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Preparation and characterization of cross-linked enzyme aggregates (CLEAs) of recombinant thermostable alkylsulfatase (SdsAP) from *Pseudomonas* sp. *S9*

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Acetonitrile (PubChem CID: 6342)
Ammonium sulfate (PubChem CID: 6097028)
PEG4000 (PubChem CID: 448032)
Glutaraldehyde (PubChem CID: 3485)
Dichloromethane (PubChem CID: 6344)
DMF (PubChem CID: 6228)
Chloroform (PubChem CID: 6212)
DMSO (PubChem CID: 679)

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ABSTRACT

SdsAP, an efficient SDS degradation alkylsulfatase from *Pseudomonas* sp. *S9*, was immobilized in the form of cross-linked enzyme aggregates (CLEAs). Preliminary results revealed that over 80% activity of SdsAP-CLEAs was recovered using PEG4000 as the precipitating agent. Conditions for enzyme precipitation and cross-linking were further optimized. Compared to free SdsAP, SdsAP-CLEAs showed higher pH and temperature stability, and better tolerance to a certain organic solvents. Kinetic characterization analysis showed that SdsAP-CLEAs had higher affinity and catalytic efficiency than its soluble counterpart. Furthermore, SdsAP-CLEAs retained more than 60% of their initial activity after 10 batches of re-use at 50 °C and little or no loss of activity after one month at 4 °C. These results suggested that immobilization with CLEAs could improve the stability and operability of SdsAP, exhibiting a great potential application of SdsAP-CLEAs on SDS degradation in industry wastewater treatment.

1. Introduction

In recent years, surfactants have been used in a wide range of industries including, pharmaceuticals, cosmetics, textile, agriculture, biotechnology and advances for their interfacial functional capabilities [1]. Which have results in large quantities of surfactants and their derivatives discharged into aquatic and/or terrestrial environments [2]. Waste water contamination caused by excessive use of detergents is becoming a serious issue of concern, due to lower oxygenation potentials and consequent waterborne organism toxicity [3–5]. SDS is a widely used anionic surfactant that accounts for approximately 25–30% of the world's total synthetic surfactants [1]. Due to the increased use of SDS, its bioremediation has become important and gained much attention [6].

The application of biocatalysts in sewage treatment has increased considerably over the years, not only on a laboratory but also commercially [7–9]. Till date, some SDS-degrading strains have been found in *Pseudomonas* as well as in *Klebsiella*, *Acinetobacter*, *Pantoea* and so on [10–12]. And alkylsulfatases, which were defined as members of the metallo-β-lactamase superfamily that cleaves the sulfate ester bond to liberate inorganic sulfate and the corresponding alcohol, have been utilized as an ideal bio-enzyme for SDS degradation [13]. As reported, the alkylsufatase from *Pseudomonas* C12B specific to primary alkyl sulfates was able to degrade

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alkyl sulfate, alkyl ethoxy sulfate surfactants and aryl sulfonates; and the immobilized enzyme could efficiently degrade surfactants up to a concentration of 750 mg/dm^3 [14].

Generally, compared to whole cells, isolated enzymes are easier to retain activity and have higher catalytic efficiency in complex environment. However, few alkylsulfatases to our knowledge have been used in the biodegradation of waste SDS. Recent studies indicates that SdsAP, an alkylsulfatase isolated from *Pseudomonas* sp. *S9*, showed high catalytic activity in degrading SDS and could be overexpressed in a large quantity as the recombinant enzyme in *E. coli* [15], indicating a huge potential application in waste SDS treatment. However, maintaining the stability and activity with different parameters, including varied range of temperature, pH or other factors, are essential for the repeated and sustainable application of an enzyme in an industrial "green" process.

The enzyme immobilization usually results in catalyst stabilization against thermal and chemical denaturation [16,17]. In principle, enzymes are immobilized via three major routes: (i) binding to a support, (ii) encapsulation or entrapment, (iii) cross-linking (carrier-free, CLEAs) [18]. As a new carrier-free immobilization strategy proposed as an alternative to conventional immobilization methods on solid carriers [19–21], cross-linking of enzyme aggregates (CLEAs) are prepared by a regular procedure that involves salt- or solvent-induced aggregation of the enzyme or a desired combination of several enzymes, followed by chemical cross-linking to obtain a stable all-protein precipitate [22]. This kind of immobilization method has several advantages over the conventional ones, such as highly specific enzyme activity and low production cost due to the exclusion of an additional support [23,24]. It has been successfully used in some enzyme immobilization of CLEAs [17,19,25–27].

In this study, we present the manipulation of SdsAP-CLEAs with the refined immobilization parameters, which affect the final activity recovery of the biocatalyst. As the first reported immobilization for alkylsulfatase, the functional characterization has also been assessed.

2. Methods

2.1. Materials

The plasmid for pET-His-SdsAP recombinant construct was provided by Professor Xun Xu. *E. coli* bacterium BL21 (DE3) was used for plasmid expression. PEG4000 and PEK6000 were purchased from Sigma (St. Louis, USA). And all other reagents were from Sangon Biotech (China).

2.2. Expression and purification of SdsAP

Strains harboring the recombinant plasmid were cultured in the Luria-Bertani (LB) medium supplemented with 100 mg/L Amplicine at 37 °C. Protein expression was induced by 0.3 mM Isopropyl β-D-1-thiogalactopyranoside overnight at 16 °C when cells grown to an OD_{600} of 0.5–0.6. Cells were harvested by centrifugation, and cell pellets were resuspended in lysis buffer (50 mM pH 8.0 Tris-HCl, 300 mM NaCl and 5% glycerol). The resuspended cells were lysed by ultrasonication on ice, and the cell debris and protein aggregates were removed by centrifugation. Purification was achieved by Ni-NTA affinity chromatography (GE Healthcare). SDS-PAGE analysis was used to examine the molecular weight and purity of the purified SdsAP.

2.3. Preparation of SdsAP-CLEAs

The purified SdsAP protein was concentrated to about 30 mg ml^{-1} with an ultrafiltration system (Millipore, 30 kDa cut-off). Protein concentration was determined by the Bradford method using bovine serum albumin (BSA) as standard [28]. Cross-linked aggregates of SdsAP were prepared by mixing 100 μl SdsAP with 900 μl 50% (v/v) methanol, ethanol, isopropanol, *tert*-butanol, acetonitrile and acetone, 50% (m/v) ammonium sulfate, and 25% (m/v) PEG4000 and PEG6000, separately. After incubating at room temperature for 30 min, adding 10 mM final concentration of glutaraldehyde as cross-linker and incubating them at room temperature for 1 h. Then, CLEAs were collected by centrifuging at 8000 g for 10 min and washed with lysis buffer till no alkylsulfatase activity was detectable in the supernatant. Lysis buffer was added to a final volume of 1 ml for suspending the CLEAs.

Conditions for cross-linking were also optimized by modifying the concentration of SdsAP and cross-linked agent, regulating the cross-linked time and temperature, adding some BSA or sugar as cross-linked protective agent et al.

2.4. Determination of enzyme activities and calculation of activity recovery

The activity of alkylsulfatase was assayed using the stains-all solution method [15,29]. 10 μl diluted enzyme solution (150 μg ml^{-1}) was mixed with 490 μl of 50 mM Tris–HCl buffer (pH 8.0) containing SDS with a final concentration of 0.01% (w/v). After incubation at 70 °C for 5 min, the reaction was terminated by adding 20 μl sample solution into 980 μl stains-all solution. The absorbance of SDS quantitation was measured at 438 nm and compared with the standard curve of SDS. Enzyme activity (U) was defined as the amount of enzyme required for degrading 1 μmol SDS per minute.

The percent activity recovery (%) in the CLEAs was calculated by total activity of CLEAs (U) divided to total free enzyme activity used for CLEAs production (U) and then multiplied 100%.

2.5. Effects of the pH and temperature on SdsAP activity and stability

The optimal temperature for SdsAP and SdsAP-CLEAs were determined by testing the enzyme activities in 50 mM Tris–HCl buffer pH 8.0 at different temperatures (30–90 °C) for 5 min. Optimization of the pH was through the assay at 70 °C with a pH range of 4.0–10.0. The activities were expressed as percentages with respect to the maximum SdsAP activities.

In addition, the thermal stability of the free SdsAP and SdsAP-CLEAs was determined by measuring the residual activity of the enzyme, exposed to three different temperatures: 60, 70, and 80 °C in 50 mM Tris–HCl buffer (pH 8.0) for 1 h. Samples were removed at certain time intervals and the remaining activity was assayed under the standard conditions. The pH stability of free SdsAP and SdsAP-CLEAs were determined by pre-incubating the enzyme under pH (4.0–11.0) gradient conditions at room temperature for 1 h, and then the activity of residual enzyme under the standard condition was measured.

2.6. Effect of organic solvents on activity of soluble and immobilized SdsAP

The effect of organic solvents on the activity of soluble and immobilized enzyme was studied by adding 10% v/v solvents under the standard condition. The organic solvents used were listed as follows: glycerol, 2-propanol, methanol, ethanol, acetonitrile, ethylen glycol, acetone, dichloromethane, DMF, chloroform and DMSO.

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