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Optimizing of the formation of active BMW-amylase after in vitro refolding

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ABSTRACT

This study was carried out to determine the optimal folding condition of α-amylase from *Bacillus megaterium* WHO using response surface methodology (RSM). A first-order model showed that three factors namely glycerol, Ca^{2+} and protein concentration had the most significant effect on refolding. Analysis of the results showed that glycerol was better than the other polyols due to its effect on protein stability. Since α-amylases are known to contain calcium ions in their structure, the presence of calcium in the refolding buffer was compulsory. The concentration of protein had the most significant quadratic effect on the response studied. A second-order polynomial model was developed to quantify the relationships between variables. It was shown that the combination of 20% (v/v) glycerol, 25 mM Ca^{2+} and 0.3 (mg/ml) protein was the most efficient condition for in vitro refolding of α-amylase. Under the optimal condition the yield of refolding was enhanced up to 7-fold. In order to analysis the size distribution in optimized and basic medium, dynamic light scattering (DLS) was fulfilled. The information gathered in this study showed that the use of solvent engineering and optimization procedure can be a general method for protein refolding.

Introduction

Overexpression of foreign proteins in *Escherichia coli* often leads to the formation of inclusion bodies (IBs),[1] which becomes the recurring obstacle in the preparation of recombinant proteins and their applications. The recovery of biologically active products from the aggregated state is typically accomplished by solubilizing with chaotropic agents or acids, followed by dilution or dialysis into optimized refolding buffers [1]. In vitro protein refolding competes with side reactions such as misfolding or aggregation which is usually the cause of decreased renaturation yields. A simple strategy to prevent aggregation by competing with intermolecular hydrophobic interactions is to use additives, small molecules that are relatively inexpensive and easy to remove once refolding goes to completion. They may stabilize the native state, by preferentially destabilizing incorrectly folded molecules, by increasing the solubility of folding intermediates and the unfolded state [2]. Additives can be polyols, sugars, polysaccharides, neutral polymers, amino acids and their derivatives. There isn't universal method for protein refolding and nature of the refolding process is unpredictable and time-consuming. In order to overcome major bottleneck, introducing systematic and rapid method that identifies refolding conditions is needed [3].

Although the formation of inclusion bodies in *E. coli* is unfavorable, this phenomenon can also be advantageous. The main advantages associated with this event are (i) high level of protein expression, (ii) rapid and easy isolation of the inclusion bodies from cell debris and soluble protein by centrifugation, (iii) being resistant to proteolytic attack, and (iv) homogeneity of the protein of interest in inclusion bodies is an initial purification to recover pure protein. Therefore, formation of inclusion bodies can be considered as a common method for the commercial production of proteins [4]. In this study, a mesophilic α-amylase from *Bacillus megaterium* WHO was expressed in *E. coli* BL21 as inclusion bodies [5]. The enzyme belongs to 1,4-α-D-glucan glucanohydrolase (EC 3.2.1.1) with a mixed enzyme specificity of α-amylase, cyclomaltodextrinase and neopullulanase. This wide range of substrate specificity will increase the applications of this special enzyme. For their high thermostability, α-amylases from *Bacillus* have found widespread use in industrial processes such as sugar, brewing, food, and detergents [6]. Ca^{2+} plays an important role in maintaining the correct conformation, thermo-stability, and activity of most α-amylases [7].

In this experiment, BMW-amylase was refolded in basic refolding condition containing fixed concentration of $CaCl_2$ (10 mM) in 50 mM Tris–HCl, pH 8 through dilution method. In order to improve the yield of refolding, influence of some chemical additives including NaCl, glycerol, sorbitol, ethylene glycol, imidazol, proline and sucrose were evaluated. When there are a large number of independent variables influencing the yield of refolding, response

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[1] *Abbreviations used:* IBs, inclusion bodies; RSM, response surface methodology; CCD, central composite design; DNS, dinitrosalicylic acid; H-FFD, half-fractional factorial design.

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surface methodology (RSM) could be an effective tool for optimizing the process, which was originally described by Box and Wilson [8]. Response surface methodology (RSM) is a set of mathematical and statistical techniques that optimizes the interest response in the presence of several selected variables [9]. The main advantage of RSM is to reduce number of experiments needed to evaluate the effect of multiple variables and their interactions. Therefore, it is cost effectiveness and time-consuming than other approaches required optimizing a process. Usually, it applies an experimental design such as Half-fractional factorial design for initial screening and central composite design (CCD) to obtain the best condition of refolding [10,11].

In this study, response surface methodology (RSM) made it possible to investigate successfully the optimal conditions of in vitro refolding and to elucidate interactions between refolding factors with a minimum number of experiments. Furthermore, dynamic light scattering was used as a proper instrument for investigating the size measurement under refolding conditions.

Materials and methods

Materials

All reagents were obtained from Sigma–Aldrich (St. Louis, MO, U.S.A.). The chemicals used were of analytical grade.

Refolding protocol

Recombinant α-amylase was expressed in *E. coli* as inclusion bodies under the control of the strong T7 promoter. Inclusion bodies were dissolved in 6 M urea solution containing 0.05 M Tris (pH 7.0), 300 mM NaCl and 4 mM imidazol. After centrifuging at 8000*g* for 30 min, the supernatant was purified by affinity chromatography. In order to form active amylase, the 100 μl denatured purified protein was added directly into 1 ml refolding buffer (50 mM Tris buffer, pH 7). In addition, different additives at different concentrations were added to the refolding buffer. Refolding was carried out in a 24-well plates at 4 °C for 24 h. Experiments were repeated three times.

Enzyme assay

α-amylase activity was determined by measuring the formation of reducing sugars released during starch hydrolysis. The reaction mixture contained 20 μl of purified enzyme and 180 μl of 1.0% (w/v) potato starch (Sigma) in 20 mM Tris–HCl buffer (pH 7.4).Subsequently, the mixture was incubated at 37 °C for 30 min. The amount of liberated reducing sugar was determined by dinitrosalicylic acid (DNS) method [12]. One unit of amylase activity is defined as the amount of enzyme that releases 1 μmol of reducing sugars (with maltose as the standard) per minute under the assay conditions specified.

Optimization of recombinant α-amylase refolding

At first, we evaluated the effect of different additives which selected according previous articles including NaCl, $CaCl_2$, glycerol, sorbitol, ethylene glycol, imidazol, proline, sucrose and enzyme on refolding condition based on the conventional method. This method of optimization involves varying one parameter at a time which is time consuming and expensive, when a large number of independent variables are to be evaluated. To overcome this difficulty and to understand the interactions between one or more variables, response surface methodology (RSM) has been widely used in this study.

Response surface methodology is a statistical method to explore the relationship between the controlled experimental factors to optimize the desirable response. The first step of the optimization is screening of the factors influencing the response which selected based on conventional method, using Half-fractional factorial design (H-FFD).Then in order to obtain optimal values for selected factors, central composite design was employed.

Half-fractional factorial design (H-FFD)

A two-level fractional factorial design can be used for screening of factors to find significant parameters in a minimal number of experiments. Here, we selected some factors from conventional method that were capable of influencing the studied reaction yield. The experiments were fulfilled in triplicate and the results were analyzed using Design Expert software (version 8.0, Stat-Ease, Inc., Minneapolis, MN).

Central composite design (CCD)

The most popular response surface method (RSM) design is the central composite design (CCD) that is applied to determine the optimal conditions for refolding of recombinant amylase, with the assistance of design experimental software. The range and levels of experimental variables investigated in this study are presented in Table 1. The actual values of independent variables (Xi) were coded to xi according to Eq. (1) by assigning the lowest values listed in Table 1 as −2 and the highest values as + 2:

$$X_i = \frac{X_i - \overline{x}_i}{\Delta x_j} \quad i = 1, 2, 3, .., k \tag{1}$$

where x_i is the coded value of an independent variable, X_i represents the corresponding natural value of the independent variable, $\overline{xi}$ is the natural value in the center of the domain and Δx_j is the step change.

The CCD permits the response surface to be modeled by fitting a classical second-order polynomial with the number of experiments equal to $2f + 2f + n$, where f and n are the number of factors and center runs, respectively ($f = 4$, $n = 6$). The repetition of central runs was carried out to provide information on the variation of the responses about the average, the residual variance, and eventually estimate the pure experimental uncertainty. In this study, it consisted of 30 experiments organized in a 25-1 fractional factorial design. A four factor-five coded level (Table 1) CCD, 30 runs, was carried out to fit to the general model of Eq. (2) and to obtain optimum conditions for refolding of amylase.

$$Y = \beta_o + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum_{i=1}^{4} \sum_{j=i+1}^{4} \beta_{ij} X_i X_j \tag{2}$$

where Y is the observed reaction yield of refolding; β_o is the grand average response; β_{is} are model coefficients and X_{is} are variables under study calculated from experimental data. The data represents in Table 2 are means of three repeated experiments. Thus, the average values of them were used as final values for developing the model. The Design Expert software (version 8.0, Stat-Ease, Inc., Minneapolis,

Table 1
Coded levels and range of independent variables for experimental design.

Variable	Coded level				
	−2	−1	0	+1	+2
Enzyme (mg/ml) ($i = 1$, X_1)	0.01	0.05	0.175	0.3	0.4
$CaCl_2$ (mM) ($i = 2$, X_2)	1.85	5.00	15.0	25.0	28.1
Glycerol (%) ($i = 3$, X_3)	0.00	0.00	10.0	20.0	23.1