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Effect of additives on the purification of urease

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ABSTRACT

The effect of additives on the purification of proteins was investigated. The target protein studied here is the enzyme urease. Studies on the purification of urease from jack bean meal were carried out. 32% (v/v) acetone was utilized to extract urease from the jack bean meal. Further purification by crystallization with the addition of 2-mercaptoethanol and EDTA disodium salt dehydrate was carried out. It was found out that the presence of additives can affect the selectivity of the crystallization. Increases in both purity and yield of the urease after crystallization were observed in the presence of additives, which were proven using both SDS-PAGE and activity. Urease crystals with a yield of 69.9% and a purity of 85.1% were obtained in one crystallization step in the presence of additives. Furthermore, the effect of additives on the thermodynamics and kinetics of urease crystallization was studied.

1. Introduction

Proteins, especially, crystalline proteins, are of growing importance in areas of medical applications, chemical and food industries [1,2]. In general, proteins cannot be used without further purification [3]. Especially, in the pharmaceutical area, highly purified proteins are strongly needed and also of high value. As a commonly used purification method in industry, crystallization can also be utilized in the purification of proteins. For products used in technical applications, industrial crystallization can serve as an inexpensive alternative for purification of proteins [4].

Additives have been a very attractive tool in the area of protein crystallization. Utilization of additives is a common practice in crystallization. A large number of scientific and industrial crystallization processes are carried out using additives [5,6]. It was reported that additives can be applied to protein crystallization as well [2]. Additives as ionic liquids, nanoparticles and small molecule chemicals have been proven crucial to the crystallization of proteins [2,7,8]. The effects of additives to increase the crystal size and to preserve the protein structure have also been reported in the literature [8]. Additives in form of ionic liquids were found to improve the efficiency of protein crystallization as well [2].

The effect of additives on the selectivity of protein purification was studied here. A naturally outcome protein mixture (from the before used acetone extraction) was used for the investigations. The purification of urease from jack bean meal by crystallization has been carried out. The enzyme urease, obtained from jack bean meal (*Canavalia ensiformis*), was first crystallized by Sumner in 1926 [9]. The total protein content of jack bean meal is approximately 22% and the urease content varies between 0.07% and 0.14% of total mass [10]. Besides urease, other main protein components inside jack bean meal are canavalin, concanavalin A and concanavalin B. Canavalin, the major protein storage of jack bean, makes up almost half of the protein content [11]. Concanvalin A occurs in high concentration in jack bean as well [12] and concanavalin B constitutes 0.9% of total protein [13].

Since the first discovery of urease crystals, there have been many works concerning the crystallization of this enzyme [14]. In order to improve the purity of urease and the process reproducibility, multi-step recrystallization was introduced [15]. However, for industrial crystallization, one of the most important criteria is to obtain maximum product in minimum steps [14]. The use of additives, EDTA and 2-mercaptoethanol were found essential to improve the efficiency of urease purification [16]. In the work of Weber et al. [14], the yield of urease was increased to 55% with a specific activity of 550 U/mg in the presence of 50 mM 2-mercaptoethanol. In their works, the influence of additives was

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mainly considered to bind the metal ions to maintain the enzymatic activity [14]. Whether the additives have an effect on the crystallization process itself still remains unanswered.

As a consequence, the present work deals with (a) the effect of additives on the purification of urease, (b) the influence of additives on the thermodynamics and kinetics in urease crystallization.

2. Material and methods

2.1. Materials

All chemicals used in this work were reagent grade or higher. Jack bean meal (JBM) was purchased from A. L. Jowitt (612 Runway Bay Drive, Bridgeport Texas 76426 USA, screen 40#). Acetone (Carl Roth, Germany) was used for the extraction. Sodium citrate buffer was prepared with a pH value of 6.5 and a concentration of 25 mM. Poly(ethylene glycol) (molecular weight 6000 g/mol, Carl Roth, Germany) was used as the precipitant for urease. The additives studied here were 2-mercaptoethanl (Carl Roth, Germany) and EDTA disodium salt dehydrate (Carl Roth, Germany). Urea was purchased from BASF and used for urease activity assay.

2.2. Urease crystallization

The crystallization of urease was carried out using batch crystallization. The experimental procedures were designed based on the recrystallization method described in literature [17]. Before crystallization, urease was extracted from the jack bean meal (JBM) using 32% (v/v) aqueous acetone. The extraction was carried out at 28 °C for 7 min under 700 rpm of mechanic stirring. The suspension was then separated using centrifugation (Sigma 2-16KL; Sigma Laborzentrifugen GmbH) and the supernatant was positioned at 4 °C overnight. The protein mixture (containing urease and impure proteins) obtained were then separated and dissolved in a buffer solution at 50 °C for 4 h. The undissolved proteins were separated by centrifugation. The clear protein solution was used for crystallization.

Crystallization was carried out by mixing protein solution and PEG6000 solution together at 4 °C. Different initial protein concentrations and PEG6000 concentrations were studied here. For the experiments with the additives, 2-mercaptoethanol and EDTA disodium salt dehydrate were added to the PEG6000 solution. After the dissolution of additives, the precipitant solution and protein solution were mixed together for crystallization. The final concentrations of additives were 0.5 μL/mL 2-mercaptoethanol and 0.5 mg/mL EDTA disodium salt dehydrate. All other parameters remained the same. The crystallization behaviors were analyzed using a microscope (Keyence VHX-500F). The crystals obtained from the batch crystallization were separated and used for further studies.

2.3. Nucleation point measurement

The nucleation point was investigated using microtiter plates. The nucleation point studied here is defined as the protein concentration at which crystallization of urease started to occur (the upper limit of the metastable zone) at a certain temperature. The maximal waiting time was 24 h. The measurements have been carried out using a volume of 300 μL per well. Protein and precipitant solutions were prepared separately and mixed to reach a desired final composition of the solution. A protein crystal mixture (obtained from the acetone extract) was used to prepare the protein solution. Subsequently, the microtiter plates were sealed and immersed in a water bath for 24 hours. Proteins solutions were visually inspected by the means of an optical microscope (Keyence VHX-500F). The data were collected for the urease-PEG6000 systems in 25 mM sodium citrate buffer pH 6.5 with protein concentrations up to 1 mg/mL and PEG6000 concentrations of 10 wt%. Experiments were divided into three groups. In group 1, no additives were involved in both the dissolution and the crystallization step. The protein and the precipitant solutions were prepared using a buffer without additives. In group 2, the additives were included in the crystallization step. In this case, the protein solution was prepared with a usual buffer, while the PEG6000 solution was prepared using a buffer containing additives. In group 3, additives were included in the dissolution step only. Proteins were dissolved using a buffer solution containing additives and a usual buffer was utilized to dissolve PEG6000. The concentration of additives was adjusted to 0.5 μL/mL 2-mercaptoethanol and 0.5 mg/mL EDTA disodium salt dehydrate as the abovementioned.

2.4. Urease solubility measurement

The solubility of urease crystals was determined using the isothermal batch method. The urease crystals utilized for solubility measurement were recrystallized 3 times, the purity of which was verified using a SDS-PAGE and the specific activity of which was 530 U/mg. As performed in literature [18], the solubility measurements were performed in temperature controlled double-walled beakers. A slurry of urease crystals was added to the beaker with a solution at defined pH and precipitant concentration. The solution was kept gently agitated for 24 h. 200 μL suspension was taken for analysis. Centrifugation was performed under the same temperature as the solubility measurement. The amount of protein in the supernatant was determined by UV spectrophotometer. The solubility data were determined in a 25 mM sodium citrate buffer with 10 wt% PEG6000. The effect of additives on the solubility of urease was investigated here.

2.5. Urease assays

The concentration of proteins was measured using a Bradford assay. The enzymatic activity of urease crystals and urease solutions was determined by measuring the amount of ammonia produced by the decomposition of urea using the Berthelot color reaction, and the steps of which were described in literature [3]. One urease unit is defined as the amount of enzyme which hydrolyzes 1 μmol of urea per min at 38 °C. The purity of urease crystals was evaluated by SDS-PAGE.

3. Results and discussion

3.1. Urease solubility

Due to the strong absorbance of PEG6000 at 280 nm, the miniature column [20,21] technique is not suitable for determining urease solubility in own case. The isothermal batch method, which is widely used in small molecular chemicals, was utilized here in the determination of solubility urease. The data was measured in two groups, one with additives and one without additives. As shown in Fig. 1, the solubility of urease increases with the increase of temperature in both groups and the protein concentrations in both groups are approximately the same, indicating that the presence of additives does not affect the solubility of pure urease.

3.2. Effect of additives on the crystallization behavior of urease

The effect of additives on urease crystallization was investigated using batch crystallization. The crystallization was carried out using solutions containing different amounts of protein and PEG6000. The result is shown in Fig. 2. The images in the upper row A, B and C show