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Engineering the substrate-binding domain of an esterase enhances its hydrolytic activity toward fatty acid esters

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ABSTRACT

The poor solubility and dispersibility of fatty acids in aqueous reaction media may limit the catalytic activity of fatty acid transformation enzymes. Therefore, we studied a novel method to increase the catalytic activity of an esterase by introducing a presumed substrate-binding domain. The primary structure of an esterase from *Pseudomonas fluorescens* WI SIK (PFEI) is similar to that of an esterase in *P. fluorescens* DSM 50106 (PFEII) but not *Bacillus subtilis* DSM 402 (BS2). However, the reaction kinetics for the formation of octylacetate and a ricinoleic acid-derived ester (3) were more similar to the kinetics in BS2. For instance, the k_{cat} value of PFEI with 3 was similar to that of BS2, which was approximately 12-fold lower than the k_{cat} value of PFEII. Furthermore, fusion of PFEI to the N-terminal hydrophobic domain of PFEII led to a substantial increase (an approximate 6-fold increase in the k_{cat} value) in its hydrolytic activity of 3. These results indicate that the N-terminal domain of PFEII, which is assumed to be involved in anchoring the enzyme in the membrane, interacts with fatty acid-like substrates, resulting in an improved enzymatic activity. Therefore, we conclude that the membrane-anchoring domains can be used to increase the catalytic activity of fatty acid transformation enzymes.

1. Introduction

Fatty acids are second only to plant-derived carbohydrates as the most abundant renewable biomass, and they can be used for the production of chemicals, fuels, and plastics [1]. Large amounts of fatty acids are currently used to manufacture biodiesel and as monomers for plastics such as polyamides, polyesters, and polyurethanes. They are also used in the preparation of specialty chemicals including lactones, surfactants, plasticizers, and flavors [1–3].

Although the majority of fatty acid-derived products are currently made via chemical transformation processes, biocatalytic processes are steadily becoming a competitive alternative [3–10]. For instance, enzymatic conversions of long chain fatty acids into C9 to C13 carboxyl synthons such as ω-hydroxyfatty acids, ω-aminofatty acids, and α,ω-dicarboxylic acids have been reported; these synthons are usually produced via caustic pyrolysis or ozonolysis [8–10]. A representative example is the microbial production of ω-hydroxyundec-9-enoic acid and n-heptanoic acid from ricinoleic acid. The volumetric productivity and product yield of whole-cell biocatalysts have been reported as >0.6 g/L/h and 80%, respectively [8].

Two of the hurdles in the biocatalysis of fatty acids include poor solubility and dispersibility of the substrates in an aqueous environment, which is similar to the physiological environment of most enzymes. Different fatty acid states (e.g., oleic acid) have been reported in aqueous solutions such as emulsified microemulsions, micellar cubosomes, hexosomes, bicontinuous cubosomes, and vesicles [11,12]. These various states may affect substrate transport to the active site of enzymes. Therefore, it is important to increase the interactions between fatty acids and the enzyme to improve substrate access to the enzymes.

There are multiple ways to increase the interaction of hydrophobic molecules and soluble enzymes. One approach is to use surfactants to allow dispersion of the hydrophobic substrates into the reaction medium [13,14]. Another way involves introducing a substrate-binding domain that could interact with insoluble substrates. For example, the introduction of a carbohydrate-binding module to endoglucanases led to a significant increase in its catalytic activity on insoluble cellulose substrates when compared to wild type endoglucanases [15].

Lipases (EC 3.1.1.3, triacylglycerol hydrolases) are well known to convert hydrophobic fatty acid esters such as triglycerides that bear long-chain fatty acids [16]. Lipases are also very useful enzymes

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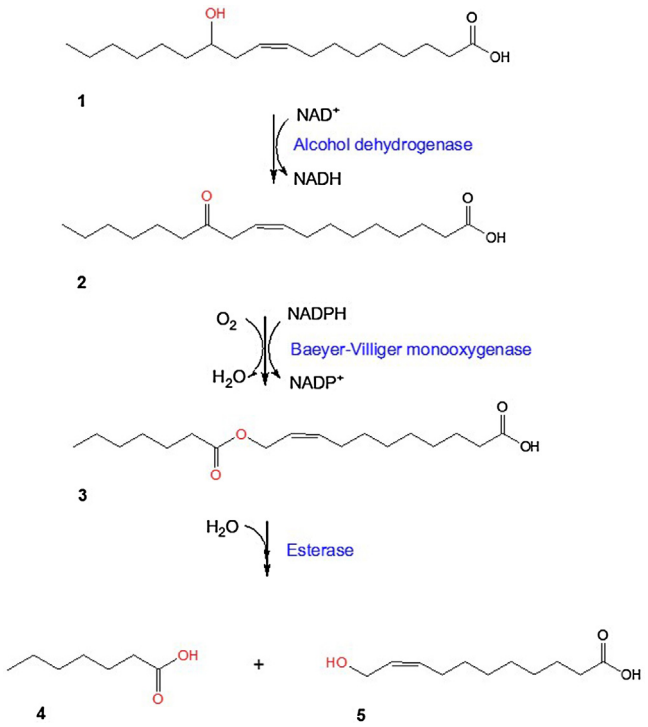
for organic synthesis, where they have been applied in the synthesis of a broad variety of chiral primary, secondary and tertiary alcohols or chiral carboxylic acids via kinetic resolution as well as by desymmetrization to access building blocks for pharmaceuticals. Furthermore, lipases have been also used for the synthesis of polyesters, sugar esters etc. This is summarized in an extensive book chapter [17] and many reviews [18–20]. Moreover, they are highly active in organic solvents as well as in non-conventional solvents such as ionic liquids [21]. Their high activity toward hydrophobic substrates and their ability to be active in the presence of hydrophobic organic solvents could be structurally related to the presence of a lid covering the entrance to the active site [22]. In the presence of a hydrophobic environment, this lid translocates, facilitating substrate entry into the active site and increasing the activity of the enzyme; this process is called interfacial activation. In contrast, typical esterases (EC 3.1.1.1, carboxyl esterases) prefer water-soluble substrates and do not exhibit interfacial activation [23]. Several years ago, we identified an esterase from *Pseudomonas fluorescens* DSM50106 (PFEII) [24]. PFEII was predicted to be a membrane-associated protein because the enzyme has a highly hydrophobic N-terminal region and because the isolation of the enzyme in its soluble and active form was facilitated by the addition of a detergent after recombinant expression in *Escherichia coli*.

To elucidate the molecular reasons for the unusual properties of PFEII, we investigated the conversion of several fatty acid-like substrates using PFEII and related carboxylesterases while focusing on the role of the highly hydrophobic N-terminal region of PFEII. Fusion of a soluble esterase from *P. fluorescens* SIK WI to the hydrophobic N-terminal domain of PFEII resulted in a significant improvement in the hydrolysis of the fatty acid ester **3** (approximately 6-fold increase in the k_{cat} value, Scheme 1).



Scheme 1. n-Heptanoic acid (**4**) and ω-hydroxyundec-9-enoic acid (**5**) were produced by esterases from ester **3**, which was obtained from ricinoleic acid (**1**) via 12-ketooleic acid (**2**) as described in Song et al. [9].

2. Materials and methods

2.1. Preparation of enzymes

The esterases from *P. fluorescens* WI SIK (PFEI), *P. fluorescens* DSM 50106 (PFEII), and *Bacillus subtilis* DSM 402 (BS2) were prepared as described in previous reports [24–26]. In brief, recombinant *E. coli* BL21 (DE3) was cultivated in LB broth, and cells were lysed via sonication. Then, the enzymes were purified via affinity chromatography on a Ni-NTA gel matrix (Qiagen, Crawley, United Kingdom). A column containing 3 mL of Ni-NTA resin was equilibrated with 15 volumes of buffer (20 mM Tris, 500 mM NaCl, and 5 mM imidazole), and the supernatant was loaded onto the column. The column was washed with 10 volumes of washing buffer (20 mM Tris pH 8.0, 500 mM NaCl, and 20 mM imidazole). The proteins were then eluted by increasing the imidazole concentration to 0.3 M. Fractions containing the recombinant proteins were pooled and dialyzed to remove imidazole. To isolate recombinant PFEII, a detergent (Emulgen 913 [2-[2-4-(nonylphenoxy)ethoxy]ethanol] from Sigma–Aldrich, St. Louis, MO, USA) was added prior to cell lysis at a concentration of 5 g/L to facilitate the extraction of the enzymes from cell membranes [24].

2.2. Protein electrophoresis and Western blot analysis

Cell lysates were prepared using a bacterial cell lysis buffer supplemented with a protease inhibitor cocktail (Roche, Zürich, Switzerland). Whole-cell lysates were separated on 10% sodium dodecyl sulfate-polyacrylamide gels, and the proteins were stained with Coomassie Brilliant Blue R-250. Western blot analysis was performed by semi-dry electroblotting. The QIAexpress detection system using a Ni–NTA–alkaline phosphatase conjugate (Qiagen, Hilden, Germany) was used to detect His-tagged proteins according to the manufacturer's instructions.

2.3. Measurements of initial esterase reaction rates

The initial reaction rates of the esterases were determined by measuring concentrations of the final products at different time points by gas chromatography/mass spectrometry (GC/MS). The concentrations of n-octanol, octanoic acid, and ω-hydroxyundec-9-enoic acid were measured to determine the hydrolysis rates of octylacetate, ethyloctanoate, and ester **3**. Enzymatic hydrolysis was performed in rotating (200 rpm) 1.5 mL Eppendorf tubes containing 50 mM sodium phosphate buffer (pH 7.5) at 30 °C. The purified enzymes (2.3 μg/mL) were added to the buffer solution containing various concentrations of the substrate (0.5, 1.5, 3.5, and 5.5 mM) and Tween 60 (1 g/L). The concentration of reaction products in the reaction medium was measured at $t = 1$, 3, 5, 10, and 20 min by GC/MS. One unit (U) of esterase activity was defined as the amount of enzyme releasing 1 μmol of n-octanol, octanoic acid, or ω-hydroxyundec-9-enoic acid under the reaction conditions.

2.4. Determination of the cell concentration

The cell concentration was determined by measuring the optical density (OD) at 600 nm and converting the OD value to cell dry weight using a conversion factor of 0.36.

2.5. Product analysis by GC/MS

The substrate and product concentrations were determined as described previously [9]. The reaction medium was mixed with an equal volume of ethyl acetate containing palmitic acid (5 g/L) as an internal standard. The organic phase was harvested after vigorous vortexing and then subjected to derivatization with N-methyl-N-(trimethylsilyl) trifluoroacetamide (TMS). The TMS derivatives were analyzed using a Thermo Ultra Trace GC system connected