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Characterization of the activity of β-galactosidase from *Escherichia coli* and *Drosophila melanogaster* in fixed and non-fixed *Drosophila* tissues

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Abstract

β-Galactosidase encoded by the *Escherichia coli lacZ* gene, is widely used as a reporter molecule in molecular biology in a wide variety of animals. β-Galactosidase retains its enzymatic activity in cells or tissues even after fixation and can degrade X-Gal, a frequently used colormetric substrate, producing a blue color. Therefore, it can be used for the activity staining of fixed tissues. However, the enzymatic activity of the β-galactosidase that is ectopically expressed in the non-fixed tissues of animals has not been extensively studied. Here, we report the characterization of β-galactosidase activity in *Drosophila* tissues with and without fixation in various experimental conditions comparing the activity of two evolutionarily orthologous β-galactosidases derived from the *E. coli lacZ* and *Drosophila melanogaster DmelGal* genes. We performed quantitative analysis of the activity staining of larval imaginal discs and an *in vitro* assay using larval lysates. Our data showed that both *E. coli* and *Drosophila* β-galactosidase can be used for cell-type-specific activity staining, but they have their own preferences in regard to conditions. *E. coli* β-galactosidase showed a preference for neutral pH but not for acidic pH compared with *Drosophila* β-galactosidase. Our data suggested that both *E. coli* and *Drosophila* β-galactosidase show enzymatic activity in the physiological conditions of living animals when they are ectopically expressed in a desired specific spatial and temporal pattern. This may enable their future application to studies of chemical biology using model animals.

Keywords: β-Galactosidase; *lacZ*; *Drosophila*; X-Gal

1. Introduction

β-Galactosidase encoded by the *Escherichia coli lacZ* gene, is widely used as a reporter molecule in molecular biology in a wide variety of animals [1]. β-Galactosidase retains its enzymatic activity in cells or tissues even after fixation and can degrade X-Gal (5-bromo-4-chloro-3-indolyl-*β*-D-galactoside), a widely used colormetric substrate, producing a blue color. Therefore, it can be used for the activity staining of fixed tissues. X-Gal is an indole derivative and is transformed into an indoxyl monomer and galactose when its glycosidic linkage is cleaved by β-galactosidase. Subsequently, two indoxyl monomers form a dimer that is a stable and insoluble blue-colored substance through nonenzymatic oxidation [2,3]. This process can be enhanced by an oxidation catalyst; for example, it is effectively enhanced by the addition of ferri-ferrocyanide [4]. Without ferri-ferrocyanide, indoxyl monomers become diffused from the original enzymatic sites so that slowly oxidated indoxyl monomers become deposited as blue-colored dimers at the active sites of endogenous peroxidase. Therefore, activity staining is usually performed in a staining buffer that contains ferri-ferrocyanide.

In *Drosophila*, the *Gal4-UAS* (upstream activation sequence) system is used to express *lacZ* in specific tissues or cells. By combining various *Gal4* strains that confer a variety of expression patterns of *Gal4* with *UAS-lacZ*, β-galactosidase

Abbreviations: CPRG, chlorophenol red-β-D-galactopyranoside; PBS, phosphate-buffered saline; UAS, upstream activation sequence.

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can be expressed with a desired specificity [5]. Although no background activity of β-galactosidase is detected in frequently examined *Drosophila* tissues such as the third instar larval imaginal discs in standard experimental conditions, it is reported that there is endogenous activity of β-galactosidase in certain wild-type *Drosophila* tissues [6]. When whole wild-type third instar larvae were fixed, sectioned by cryostat and stained by X-gal, β-galactosidase activity was detected in tissues including the spiracles, lymph glands and certain parts of the intestine. Additionally, in adult wild-type flies, endogenous β-galactosidase activity was detected in certain tissues. β-Galactosidase activity is also reported to be induced in some *Drosophila* cultured cells, such as Kc cells, by the administration of ecdysterone [7]. Endogenous β-galactosidase activity was shown to be elevated in association with cellular senescence induced by the ectopic activation of Ras [8]. As a candidate for the origin of this endogenous β-galactosidase activity, the genome of *Drosophila melanogaster* has an orthologous β-galactosidase-encoding gene called the *β-Gal-1*, *Gal* or *CG9092* gene [9]. Hereafter, we call this gene *DmelGal*. This locus encodes an 80 kDa protein. DmelGal protein biochemically purified from adult flies was found to be a 160 kDa homodimer, which showed an optimum enzyme activity at pH 6.0 when it was assayed by the hydrolysis of *p*-nitrophenyl-*β*-D-galactopyranoside *in vitro* [10].

Despite the wide usage of β-galactosidase in fixed tissues, the enzymatic activity of β-galactosidase ectopically expressed in non-fixed tissues of animals has not been extensively studied. Previously, β-galactosidase encoded by *lacZ* has been suggested to show enzymatic activity in living *Drosophila* embryos under physiological conditions without fixation [11]. In this study, the activity of β-galactosidase encoded by *lacZ* was visualized in living embryos by injecting a fluorescent substrate. Another fluorescent substrate of β-galactosidase was also demonstrated for use in the detection of ectopically expressed *lacZ* activity in imaginal discs without fixation in phosphate-buffered saline (PBS) [12].

Here, we report the characterization of β-galactosidase activity in *Drosophila* tissues with and without fixation in various experimental conditions, comparing the activity of *E. coli lacZ* and *Drosophila DmelGal*. Our data showed that both *E. coli* and *Drosophila* β-galactosidase can be used for cell-type-specific activity staining, but they have different preferences in regard to conditions.

2. Materials and methods

2.1. Fly genetics

Flies were reared at 25°C, in 60% humidity and 12 h light/dark cycles. *dpp-Gal4* (stock number 1553) was obtained from the Bloomington Drosophila Stock Center. The *UAS-DmelGal* transgenic fly was created as follows. A full-length cDNA clone (LP09580) of the *D. melanogaster DmelGal* (*CG9092*) gene was obtained from the BAC PAC Resources Center at Children's Hospital Oakland Research Institute (Oakland, CA, USA). The 2.3 kb *Xho*I/*Eco*RV DNA fragment containing the full-length *DmelGal* gene was subcloned to the *Xho*I and *Eco*RV sites of pBluescriptII SK-(Agilent Technologies). Then the 2.3 kb *Xho*I/*Bam*HI fragment was subcloned to the *Xho*I and *Bgl*II sites of the pUAST vector plasmid [5]. After the full-length sequence of the *Gal* gene was checked, it was used to create a transgenic fly by P-element mediated transformation. Six transgenic lines were successfully recovered. Among them, the *UAS-DmelGal 2M* strain, in which the transgene was inserted into the second chromosome, was chosen for use in the further experiments because it showed the highest expression when driven by ubiquitous *actin5C-Gal4*, as assayed by staining imaginal discs with X-Gal.

2.2. Staining imaginal discs

The activity staining of imaginal discs with X-Gal was performed essentially as previously described [13]. Wandering third instar larvae were collected and washed in PBS, and leg imaginal discs were dissected. They were fixed in 0.25% glutaraldehyde in 130 mM NaCl, 7 mM Na_2HPO_4, and 3 mM NaH_2PO_4 for 2 min at 25°C, when necessary. Then, the disc was transferred into 10 μl X-Gal staining solution (0.2% X-Gal in appropriate buffers) on the glass slide between two cover glasses that were used as "pillows". The cover glass was set on the staining solution containing a sample between two cover glasses above the thickness of the cover glass. X-Gal stock solution (10% in dimethylformamide) was prewarmed at 65°C for 15 min before being dissolved in the appropriate buffers. The buffers used were Fe/NaP buffer (5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 150 mM NaCl, 1 mM $MgCl_2$, 10 mM sodium phosphate buffer, pH 7.0 or 6.0), PBS (4 M NaCl, 27 mM KCl, 97 mM Na_2HPO_4, 15 mM KH_2PO_4, pH 7.3) and HL3 (70 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 20 mM $MgCl_2$, 10 mM $NaHCO_3$, 5 mM trehalose, 115 mM sucrose, 5 mM HEPES-NaOH, pH 7.1). The samples were observed using Axioskop2 (Zeiss), and photos were taken using DP73 camera (Olympus) with a 20× objective lens and Nomarski optics, while incubating at room temperature (25°C). Staining intensities were quantified using CellSens Dimension software (Olympus). 3 to 8 discs were stained for each experimental condition.

2.3. Enzymatic assay using larval lysate

An enzymatic assay of β-galactosidase activity using tissue lysate was performed as described [14,15]. Wandering third instar larvae were collected and washed with PBS, and each whole larva was collected into a BioMasher II tube (Nippi Inc., Japan). An additional 100 μl appropriate buffer was added and homogenized by 20 strokes with PowerMasher (Nippi Inc., Japan). Additional 100 μl buffer was added and vortexed for 30 s. Then, 2 μl larval lysate was added to 200 μl assay solution (1 mM chlorophenol red-β-D-galactopyranoside; CPRG; Wako pure chemicals, Japan in appropriate buffers) and incubated at 25°C for 30 min. An absorbance of 574 nm was measured. 4 to 10 larvae were assayed for each experimental condition. The background value was obtained