Journal of Molecular Catalysis B: Enzymatic 63 (2010) 109–115

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Immobilization of *Geobacillus pallidus* RAPc8 nitrile hydratase (NHase) reduces substrate inhibition and enhances thermostability

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ARTICLE INFO

Article history:
Received 26 September 2009
Received in revised form 9 December 2009
Accepted 10 December 2009
Available online 21 December 2009

Keywords:
Nitrile hydratase
Geobacillus pallidus RAPc8
Immobilization
Eupergit
Thermostable enzyme
Thermophilic

ABSTRACT

The nitrile hydratase (NHase) from the thermophilic strain *Geobacillus pallidus* RAPc8 was investigated for its potential application in the biocatalytic production of amides from nitriles. The recombinant NHase was immobilized to a range of insoluble matrices using various cross-linking agents. The immobilized preparation using Eupergit® C with 1-ethyl-3-(dimethylamino-propyl) carbodiimide (EDAC) cross-linking exhibited the highest immobilization efficiency (93%). The pH range and optimal temperature for activity were unchanged by immobilization but the thermostability of the Eupergit® C-NHase was improved compared with the soluble enzyme; at 60 °C the half-life of the immobilized NHase was 330 min as compared with 54.5 min for the soluble enzyme. Kinetic parameters V_{max} (4.5 μmol mL^{-1} min^{-1}), K_m (17.3 mM) and k_{cat} (3543.3 min^{-1}) were obtained for the immobilized NHase at 50 °C, as compared with 48.8 μmol mL^{-1} min^{-1}, 10.2 mM and 37777.1 min^{-1} respectively for the soluble enzyme. The operational stability was improved significantly by immobilization, with 85.7% of initial activity maintained after reuse for eight cycles. Most notably, the Eupergit® C-immobilized NHase showed substantially lower substrate inhibition (K_i = 194.7 mM) than the soluble enzyme (K_i = 101.0 mM). In the presence of various co-organic solvents, Eupergit® C-EDAC NHase showed statistically higher retention of activity than the non-immobilized control.

1. Introduction

The nitrile hydratases (NHases: EC3.5.5.1) catalyze the hydration of nitriles to their corresponding amides [1]. The versatile nature of these enzymes, associated with their very broad substrate specificity, has resulted in a number of commercial applications, including manufacture of the commodity chemicals acrylamide [2], nicotinamide [3] and 5-cyanovaleramide [3], and in treatment of organocyanide industrial effluents [4]. However, the instability of many NHases currently limits successful application at commercial scale, particularly in the production of acrylamide and nicotinamide [5–7]. Consequently, whole-cells are often employed, in processes which are operated at relatively low temperatures [3], resulting in reduced reactivity due to diffusion limitation and low specific activity [8]. Further, many NHases are inhibited at high concentrations of substrate and product [9]. Attempts to overcome substrate and product inhibition have been made by keeping these concentrations low [10], but this compromises industrial capacity for bulk amide production.

More recently, isolated enzymes with high selectivity have been used effectively to catalyse industrial reactions and high volumetric productivity has been achieved by increased biocatalyst loading [11]. However, in view of the noted instability of NHases, if isolated and purified NHases are to be applied in industry, enhancing their stability is seen as one prerequisite for their effective use [12]. The use of thermostable enzymes isolated from thermophilic microbial sources presents one opportunity to achieve higher stability. Further, immobilized biocatalysts are often regarded as the appropriate form for application of enzymes in industry as they can be reused, are readily separated from reaction mixtures, are convenient to handle, and allow reduced effluent disposal problems [13]. In laboratory applications, NHases have commonly been used as immobilized whole cell biocatalysts entrapped in calcium alginate [14,15] or polyacrylamide gels [16].

A thermostable NHase was isolated from the moderate thermophile *Geobacillus pallidus* RAPc8 [17], the gene has been cloned and over-expressed in *E. coli* BL21 (DE3) [18]. The NHase consists of two subunits (α and β) having M_r of 28 and 29 kDa where the functional structure is heterotetrameric $(\alpha\beta)_2$ [19]. The active site of the *G. pallidus* RAPc8 NHase, which is located at interface of the α- and β-subunits, contains a non-corrinoid cobalt (Co^{III}), chelated cysteine sulfinic acid residues and main-chain nitrogen

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doi:10.1016/j.molcatb.2009.12.011

atoms (αSer114 and αCys115) in what is known as a 'claw setting' [20]. In preliminary studies, the enzyme was shown to exhibit a broad specificity for aliphatic nitriles, and to be highly active towards heteroaromatic substrates (such as 3-cyanopyridine), but it showed no reaction with aromatic nitriles [17,18]. Both native and recombinant enzymes are subjected to considerable substrate and product inhibition [17,18].

In this study, immobilization of the purified thermostable *G. pallidus* RAPc8 NHase was investigated using a wide variety of support materials. Here, we show that *G. pallidus* NHase immobilized to Eupergit®C and cross-linked with 1-ethyl-3-(dimethylamino-propyl) carbodiimide (EDAC) shows substantially modified properties, including, importantly, approximately 50% reduction in substrate inhibition.

2. Materials and methods

2.1. Chemicals

Carrier materials tested for immobilization included: Eupergit®C and CM (Röhm, Pharma Polymers, Germany), Sepabeads Series EC (Resindion, Mitsubishi Chemicals Corporation, Japan), SE and DEAE-Sephadex A 50 (Pharmacia), Dowex (Serva Feinbiochemica), and Super-Q-Toyopearl (Tosoh Corporation), glass beads (Supelco), and Bio-Beads (Bio-Rad). Other reagents included 50% glutaraldehyde (Aldrich), 3-aminopropyltriethoxysilane (Sigma), ethylenediamine (EDA) (Aldrich), 1-ethyl-3-(dimethylamino-propyl) carbodiimide (EDAC) (Aldrich), trichloroacetic acid (Saarchem), $ZnSO_4$ (Aldrich), $AgSO_4$ (Saarchem), $CoCl_2$ (NT laboratories Suppliers) and $CuCl_2$ (Holpro Analytics (Pty) Ltd.

2.2. Microorganism and cultivation

The *G. pallidus* RAPc8 NHase, cloned and expressed in *E. coli* BL 21 (DE3) pLysS, was used throughout the study. Cultures were grown in a medium consisting of 10 g/L bacto-tryptone, 5 g/L yeast-extract and 10 g/L NaCl in dH_2O (pH 7.0). A 2 L flask containing 1000 mL medium with 50 μg mL^{-1} ampicillin was inoculated and incubated at 37 °C with shaking at 220 rpm, then induced by addition of IPTG to 0.4 mM final concentration. Approximately 30 min prior to induction, cobalt chloride solution was added to a final concentration of 0.1 mM. At 30 min intervals, cell growth was estimated spectrophotometrically at A_{600}. After 4 h of cell growth (OD_{600} = 0.8), cells were harvested by centrifugation at 7000 × *g*, 4 °C for 15 min and washed with 50 mM KH_2PO_4–K_2HPO_4 buffer, pH 7.2. Harvested cells were suspended in 50 mM KH_2PO_4–K_2HPO_4 buffer total volume of 25 mL, pH 7.2, and sonicated at 0 °C using a VirTis Ultrasonic Cell Disrupter (for 10 min, at 30 s intervals breaks). Disrupted cells were centrifuged at 7500 × *g* for 30 min, and the supernatant was retained as cell-free enzyme extract.

2.3. Protein purification

The cell-free enzyme extract was heat-treated at 55 °C for 45 min and centrifuged (7500 × *g*, at 4 °C for 15 min) to remove precipitated protein. The supernatant was fractionated with $(NH_4)_2SO_4$. Proteins precipitating at 20% saturation were collected by centrifugation (7500 × *g*, at 4 °C for 15 min) and discarded, and the supernatant was loaded onto a pre-packed Hiload 16/10 Phenyl-Sepharose column on a Pharmacia FPLC system. The column was equilibrated with 1.0 M ammonium sulphate in 50 mM potassium phosphate buffer, pH 7.2. Elution of bound protein was with a 1.0–0 M linear gradient of ammonium sulphate in 50 mM potassium phosphate buffer, pH 7.2 at a flow rate of 3.0 mL min^{-1}. The NHase-containing fractions were combined and dialysed overnight against 25 mM potassium phosphate buffer, pH 7.2. The NHase extract from HIC was loaded onto a pre-packed HiPrep 16/10 Q-Sepharose FF column equilibrated with 25 mM potassium phosphate buffer, pH 7.2. Bound protein was eluted with a linear gradient of NaCl (0–0.5 M) in 20 mM potassium phosphate buffer, pH 7.2. Fractions of 2 mL were collected and concentrated by dialysis against 50 mM potassium phosphate buffer pH 7.2.

2.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

At each stage of purification, active proteins were analyzed by a 12% SDS-PAGE gel as described previously [17]. Protein bands were stained with a solution of Coomassie Blue R-250 and the excess dye was removed with destaining solution.

2.5. NHase activity assay

The conversion of 3-cyanopyridine to nicotinamide was used as the model reaction to monitor NHase activity. The standard reaction mixture (3 mL) contained 50 mM of 3-cyanopyridine and 50 mM KH_2PO_4–K_2HPO_4 buffer (pH 7.2). The reaction was initiated by addition of 100 μL enzyme solution. All assays were carried out at 50 °C, with stirring (150 rpm), in duplicate. Reactions were terminated by addition of 0.2 mL of 3 M HCl. Nicotinamide and 3-cyanopyridine concentrations in the reaction mixture were determined by analytical HPLC using a Merck Hitachi LaChrom system with 4.6 mm × 150 mm C18 column at a flow rate of 1.0 mL min^{-1}. Separations were performed at room temperature with the following mobile phase system: acetonitrile-10 mM KH_2PO_4–H_3PO_4 buffer (pH 2.8), 1:4 (v/v). The absorbance was measured at 235 nm using a UV detector.

One unit of enzyme activity was defined as the amount of enzyme that catalyzed the production of 1 μmol of nicotinamide per minute under the standard assay conditions.

2.6. Protein detection assay

Protein concentration was determined using the bovine serum albumin as the standard and measuring absorbances at 595 nm [21].

2.7. Methods of immobilization

Entrapment in Ca-alginate beads: 20 mL enzyme solution (containing 10 mg protein) was added to 10 mL of 2 or 4% (w/v) sodium alginate solutions as described previously [22].

Eupergit®C and CM: 10 mg of enzyme dissolved in 20 mL of 50 mM potassium phosphate buffer (pH 7.2), were added to 5 g of beads.

Glutaraldehyde- and ethylene diamine-activated Amberlite XAD 4: 10 mg of NHase dissolved in 50 mM potassium phosphate buffer (pH 7.2) was added to 5 g of glutaraldehyde- or ethylene diamine-activated Amberlite-XAD-4 beads (as described previously [23]).

Epoxy-Sepabeads (EC-EP), amino-epoxy Sepabeads (EC-HFA), ethylendiamino Sepabeads (EC-EA), hexamethylendiamino Sepabeads (EC-HA) and metal ion activated Sepabeads (EC-IDA): 10 mg of enzyme solution in 50 mM potassium phosphate buffer (pH 7.2), was added to 5 g of each, pre-activated by Ag^{2+}, Zn^{2+}, Cu^{2+} or Co^{2+} ions (Resindion, Mitsibishi Chemical Corporation).

Cation and anion exchangers: 10 mg of enzyme solution in 50 mM potassium phosphate buffer (pH 7.2) was added to 5 g each of Super Q-Toyopearl, DEAE-Sephadex, SE-Sephadex C 50 and Dowex beads.

Glass beads: 10 mg of enzyme solution in 20 mL of potassium phosphate buffer was added to 5 g of 3-aminopropyltrimethoxysilane-activated glass beads [24].

In all experiments, the ratio of enzyme solution to beads was kept at 4:1 and the mixtures were incubated with stirring at 150 rpm (in an orbital shaker) at room temperature for specified time periods: 72–96 h for Eupergit®C, Eupergit®CM, EDA-Amberlite XAD-4, glutaraldehyde-Amberlite XAD-4, Epoxy-Sepabeads (EC-EP), amino-epoxy Sepabeads (EC-HFA), ethylendiamino Sepabeads (EC-EA), hexamethylendiamino Sepabeads (EC-HA) and metal ion-activated Sepabeads (EC-IDA); 42–48 h for cationic and anionic exchanger resins, glass beads and bio-beads and 30 min for Ca-alginate. During incubation, residual activity was assayed periodically. After incubation the supernatant was removed by decanting, the residual activity re-assayed and the immobilized biocatalyst washed three times with 50 mM potassium phosphate buffer, pH 7.2. In a separate procedure, EDAC or glutaraldehyde 0.5 w/v (%) were added to the Eupergit®C-enzyme biocatalyst to allow cross-linking of the immobilized NHase [23]. The mixture was further incubated for an additional 3 h. Filtrates were assayed for protein concentration and enzyme activity to determine the effects of cross-linking on binding and activity yields.

2.8. Characterization of Eupergit®C (EDAC)-immobilized G. pallidus RAPc8 NHase

2.8.1. Loading efficiency

Protein loading was determined by measuring the amount of protein before and after immobilization, and was expressed as loading efficiency (%). The loading efficiencies were calculated as:

$$\text{Loading efficiency}\,(\%) = \left(\frac{P_\text{i} - P_\text{f}}{P_\text{i}}\right) \times 100 \quad (1)$$

where P_i is the initial amount of protein in the enzyme solution, P_f is the final amount of protein in enzyme solution after immobilization.

2.8.2. Immobilization efficiency

The extent of immobilization (immobilization efficiency) was determined by measuring the NHase activity before and after the immobilization process. The immobilization efficiencies were calculated as:

$$\text{Immobilization effeciency}\,(\%) = \left(\frac{a_\text{imm}}{a_\text{free}}\right) \times 100 \quad (2)$$

where a_imm is apparent specific activity of immobilized enzyme (U/mg bound protein) and a_free is specific activity of free enzyme (U/mg protein).

2.9. Effect of pH on soluble and Eupergit®C (EDAC)-immobilized G. pallidus RAPc8 NHase

The effect of pH on the stability of soluble and immobilized NHase was determined using four buffer systems: acetate/citric acid (pH 4–6), potassium phosphate (pH 6–8), Tris–HCl (pH 8–10), and sodium hydrogen phosphate/sodium hydroxide (pH 10–12). Purified soluble or immobilized NHase (100 μL or 0.15 mg protein) was added to 3.0 mL of 50 mM buffer of specified pH. The mixture was pre-incubated at 50 °C for 1 min. 3-Cyanopyridine was then added to a final concentration of 50 mM and the reaction mixture incubated for a further 5 min. The reaction was stopped by addition of 200 μL of 3 M HCl. Samples were centrifuged, and the supernatant was analyzed by HPLC. Relative activity is expressed as a percentage of the enzyme activity at pH 7, taken as 100%.

2.10. Effect of temperature on soluble and Eupergit®C (EDAC)-immobilized NHase

Reactions were carried out between 20 and 70 °C. 100 μL of purified soluble or immobilized NHase (100 μL or 0.15 mg protein) was added to 2.9 mL of 50 mM phosphate buffer, pH 7.2. The reaction mixture was pre-incubated for 1 min at the desired temperature. 3-Cyanopyridine was then added to a final concentration of 50 mM and the reaction mixture incubated for a further 5 min. Reactions were terminated and reaction mixtures analysed as described above. Relative activity is expressed as percentage of the enzyme activity at 50 °C, taken as 100%.

2.11. Effect of co-organic solvents on the NHase activity

Purified soluble or immobilized NHase (100 μL or 0.15 mg protein) was added to 2.9 mL of 50 mM phosphate buffer, pH 7.2 containing the specified organic solvent at 10 (v/v)%. Reactions were incubated, terminated and reaction mixtures analysed as described above. The residual activity in the samples without organic solvent (buffer only) was taken as 100%.

2.12. Thermal stability of the soluble and Eupergit®C (EDAC)-immobilized NHase

Enzyme thermostability was determined by pre-incubating soluble and immobilized NHase at 40, 50, 60, and 70 °C for up to 90 min. Aliquots of 100 μL of soluble enzyme or biocatalyst (equivalent to 0.15 mg) were withdrawn at 10 min intervals and the residual activities measured under the standard assay conditions. Relative activity is expressed as percentage of the enzyme activity prior to incubation, taken as 100%.

2.13. Recycled use of Eupergit®C (EDAC)-immobilized NHase

The operational stability of the Eupergit®C (EDAC)-immobilized NHase was measured by repeated 5 min reactions at 50 °C using a 3-cyanopyridine substrate with single batches of biocatalyst prepared as described above. At the end of each cycle, a 1 mL sample was withdrawn, centrifuged, the amount of product formed analyzed by HPLC and residual activity calculated. The immobilized enzyme sample was washed with 100 mL KH_2PO_4–K_2HPO_4 buffer (pH 7.2) before re-use in a subsequent cycle. Volumetric productivity of each cycle was measured as expressed as g/L/h.

2.14. Effect of substrate concentration on NHase activity

The effect of substrate concentration on activity for both soluble and immobilized NHase was measured using the 3-cyanopyridine at various concentrations under otherwise standard reaction conditions.

2.15. Substrate specificity and kinetic analysis of NHase

The kinetic constants K_m, and V_max for conversion of heteroaromatic nitriles by the soluble and immobilized NHase were determined by measuring initial reaction rates over a substrate concentration range of 5–100 mM using 2-cyanopyridine, 3-cyanopyridine and 4-cyanopyridine. Kinetic constants were calculated using the Hanes–Woolf plot. Experiments were carried out in duplicate and experimental error was no more than ±4% (S.D.).

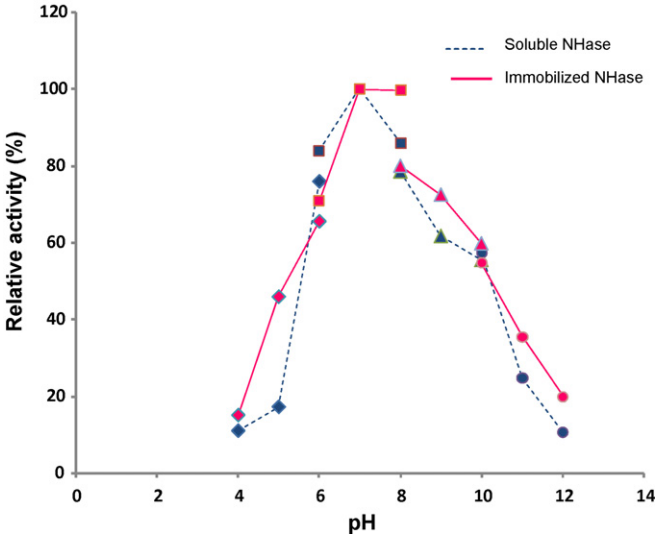

Fig. 1. pH-activity profiles of soluble and Eupergit®C (EDAC)-immobilized NHase. Buffers used include: CH_3COOH/CH_3COONa for pH 4–6 (♦); K_2HPO_4/KH_2PO_4 pH 6–8 (■); Tris–HCl pH 8–10 (▲) and $NaHPO_4$–NaOH pH 10–12 (●).

3. Results and discussion

3.1. Purification and molecular weight determination of G. pallidus RAPc8 NHase

Based on methods published previously [17,18] the recombinant *G. pallidus* RAPc8 NHase was purified to homogeneity from *E. coli* cells with a 67% yield and 1.2-fold purification. SDS-PAGE showed that the enzyme to be more than 95% homogeneous (data not shown). The enzyme was immobilized and the biocatalyst characterised with the objective of developing its application for production of amides of industrial interest.

3.2. Immobilization of G. pallidus RAPc8 NHase

The *G. pallidus* RAPc8 NHase exhibited its highest activity at pH 7 (Fig. 1) and temperature 60 °C, and the activity was demonstrated over the temperature range 40–70 °C (Fig. 2). However, the activity was not retained for more than 20 min at temperatures above 50 °C (Fig. 3a). Since immobilization is one approach to enhance the stability of enzymes for use as biocatalysts, this was investigated in the case of the *G. pallidus* RAPc8 NHase. In order to select an optimal immobilization method, various support materials were investigated, including Eupergit®C and CM, Sepabeads Series EC, SE and DEAE-Sephadex A 50 (Pharmacia), Dowex, and Super-Q-Toyopearl, glass beads, and Bio-Beads. Cross-linking and activating reagents including 50% glutaraldehyde, 3-aminopropyltriethoxysilane, ethylenediamine (EDA), and 1-ethyl-3-(dimethylamino-propyl) carbodiimide (EDAC) were also investigated. Loading and immobilization efficiency data for each support are given in Table 1.

Hexamethylendiamino-activated Sepabeads (EC-HA), (100%), amino epoxy-activated Sepabeads (EC-HFA) (96.2%), ethylendiamino-activated Sepabeads (EC-EA) (91.5%) and Eupergit®C (90.5%) showed the highest loading efficiencies, respectively. We attribute this to the presence of the extended spacer arm affording increased access for the protein. However, the total protein loading did not correlate with immobilization efficiency of the NHase (Table 1). With respect to immobilization efficiency, Eupergit®C with either EDAC or glutaraldehyde cross-linking was the optimum support material tested. The higher immobilization efficiencies obtained with cross-linked matrices

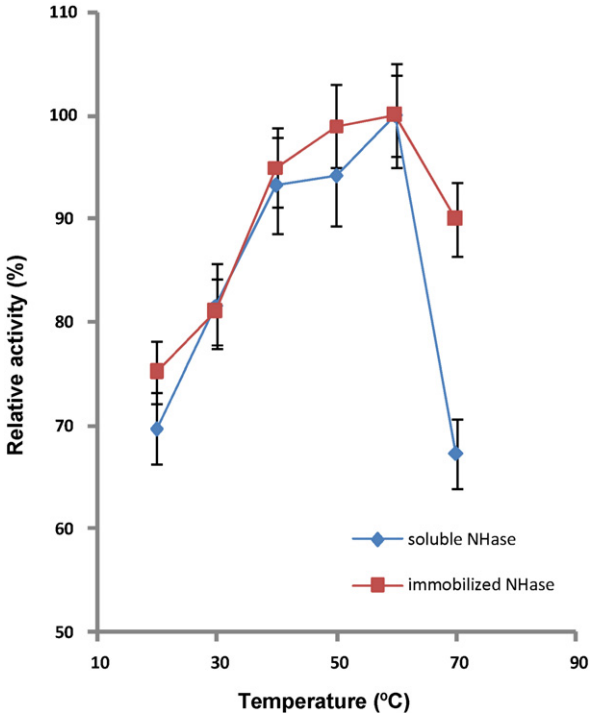

Fig. 2. Temperature-activity profiles for soluble and Eupergit®C (EDAC)-immobilized NHase at pH 7.2.

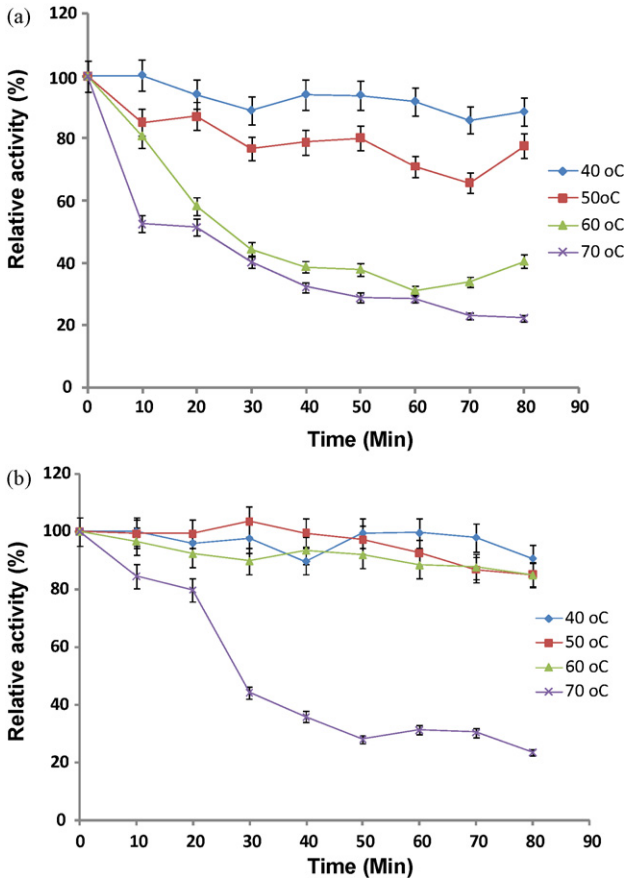

Fig. 3. Thermostability of soluble [A] and Eupergit®C (EDAC)-immobilized [B] NHase.

Table 1
Loading and immobilization efficiencies for *G. pallidus* RAPc8 NHase prepared by various techniques.

Support	Technique	Total protein loading (mg)	Loading efficiency (%)	Immobilization efficiency (%)
Eupergit C (EDAC)	Covalent	7.7	77.1	92.3
Eupergit C (GA)	Covalent	5.8	58	83.4
EDA-Amberlite XAD 4	Covalent	1.8	18.5	65.4
GA-Amberlite XAD 4	Covalent	1.6	15.5	53.5
Eupergit C	Covalent	9.1	90.5	53.1
DEAE-Sephadex A 50	Anionic	3.5	35.2	47.2
SE-Sephadex A 50	Cationic	7.2	72.2	42.8
Eupergit CM	Covalent	8.2	82.3	32.1
Dowex®50WX8	Cationic	7.4	73.6	36.9
EC-IDA-Ag	Metal chelation	8.1	81.2	34.6
EC-IDA-Co	Metal chelation	3.3	32.5	32.4
EC-EA	Covalent	9.2	91.5	30.1
Controlled pore glass	Simple adsorption	8.8	88.4	28.7
EC-HFA	Covalent	9.6	96.2	28.5
EC-EP	Covalent	8.1	81.2	28.4
Bio-beads	Simple adsorption	6.2	61.7	25.6
EC-IDA-Cu	Metal chelation	8.5	85	24.8
EC-HA	Covalent	10	100	24.6
EC-IDA-Zn	Metal chelation	7.3	73.2	24
Super-Q-Toyopearl	Anionic	9.5	95.2	20.9
4% Sodium alginate	Entrapment	9.6	95.8	nd[a]
2% Sodium alginate	Entrapment	8.1	80.8	nd

[a] nd: not determined.

Table 2
Comparative thermostability of thermophilic NHases (expressed as % residual activity following incubation at given temperatures).

Microorganism	Temperature (°C)	Incubation time (min)	Residual activity (% initial)	Reference
Bacillus BR 449	60	120	100	[44]
G. pallidus RAPc8	50	150	50	[17]
G. pallidus DAC521	50	–	50	[45]
Bacillus smithii	55	90	50	[46]
Ps. thermophila	50	120	100	[47]
Eupergit®C (EDAC)-immobilized *G. pallidus* RAPc8	60	90	80	(This paper)

might be attributed to stabilization of the NHase quaternary structure. It has been established that chemical cross-linking, either by use of bifunctional reagents or multipoint covalent immobilization, will commonly stabilize both monomeric and multimeric protein structures [25–28]. Based on immobilization efficiency as a criterion for production of an efficient biocatalyst, Eupergit®C cross-linked with EDAC was selected for further characterization.

3.3. Performance of the Eupergit®C (EDAC)-immobilized NHase

A comparison of the pH-activity profiles of both soluble and immobilized *G. pallidus* RAPc8 NHase showed no significant differences, with an optimum pH of approximately 7.0 (Fig. 1), consistent with the report of Pereira et al. [17].

The effect of temperature on the activity of soluble and immobilized NHase was determined over the temperature range 20–70 °C (Fig. 2). The immobilized preparation showed better retention of activity after incubation at higher temperatures. This behavior is consistent with conformation stabilization associated with immobilization/cross-linking. The temperature optima for the soluble and immobilized enzyme preparations were similar (60 °C).

3.4. Thermostability of soluble and Eupergit®C (EDAC)-immobilized NHases

The thermostability of the soluble and Eupergit®C (EDAC)-immobilized NHases was compared over a temperature range of 40–70 °C. In both cases, NHase was functionally stable in the temperature range of 40–50 °C over 80 min of incubation (Fig. 3a and b). However, at 60 °C, the immobilized NHase exhibited significantly higher stability than that of the soluble enzyme. Plots of the natural log of relative activity as a function of time for both free and immobilized NHase showed first order kinetics and were used to estimate deactivation constant and half-life values (data not shown). The estimated parameters were k_d (immobilized) = 0.0021 min^{-1}, $t_{1/2}$ (immobilized) = 330.1 min, k_d (soluble) = 0.0127 min^{-1} and $t_{1/2}$ (soluble) = 54.5 min. While it is noted that comparisons of thermal stability in NHases are complicated by the fact that researchers have used diverse approaches [29], comparative data shown in Table 2 indicate that the immobilized *G. pallidus* RAPc8 NHase compares favourably with other thermophilic NHases.

3.5. Reusability of Eupergit®C (EDAC)-immobilized NHase

The potential for recycling of the immobilized biocatalyst was measured by conducting repeated reactions with the same batch of biocatalyst under otherwise constant conditions. The Eupergit®C (EDAC)-immobilized NHase showed only a small decrease in volumetric productivity after repeated use, from 45 to 40 g/L/h (50–44 mM/h) after 8 cycles; the retention of activity indicates only approximately 10% loss of activity in this protein over the 8 cycles. These reactions were conducted using the equivalent of 5 g protein per litre of solution in the reactor, which compares favourably with other reports [10]. In general, this result is consistent with the stability of the immobilized enzyme at 50 °C (Fig. 3).

3.6. Effect of substrate concentration on G. pallidus RAPc8 NHase

The effect of substrate concentration on NHase activity was studied by using varying concentrations of 3-cyanopyridine (5, 10, 20, 50, 100, 200, 300, 400, and 500 mM) under standard assay conditions. Profiles of soluble and immobilized NHase are shown in Fig. 5. Maximal rates were achieved at 100 mM substrate for the

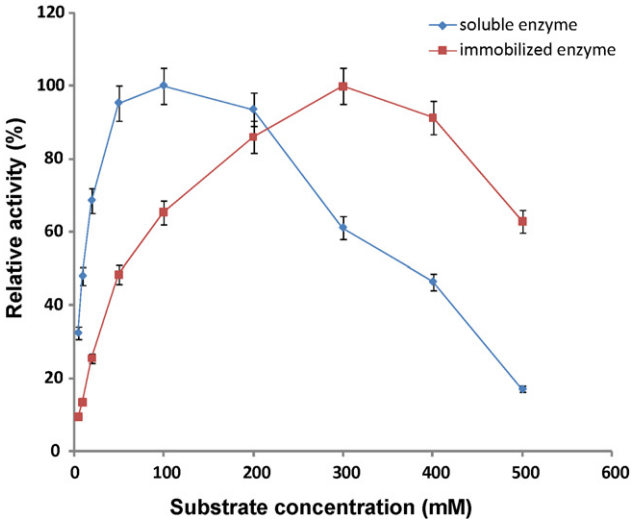

Fig. 4. 3-Cyanopyridine substrate inhibition of soluble and Eupergit®C (EDAC)-immobilized NHase.

soluble enzyme, with higher substrate concentrations leading to a rate reduction. This is consistent with findings reported for other NHases which also exhibit substrate inhibition at high substrate concentrations [36].

Experiments using immobilized *G. pallidus* RAPc8 NHase over the same substrate range (5–500 mM) showed a markedly increased optimal substrate concentration (300 mM) with enhanced enzyme performance (compared with the soluble enzyme) at even higher substrate concentrations (Fig. 4). The mechanism by which immobilization reduces substrate inhibition is unclear. We speculate that conformational stabilization resulting from covalent immobilization and cross-linking may be responsible for preventing deleterious conformational changes that occur in the NHase structure during the substrate–enzyme binding process [33], particularly at higher substrate concentrations.

Experimental data were fitted to the Haldane model (Fig. 5a and b) and inhibition constants were determined using GraphPad prism 5 software (Hearn Scientific). Calculated inhibition constants for the soluble and Eupergit®C (EDAC)-immobilized NHase preparations were 110 and 195 mM, respectively.

3.7. Effect of biphasic organic and aqueous solvents on NHase activity

The use of organic solvents may be desirable in biocatalysis involving the conversion of non-polar substrates such as aromatic nitriles. Since the *G. pallidus* RAPc8 NHase showed selectivity for aromatic substrates [18], its activity in a range of organic media was investigated. When different water–miscible organic solvents were used as co-solvents (at 10%, v/v), the soluble NHase generally exhibited reduced activity compared to the co-solvent free control (Fig. 6). Solvent-dependent enzyme inactivation is generally attributed to disruption of the protein hydration layer, either directly impacting catalytic function or reducing observed catalytic rates as a result of protein denaturation [31–33]. *G. pallidus* RAPc8 NHase has seven bridging water molecules involved in maintenance of the geometry of the α and β dimeric structures [34]. It is suggested that disruption of water molecules involved either the dimer interface or the dimer–dimer association may accelerate inactivation *via* dissociation of the quaternary structure. Notably, bi-phasic organic/aqueous systems (such as with benzene and toluene) did not result in significant losses of enzyme activity (Fig. 6). In almost all cases, the immobilized enzyme preparation retained higher levels of activity under identical incubation conditions (Fig. 6). These data are consistent with the stabilization of quaternary structure resulting from multi-point covalent linkages and intra-protein crosslinking [28].

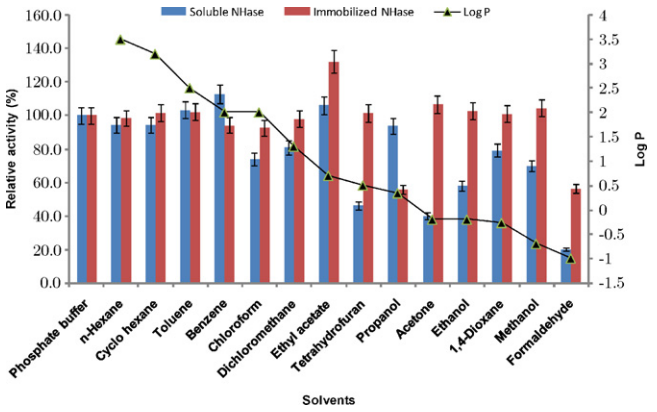

Fig. 6. The effect of organic solvents on the activity of soluble and Eupergit®C (EDAC)-immobilized *G. pallidus* RAPc8 NHase.

3.8. Kinetic parameters for G. pallidus RAPc8 NHase

Kinetic constants for *G. pallidus* RAPc8 NHase were determined using three heteroaromatic nitrile substrates; 2-cyano-, 3-cyano- and 4-cyanopyridine over a concentration range of 5–100 mM (Table 3). V_{max} values for the soluble NHase were estimated as 45.6, 31.3, and 13.1 μmol mL^{-1} min^{-1} respectively for substrates 3-cyanopyridine, 2-cyanopyridine, and 4-cyanopyridine. The relative values for the three substrates are consistent with the extent of activation of the nitrile carbon resulting from electron delocalization across the heteroaromatic ring.

A comparison of constants of the soluble and Eupergit®C (EDAC)-immobilized NHase preparation on 3-cyanopyridine (Table 3) shows approximately 10-fold reduction in V_{max} and 2-fold increase in K_m. Such a substantial reduction in catalytic efficiency is attributable to the immobilization of enzyme molecules

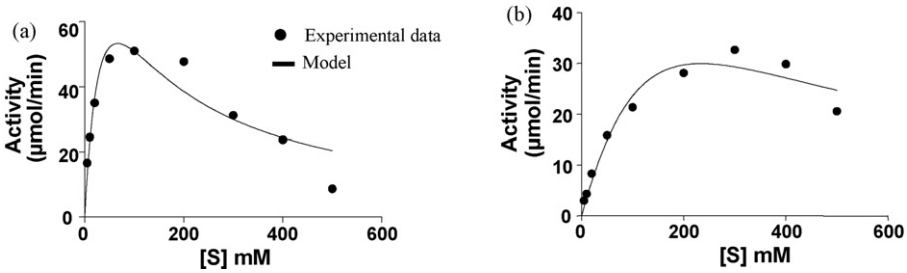

Fig. 5. Non-linear regression Haldene model fitted to substrate inhibition data of (a) soluble [R^2 = 0.857, Sy. x = 1 2.8] and (b) Eupergit®C (EDAC)-immobilized [R^2 = 0.9504, Sy. x = 2.85] *G. pallidus* RAPc8 NHAse.

Table 3
Kinetic parameters for soluble and Eupergit®C (EDAC)-immobilized *G. pallidus* RAPc8 NHase.

Substrate	V_{max} (μmol mL^{-1} min^{-1})	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})	R^2
2-Cyanopyridine	33.8	5.3	26598	5018	0.9894
3-Cyanopyridine	48.1	10.2	37777	3703	0.9951
4-Cyanopyridine	15.3	8.7	12078	1388	0.9802
3-Cyanopyridine (immobilized NHase)	4.5	17.3	3543	204	0.9830

in inactive or substrate-blocked configurations, and to diffusional limitations typically associated with the use of high surface area porous immobilization matrices [41–43].

4. Conclusion

The *G. pallidus* RAPc8 NHase, previously found to be active on cyano-pyridines as substrates, is a potentially useful enzyme for production of nicotinamide and related aromatic amides. However, its relatively poor thermostability and notable substrate inhibition are disadvantageous in this regard. The results presented above confirm that immobilization of the NHase lead to considerably enhanced thermostability and reduced substrate inhibition. Further, the retention of the activity of the immobilized NHase biocatalyst in repeated reactions and in the presence of organic solvents as demonstrated here provide evidence of its potential usefulness.

Acknowledgement

The financial support of the National Research Foundation (NRF) of South Africa is gratefully acknowledged.

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