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Short communication

Catalytic activity and structural stability of three different *Bacillus* enzymes in water/organic co-solvent mixtures

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ABSTRACT

Effect of various organic co-solvents on catalytic activity and structural stability of three different *Bacillus* enzymes, including *Bacillus licheniformis* γ-glutamyltranspeptidase (*Bl*GGT) and aldehyde dehydrogenase (*Bl*ALDH), and *Bacillus stearothermophilus* aminopeptidase II (*Bs*APII) were investigated. The experimental results revealed that almost all co-solvents had a detrimental effect on catalytic activity and molecular structure of enzymes at high concentrations. Acetonitrile and dimethylformamide caused a highest degree of enzyme inactivation; however, other more hydrophilic co-solvents, such as ethylene glycol and dimethyl sulfoxide, were better tolerated. High residual activity of *Bl*GGT was obtained in the majority of organic co-solvents tested, but catalytic activities of *Bl*ALDH and *Bs*APII were significantly decreased by the respective concentrations of the same co-solvents. Collectively, the favorable influence of these co-solvents on both catalytic activity and structural stability of *Bl*GGT makes this enzyme more suitable for biocatalytic applications.

1. Introduction

Biocatalysis has emerged as an elegant methodology that allows us to develop eco-friendly processes within the basic principles of sustainable chemistry [1]. Despite initial concerns from members of the scientific community, biocatalysis has gained increasing attention and popularity in synthetic chemistry over the last three decades [2,3]. In particular, hydrolases, a group of biocatalysts that originally catalyze bond cleavage using water as nucleophile, have contributed significantly to the industrial production of agrochemicals, pharmaceuticals, and high value-added compounds [4,5].

The detailed investigations of enzymes in water-miscible organic co-solvents were initially demonstrated by Klibanov and co-workers [6]. Their findings shortly attracted positive attention of enzyme-related researchers from both academia and industry. Since then it became apparent that enzymes are not only able to work with water-miscible organic co-solvents, but also can acquire some new traits, such as altered regioselectivity [7], increased enantioselectivity or improved thermostability [8], in water/organic co-solvent systems. The possibility to influence enzyme properties by changing the nature of working solvents was termed medium engineering. Nowadays, medium engineering represents a well-established alternative with respect to the protein engineering and the time-consuming exploration of new catalysts [9]. Many examples for the use of enzymes, mainly hydrolases, in water-miscible organic co-solvents have been documented [10,11]. However, exploring advantages with aqueous-organic systems or even neat organic solvents are limited by the risk of enzyme inactivation and the environmentally hazardous nature of solvents. Despite this, significant progress has been made towards the development of environment-friendly strategies for stabilizing enzymes in organic co-solvents [9,12].

In this study, we focused our attention on effect of 14 organic co-solvents on biocatalytic activity, conformational change, and thermal stability of γ-glutamyltranspeptidase (*Bl*GGT) and aldehyde dehydrogenase (*Bl*ALDH) from *Bacillus licheniformis*, and aminopeptidase II (*Bs*APII) from *Bacillus stearothermophilus*. *Bl*GGT can be employed for the biocatalytic synthesis of various γ-glutamyl compounds with great potential for pharmaceutical and biotechnological applications [13–15], while the use of *Bl*ALDH- and *Bs*APII-related enzymes has been seen in various industrial processes, including the production of 3-hydroxypropionic acid [16], the preparation of debittering protein hydrolysates [17], and the conversion of L -homophenylalanyl amide into L -homophenylalanine [18], a key building block for the enantioselective synthesis of angiotensin-converting enzyme inhibitors. Giving the fact that the selected organic co-solvents are the major representatives of biocatalytic processes [19,20], the ability of aforementioned *Bacillus* enzymes to retain their respective functions in

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organic-aqueous media was evaluated to identify which of 14 organic co-solvents is most appropriate for the catalytic activity of each enzyme. The experimental data will offer a practical perspective for the broader use of these enzymes in the industrial processes.

2. Methods

2.1. Materials

L -γ-Glutamyl-*p*-nitroanilide (Glu-*p*-NA), *p*-nitroaniline (*p*-NA), NAD^+, NADH, L -leucine-*p*-nitroanilide (Leu-*p*-NA), and imidazole were obtained from Sigma–Aldrich Fine Chemicals (St. Louis, MO, USA). Ni-nitrilotriacetate (Ni-NTA) resin was acquired from Qiagen Inc. (Valencia, CA, USA). The organic co-solvents, including polar polyprotic – glycerol, ethylene glycol, polyethylene glycols (PEGs); polar protic – methanol, ethanol, isopropanol, formamide; polar aprotic – acetonitrile, acetone, dimethylformamide (DMF), dimethyl sulfoxide (DMSO), tetra-hydrofuran (THF); and non-polar – 1,4-dioxane, were commercial products of analytical or molecular biological grade.

2.2. Protein expression and purification

The His_6-tagged *Bl*GGT, *Bl*ALDH, and *Bs*APII were all over-expressed in *Escherichia coli* M15 cells according to the procedures described previously [21–23]. The expressed enzymes were individually purified from the cell-free extracts using the Ni-NTA Superflow Cartridge (Qiagen). The adherent enzymes were eluted from the cartridge by a buffer containing 0.5 M imidazole, 0.5 M NaCl, and 20 mM Tris–HCl buffer (pH 7.9). Thereafter, the active fractions from each enzyme were pooled and dialyzed overnight against ice-cold Tris–HCl buffer (20 mM, pH 7.9). Protein concentration was determined by the Bradford reagent (Bio-Rad) with bovine serum albumin as a standard. The purity of isolated enzymes was checked by SDS–12 % polyacrylamide gel. Enzyme aliquots (1 mL each) were stored at −20°C until used.

2.3. Activity assays

γ-Glutamyltranspeptidase activity was determined with Glu-*p*-NA as substrate [21]. The enzymatic activity of aldehyde dehydrogenase was measured as described earlier [22]. Aminopeptidase activity was determined with Leu-*p*-NA as substrate [23]. The stock solution of each enzyme was diluted to a suitable concentration prior to activity measurement and the assays were performed in three independent replicates.

2.4. Circular dichroism (CD)

All far-UV CD studies were performed on a JASCO-815 spectrometer equipped with a temperature-controlling liquid nitrogen system. Prior to spectral analyses, enzyme samples with defined amount of organic co-solvents were adjusted with 20 mM Tris–HCl buffer (pH 7.9) to a final concentration of approximately 200 μg/mL. Far-UV CD spectra were acquired over the wavelength range from 250 to 190 nm using a 2 mm path length cell. The photomultiplier absorbance was always kept below 600 V in the analyzed region. Ten spectra were averaged for each protein sample and the data were corrected for the background contribution. The spectral data were expressed as molar ellipticity (deg / cm^2 / dmol) based on the mean molecular weight (MRW) of each enzyme. Molar ellipticity was calculated as $[\theta] = [100 \times (\mathrm{MRW}) \times \theta_{obs}/(c \times l)]$, where θ_{obs} represents the observed ellipticity in degree at a given wavelength, c is the protein concentration in mg/mL, and l is the length of light path in cm.

Temperature-induced unfolding of enzymes (200 μg/mL each) in water/organic co-solvent mixtures was monitored by following the change in ellipticity at 222 nm. Samples were heated at a scan rate of 1 °C/min. The changes in ellipticity (θ) at 222 nm were analyzed according to Eq. (1) [24].

$$\theta_{222} = \frac{\theta_N + \theta_U \cdot \exp\left[-\frac{\Delta H_U}{RT}\cdot\left(1-\frac{T}{T_m}\right)+\frac{\Delta C_{PU}}{RT}\cdot\left(T\ln\left(\frac{T}{T_m}\right)+T_m-T\right)\right]}{1+\exp\left[-\frac{\Delta H_U}{RT}\cdot\left(1-\frac{T}{T_m}\right)+\frac{\Delta C_{PU}}{RT}\cdot\left(T\ln\left(\frac{T}{T_m}\right)+T_m-T\right)\right]} \quad (1)$$

where θ_{222} is the relative ellipticity at 222 nm, θ_N and θ_U are the calculated ellipticities of the native and unfolded states, respectively, ΔH_U is the free enthalpy of unfolding, ΔC_{PU} is the heat capacity of unfolding, T_m represents the transition midpoint temperature, T is temperature, and R represents the universal gas constant.

2.5. Fluorescence spectroscopy

Fluorescence emission spectra of enzyme samples were monitored at 25 °C on a JASCO FP-6500 fluorescence spectrophotometer with an excitation wavelength of 295 nm. The spectra were measured in a 0.1 cm quartz cuvette containing enzyme samples (*Bl*GGT, 120 μg/mL; *Bs*APII, 43 μg/mL; *Bl*ALDH, 71 μg/mL) and defined amount of organic co-solvents. All spectra were recorded from 305 to 500 nm at a scanning speed of 240 nm/min and corrected for the background contribution. The maximal peak of fluorescence spectrum and the change in fluorescence intensity were used in monitoring the unfolding processes of protein samples.

3. Results and discussion

3.1. Activity of the tested enzymes in organic co-solvents

Concentration effects of 14 organic co-solvents that confer different physico-chemical aspects on the enzymatic activities of *Bl*GGT, *Bl*ALDH, and *Bs*APII were investigated with their respective substrates. As shown in Fig. 1, *Bl*GGT, *Bl*ALDH, and *Bs*APII exhibited different responses to the selected organic co-solvents even though these enzymes all come from either the same species or genus. The tolerance of enzymes toward the selected organic co-solvents could be ordered as follows: *Bl*GGT > *Bl*ALDH > *Bs*APII. It is noteworthy that the catalytic function of *Bl*GGT was well preserved in most of the organic co-solvents tested up to concentrations of 15 % (v/v). Strikingly, 1,4-dioxane was the only co-solvent that completely inhibited *Bl*GGT activity at concentrations above 20 % (v/v). In contrast, the enzymatic activity of *Bs*APII was significantly decreased by the majority of organic co-solvents, even under low concentrations (<15 %, v/v). Consistent effects were observed in *Bl*ALDH, except for a concentration-dependent activation by PEG1000 and acetone.

Glycerol, ethylene glycol, and PEGs were found to be the most compatible co-solvents to all enzymes evaluated (Fig. 1). This finding is consistent with a previous investigation in which polyols have been recommended as excellent protein stabilizers [25]. However, chemical compatibility between enzymes and polyols cannot be fully generalized due to PEG6000 was observed to have a deleterious effect on *Bacillus* enzymes, especially *Bs*APII. Among the remaining co-solvents, the least harmful to the catalytic functions of *Bl*GGT, *Bs*APII, and *Bl*ALDH was DMSO, followed by methanol and ethanol. DMSO at low concentrations (<10 %, v/v) has been shown to stabilize some protein samples [26–28]. Although the protective mechanism of DMSO remains obscure, protein-solvent preferential interactions may explain its beneficial effect on the conformational stability of proteins in aqueous solutions [27,29]. Conversely, these three enzymes were weakly tolerated towards the water-miscible organic co-solvents with hydrophobicity parameter higher than −0.35 (such as THF, 1,4-dioxane and acetonitrile). These observations agree well with the hypothesis proposed by Mozhaev et al. [30]; it elucidates that the best solvents for the water/organic co-solvent systems are