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Direct and simple detection of recombinant proteins from cell lysates using differential scanning fluorimetry

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ABSTRACT

A simple, inexpensive, and universal method to quantify the recombinant proteins in *Escherichia coli* cell lysate using differential scanning fluorimetry (DSF) is reported. This method is based on the precise correlation between Δ(fluorescence intensity) determined by DSF and the amount of protein in solution. We first demonstrated the effectiveness of the DSF method using two commercially available enzymes, α-amylase and cellobiase, and then confirmed its utility with two recombinant proteins, amylosucrase and maltogenic amylase, expressed in *E. coli*. The Δ(fluorescence intensity) in DSF analysis accurately correlated with the concentration of the purified enzymes as well as the recombinant proteins in *E. coli* cell lysates. The main advantage of this method over other techniques such as Western blotting, enzyme-linked immunosorbent assay (ELISA), and green fluorescence protein (GFP) fusion proteins is that intact recombinant protein can be quantified without the requirement of additional chemicals or modifications of the recombinant protein. This DSF assay can be performed using widely available equipment such as a real-time polymerase chain reaction (RT–PCR) instrument, microplates or microtubes, and fluorescent dye. This simple but powerful method can be easily applied in a wide range of research areas that require quantification of expressed recombinant proteins.

During recent years, the use of recombinant protein expression in the life sciences has increased dramatically not only for agricultural and medicinal applications but also for academic research [1]. As a result of advancements in recombinant DNA technology, a variety of host systems, including phage, bacteria, yeast, filamentous fungi, plants, insects, and mammalian cells, are currently available [2–4]. The choice of host system usually depends on the particular requirements of the target protein such as secretion, glycosylation, and its application. In general, however, the first choice for heterologous protein expression system is inevitably *Escherichia coli* [5,6]. The most important goal of heterologous protein expression in *E. coli* is to acquire a soluble and active protein with maximized protein yield [7].

Therefore, direct or indirect quantitation methods to determine the status of expressed target protein in heterologous host cells are very important. Conventional methods for detecting recombinant proteins are Western blot analysis and enzyme-linked immunosorbent assay (ELISA).[1] These methods require a specific antibody for the target protein or a protein tag as well as high-level skills and a relatively long experimental time. Furthermore, direct quantitative detection of recombinant proteins in cell lysates is not possible. Consequently, a number of methods have been developed to directly assay recombinant proteins in the cell lysate. Reporter proteins such as green fluorescent protein (GFP) can be used to tag the target protein, allowing easy detection of recombinant protein expression [8]. GFP is a protein composed of 238 amino acid residues that displays bright green fluorescence when exposed to light. Although GFP tagging is a powerful tool in recombinant protein expression, there are several drawbacks to its use [9,10]. The expressed protein must be physically modified by the addition of GFP, necessitating removal of the attached GFP from the target protein for further application if use of the original form of the protein is mandatory [11]. In addition, the relatively large size of GFP occasionally affects the folding and function of the recombinant proteins [12].

Recently, Tan and coworkers [13] developed a rapid, inexpensive, and simple method to detect and quantify recombinant proteins using an anti-His-tag molecular beacon aptamer (HMBA). Aptamers are single-stranded nucleic acids created by SELEX (systematic evolution of ligands by exponential enrichment). They form distinct stem and loop three-dimensional structures that allow specific binding to a variety of targets, such as the His-tag, with high affinity and specificity comparable to that of antibodies. Therefore, the recombinant protein fused with a His-tag can be directly measured by the interaction between the His-tag and

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[1] *Abbreviations used:* ELISA, enzyme-linked immunosorbent assay; GFP, green fluorescent protein; HMBA, anti-His-tag molecular beacon aptamer; T_m, melting temperature; DSF, differential scanning fluorimetry; RT–PCR, real-time polymerase chain reaction; DGAS, *Deinococcus geothermalis* amylosucrase; ThMA, *Thermus sp.* maltogenic amylase; DNS, dinitrosalicylic acid; G3, maltotriose; GOD–POD, glucose oxidase–peroxidase; A.U., arbitrary units.

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HMBA. When bound to His-tagged proteins, the stem portion of HMBA is reorganized to physically separate the fluorophore from the quencher, permitting a fluorescent signal to be emitted on excitation. Although the HMBA method is powerful enough to detect recombinant proteins, it has certain weaknesses such as the need for both His-tagged recombinant proteins and HMBA.

The melting temperature (T_m) of a protein is defined as the temperature at which the native and denatured states are equally present at equilibrium. It is an important characteristic of a protein that is entirely dependent on the folding and unfolding status. T_m can be measured using various analytical instruments, including circular dichroism (CD) spectroscopy [14], differential scanning calorimetry (DSC) [15], differential static light scattering (DSLS) [16], and differential scanning fluorimetry (DSF) [17]. Among those, DSF is a rapid and cost-effective method that has been used to study protein–ligand binding and the stability of proteins in various conditions [18–20]. With DSF, protein unfolding is monitored by an increase in the fluorescence of a specific dye with enhanced affinity for the hydrophobic parts of the protein, which are exposed during the unfolding process. This is easily measured using a conventional real-time polymerase chain reaction (RT–PCR) instrument. Ericsson and coworkers have employed the DSF method as a high-throughput approach for identifying optimal protein formulation for crystallization. It was suggested that the identification of potential ligands, physiological or nonphysiological, that can be used in subsequent crystallization trials was allowed by this DSF method [21].

In the current study, we demonstrate a possible application of DSF in the quantitation of recombinant proteins expressed in *E. coli*. With this rapid, inexpensive, and simple method, the recombinant proteins are easily detected in cell lysates. Moreover, the original recombinant protein can be easily quantified by RT–PCR without the need for tagging proteins such as GFP or modification by His-tag.

Materials and methods

Materials

α-Amylase (EC 3.2.1.1, A6380) and cellobiase (EC 3.2.1.4, A6105) were purchased from Sigma–Aldrich Chemical (St. Louis, MO, USA). SYPRO orange dye (5000× concentrate in dimethyl sulfoxide [DMSO]; Life Technologies, Carlsbad, CA, USA), MicroAmp Fast Optical 96-Well reaction plate (Applied Biosystems, Foster City, CA, USA), and MicroAmp Optical adhesive film (Applied Biosystems) were used for DSF analysis. All other chemicals were of reagent grade and obtained from Sigma–Aldrich Chemical and Duchefa Biochemie (Haarlem, The Netherlands).

Fluorescence measurements

The complement unfolding point of proteins was measured by DSF. The sample was prepared by mixing 10 μl of SYPRO orange (10×) with 10 μl of enzyme solution or *E. coli* cell lysate. The mixture of proteins and SYPRO orange was incubated in an RT–PCR system using a temperature gradient from 30 to 99 °C with 1 °C increments. All fluorescence measurements were carried out using an Applied Biosystems 7500 Fast RT–PCR system (Applied Biosystems) and were collected using TAMRA (tetramethyl-6-carboxyrhodamine) dye detection (λ_{ex} = 477 nm/λ_{em} = 549 nm) installed on the instrument. The value of Δ(fluorescence intensity) was obtained from the difference between the highest fluorescence intensity and the lowest fluorescence intensity in the DSF curve. SigmaPlot 12.0 software (Systat Software, San Jose, CA, USA) and Microsoft Excel program (Microsoft, Redmond, WA, USA) were used for all graphic analyses and data statistics.

Expression and purification of recombinant protein in E. coli

Amylosucrase from *Deinococcus geothermalis* DSM 11300 (DGAS) [22] and maltogenic amylase from *Thermus* sp. (ThMA) [23] were used as model proteins to examine the efficacy of the DSF method to quantify recombinant protein expression in *E. coli*. Construction of the constitutive DGAS and ThMA expression vectors, pHCDGAS and pHisThMA, was described previously [24,25]. Recombinant *E. coli* MC1061 [F$^-$*ara*D139 Δ(*ara-leu*)7696 *gal*E15 *gal*K16 Δ(*lac*)X74 *rps*L(Str^r) *had*R2 ($r_k^-m_k^+$) *mrc*A *mrc*B1] cells harboring pHCDGAS and recombinant *E. coli* DH10B [F$^-$Φ80*lac*Z M15 (*lac*ZYA-*arg*F) U169 *rec*A1*end*A1 *hsd*R17(r_k^+, m_k^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1λ$^-$] cells harboring pHisThMA were cultured in 100 ml of LB broth at 37 °C for 24 h with agitation at 200 rpm. The cells were harvested by centrifugation (7000*g* for 20 min at 4 °C) and washed with a binding buffer containing 20 mM Tris–HCl, 500 mM NaCl, and 20 mM imidazole (pH 7.0). The bacterial pellet was resuspended in binding buffer and disrupted by sonication (output 4, 6 × 10 s, constant duty; Sonifier 450, Branson, Danbury, CT, USA) in an ice bath. The supernatant acquired by centrifugation (10,000*g* for 10 min at 4 °C) was used as a starting material to obtain recombinant protein and cell lysate samples.

Purification of recombinant DGAS and ThMA was performed using a fast-performance liquid chromatography (FPLC) system with a HisTrap HP column (GE Healthcare, Uppsala, Sweden). The cell lysate was loaded into the HisTrap HP column and eluted with elution buffer (20 mM Tris–HCl, 500 mM NaCl, and 500 mM imidazole, pH 7.0) at a flow rate of 1.0 ml/min. All fractions were collected and confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with a 10% (w/v) acrylamide gel. The protein concentration was determined using a BCA (bisinchoninic acid) protein assay kit (Pierce Biotechnology, Rockford, IL, USA) with bovine serum albumin (BSA) as a standard.

Assay for DGAS and ThMA activities

DGAS activity was measured by determining hydrolysis activity by the dinitrosalicylic acid (DNS) method [22]. The reaction mixture was composed of 20 μl of 5% sucrose, 20 μl of deionized water, and 50 μl of 100 mM Tris–HCl buffer (pH 7.0). The reaction was started by adding 10 μl of the enzyme solution to the substrate solution and was continued for 10 min at 45 °C. The presence of reducing sugar in the reaction mixture was determined by adding 300 μl of DNS solution, followed by boiling for 5 min. The absorbance of the final reaction solution was measured at 550 nm using an Ultra multifunctional microplate reader (InfinteM200, Tecan, Durham, NC, USA). Reducing sugar concentration was calculated using fructose as a standard. One unit of DGAS was defined as the amount of enzyme that produces 1 μmol of fructose per minute in the assay conditions.

Enzymatic activity of ThMA was determined using 1% maltotriose (G3) in 50 mM sodium–citrate buffer (pH 6.0). ThMA (50 μl) diluted in the same buffer was reacted with 50 μl of 2% G3 at 60 °C for 10 min. The reaction was stopped by boiling for 10 min. Release of glucose from G3 was assessed using a glucose oxidase–peroxidase (GOD–POD) method [26]. The presence of D-glucose in the reaction mixture was evaluated by adding 900 μl of GOD–POD solution and incubating at 37 °C for 5 min. The absorbance of the final mixture solution was measured at 505 nm using an ultraviolet/visible (UV/Vis) spectrophotometer (DU 730, Beckman Coulter, Fullerton, CA, USA). One unit of ThMA was defined as the amount