stages the unreacted substrate began to elute but the conversion rate did not decrease below 80% up to 9.5 h and it was still about 70% after 15 h (see Fig. 2).

4-Cyanopyridine was a superior substrate of the nitrilase, being transformed by about 250% more rapidly than benzonitrile. The conversion of 4-cyanopyridine was carried out under the same conditions as that of 3-cyanopyridine but the operation time was prolonged. Due to the high activity of the nitrilase for 4-cyanopyridine, no unreacted substrate was eluted up to

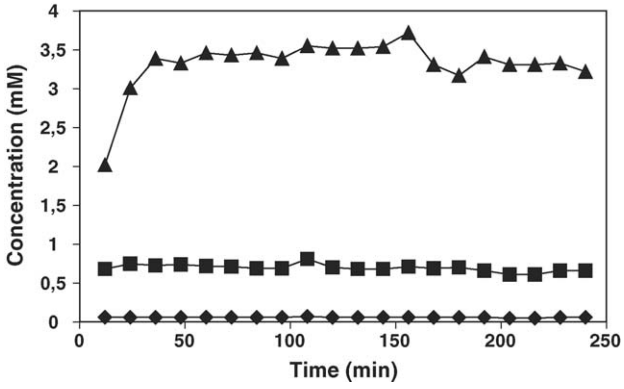

Fig. 1. Conversion of 5 mM benzonitrile (▲) into benzoic acid (■) and benzamide (♦) by the immobilized nitrilase (0.4 U per 1 mL of Butyl Sepharose). The column was operated at 35 °C at a flow rate of 0.5 mL min^{-1}. See Section 2 for details.

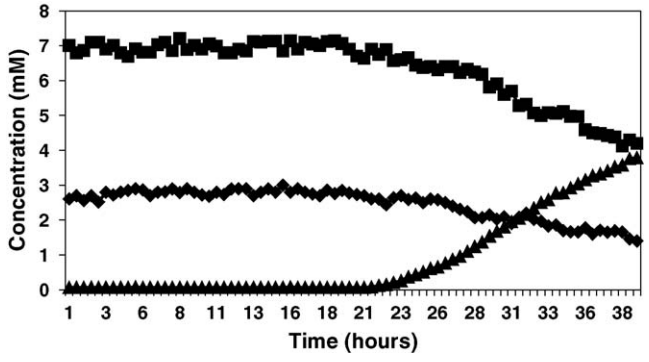

Fig. 3. Conversion of 10 mM 4-cyanopyridine (▲) into isonicotinic acid (■) and isonicotinamide (♦) by the immobilized nitrilase (7.2 U per 1 mL of Butyl Sepharose). The column was operated at 35 °C at a flow rate of 0.25 mL min^{-1}. See Section 2 for details.

22 h. However, at later stages the conversion rate decreased (to approximately 60% after 39 h, see Fig. 3). The products of the reaction were isonicotinic acid and isonicotinamide. The amide formed a larger ratio (about one-fourth) of the total product than in the case 3-cyanopyridine.

Small deviations from the sum of substrates and products (theoretically 10 mM) may be explained by the partial consumption of the products by enzymes present in the cell free extract. This phenomenon was reported for the whole cells of *A. niger* K10 which partially metabolized the products of nitrile hydrolysis, benzoic and nicotinic acid [8].

4. Conclusions

A mild immobilization method, adsorption on a hydrophobic carrier (Butyl Sepharose) was applied to a fungal nitrilase. The high specific activity of the enzyme, which was induced by 2-cyanopyridine and valeronitrile, was recovered in the immobilized preparation. Due to its good operational stability, the immobilized nitrilase appears to be suitable for the continuous hydrolysis of (hetero)aromatic nitriles.

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