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Optimization of Recombinant Novel Esterase Expression from Extremophiles

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Abstract

Esterases, which are a sub-group of lipolytic enzymes, are important biocatalysts for many industrial applications. In this study, optimization for the recombinant expression of a novel esterase, which was previously obtained by metagenomic approach, was studied. To optimize the expression, 0.1, 0.5 and 1 mM of isopropyl β-D-1 thiogalactopyranoside (IPTG) concentrations were applied. In addition, induction at 25 ºC for 16 hours, 30 ºC for 6 hours and 37 ºC for 3 hours were tested. According to the results, induction at 30 °C for 6 hours by 0.1 mM of IPTG yielded high amount of protein with maximum catalytic activity. After the gene was successfully expressed, purification studies were conducted. The protein was purified using His-tag method. Native and SDS-PAGE analysis showed that protein which is present as a monomer was successfully purified.

Keywords: Protein expression, esterase, optimization

INTRODUCTION

Lipolytic enzymes are widely used biocatalysts in many industries such as pharmaceutical, detergent, food and paper industry. They find application in a wide variety of reactions such as hydrolysis, esterification, interesterification, alcoholysis and acidolysis [1]. These enzymes are widely distributed in nature and present in animals, plants and microorganisms. Lipolytic enzymes are divided into two groups as lipases and esterases according to their substrate preference. Esterases (EC 3.1.1.1) are the enzymes that catalyze the hydrolysis of ester bonds of partly water soluble molecules, with relatively short carbon chain [2].

Metagenomics is the culture independent study of mixed microbial populations, without using the traditional culturing techniques [3]. Metagenomics is an effective approach for studies in which the culturing is troublesome, especially for studies with extremophiles. Halophiles are extremophilic microorganisms that can live in high salt concentrations [4]. From industrial aspect, they are especially important for their enzymes which are usually exploitable in organic solvents and receive the attention of researchers due to their ability to maintain the activity in low water content medium.

The gram negative bacterium *E. coli* has been used for many years as a host organism to produce recombinant proteins rapidly and with a high yield [5]. Its physiology is well known and due to its rapid growth kinetics, high cell densities can be achieved in a relatively short time. In addition, procedures for transformation with exogenous DNA are well established and efficiency is usually high [6]. There are different strains of *E. coli* available for recombinant protein expression. The strain C43 (DE3) used in this study was generated from *E. coli* BL21 (DE3) strain by Mirous and Walker [7]. The strain was especially designed for the expression of toxic or membrane proteins at high levels. To minimize the risk of toxicity of the recombinant protein to the host cell, *E. coli* C43 (DE3) was used [6]. pET vector systems, which are used in this study, are widely used for recombinant protein expression. They use T7 promoter system and in some cases, the recombinant protein can possess 50% of total proteins within the cells [8]. In this system, the transcriptional control in under the *lac*UV5 promoter, which makes the system inducible by IPTG [6]. Along with IPTG concentration, induction temperature has important effect on the production of recombinant protein. It is known that although it is a highly efficient host, recombinant proteins produced by *E. coli* tend to form inclusion bodies, which obligate more steps to be applied in downstream processing to obtain a soluble and active protein. An effective way to reduce the formation of inclusion bodies is to reduce the expression temperature and extend the time [9]. As can be concluded, recombinant protein production requires optimization of inducer concentration and time/temperature for induction to be able to obtain active proteins with a high yield.

The novel esterase enzyme related to this study was previously isolated from the metagenome of a hypersaline lake, Acıgol (Denizli), by metagenomic approach [10]. Within the scope of this study, it was aimed to optimize the conditions for the recombinant expression and purification of this protein in active form with a high yield.

MATERIALS AND METHODS

Bacterial strains and plasmids

The gene encoding the esterase was previously cloned in pET-28(+) vector (Novagen, USA) and transformed into OverExpress™ C43 (DE3) strain of *E. coli* (Lucigen, USA).

Optimization of recombinant protein expression

Single colony from transformed *E. coli* C43 (DE3) cells was inoculated into 10 mL of LB containing 40 µg/mL ka-

namycin. After 16 hours of incubation at 37°C with 200 rpm orbital shaking, 200 mL of medium was inoculated with the starter culture. The plasmid used in cloning (pET-28a(+)) contains a *lac* operon and the expression is inducible with IPTG. To determine the optimum IPTG concentration, cells were induced with 0.1, 0.5 and 1 mM of IPTG when OD_{600} was 0.8 and incubated at 37 °C for 1, 3 and 4 more hours.

After optimum IPTG concentration was found, the expression was conducted at different time and temperatures (16 hours at 25 ºC, 6 hours at 30 ºC and 3 hours at 37 ºC) to assess the effect of expression temperature on the activity of esterase enzyme. Recombinant protein was purified as explained below, analyzed on SDS-PAGE and kinetic analysis was performed in the presence of para nitro phenyl octanoate as substrate. After optimization of the IPTG concentration, expression time and expression temperature were also optimized. The volume of the starter culture was fixed to 50 mL and proteins were expressed in 1L of medium. At the end of incubation, cells were harvested by centrifugation at 5000 rpm for 20 min at 4°C. Cell pellets were stored at -20°C.

Protein purification

Cell pellet from 1 L of culture was resuspended in 15 mL of ice cold lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8). Lysozyme was added and incubated on ice for 30 min. Cell suspension was sonicated (Bandelin SonoPuls) 10 times with 10 s on / 20 s off cycle. Cell debris was pelleted at 12000g for 15 min at 4 °C. To bind the histidine-tagged proteins, 1.2 mL of Ni-NTA Agarose (Qiagen, #30210) was added to the cell free lysate and incubated for 1 hour at 4 °C on rotary shaker. Proteins bound to resin was pelleted by centrifugation at 3000 g at 4 °C. Resin was washed with 4 mL of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8) for three times and proteins were eluted with 1 mL of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8) for six times.

SDS and native polyacrylamide gel electrophoresis

Crude lysates or purified protein samples were electrophoresed on polyacrylamide gel. Stacking gel concentration was 5% and resolving gel concentration was 12%. For SDS-PAGE, 20 µL of samples were mixed with 5 µL of sample application buffer and heated at 95ºC for 15 min. Molecular weight marker (Pierce™, #26610) was denatured at 95 ºC for 5 min. For native PAGE, same buffers were used except that denaturing agents were not included in any buffer and heating step was omitted. Electrophoresis was initiated with a voltage of 80 V, and after proteins enter the resolving gel, a constant voltage of 120 V was applied until the front dye reached at the end of the gel. After electrophoresis, proteins were stained with Coomassie Brilliant Blue R-250.

After purification of the protein was verified by SDS-PAGE, pure protein fractions were collected and applied to ultrafiltration tubes (Amicon®, # UFC901008) with 10 kDa molecular weight cut-off value. Elution buffer was changed to 50 mM Tris-HCl, containing 10% NaCl, pH 8 and protein was concentrated to a total volume of 1.5 mL. For protein quantification, Bradford method was used [11]. 5 µL of sample was mixed with 195 µL of Bradford reagent (Sigma, #B6916). BSA was used as standard and absorbance measurement at 595 nm was done triplicate.

Activity Assay

The activity assay was performed according to Winkler and Stuckman [12]. 1 µM of enzyme and 1 mM of para nitrophenyl octanoate was used in the reaction. Reaction was incubated at 30 °C and the absorbance at 410 nm was measured at 20th minute.

RESULTS AND DISCUSSION

A novel metagenome derived esterase gene was previously cloned into pET-28(a) and transformed into *E. coli* C43(DE3) cells [10]. To determine the optimum conditions for protein expression, different IPTG concentrations (0.1, 0.5 and 1 mM IPTG) were tried. Expression was conducted for 1, 3 and 4 hours to determine the optimum duration of expression. At the end of incubation, cells were harvested, sonicated and cleared lysates were analyzed on SDS-PAGE (Figure 1).

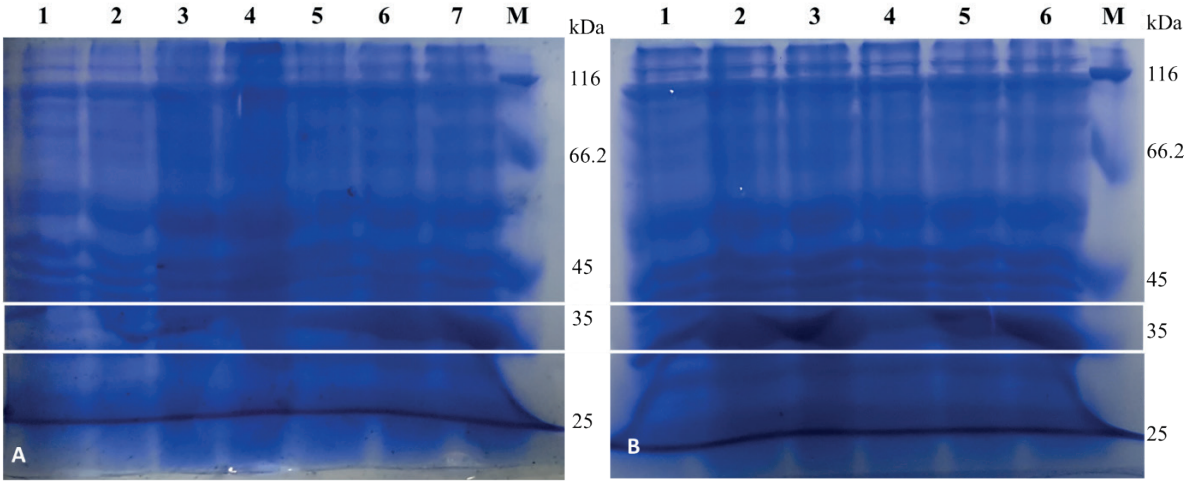

Figure 1. SDS-PAGE analysis of crude lysates samples for IPTG optimization. A. Lane 1, 2, 3 and 4: no IPTG was added, incubation time was 0, 1, 3 and 4 hours, respectively. Lane 5, 6 and 7: 0.1 mM of IPTG was added, incubation time was 1, 3 and 4 hours, respectively. B. Lane 1, 2 and 3: 0.5 mM of IPTG was added, incubation time was 1, 3 and 4 hours, respectively. Lane 4, 5 and 6: 1 mM of IPTG was added, incubation time was 1, 3 and 4 hours, respectively. Lane M: Molecular weight marker. Position of respective bands are shown inside the white rectangle.

As shown in Figure 1, protein was not expressed in the absence of IPTG (Figure 1A, Lane 1-4) as expected. The protein was expressed in high amount with 0.1 mM IPTG. The yield did not improved significantly with 0.5 mM and 1 mM of IPTG. Protein amount expressed in 1 hour was lower than 3 and 4 hours, as expected. Optimum conditions at 37 ºC was found to be 0.1 mM IPTG and 3 hours of incubation. However, it is known that the recombinant proteins expressed in *E. coli* tend to form inclusion bodies [6]. To overcome this problem, induction at lower temperatures are recommended. In this study, expression level and activity of the enzyme expressed at 25 ºC for 16 hours, at 30 ºC for 6 hours and 37 ºC for 3 hours were also compared. Following the appropriate amount of expression, the recombinant protein from each sample was purified by His-tag method. The results are given in Figure 2.

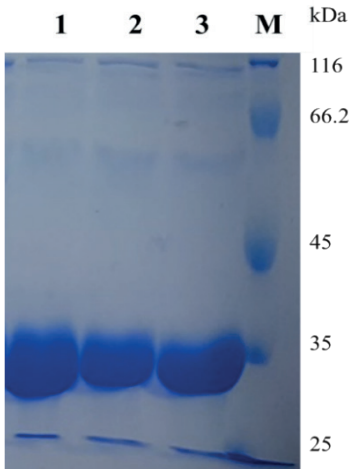

Figure 2. SDS-PAGE analysis of purified proteins for optimum induction temperature. Lane 1: 25 °C for 16 hours; Lane 2: 30 °C for 6 hours, Lane 3: 37 °C for 3 hours, Lane M: molecular weight marker.

The concentration of the purified proteins were measured by Bradford method, using BSA as standard. The protein amounts were calculated as 8.8 g/mL, 7.7 g/mL and 11.8 g/mL for the proteins expressed at 25°C for 16 hours, 30°C for 6 hours and 37°C for 3 hours, respectively. As can be interpreted from the concentration values, the recombinant protein was successfully expressed in all conditions. However, expression of the protein does not always guarantee the high activity of the protein. To assess the effect of incubation temperature on enzyme activity, purified enzymes were used in kinetic assay using pNP-octanoate as substrate. As can be seen in Figure 3, the enzyme expressed at 30 °C showed maximum activity. Lower activity in the protein expressed at 37 °C can be explained with improper folding of the protein. However, reducing the temperature to 25 °C did not improve the activity of the protein. This enzyme was isolated from enrichment culture of organisms which are grown at 30 °C. Additionally, optimum temperature of the enzyme was found as 30 °C [10]. So, it is predicted that the enzyme can gain its best conformation when the temperature is fixed to 30 °C.

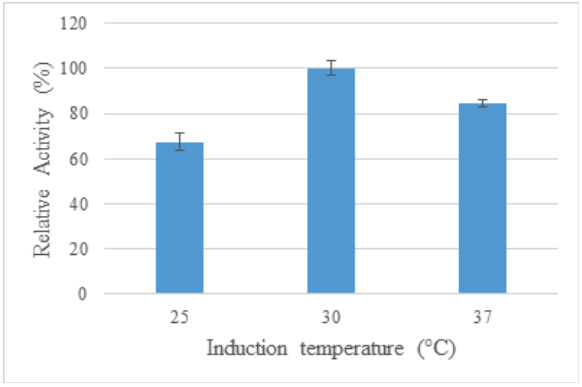

Figure 3. Effect of induction temperature on the activity of the enzyme.

The optimized conditions for recombinant protein expression in *E. coli* varies according to the host strain, vector type and the nature of the protein. In a similar study, in which a metagenomic esterase was isolated, expression was induced by 0.4 mM of IPTG for 18 hours at 20 °C [13]. In another study, *E. coli* BL21 cells bearing a metagenomic esterase coding gene, which was cloned into pET-22b, was induced by 1 mM of IPTG at 20 °C for 20 hours [14]. Another metagenome derived esterase was expressed in *E. coli* BL21 (DE3) using pET28a(+) and induced by 0.1 mM IPTG at 30 °C for 6 hours, which are the same conditions used in present study [15].

Affinity tags have been widely used for recombinant protein purification. In this work, we used an N-terminal 6 histidine tag and purified the protein using the principle of affinity of nickel to histidine. Proteins were bound to Ni-NTA matrix, then eluted with high amount of imidazole. During purification, samples from each fraction were collected and analyzed on SDS-PAGE. Elution samples were combined and concentrated by ultrafiltration membranes. 20 µg of protein samples were analyzed on both native and SDS-PAGE, as results are shown in Figure 4.

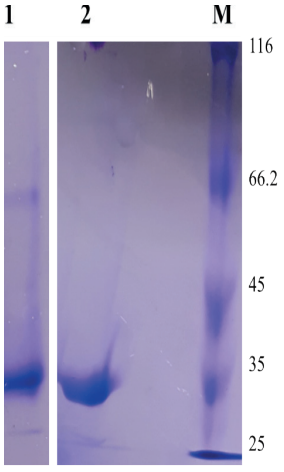

Figure 4. Native (Lane 1) and SDS PAGE (Lane 2) result of purified protein. Lane M: Molecular weight marker.

According to the gel images obtained after native and SDS-PAGE analysis, it is concluded that the enzyme is present as a monomer. The molecular weight of microbial esterases range from 27 to 54 kDa [16]. It is observed that the band corresponding to the purified protein on SDS-PAGE is between 35 and 25 kDa, compatible with the estimated molecular weight which is 28.4 kDa.

As a conclusion, optimum parameters for the expression of an esterase were evaluated with this study. Different IPTG concentrations, expression durations and temperatures were tried to find the optimum condition that yields not only highest amount of purified protein, but also highest activity. According to the results, the best condition was determined as 0.1 mM of IPTG, and induction at 30 °C for 6 hours. Although the amount of purified protein is much higher when induced at 37 °C for 3 hours, highest activity was observed when induction was done at 30 °C for 6 hours. In addition, native and SDS-PAGE analysis of the purified protein revealed that the protein is a monomer, giving a single band between 25 kDa – 35 kDa. After optimization of the expression conditions, studies focusing on detailed biochemical characterization of the enzyme are planned as future work.

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