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SCANNING ASSAY OF β-GALACTOSIDASE ACTIVITY

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β-galactosidase, encoded by the *lacZ* gene in *E. coli*, can cleave lactose and structurally related compounds to galactose and glucose or structurally related products. Its activity can be measured using an artificial substrate, o-nitrophenyl-β-D-galactopyranoside (ONPG). Miller firstly described the standard quantitative assay of β-galactosidase activity in the cells of bacterial cultures by disrupting the cell membrane with the permeabilization solution instead of preparing cell extracts. Therefore, β-galactosidase became one of the most widely used reporters of gene expression in molecular biology to reflect intracellular gene expression difference. But the Miller assay procedure could not monitor the β-galactosidase reaction in real time and its results were greatly influenced by some operations in the Miller procedure, such as permeabilization time, reaction time and concentration of the cell suspension. A scanning method based on the Miller method to determine the intracellular β-galactosidase activity in *E. coli* Tuner (DE3) expressing -galactosidase in real time was developed and the permeabilization time of cells was optimized for that. The comparison of 3 assays of β-galactosidase activity (Miller, colorimetric and scanning) was made. The results proved that scanning method for the determination of enzyme activity with using ONPG as substrate is simple, fast and reproducible.

β-Galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23), encoded by the *lacZ* gene of the *lac* operon in *E. coli*, is a large (120 kDa, >1000 amino acids) protein that forms a tetramer. It cleaves lactose and structurally related compounds to galactose and glucose or structurally related products [1, 2]. Since its activity can be monitored using a variety of chromogenic and fluorogenic substrates [3, 4], it has been a versatile reporter for both prokaryotes and eukaryotes [5–10]. The standard quantitative assay of β-galactosidase activity in cells, originally described by Miller [11] for assay in bacterial cultures, involves spectrophotometric measurement of the formation of the yellow chromophore *o*-nitrophenol (**ONP**) as the hydrolytic product of the β-galactosidase reaction from the colorless substrate o-nitrophenyl-β-D-galactopyranoside (**ONPG**). The amount of ONP produced as a function of reaction time per volume of cell culture is divided by the optical density of the culture to generate a value of specific enzyme activity in Miller units [11]. Subsequently, some derivative methods based on Miller's method were reported [12–14].

In the Miller method and its derivative methods, the bacteria cells are treated by some surfactant and/or organic solvents, e.g. chloroform and SDS, which partially disrupts the cell membrane and after that small molecules like ONPG can freely diffuse into the cell instead of preparing cell extracts, e.g., by sonic disruption. Therefore, β-galactosidase became one of the most widely used reporters of gene expression in molecular biology to reflect intracellular gene expression difference. But the Miller procedure and its derivative methods are colorimetric which cannot monitor the reaction in real time. Therefore, the results of the β-galactosidase activity assay are influenced by some operations in the Miller procedure, such as permeabilization time, reaction time and cell suspension concentration.

The aim of the study was to develop a scanning method based on the Miller procedure and its derivative methods to determine the intracellular β-galactosidase activity using *E. coli* Tuner (DE3) in which β-galactosidase was expressed.

MATERIALS AND METHODS

Materials. *E. coli* DH5α [*endA1 hsdR17 supE44 thi-1 recA1 gyrA (Nal^r) relA1D (lacZYA-argF) U169 (f80dlacd(lacZ)M15)*] was obtained from Invitrogen (USA). *E. coli* Tuner (DE3) [F$^-$ *ompT hadSB (rB$^-$mB$^-$) gal dcm lacY1* (DE3)] and plasmid pET28a were purchased from Novagen (Germany). DNA modification enzymes, restriction enzymes and polymerase chain reaction (PCR)-related materials were obtained from Takara Bio (Japan). Oligonucleotides synthesis and DNA sequence were purchased from Invitrogen (USA).

Bacterial strains and growth conditions. *E. coli* DH5α and *E. coli* Tuner (DE3) were used for cloning experiments and for protein expression, respectively. They were routinely grown in Luria-Bertani (LB) broth at 37°C. 15 μg/ml of kanamycin was added when required.

For protein expression experiments, cells were grown in M9 defined medium with kanamycin at 37°C and 200 rpm [15]. Overnight culture, accounting for a

1/100 volume of the fresh medium, was used for inoculation. When the OD_{600} of the cultured cells reached 0.5 ± 0.01, protein expression was induced by adding IPTG to a final concentration of 0.5 mM.

Plasmid construction. Standard PCR was performed with Pfu DNA polymerase (Promega, USA) according to the manufacturer's instructions. General techniques for plasmid DNA preparation, restriction enzyme manipulation, molecular cloning, and agarose gel electrophoresis were carried out as described in [15].

The lacZ gene was PCR-amplified with two primers lacZ-NcoI (5'-AACAGCCATGGGCATGATTACGGATTC-3') and lacZ-XhoI (5'-ACCAGCTCGAGTTATTTTTGACACCAGACC-3') from *E. coli* K-12 genome. The PCR product was digested with restriction enzymes *Nco*I and *Xho*I and cloned into the restriction enzyme sites of *Nco*I and *Xho*I in vector pET28a to form pET28a-lacZ.

β-Galactosidase activity assays. β-Galactosidase activities were assayed in at least triplicate as described [11, 12] with the following modifications.

Colorimetric method and Miller's method. 20 μl of cell suspension mixed with 80 μl of permeabilization solution (PS) preheated at 30°C, containing 100 mM Na_2HPO_4, 20 mM KCl, 2 mM $MgSO_4$, 0.8 mg/ml of hexadecyltrimethylammonium bromide, 0.4 mg/ml of sodium deoxycholate and 5.4 μl/ml of β-mercaptoethanol. Then 600 μl of substrate solution (SS) preheated at 30°C, containing 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 1 mg/ml of ONPG and 2.7 μl/ml of β-mercaptoethanol, was added to initiate the reaction. The reaction was conducted at 30°C and stopped by adding 700 μl of 1 M Na_2CO_3 when a faint yellow color was observed. The reaction time was accurately recorded and not less than 1 min. Then both OD_{420} and OD_{550} were measured.

Scanning method. 20 μl of cell suspension mixed into 80 μl PS preheated at 30°C. Then 600 μl SS preheated at 30°C was added to start the reaction. The reaction was carried out at 30°C and monitored at 420 nm. The activities were determined from the increase rates of the ONP formation.

One unit of β-galactosidase activity determined by colorimetric and scanning methods was defined as the amount of cells produced 1 μmol of ONP per min under the assay conditions. The specific activity was expressed in μmol/min OD_{600} l. The β-galactosidase activities measured by Miller's method were calculated in Miller Units which give the change in OD_{420}/min/ml for cell density at OD_{600}.

The absorbance value was read on a Cary50 spectrophotometer (Varian, USA). All initial rates were corrected for the background (nonenzymatic) reaction.

Standard curve of ONP. *Standard curve of ONP for colorimetric method.* 0–200 μl of ONP standard solution (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 1.0 mM ONP and 2.7 μl/ml of β-mercaptoethanol) were pipetted to 10 tubes respectively and sodium phosphate buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, and 2.7 μl/ml of β-mercaptoethanol) was added to 620 μl. 80 μl PS preheated at 30°C and 700 μl of 1 M Na_2CO_3 was added to each tube. Absorbance readings were spectrophotometrically determined at 420 nm.

Standard curve of ONP for scanning method. The standard curve of ONP for scanning method was carried out as for colorimetric method but without adding Na_2CO_3. Absorbance readings were spectrophotometrically determined at 420 nm. The values obtained were converted to molar concentrations using ONP standard curve.

RESULTS

Calculations of β-galactosidase activities. *β-Galactosidase activities calculated in Miller's method.* The following equation to calculate units of enzyme activity assayed with Miller's method:

$$\text{Miller Units} = \\ = 1000 \times [(OD_{420} - 1.75OD_{550})]/(T \times V \times OD_{600}), \quad (1)$$

where OD_{420} and OD_{550} were read from the reaction mixture; OD_{600} reflected cell density in the washed cell suspension; T was the time of the reaction in minutes; V (ml) was the volume of culture used in the assay. The units gave the change in OD_{420}/min/ml for cell density at OD_{600}.

β-Galactosidase activities calculated in colorimetric method. The standard curve of ONP for colorimetric method resulted in the straight line $OD_{420} = -0.00256 + 0.00319 \times$ ONP (nmol) with a correlation coefficient of 0.99995 (Fig. 1a). One unit of β-galactosidase activity determined by this method was defined as the amount of cells that produces 1 μmol of ONP per min under the assay conditions. Therefore, β-galactosidase activity measured by colorimetric method was calculated by following equation:

$$\text{Colorimetric activity Units} = (OD_{420} + \\ + 0.00256)/(0.00319 \times 1000 \times T \times V \times OD_{600}), \quad (2)$$

where OD_{420} was read from the reaction mixture; OD_{600} reflected cell density of the cell suspension; T was the time of the reaction in min; V (l) was the volume of the cell suspension used in the assay. The specific activity was expressed in μmol/min · OD_{600} · l.

β-Galactosidase activities calculated in scanning method. The standard curve of ONP for scanning method resulted in the straight line $OD_{420} = 0.01512 + 2238.1 \times$ [ONP] (mol/l) with a correlation coefficient of 0.99957 (Fig. 1B), so the molar extinction coefficient of ONP in the assay system was 2238.1 M^{-1} cm^{-1}. One unit of β-galactosidase activity of scanning method was defined as the amount of cells that produced 1.0 μmol of ONP per min under the assay conditions.

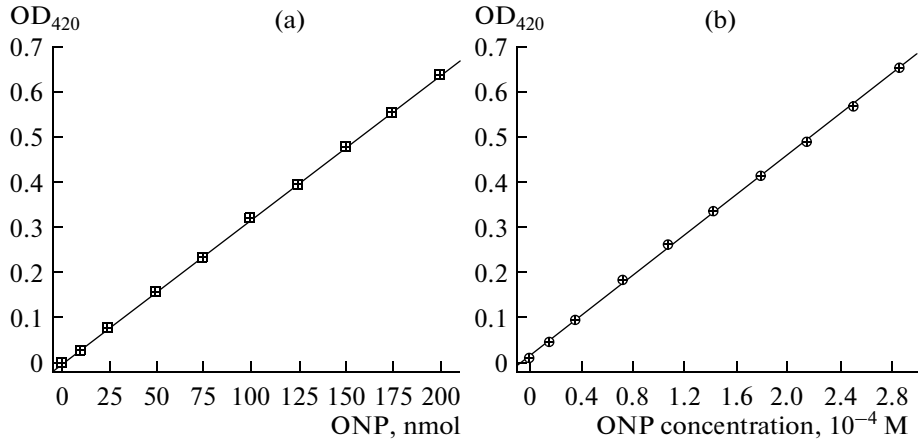

Fig. 1. The standard curves of β-galactosidase assay determined by colorimetric (a) and scanning (b) methods. a – standard curve of the ONP is presented by plotting OD_{420} versus the ONP in nmol. The fitting line was expressed by formula $OD_{420} = -0.00256(\pm 9.05996 \times 10^{-4}) + 0.00319(\pm 1.04561 \times 10^{-5}) \times$ ONP (nmol) with a correlation coefficient of 0.99995. b – standard curve of the ONP is presented by plotting OD_{420} versus the ONP concentration. The fitting line was expressed by formula $OD_{420} = 0.01512(\pm 5.40908 \times 10^{-4}) + 2238.1(\pm 3.89442) \times$ [ONP] (M) with a correlation coefficient of 0.99957. All measurements were done in at least three times. The error bars are smaller than the symbol size and therefore barely visible.

Therefore, β-galactosidase activity measured by scanning method was calculated by following equation:

$$\text{Scanning activity Units} = \\ = OD_{420} \times V_r \times 1000000/ (\varepsilon \times V \times OD_{600}), \quad (3)$$

where OD_{420} was the variation of OD_{420} per min; OD_{600} reflected cell density of the cell suspension; V_r was the volume of the reaction system; ε was the molar extinction coefficient of ONP in the assay system, 2238.1 M^{-1} cm^{-1}; V was the volume (l) of the cell suspension used in the assay. The specific activity was expressed in μmol/min OD_{600} l.

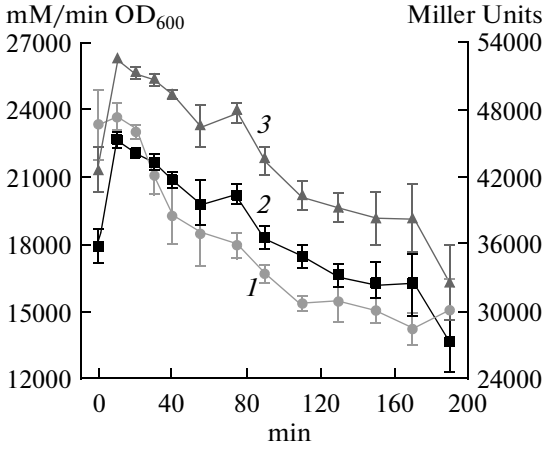

Fig. 2. The β-galactosidase activities of the *E. coli* Tuner(DE3)-pE28Z cells were determined using 3 methods at different incubation time of cell suspension with PS. Time of induction of protein expression was 2 h. *1* – β-galactosidase activity assayed by scanning method, μmol/min OD_{600} l; *2* – β-galactosidase activity assayed by colorimetric method, μmol/min OD_{600} l; *3* – β-galactosidase activity assayed by Miller's method, Miller Units.

Effect of the PS treatment time on the β-galactosidase activity. The *E. coli* Tuner (DE3)-pE28Z cells were cultured to $OD_{600} = 0.5 \pm 0.01$, and protein expression was induced by adding IPTG to a final concentration of 0.5 mM. After 2 h of induction at 37°C the suspension was taken out for enzyme assay. β-Galactosidase activities were determined using above-mentioned 3 methods at different incubation time of the cell suspension with PS. The results are shown in Fig. 2. No matter which method was used, the β-galactosidase activity of the mixture of cell suspension with PS incubated for 10 min was the highest. Then, the enzyme activities were decreased with increasing the PS treatment time. The curve of the β-galactosidase activities determined by Miller's method was similar to that determined by colorimetric method. They declined slowly, but the curve of the β-galactosidase activity determined by scanning method sharply dropped.

Effect of the dilution of cell suspensions on the β-galactosidase activity. The *E. coli* Tuner (DE3)-pE28Z cells were cultured to $OD_{600} = 0.5 \pm 0.01$, and protein expression was induced by adding IPTG to a final concentration of 0.5 mM. After 1 h of induction at 37°C the suspension was taken out for β-galactosidase assay. The mixture of the cell suspension with PS was incubated for 10 min. β-galactosidase activities of the cells taken in different dilutions were determined using above-mentioned 3 methods. The results are presented in Fig. 3. The enzyme assay does not depend on dilution of the cell suspension.

β-Galactosidase activities determined at different induced times using 3 methods. The *E. coli* Tuner

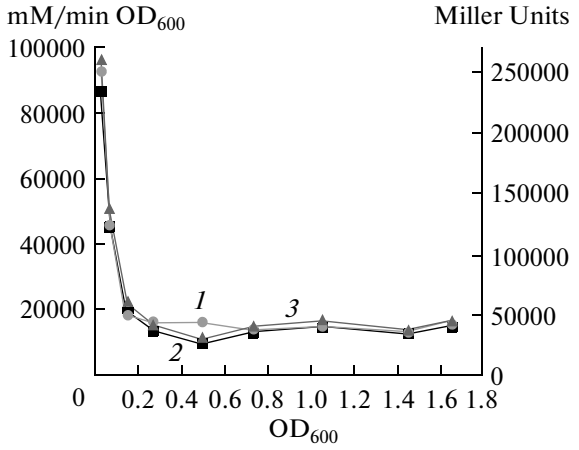

Fig. 3. The β-galactosidase activities of the of the *E. coli* Tuner(DE3)-pE28Z cells taken in different dilutions determined using 3 methods. Time of the induction of protein expression was 1 h. Legend to the curves is the same as in Fig. 2.

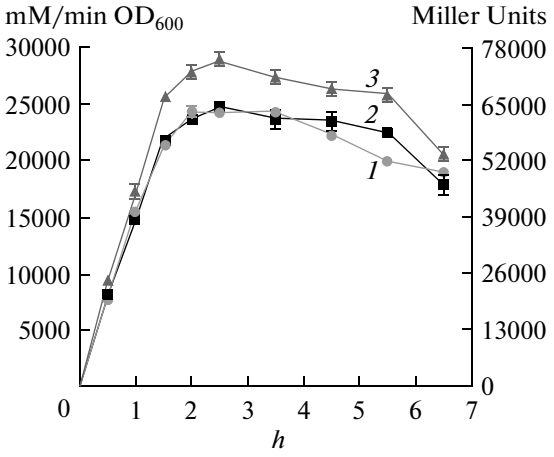

Fig. 4. The β-galactosidase activities of the *E. coli* Tuner(DE3)-pE28Z cells determined at different time of induction of protein expression using 3 methods. The cell suspension was incubated with permeabilization solution for 10 min. Legend to the curves is the same as in Fig. 2.

(DE3)-pE28Z cells were cultured to $OD_{600} = 0.5 \pm 0.01$, and protein expression was induced by adding IPTG to a final concentration of 0.5 mM. The cell suspension was used for different time of induction in β-galactosidase assay using above-mentioned 3 methods. The mixture of the cell suspension with permeabilization solution was incubated for 10 min. The results are shown in Fig. 4.

After the beginning of induction the β-galactosidase activity increased rapidly and the intracellular enzyme accumulated to a high level; then after about 6 h of induction, the rate of reaction gradually slowed down and the intracellular β-galactosidase maintained this high level. The curves of the β-galactosidase activities determined by 3 methods were similar and the data determined by Miller' method were higher.

DISCUSSION

ONPG is an artificial chromogenic substrate for β-galactosidase. It is colorless, while its hydrolytic product of the action of β-galactosidase, ONP is yellow ($OD_{max} = 420$ nm). Therefore, enzyme activity of β-galactosidase can be measured by the rate of formation of yellow color using a spectrophotometer.

However, the color intensity of ONP is pH dependent. Only when its hydroxyl is deprotonated, ONP becomes yellow, so the strongest color appears at pH > 8.0. Obviously, such a high pH may inhibit the enzyme in the process of assay. A stopped-time assay can avoid this problem because it can be run at the optimum pH for the enzyme, and then the pH can be raised when the reaction is stopped, which was usually done by the addition of sodium carbonate. Therefore, colorimetric methods were popular in enzyme assay of β-galactosidase.

Although its color is not as strong as that at pH > 8.0, ONP also can be chromogenic at pH about 7.0 and OD_{420} value is linear to the ONP concentration when it is less than 0.3 mM. Therefore, ONPG as substrate can be feasible for a real-time scanning assay of β-galactosidase activity with controlling the assay buffer at pH about 7.0.

A critical step in the Miller procedure is the permeabilization of cells. Rather than preparing cell extracts, e.g., by sonic disruption, the bacteria could be treated by the permeabilization solution that partially disrupts the cell membrane such that ONPG can freely diffuse into the cell. On the other hand, other molecules in the permeabilization solution also can enter into cells and denaturalize the β-galactosidase since the permeabilization solution usually contains surfactant and/or organic solvents. Therefore, it is important to fix the incubation time of the cell suspension with permeabilization solution, which not only ensure the cell membrane can be disrupted and ONPG can freely diffuse into the cell, but also the intracellular β-galactosidase is denaturalized as less as possible. The results obtained revealed that the incubation time of the cell suspension with permeabilization solution should better be controlled in 10 min.

In the Miller and colorimetric methods, the color of reaction solution must be kept close watch on and the reaction time must be noted. If there is too much β-galactosidase in incubation mixture, the reaction solution will turn yellow in few minutes or even seconds. The reaction time is too short to be exactly noted and it is one of the greatest contributions to error. Therefore, the reaction time is usually adjusted to a long time, 45–60 min, so that the time error becomes insignificant. Cells containing β-galactosidase had to be diluted in order to delay the reaction time. Howev-

er, it is necessary to take into account that diluting cell suspension would seriously affect the β-galactosidase activity, especially when the OD_{600} of the cell suspension is less than 0.1.

Besides that, the reaction is stopped usually by the addition of sodium carbonate. In this case the end of the reaction depends on the observation of experimenters, and the color of reaction solution will change after adding sodium carbonate, which makes the assay results by the Miller and colorimetric methods poor reproducible.

It is very important that colorimetric method gives the product, ONP, being produced at a linear rate. Actually, the reactions are often not linear. Because colorimetric method is not real-time monitor assay, the measurement of enzyme activities usually is inaccurate.

Due to development of scan method every assay could be completed in 1 ~ 5 min without considering the reaction time or the color of reaction solutions. Therefore, scanning method for the detection of β-galactosidase activity with ONPG as substrate is simple, fast and reproducible.

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