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Optimization of reversed micellar extraction of chitosanases produced by *Bacillus cereus*

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Abstract

The fermentation broth of *Bacillus cereus* NTU-FC-4 was precipitated with 70% acetone to obtain crude enzyme. Chitosanases in the crude enzyme were then extracted by reversed micelles. It was found that proper amount of crude enzyme should be first dissolved in the 50.0 mM phosphate buffer containing 96.0 mM sodium chloride to make a 1.0 mg/ml protein solution. After adjusting the pH of the crude enzyme solution to a value of 4.0, the aqueous solution was mixed with an organic solution, the isooctane containing 102.3 mM of the anionic surfactant AOT (sodium 1,2-bis(-2-ethylhexyl) sulfosuccinate). The mixture was shaken in reciprocating shaker bath at 15 °C for 85 min to solubilize the target enzymes in the reversed micelles formed in the organic phase, thus completed the forward extraction. Then, the reversed micellar phase was separated from the aqueous phase, and allowed to mixed with 50 mM phosphate buffer containing 1.0 M potassium chloride at pH 10. After mixing the two solutions at 40 °C for 40 min, the target enzymes in the reversed micelles transferred back to the aqueous solution. The processes recovered approximately 70% of total activity of chitosanases. The purity of the chitosanases was increased to 30-fold as compared to that of the fermentation broth, and the specific activity of the final product was 60.3 unit/mg.

Keywords: Chitosanases; *Bacillus cereus*; Reversed micelles; Extraction; Optimization; Enzyme

1. Introduction

Chitosan has been recognized as a health promoting food supplement since it possesses antibacterial activity [1–5], hypocholesterolemic activity [6–8], and anti-hypertensive action [9]. However, increasing attention has recently been given to the conversion of chitosan to oligosaccharides. Chito-oligomers show interesting biological activities, such as antitumor activity [10–12], immuno-enhancing effects [13], protective effects against infection with some pathogens [14,15], antifungal activity [16], and antimicrobial activity [3,4].

Chitooligosaccharides can be prepared by chemical or enzymatic hydrolysis. However, drawbacks, such as acid corrosion, the need for neutralization after reaction, and low yield of products with degree of polymerization (DP) equal or larger than 6 (DP) limit the practical application of acid hydrolysis. Chitosanases, which represent a class of hydrolytic enzymes, are found in bacteria, fungi, and plants [17]. Among these, bacterial chitosanases appear to be especially useful for the production of chito-oligomers. *Bacillus cereus* NTU-FC-4, a strain originally isolated from Taiwan soil by Hung [18] was found to be able to produce high amounts of extracellular chitosanases along with a minor amount of chitinase during fermentation. However, a practical method for extraction and purification of these enzymes from the culture broth needs to be established in order to fully explore the industrial applications of these enzymes. The chitosanases from various sources have been purified using the conventional protein purification techniques including ammonium sulfate fractionation, gel filtration, ion-exchange chromatography, and isoelectric focusing [17]. These methods are often used in laboratory practice, but scaling-up of them for commercial production might encounter the problem of limited processing capacity.

Reversed micelles are the aggregates of amphiphilic molecules in an organic solvent. When the reversed micelles are formed with an anionic surfactant, such as AOT, they would display a surface of negative charge surrounding an aqueous

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polar core. Because of the electrostatic interactions, the positively charged proteins could transfer from the aqueous phase to the inner core of the reversed micelles, thus effect a separation [19–25]. Reversed micellar extraction is an attractive separation method for large-scale operation because the process could be carried out using the existing liquid–liquid extraction system in the chemical and biochemical industries. Factors affecting the performance of the reversed micelle system are rather complicated, including the nature and concentration of target protein, pH, and ionic strength of the aqueous phase, extraction temperature, type and concentration of the surfactant, and the processing time [26–28]. Therefore, investigation of the effects of these processing parameters on the performance of reversed micellar extraction often requires tedious experimental works. In this study, all of the processing parameters were considered and pre-tested to screen the factors that had a dominant effect on the process performance. Then, the response surface methodology was used to develop the mathematical functions describing the relationships between these factors and the recovery rate of chitosanases during extraction. Thus, the optimal processing conditions for the purification of chitosanases by the reversed micellar extraction could be established.

2. Materials and methods

2.1. Materials

Chitin, glucosamine, dioctyl sulfosuccinate sodium salt (AOT), potassium chloride, polyacrylamide, and Coomassie brilliant blue R-250 were purchased from Sigma Chemical Co. (St. Louis, MO). Crab chitosan with 66% deacetylation was obtained from Ohka Enterprises Co. (Kaohsiung, Taiwan). Other materials used in this study included Soyton and yeast extract (Difco Lab. Sparks, MD), 2,2,4-trimethylpentane (isooctane) (Mallinckrodt Baker Inc., Phillipsburg, NJ), Bio-Rad Dc protein assay kit (Bio-Rad Lab., Hercules, CA), and various chemicals of reagent grade.

2.2. Crude enzyme preparation

The *B. cereus* isolated from Taiwan soil and was kindly supplied by Professor Lee of the Department of Agricultural Chemistry of National Taiwan University. The microbe was cultured in a 500-ml glass jar containing 150 ml of the medium composed of 0.3% colloidal chitin, 0.5% yeast extract, 0.5% soyton, 0.1% potassium dihydrogen phosphate, and 0.5% magnesium sulfate at pH 6.24. The jars were incubated in reciprocating shaker at 30 °C for 48 h. The fermentation broth was centrifuged at 6500 × *g* for 40 min at 4 °C, and acetone was added to the supernatant until its concentration reached 70%. The resulting solution was centrifuged at 7000 rpm for 10 min at 4 °C. The precipitate was dried by lyophilization and used as crude enzyme.

2.3. Reversed micellar extraction

The aqueous solutions were prepared by dissolving an appropriate amount of the freeze-dried crude enzyme in 50 mM of sodium phosphate buffers at pH 3, 4, or 5. Sodium chloride was added to the aqueous solution to adjust the ionic strength. The organic solution was prepared by dissolving a designated amount of AOT in isooctane. For the forward extraction (i.e. inclusion of enzyme in the reversed micelles), equal volumes (ca. 5 ml) of the organic solution and aqueous solution were mixed in a centrifugal tube (15 ml) at approximately 200 rpm in a reciprocating shaker bath for various time periods and temperatures. The resulting mixture was then centrifuged at 1000 × *g* for 10 min to separate the two phases. The upper layer (reversed micellar solution, the organic phase) was further processed by the subsequent backward extraction (i.e. release of the enzyme from the reversed micelles to the aqueous solution). For backward extraction, the organic solution from forward extraction and equal volume of 50 mM phosphate solution at pH 10.0 containing 1 M KCl were mixed. The mixture was held at 40 °C in a water bath for 5 min, shaken at 150 rpm for 40 min, and centrifuged at 1000 × *g* for 5 min to separate the two phases. Samples of aqueous phase were then taken for analysis.

2.4. The experimental design

There were six experimental factors that might have affected the recovery of chitosanase activity during reversed micellar extraction. This include protein concentration, pH, and NaCl concentration in aqueous phase; AOT concentration in the organic phase; and extraction temperature and time. To reduce the number of experimental variables to the level that can be handled practically, initial studies were focused on determining the proper protein concentration (0.5–5.0 mg/ml), extraction temperature (10–30 °C), and time (15–155 min). These factors were determined using the aqueous solution containing 50.0 mM of NaCl at pH 4, and the organic solution was 100.0 mM of AOT in isooctane. For studying the proper initial protein concentration, the extractions were carried out at 15 °C for 85 min. Once these variables were determined, the effects of the other three factors on the recovery of chitosanase activity were further determined experimentally based on a Box–Behnken design [29]. Two sets of experiments were designed and carried out. For the first set of experiment, the pH were set at 3, 4, or 5; AOT concentrations were 50, 200, or 350 mM, and sodium chloride concentrations were 50, 200, or 350 mM. The pH for the second set of experiment were 4.0, 4.5, or 5.0; AOT concentrations were 50, 100, or 150 mM; and sodium chloride concentrations were 30, 90, or 150 mM. The mathematical equations giving the activity recovery as functions of these variables were then developed.

2.5. Model building and data analysis

A regression procedure in the SAS package (SAS Institute Inc., Cary, NC) was used to fit the activity recovery data into second-order polynomial equations with interaction terms:

$$Y = B_0 + B_i \sum X_i + B_{ii} \sum X_i^2 + B_{ij} \sum X_{ij} \quad (i \neq j) \tag{1}$$

where Y is the dependent variable, B_0, B_i, B_{ii}, and B_{ij} regression coefficients of the model and X_i are magnitudes of the selected critical variables. An F-test for lack of fit was used to determine whether the regression models adequately fit the experimental data. Once the regression models were developed, non-linear programming techniques were used to search the maximum recovery of chitosanase activity. A commercial linear and non-linear programming package "AMPL" (The Scientific Press, San Francisco, CA) [30] was used to search for the optimal conditions.

2.6. Analytical methods

The protein concentration was determined by the modified Lowry method using Bio-Rad protein Dc protein assay kit [31]. SDS-polyacrylamide gel electrophoresis using 10% acrylamide was performed and stained by Coomassie blue R-250 [32]. The sheets were destained with acetic acid/methanol/water solution (1/3/6, v/v/v). A pre-stained protein standard (SeeBlue Plus2, Invitrogen Co., Carlsbad, CA) was used during SDS-PAGE for determining the molecular weights of the separated proteins. Chitosanase activity was determined by measuring the reducing sugar produced from chitosan. Chitosan was dissolved in the 0.2 M acetate buffer at pH 5 to make a 1% (w/v) chitosan solution. A mixture consisting of 1 ml of 1% chitosan solution, 3.5 ml of 0.2 M acetic acid solutions, and 0.5 ml of enzyme solution was then prepared and incubated at 45 °C for 30 min, then boiled for 15 min to stop the reaction. A portion of the mixture (0.5 ml) was mixed with 1.8 ml of water and 2 ml of alkaline ferri-cyanide solution, and the reducing sugar produced was measured colorimetrically [33] using a standard curve constructed by pure compound of glucosamine. One enzyme unit was defined as the amount of enzyme that hydrolyzed 1% chitosan solution to yield 1 μmol of reducing sugar per minute at 45 °C.