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Chemical modification of fatty acid decarboxylases to pursue a anhydrous biofluid system for drop-in advanced biofuels

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ABSTRACT

This study introduces a protein chemical modification technique employing the chimera enzyme OleTP$_{RN}$-AldO, aimed at decarboxylating fatty acids to produce alkenes. This process is facilitated by the in-situ generation of hydrogen peroxide through polyol oxidation, catalyzed by AldO. The primary objective of this modification is to enable non-aqueous reactions, wherein enzymes are stabilized within ionic liquids exhibiting enhanced temperature resistance and improved activity, as previously demonstrated by Dr. Brogan and his research team with lysozyme and myoglobin enzymes. The chemical modification of OleTP$_{RN}$-AldO was assessed based on ζ.-potential and particle size data, revealing no significant alterations to the secondary structure composition. Future investigation into the enzyme's activity in ionic liquid environments is needed to deepen our understanding of the modified OleTP$_{RN}$-AldO mechanism.

Keywords: OleTP$_{RN}$-AldO. Chemical modification. Alkenes.

1 INTRODUCTION

In the past century, environmental concerns such as rising temperature and fossil fuel depletion have become paramount issues to humankind[1]. In response, the development of renewable energy technologies from different sources has gained momentum, offering solutions to reduce greenhouse gas (GHG) emissions in both the power generation industry and the transportation sector[2]. Advanced biofuels, also called drop-in biofuels, such as butanol, alkanes, and alkenes, are emerging as promising alternatives to traditional fuels, addressing this growing demand[3]. Concurrently, biological pathways such as utilizing enzymes are being increasingly applied in processes characterized by mild conditions of temperature and pH. Enzymes present an attractive technological solution due to their environmental and economic benefits, as well as the potential to utilize renewable energy sources[4].

Given the importance of this line of research, our research group has dedicated the past years to studying enzymes from the P450 superfamily. We have identified a novel cytochrome P450-based mechanism from *Rothia nassimurium* (OleTP$_{RN}$), which exhibits a preference for fatty acids decarboxylation over hydroxylation, forming alkenes[9]. OleTP$_{RN}$ demonstrated the ability to decarboxylate a variety of saturated and unsaturated substrates, offering advantages for biotechnological applications as oleic and linoleic acids are widely available in nature[9]. Additionally, a notable distinction of OleTP$_{RN}$ is its dimeric conformation, a characteristic not previously observed in the CYP152 family, but crucial for its activity[9]. We have also constructed the chimeric OleTP$_{RN}$-AldO, which was heterologously produced and shown to be active in the presence of glycerol, generating in situ hydrogen peroxide.

While biocatalysis offers numerous sustainability advantages, the necessity to operate in an aqueous environment poses challenges for industrial applications due to difficulties of product separation and purification[10,11]. Recent studies have focused on enabling enzymatic biocatalysis in non-aqueous environments. Anhydrous liquid proteins, a biomaterial technology developed by Dr. Brogan's research group, have shown promising results by conferring new properties to enzymes, enhancing their versatility[10]. Recent study utilizing anhydrous liquid protein technology with lysozyme and myoglobin model enzymes demonstrated significant improvements in temperature stability and catalytic activities[11]. Considering all the exceptional results in expanding enzyme activity into ionic liquids using anhydrous proteins, this work aims to apply this technique to the bifunctional enzyme OleTP$_{RN}$-AldO. Our goal is to develop a robust and sustainable alternative applicable to industrial conditions for producing alkenes from fatty acids, the precursors of advanced biofuels.

2 MATERIAL & METHODS

2.1 Enzyme production: A single-cell colony was cultured in modified Terrific Broth medium (TB) supplemented with appropriate antibiotics. Upon reaching an optical density (OD600) of 0.6, the temperature was reduced to 20 °C and maintained for 30 min. Then, the culture was induced with 200 µM isopropyl-β-D-thiogalactopyranoside (IPTG) (Invitrogen), 5 µM of $FeCl_3$, 0.5 M riboflavin, and 10 µg/mL tetracycline and grown overnight at 20 °C. The cells were harvested by centrifugation, and the resulting

pellet was resuspended in buffer (30 mM sodium phosphate, pH 7.5, 300 mM NaCl, 20 mM imidazole) and disrupted by sonication. The enzyme was purified using a Hi-Trap chelating HP column followed by a HiLoad 16/600 Superdex 200 pg column, with subsequent concentration by ultrafiltration. Heterologous expression and purification were confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12 %).

2.2 Chemical modification of enzyme: The enzyme modification comprised three main steps: cationization, nanoconjugation, and dehydration. Cationization of OleTP$_{RN}$-Aldo was carried out following a literature protocol applied to myoglobin enzymes, given their shared heme-containing nature[12]. This process involves homogenizing the surface charge by coupling a diamine to surface-oriented acidic residues mediated by EDC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide), resulting in a positive surface charge[10]. To assess the effectiveness of enzyme cationization, changes in the ζ.-potential value were monitored throughout the modification process. The ζ.-potential value represents the surface charge by quantifying electrostatic interactions between the enzyme and matrix particles, commonly used in immobilization studies[13]. Reaction was performed with 0.5 mg/mL of protein, using 250 mM of N,N,N′-Trimethyl-1,3-propanediamine, 150 mM of the peptide coupling reagent 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 50 mM of N-Hydroxysuccinimide, for 5 hours, with controlled pH at 7.2. The cationized protein was then added to a solution of anionic surfactant at pH 7.2 to allow the surfactant to conjugate to the surface of the protein overnight, creating an organic corona around the protein. The protein-surfactant nanoconjugate was then buffer exchanged, to remove excess surfactant, by repeated centrifugal concentration and resuspension in aqueous buffer (30 mM phosphate buffer pH 7.2). The nanoconjugate solution underwent dehydration through freeze-drying overnight. The modification of OleTP$_{RN}$-Aldo was characterized by measuring ζ.-potential and particle size using Dynamic Light Scattering (DLS).

2.3 Circular Dichroism: The secondary structure profile of OleTP$_{RN}$-Aldo was assessed in its native, cationized, and nanoconjugated forms using a sample concentration of 0.02 mg/mL. The molar ellipticities were calculated and analyzed using the online algorithm BestSel[14] to predict secondary structure composition.

3 RESULTS & DISCUSSION

3.1 Enzyme production

The purified enzyme demonstrated a satisfactory yield of 5.7 mg per liter of medium and exhibited a red color characteristic of heme enzymes.

3.2 Chemical modification and characterization of OleTP$_{RN}$-Aldo (Dynamic Light Scattering and Circular Dichroism)

The chemical modification of the native enzyme to transform it into an anhydrous liquid protein involves three main steps: cationization, nanoconjugation, and dehydration. Cationized OleTP$_{RN}$-Aldo exhibited an increase in ζ.-potential from -24 mV in its native form to 23.1 mV of the cationized, which confirms that the cationization reaction works.

In the nanoconjugation reaction, electrostatic complexation occurs as anionic polymer surfactant molecules stoichiometrically binds to form a nanoconjugated enzyme[10]. Following successful cationization, nanoconjugation promotes the conjugation of a surfactant to the surface of OleTP$_{RN}$-Aldo, forming an organic corona coat[11]. This step was also validated through ζ.-potential measurement, which reached -29 mV. The measurements revealed similar number-weighted hydrodynamic diameters for the native, cationized, and nanoconjugated forms (6.22±0.9, 6.97±1.16, and 6.95±0.76, respectively), indicating no aggregation or crosslinking of the enzyme during the modification process.

Circular dichroism (CD) profiles were measured during the modification process of OleTP$_{RN}$-Aldo to identify any changes in its secondary structure. As shown in Figure 1, the secondary structure profiles for the cationized and nanoconjugated steps closely resemble the native profile of OleTP$_{RN}$-Aldo. This was evident from the negative peaks in 208 and 222 nm and a positive peak in 198 nm, indicative of the predominance of α-helices[15]. Percentage rates of secondary structure (Table 1) revealed minimal changes between the modification steps. This confirms that both cationization and nanoconjugation preserved the enzyme's structure, as there were no significant alterations in the secondary structure.

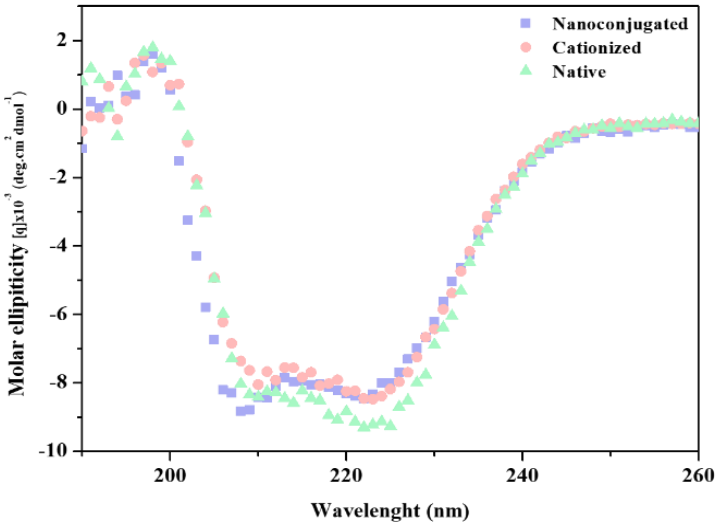

Figure 1: Circular dichroism spectrum of OleTP$_{RN}$-AldO in its native form (green), cationized (pink), and nanoconjugated (purple)

Table 1: Secondary structure composition of native, cationized, and nanoconjugated OleTP$_{RN}$-AldO

(%)	A-Helices	B-Sheets	Turns and others
Native	19.3	23.4	57.3
Cationized	18.3	22.5	59.2
Nanoconjugated	15.5	20.8	63.7

4 CONCLUSION

The chemical modification of $OleTP_{RN}$-AldO was successfully achieved, as evidenced by ζ.-potential and particle size data. Moreover, circular dichroism analysis revealed no significant alterations in the secondary structure composition across the native, cationized, and nanoconjugated profiles. To further elucidate the stability and activity of the enzyme post-modification, additional assay experiments will be conducted in ionic liquids. This ongoing research will provide valuable insights into the enzyme's performance under these conditions, facilitating the optimization of its applications in various biotechnological processes.

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