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Stabilization of an amine transaminase for biocatalysis

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ABSTRACT

The amine transaminase from *Chromobacterium violaceum* (*Cv*-ATA) is a well-known enzyme to achieve chiral amines of high enantiomeric excess in laboratory scales. However, the low operational stability of *Cv*-ATA limits the enzyme applicability on larger scales. In order to improve the operational stability of *Cv*-ATA, and thereby extending its applicability, factors (additives, co-solvents, organic solvents and different temperatures) targeting enzyme stability and activity were explored in order to find out how to store and apply the enzyme. The present investigation shows that the melting point of *Cv*-ATA is improved by adding sucrose or glycerol, separately. Further, by storing the enzyme at higher concentrations and in co-solvents, such as; 50% glycerol, 20% methanol or 10% DMSO, the active dimeric structure of *Cv*-ATA is retained. Enzyme stored in 50% glycerol at −20 °C was e.g., still fully active after 6 months. Finally, the enzyme performance was improved 5-fold by a co-lyophilization with surfactants prior to usage in isooctane.

1. Introduction

Enzymes have acquired extensive attention as catalysts in applied chemistry due to their wide range of applications in the synthesis of pharmaceuticals, agro-chemicals, biofuels or fine chemical building blocks. Although biocatalysts have numerous advantages, such as high chemo-, regio- and enantioselectivity, high turnover and ability to operate in mild reaction conditions, the poor operational stability of enzymes still restricts their application in industrial process conditions [1,2]. Generally, enzyme stability can be defined as: thermodynamic (conformational) stability and kinetic (long-term) stability [1,2]. Thermodynamic stability concerns the reversible unfolding of the protein conformation, while kinetic stability determines how long enzyme activity is maintained before irreversible denaturation occurs. The operational stability, which involves thermal stability, stability in organic solvents and stability over time, is related to both thermodynamic- and kinetic stability [3–5]. In order to improve the operational stability of an enzyme both of these factors need to be considered.

Enzymes dependent on the cofactor pyridoxal-5≠phosphate (PLP) are a large class of enzymes consisting of several protein fold types and activities. The versatility of PLP as a cofactor leads to the PLP-dependent enzymes being able to catalyze a wide range of reactions such as transamination, racemization, decarboxylation, substitution and elimination [6,7]. One largely represented activity among the PLP-dependent enzymes is transamination. The largest substrate group within that activity is amino acids but amine transaminases (ATAs) have recently gained a lot of interest as catalysts for the production of chiral amines. ATAs can be used to perform asymmetric synthesis of chiral amines from the corresponding prochiral ketones with high yields and excellent enantioselectivities in mild reaction conditions [8,9]. The operational stability of ATAs is however poor and attempts to improve it using various strategies have been reported. Immobilization is a common method to improve enzyme stability and several methods for ATA immobilization have been reported over the last decades [10–23]. Also, directed evolution has been successfully applied to improve both the stability of *Arthrobacter citreus* ATA and of an (*R*)-selective ATA for the production of substituted 2-aminotetralin [24] and the anti-diabetic compound Sitagliptin [25]. Both these cases show that improvement of operational stability is crucial for the application of ATAs in industrial processes. Another recently published method to improve ATA stability is the global incorporation of fluorotyrosine, which enhanced the stability of *Vibrio fluvialis* ATA [26].

The ATA from *Chromobacterium violaceum* (*Cv*-ATA, CV2025) is an extensively studied enzyme with a broad substrate scope toward aromatic and aliphatic amines [27–30]. Recently, the *Cv*-ATA activity toward serine was improved by different single point mutants [31]. We have previously reported on important characteristics of the *Cv*-ATA enzyme, such as thermostability [32] and enantioselectivity [33], as well as how to perform active-site

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titration [34]. However, our previous experiments in harsh conditions have shown that the operational stability of *Cv*-ATA is low and the wild type enzyme can therefore not be applied in an industrial process with satisfying results. In addition, we have previously observed that this enzyme activates over time upon storage in room temperature, which sometimes results in reproducibility problems.

Previously, we explored the crystal structure of *Cv*-ATA [32]. To promote protein stabilization prior to crystallization, a buffer screen was made to explore individual stabilization effects of various buffers supplemented by additives and/or co-solvents by melting point (T_m) measurements using differential scanning fluorimetry. In that study, the enzyme displayed a high T_m of 78 °C in HEPES (100 mM, pH 7.4, 100 mM NaCl) buffer. The enzyme was considered to be thermostable due to this high melting point. However, it turned out that the enzyme is not active and cannot perform catalysis at this high temperature. Therefore, we decided to further explore parameters that may affect the stability of *Cv*-ATA. The use of additives and co-solvents showed positive stabilization effects in our previous study and was therefore used as a starting point in this investigation. The reasons for *Cv*-ATA activation and inactivation in buffer solutions have been explored through various stability investigations combining enzyme activity measurements and the amount of active dimer present.

There are only a few reports of cell-free ATA-catalyzed reactions in hydrophobic organic solvents [35–39]. Generally, enzymes are structurally considered to be more rigid in organic solvents. Also, the enzyme activity is commonly reduced if the enzyme preparation is not lyophilized [40]. Lyophilization is a well-established technique to prolong enzyme shelf-life [3–5]. The lyophilization process involves both freezing and dehydration, which can alter both enzyme activity and enzyme conformation by removing waters surrounding the enzyme surface or important structural waters. In the worst case, the dehydration process can result in protein denaturation [41,42]. Surfactants have shown a great potential to shield enzyme structure from denaturation during lyophilization [43,44]. Here, we show the first example of the use of an ATA enzyme co-lyophilized with surfactants which enables cell-free *Cv*-ATA-catalyzed transamination reactions in organic solvents to yield high conversions.

2. Experimental

2.1. Chemicals

All chemicals were purchased from Sigma–Aldrich and used without further purification.

2.2. Protein expression and purification

The gene encoding the *Cv*-ATA was previously inserted in a pET28a(+) vector, including an N-terminal His_6-tag [34]. For protein expression in *Escherichia coli* BL21(DE3), a 10 mL overnight culture was added to 1 L TB medium (Terrific Broth) medium (1.2% peptone, 2.4% yeast extract, 72 mM K_2HPO_4, 17 mM KH_2PO_4 and 0.4% glycerol) supplemented with 50 mg/L kanamycin. Firstly, the culture was incubated at 37 °C and 220 rpm for 2–3 h until the OD_{600} reached 0.7–0.9. Then, the culture was induced by addition of IPTG (1 mM). After 24 h of cultivation at 20 °C and 220 rpm, the cells were harvested by centrifugation (30 min, 8000 rpm) and re-suspened in IMAC binding buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0). Cells were disrupted by sonication and the cell debris was removed by centrifugation (30 min, 20,000 rpm) followed by a final filtration (0.45 μm). The IMAC purification process was performed on a Ni-NTA Sepharose column from IBA according to the manufacturerís protocol. A buffer change to HEPES buffer (50 mM, pH 8.2) was performed on a PD10 column (GE Healthcare), according to the manufacturer's protocol. After adding 3 mM of PLP, the enzyme was stored in fridge overnight. The following day, a second buffer change to HEPES buffer (50 mM, pH 8.2) was made to remove the excess of PLP, after incubation at 37 °C for 1 h. The protein concentration of the enzyme preparation was measured on NanoDrop™ 1000 Spectrophotometer at 280 nm. The enzyme was stored in fridge prior to use according to the procedures below.

2.3. Melting point measurement

Melting points were measured using differential scanning fluorimetry [45]. Samples were prepared at the desired concentrations (Fig. 1) in a 96-well PCR plate (BIO-RAD). Enzyme was added to a final concentration of 1 mg/mL. Finally, 3.75× SYPRO® Orange protein gel stain (Sigma–Aldrich, S5692) was added and the melting points were analyzed on a CFX96™ Real-Time PCR Detection System and C1000™ Thermal Cycler. Temperature was set to 25 °C and increased at a rate of 1 °C/min until it reached 95 °C. Data was extracted and MS Excel was used to perform a Boltzmann fit of the data to determine melting points.

2.4. Enzyme activity assay

To measure enzyme activity, a *Cv*-ATA-catalyzed reaction containing 2 μg (or 150 μg) enzyme, 2.5 mM (*S*)-1-phenylethylamine ((*S*)-1-PEA), 2.5 mM pyruvate and HEPES buffer (50 mM, pH 8.2) with a final volume of 1 mL was performed. The initial rate of product (acetophenone) formation was measured at 245 (or 290 nm when interference occured) [46] on a Cary® 50 UV–vis spectrophotometer.

2.5. Enzyme storage stability at room temperature or 65 °C

A purified enzyme preparation of *Cv*-ATA (0.1 mg/mL) was stored at room temperature or at 65 °C in a range of selected additives or water miscible co-solvents. At selected time points, enzyme samples (2 μg) were withdrawn for remaining enzyme activity measurement according to the enzyme activity assay.

2.6. Enzyme activity in co-solvents at 4 °C

An enzyme preparation (1.5 mg/mL) was stored in aliquots of 1 mL in different concentrations of co-solvents (glycerol and methanol) at 4 °C. Enzyme samples were taken directly from the fridge and were measured for residual activity using the enzyme activity assay at 290 nm. Thereafter, all samples were incubated in 37 °C. Samples were taken every hour to measure its re-activation.

2.7. Enzyme initial activity in co-solvents

Initial activity of *Cv*-ATA in increasing concentrations of co-solvent (5–50% of methanol, glycerol or DMSO) was determined using the enzyme activity assay. When DMSO was applied initial activity was determined at 290 nm (30 μg *Cv*-ATA) due to interference from DMSO at 245 nm. Both incubations and measurements are made in room temperature.

2.8. Enzyme stability and remaining activity after storage in co-solvents at room temperature

Aliquots of an enzyme preparation were stored in three different co-solvents (DMSO, glycerol and methanol) at room temperature with a final concentration of 1.5 mg/mL. Samples from each aliquot were taken and analyzed every day during the first week and with reduced frequency later. The samples were analyzed by the