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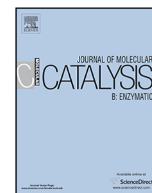

Preparation of cross-linked enzyme aggregates in water-in-oil emulsion: Application to trehalose synthase

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ABSTRACT

In this work, a novel method to prepare cross-linked enzyme aggregates (CLEA) in a water-in-oil emulsion was reported. Spherical CLEA of recombinant trehalose synthase (TreS) from *Meiothermus rubber* was obtained through the emulsion based process. By varying the experimental parameters, the optimum conditions for the preparation of spherical CLEA were determined. The optimum catalytic temperature and thermal stability of spherical CLEA were enhanced significantly compared with the free enzyme. Spherical CLEA was also more tolerant to metal ions and chemical denaturants. Optical and scanning electron microscopy analysis showed that instead of the amorphous clumping shape of conventionally prepared CLEA, spherical CLEA has a more structured ball-like appearance with a size of 20–60 μm in diameter that could be easily separated.

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1. Introduction

Trehalose is a non-reducing disaccharide composed of two glucose molecules linked by α-1,1-glycosidic linkage. Trehalose has been proven to be a good protective agent on biofilm and biological macromolecules under stress conditions [1,2]. There are five enzymatic processes of trehalose synthesis [3]: (1) trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase (TPS and TPP); (2) maltooligosyl trehalose synthase and maltooligosyl trehalose trehalohydrolase (TreY and TreZ); (3) trehalose synthase (TreS); (4) trehalose glycosyltransferring synthase (TreT) and (5) trehalose phosphorylase (TreP). In which, the enzymatic process involving TreS for catalyzing maltose to trehalose is more simple and low cost by using only TreS. Therefore, it shows great potential in producing trehalose [4]. Various TreS genes have been isolated and cloned from different bacteria and archaea [5–9]. In our previous work, a thermophilic *Meiothermus rubber* strain CBS-01 producing TreS was isolated from geothermal water, the TreS gene has been cloned and expressed in *Escherichia coli* [10,11]. However, the application of TreS is quite limited, due to the difficulty in recovering the enzyme from reaction mixtures and its intolerance and instability under extreme circumstances. All of these defects can be improved by immobilization of TreS.

The methods of enzyme immobilization can be generally divided into two types: carrier-bound (binding to or encapsulation in a carrier) or carrier-free (cross-linking of protein molecules) [12]. The immobilization of an enzyme on a carrier facilitates its separation and reuse. Moreover, improved enzyme performance, such as activity, stability, specificity or selectivity can often be achieved using a proper support and suitable enzyme–support reaction conditions [13]. However, the carrier-bound enzyme has an obvious disadvantage, which is the “dilution” of catalytic activity as the result of the large proportion of non-catalytic carriers. Besides, the carriers always have to be tailor-made, which is a laborious procedure, and costly. To date, the immobilization of TreS is limited to carrier-bound [14,15].

The technique of cross-linked enzyme aggregates (CLEA) developed by Sheldon's group is a kind of carrier-free immobilization of enzyme [16]. Generally, the procedure of preparing CLEA is composed of two steps: protein precipitation and subsequent cross-linking of the protein aggregates with a bifunctional agent (such as glutaraldehyde). CLEA is stable over wide ranges of pH and temperature and tolerance when exposure to organic solvents [17,18]. Despite the process of preparing CLEA is simple and cost effective, the CLEA prepared in a solution by conventional method forms amorphous clusters that may cause some questions. For example, the larger clusters (clumping) may result in diffusion constraints and low catalytic efficiency, while tiny clusters have poor recoverability [19].

To overcome the above problem, we developed a new method to prepare CLEA in a water-in-oil emulsion instead of in a solution.

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It involves precipitation of an enzyme in a solution, mixing the aqueous phase with an oil phase to form a water-in-oil emulsion and then chemical cross-linking of the enzyme aggregates within the emulsion droplets. It is conceivable that the CLEA obtained by emulsion-based process can form regular spherical particles with appropriate size under suitable conditions that would facilitate their recoverability and recyclability. In this work, the recombinant TreS of *M. rubber* was selected to explore the validity of this approach. The structure and some properties of prepared TreS CLEA were investigated.

2. Experimental

2.1. Materials

Trehalose and maltose were purchased from Sigma (St. Louis, MO, USA). Polyethylene glycol 8000 (PEG 8000), glutaraldehyde (25%), sorbitan monostearate 20 (Span 20), sorbitan monostearate 40 (Span 40), sorbitan monostearate 60 (Span 60) and sorbitan monostearate 80 (Span 80) were of analytical grade and obtained from Tianjin Dingguo Biotechnology Co. Ltd. (Tianjin, China). Soybean oil was purchased from local supermarket.

2.2. Production of TreS

Recombinant *E. coli* cells harboring plasmid pET-21aTreS containing the gene encoding of TreS from *M. rubber* were cultivated and induced as previously described [11]. The culture broth was centrifuged at 5000 × *g* for 10 min at 4 °C and the supernatant was decanted. The cell pellet was suspended in 50 mM phosphate buffer (pH 7.0) and cells were disrupted using a sonicator set to a power of 300 W for 10 min, 5 s bursts with 5 s intermissions. The mixture was centrifuged at 15,000 × *g* for 15 min, after that the supernatant was collected and TreS was purified with Ni-NTA column. The specific activity of purified TreS was 25 U/mg protein. Protein concentration was determined according to the method described by Bradford [20] using bovine serum albumin (BSA) as the standard protein. The purified enzyme was stored at 4 °C.

2.3. Preparation of CLEA

2.3.1. Conventional method

The method to prepare TreS CLEA in a solution was according to the procedure described by Schoevaart et al. [19]. 50 mg of TreS was dissolved in 1 mL phosphate buffer (50 mM, pH 7.0) and PEG was added up to the final concentration of 25% (w/v). The mixture was stirred for 30 min, and then glutaraldehyde was added slowly to final concentration of 2% (v/v). At the end of cross-linking 2 h at room temperature, the mixture was diluted and centrifuged at 3000 rpm for 15 min. The insoluble CLEA was collected and washed three times with phosphate buffer (50 mM, pH 7.0). Finally, the CLEA was resuspended in phosphate buffer and stored at 4 °C. This preparation was termed as conventional CLEA.

2.3.2. Emulsion based process

A certain amount of TreS was dissolved in potassium phosphate buffer (50 mM, pH 7.0), and then PEG 25% (w/v) was added. The solution was stirred for 30 min for complete precipitation of the enzyme and was used as aqueous phase. The oil phase for the emulsion was prepared by dissolving an emulsifier in soybean oil. The aqueous phase was added to the oil phase and the final solution was stirred for 1 min with magnetic stirring at 1500 rpm to form water-in-oil (W/O) emulsion. A necessary amount of glutaraldehyde was added to the W/O emulsion and the emulsion was stirred for 2 min then left to stand for 2 h. Subsequently the emulsion was centrifuged at 3000 rpm for 15 min to separate the insoluble CLEA. The CLEA was washed three times with phosphate buffer (50 mM, pH 7.0). Finally, the CLEA was resuspended in phosphate buffer and stored at 4 °C. This preparation was termed as spherical CLEA.

Different process parameters such as the range of used glutaraldehyde concentration (0.625%, 1.25%, 2.5%, 5%, 7.5% and 10%), enzyme concentration (10, 20, 30, 40, 50, 70 and 100 mg mL^{-1}), volume ratio of oil to water (5:1, 10:1, 20:1, 40:1 and 60:1), kinds of emulsifiers (Span 20, Span 40, Span 60 and Span 80) were optimized on the basis of the enzyme activity recovery.

2.4. Assay of activity

For free enzyme, 1 mL free TreS solution was added to 1 mL 2% (w/v) maltose in 10 mM, pH 6.5 of potassium phosphate buffer. The reaction mixture was incubated at 50 °C for 60 min. The reaction was terminated by heating at 100 °C for 10 min. For CLEA, 1 mL CLEA suspension was added to 1 mL 2% (w/v) maltose in 10 mM, pH 7.0 of potassium phosphate buffer. After incubation at 70 °C for 60 min, the mixture was centrifuged to collect the supernatant. The quantity of the generated trehalose was measured by a high-performance liquid chromatography (HPLC) system. The conditions of HPLC were as follows: Ø4.6 mm × 250 mm Hypersil-NH_2 column; acetonitrile–water eluent (80:20, v/v); 1.0 mL min^{-1} of flow velocity; 30 °C column temperature.

One unit (U) of enzyme activity was defined as the amount of enzyme catalyzed the formation of 1 μmol trehalose per minute.

2.5. Optimum catalytic temperature and pH

To determine the optimum temperatures and pHs of free TreS and spherical CLEA, enzyme activities were measured in the temperature range of 40–80 °C. The effect of pH on enzyme activities was determined in the pH range of 5–8. The results for optimum temperature and pH were given in relative form with the highest activity value as 100%.

2.6. Temperature and pH stabilities

Thermal stability assays were performed by incubating free TreS and spherical CLEA at various temperatures ranging from 40 to 80 °C for 2 h, and then measuring the residual activity, taking initial activity as 100%. The effect of pH on enzyme stabilities was determined at various pHs incubated at 50 °C for 1 h, then measured the residual activity, taking initial activity as 100%. In the range of 3–4 was 50 mM citric acid–sodium citrate buffer; in the range of 5–8 was 50 mM phosphate buffer; in the range of 9–11 was 50 mM sodium carbonate–sodium bicarbonate buffer.

2.7. Influences of metal ions and chemical reagents

Various concentrations of inorganic salts ($ZnSO_4·7H_2O$, $CuSO_4·5H_2O$, $AlCl_3$, $FeSO_4·7H_2O$) or chemical reagents (Tris, urea, guanidine hydrochloride) were added, and the residual activities of free TreS and spherical CLEA were examined.

2.8. Study of the morphology

The microstructures of conventional CLEA and spherical CLEA of TreS were detected by optical microscope (Olympus CX31, Japan) and scanning electron microscope (SEM, QUANTA200, FEI Co., USA). Before SEM observation, CLEAs were freeze-dried, and coated with gold in surface. The instrument accelerating voltage was set to 15 kV.

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