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CRYSTALLIZATION OF ALKALINE PROTEASE AS A MEANS OF PURIFICATION PROCESS

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Abstract – A purification protocol of alkaline protease purification using crystallization was developed by investigating the effects of pH, temperature, initial enzyme concentration, salt (as crystal inducer) concentration, and the presence of impurity proteins. A commercial alkaline protease solution was used as a starting material and NaCl was used as a crystal inducing salt. The crude enzyme solution was first diafiltered against deionized water and then concentrated by ultrafiltration. To the enzyme concentrate appropriate amount of NaCl was added to induce the crystallization which was lasted for 24 hours, and the enzyme crystals formed were filtered and washed with deionized water before being resolubilized. Crystal habit was typical needle shape, and the reaction order of its formation was estimated to be 1.53. The crystallization was strongly influenced by initial enzyme concentration. Solubility of alkaline protease at 25 °C was 24.8 mg/ml, which was about one half of that of 4 °C. Enzyme recovery yield of the purification process including the crystallization step ranged 50 to 60 %. The crystallization step was shown to successfully exclude impurity proteins from their habits as evidenced by gel permeation chromatography. The optimum condition for the crystallization was: pH 9.0, 25 °C temperature, ca. 53 mg/ml or higher enzyme concentration, and minimum 5 % (w/w) NaCl concentration. In summary, an enzyme purification protocol based on crystallization was established, which can be applied to obtain a higher-purity alkaline protease solution on a large scale.

Key words: Enzyme Crystallization, Alkaline Protease, Enzyme Purification, Crystallization Kinetics

INTRODUCTION

In the chemical and pharmaceutical industries, crystallization process has been widely used in the final processing steps, since it can provide higher-purity solids, usually over 95%, that are easier to handle and store compared to liquid. Crystallization of biomolecules such as proteins and enzymes is used to obtain a pure solid for X-ray diffraction analysis and for the studies of optical, spectroscopic, electrical, thermal, and mechanical properties. Literature reports were focused on the studies of solubility diagrams and the factors influencing them [Giege et al., 1986; Ataka and Asai, 1988; Abergel et al., 1991; Sazaki et al., 1994; Ataka, 1986; Heinrichs et al., 1993; Ries-Kautt and Ducruix, 1989; Howard et al., 1988], mechanism of crystallization [Sazaki et al., 1993], crystal growth rate [Forsythe and Pusey, 1994; Mitrovic, 1994; Durbin and Feher, 1986], and crystallization kinetics [Pusey and Naumann, 1986]. However, research articles on a large scale protein purification by crytallization are relatively scarce.

Like the crystallization process of small, inorganic molecules, protein crystallization goes through three distinct stages of nucleation, crystal growth, and growth cessation. Unlike inorganic molecules, protein crystallization is influenced by biological parameters such as the presence of contaminating biomolecules, etc. And, due to the proteins' structural flexibility and intrinsic chemical characteristics protein crystallization is very sensitive to environmental conditions [Giege and Ducruix, 1992]. The physicochemical parameters known to influence the protein crystallization process include protein concentration, pH and temperature, ionic strength, solution purity (presence of impurities), solution's density and viscosity, pressure, vibration, and magnetism.

Among these, such operational parameters as protein concentration, salt type and concentration, pH, and temperature are critical from the large scale processing point of view. Salt concentration, pH, and temperature can directly alter protein's solubility characteristics and thus the solubility curve. Initial protein concentration can determine the amount of crystal formed as well as crystal habits and purity. A solution in which a protein concentration is too low may never be crystallized regardless of the amount of the salt added. On the other hand, when the protein concentration is too high amorphous in stead of crystalline precipitation is likely to occur upon salt addition. Therefore, a certain optimum 'window' of the operating variables exists and should be identified to develop a successful crystallization process.

In this study, using alkaline protease and its detergent-grade solution as a model protein and the starting material, respectively, a purification protocol based on the enzyme crystallization process was established. The effects of the aforementioned, critical operating parameters as well as the crystallization kinetics were investigated. The crystallization process was evaluated focusing on the recovery yield (by the enzyme activity maintenance) and the crystal purity (by gel permeation chromatography).

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METHODS AND MATERIALS

The detergent-grade alkaline protease solution was Opticlean 440® obtained from Solvay Enzymes, Inc. (Elkhart, IN, USA). The protease is an serine endopeptidase and classified as bacterial alkaline protease (E.C.3.4.21). It has pH optimum of 10.0-11.0 and 10 minute half life at 70 °C [Cowan et al., 1985], 27.7 kD molecular weight, and isoelectric point of 9.4 [Takami et al., 1990]. The Opticlean 440® solution is known to contain ca. 40 % (w/w) propylene glycol as a stabilizer in addition to other low-molecular-weight substances.

The enzyme activity assay was based on its ability to hydrolyze casein substrate at 40 °C in borate buffer (pH 8.5). To 5 ml of 1.6 % (w/w) casein solution at pH 8.5 and 40 °C, 1 ml of the enzyme solution was added and incubated for 20 and 40 minutes. After the incubations, unhydrolyzed substrates were precipitated by adding 5 ml of TCA (trichloroacetic acid) solution containing 0.11 M TCA, 0.22 M sodium acetate, 0.265 M acetic acid, and aliquot of Polysorbate 80. The mixture was further incubated for 30 minutes before being centrifuged under ×1,800 g for 15 minutes at 10-15 °C. The absorbance of the supernatant was read at 275 nm. The absorbance difference between the 20 and 40 minutes' incubations were used to calculate the activity. One alkaline protease unit (APU) was defined as the enzyme activity to generate 4 micromoles of tyrosine per minute under the assay condition, and was calculated by the following equation:

$$\text{APU/ml} = \frac{\Delta A \times 11}{(1.38 \times 4) \times 20 \times V_e} \quad (1)$$

where, ΔA=absorbance difference between 20 and 40 minutes' incubations, 11=volume of the final reaction mixture including TCA solution (ml), 1.38=absorbance of 1 micromole/ml tyrosine at 280 nm, 20=incubation time differential (min), and V_e= volume of enzyme solution added (ml).

The detergent-grade alkaline protease solution (pH 5.2) was first diafiltered against 5 volumes of deionized water to remove low molecular weight stabilizers including propylene glycol using a hollow fiber ultrafiltration system equipped with 10,000 MWCO membrane (model MiniKros®-2, Microgon Inc., Laguna Hills, CA, USA). The pH of the diafiltered enzyme solution was adjusted by either 0.1 M $NaHCO_3$ or 0.1 M HCl prior to enzyme concentration by Amicon stirred cell (100 ml size) equipped with the same MWCO membrane. To the concentrated enzyme solutions, NaCl was added to various concentration levels to induce the crystallization for about 24 hours. All the crystallization experiments were performed at room temperature, except one set of experiments performed at 4 °C to investigate the temperature effect. The crystals formed were filtered by 0.45 micron membrane filter, washed with 3 ml of deionized water, and then oven-dried at 30 °C. The dried crystals were rehydrated with deionized water for activity measurement for yield calculation and for gel permeation chromatography for purity assessment. Fig. 1 shows the flow diagram of the crystallization process.

Protein assay was performed following the Bradford method using the protein assay kit from Bio-Rad Chemical Division (Richmond, CA, USA). Bovine plasma gamma globulin was used as a standard protein. Sephadex G-100 column (1.0×20 cm) was used in the gel permeation chromatography. Equilibrium and elution buffers were deionized water at 0.5 ml/min flowrate. The absorbance at 280 nm was monitored by the Econo UV detector (Bio-Rad Lab, model EM-1, Hercules, CA, USA).

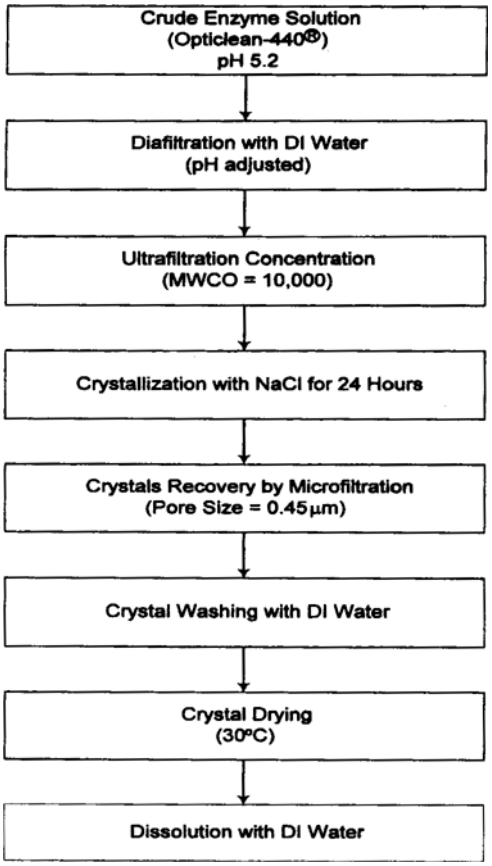

Fig. 1. Flow diagram of crystallization process.

RESULTS AND DISCUSSION

1. Effect of Temperature

Temperature effect was evaluated at two representative temperatures; refrigeration temperature (4 °C) and room temperature (25 °C). Enzyme solution of 53.3 mg/ml concentration was crystallized by adding 5 % (w/w) NaCl at pH 9.0. The crystal yield were 9.4 and 50.8 % for 4 °C and 25 °C, respectively (see Table 1). The lower yield at the lower temperature is probably due to the solubility differences, since most proteins show decreased solubility at higher temperature. One classical study on carboxyhemoglobin solubility indicated that the protein was ten times more soluble at 0 °C than at 25 °C [Dixon and Webb, 1961]. In our study, each solubility was determined by measuring the saturated enzyme concentration in the filtrate phase after filtering off the crystals. Solubilities of 24.8 and 48.3 mg/ml were obtained at 25 and 4 °C, respectively. Note that these values were the solubilities in the presence of 5 % (w/w) NaCl. The solubility at 25 °C was about one half of that of 4 °C. It strongly suggests the crystallization process be carried out at 25 °C rather than at 4 °C.

2. Effects of pH and NaCl Concentration

To the concentrated enzyme solution (53.3 mg/ml) at 25 °C,

Table 1. Effect of temperature on alkaline protease crystallization [pH 9.0; 5 % (w/w) NaCl]

Temperature (°C)	Total activity in pre-crystallization enzyme solution (APU)	Crystals		
		Mass (mg)	Total activity (APU)	Recovery yield (%)
4	5358	67	502.5	9.4
25		382	2865	50.8

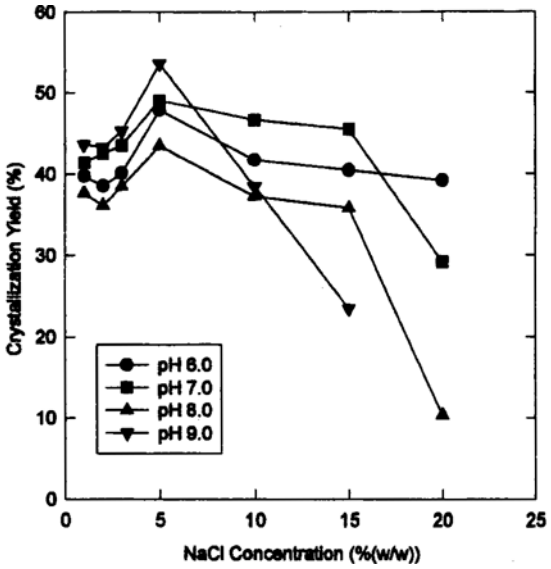

Fig. 2. Effect of pH and NaCl concentration on alkaline protease crystallization (at 25 °C).

NaCl was added to 5, 10, 15, and 20 % (w/w) at pH 6, 7, 8, and 9. NaCl was chosen as the crystal inducer because it is an effective salting-out electrolyte [Timasheff and Arakawa, 1988] and yet does not denature proteins by not binding to proteins [Arakawa and Timasheff, 1982]. Fig. 2 shows the yield data of the washed crystals under each condition. Crystallization yield was calculated as the total enzyme activity in the initial (or pre-crystallization) solution divided by that in the filtered crystalline phase. NaCl concentration of 5 % (w/w) and pH 9.0 resulted in the highest recovery yield. At each pH, the recovery yield reached a maximum at 5 % (w/w) NaCl and declined sharply as NaCl concentration increased. This result contradicts the previous reports on solubility changes as a function of NaCl concentration for lysozyme [Shih et al., 1992] and for carboxyhemoglobin [Timasheff and Arakawa, 1988]. Both groups reported the solubility decreased rather monotonously with an increase in NaCl concentration. In this study, as the salt concentration increased the yield declined more rapidly at higher pHs. At 5 % (w/w) NaCl concentration, the best pH for the crystallization appeared 9.0 which is the closest pH to the pI value (pH 9.4) among the pHs tested. This is probably because of proteins' intrinsic property that a protein has a minimum solubility around its pI where the protein has a zero net charge.

3. Effect of Initial Enzyme Concentration

By diluting the concentrated enzyme solution (53.3 mg/ml) with deionized water, the enzyme concentration was adjusted

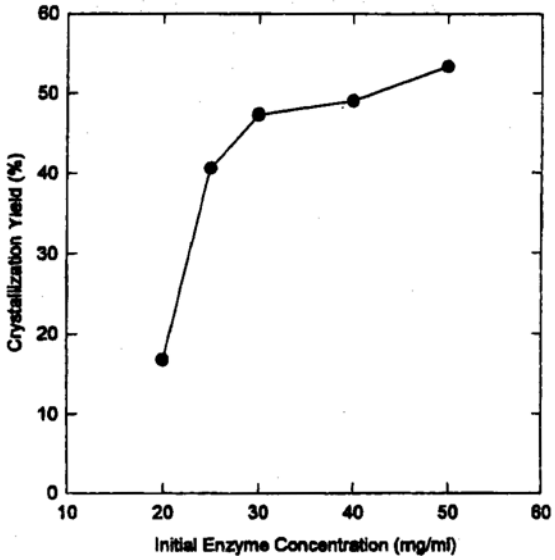

Fig. 3. Effect of initial enzyme concentration on alkaline protease crystallization [pH 9.0 and 5 % (w/w) NaCl].

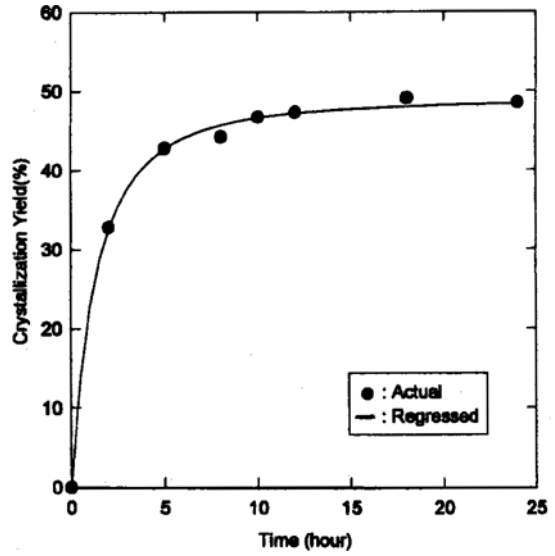

Fig. 4. Time course profile of alkaline protease crystallization [pH 6.0, 5 % (w/w) NaCl, 25 °C].

to 18.0, 26.7, 31.4, 42.7, and 53.3 mg/ml. To each solution 5 % (w/w) NaCl was added at pH 9.0 to induce the crystallization. As seen in Fig. 3, more crystals were obtained at higher initial enzyme concentration. However, the crystal yield seemed to plateau off to approximately 50 % from about 30 mg/ml concentration range. It is interesting to note that crystallization occurred to some degree at 18.0 mg/ml enzyme concentration, which is below the solubility determined (24.8 mg/ml). In protein salting-out, Shih et al. [1992] proposed that the solubility of such enzymes as α-chymotrypsin and BSA (bovine serum albumin) was strongly affected by their initial concentration prior to salting-out; higher solubility was obtained from higher initial concentration and vice versa. Our result suggests that the alkaline protease solubility is also influenced by the initial enzyme concentration in the same manner. Also, the crystal habit

took more of a needle-like shape at higher enzyme concentration.

4. Kinetics of Alkaline Protease Crystallization

To the concentrated enzyme solution (48.5 mg/ml at pH 6.0), 5 % (w/w) NaCl was added to induce the crystallization. To determine the crystallization profile for 24 hours, aliquots of the solution was sampled at various time intervals, filtered, washed, rehydrated, and measured for protease activity. The result is shown in Fig. 4. Over 90 % of the crystallization occurred within 5 hours and by 18 hours nearly all the crystallization was completed. The time necessary for complete crystallization was little affected by either pH or salt concentration (data not shown).

Assuming that the overall driving force for the crystallization process is the degree of supersaturation, i.e., the concentration difference between the solution and the saturation, a general mathematical model describing crystallization kinetics can be expressed as follows:

$$-\frac{dC}{dt} = k(C-C^*)^i \quad (2)$$

where C and C* denote the in-solution and the saturation enzyme concentrations, respectively, in mg/ml, t is the time in hour, and k is a reaction rate constant. The i value that could best fit our experimental profile in Fig. 4 was 1.53, and the k value was determined to be 0.139 hr^{-1}. The i values previously reported in glycine crystallization was 1.6 [Li and Rodriguez-Hornedo, 1992]. Our result suggests that the reaction kinetics for alkaline protease crystallization was similar to that of the amino acid.

5. Effect of Impurity Proteins

It has been reported that impurity proteins generally reduced the rates of nucleation and crystal growth and also affected the shape of crystal habit [Abergel et al., 1991]. From the crystallization study with TEWL (turkey egg white lysozyme), they showed that unless the impurity proteins had the structural similarity with the target protein the impurity proteins were not incorporated into the crystal habits, suggesting the structurally heterogeneous impurity proteins could be separated out by crystallization. In our study, BSA or BSA and α-globulin were added as impurity proteins at 15 mg/ml concentration to the alkaline protease solution (53.3 mg/ml concentration). Crystallization was induced by adding 5 % (w/w) NaCl at pH 7.0 and 25 °C, and the resulting crystals were resolubilized with deionized water and analyzed by gel permeation chromatography.

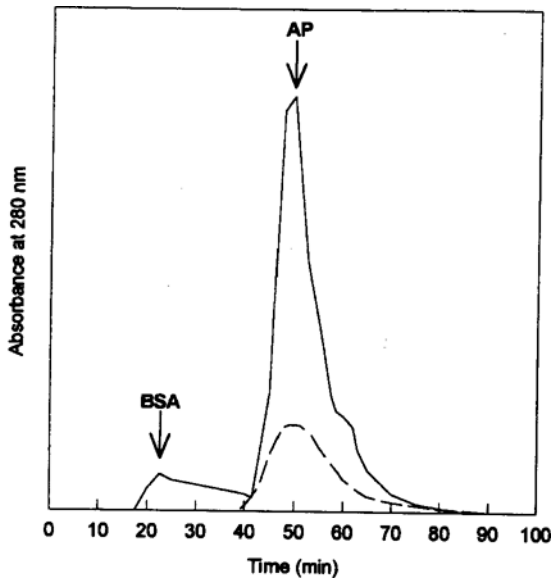

Fig. 5. Gel permeation chromatograms of rehydrated alkaline protease crystals from BSA-added solution (—: before, ···: after crystallization).

The recovery yields were 49.1, 35.7, and 30.6 % for pure (no impurity added), BSA added, and BSA and α-globulin added solutions, respectively, indicating the crystal yield decreased rather sharply in the presence of impurities. And, as seen in the gel permeation chromatograms in Fig. 5 the BSA was successfully excluded from the crystals.

CONCLUSIONS

Alkaline protease in the crude solution was successfully crystallized to a high purity by sequential steps of diafiltration, concentration, crystallization, and microfiltration. NaCl was used as the crystallization inducer. The crystal habit was of typical needle-shape. The crystallization process was completed in less than 20 hours. Recovery yield of the enzyme from the initial crude solution through the resolubilized crystals ranged 50 to 60 %. The effects of such processing parameters as pH and temperature, initial enzyme concentration, and NaCl concentration were evaluated, and the optimum condition was determined as: about 50 mg/ml initial enzyme concentration and 5 % (w/w) NaCl concentration at 9.0 pH and 25 °C. The presence of impurity proteins reduced the crystal yield by 30 to 40 % but the crystallization could successfully exclude them from the crystal habits. Thus the crystallization process could be used as a purification step to purify the crude enzymes to pharmaceutical or finer grade.

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NOMENCLATURE

- ΔA : absorbance difference between 20 and 40 minutes' incubation, in Eq. (1)
- V_e : volume of enzyme solution added [ml], in Eq. (1)
- C : enzyme concentration in solution [mg/ml], in Eq. (2)
- C* : enzyme concentration at saturation [mg/ml], in Eq. (2)
- t : crystallization time [hour], in Eq. (2)
- k : reaction rate constant, in Eq. (2)
- i : order of reaction, in Eq. (2)

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