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

Mizuki Tomizawa[1], Kohei Tsumaki[1], Masaki Sone*

Department of Biomolecular Science, Faculty of Science, Toho University, 2-2-1 Miyama, Funabashi, Chiba 274-8510, Japan

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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.

* Corresponding author. Tel./fax: +81 47 472 1709.
E-mail address: masaki.sone@sci.toho-u.ac.jp (M. Sone).

[1] These two authors contributed equally to the work.

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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

from the control assays with no larval lysate. Larvae with no UAS construct were also assayed to examine the activity of endogenous β-galactosidase.

2.4. Statistics

Statistical analyses were performed using SPSS 16.0J.

3. Results

3.1. Creation of a transgenic fly expressing the Drosophila DmelGal gene

We tested the utility of the *DmelGal* (*CG9092*) gene in transgenic flies. For this purpose, we conjugated the full-length *DmelGal* minigene under the control of the UAS regulatory sequence and used it to create transgenic fly strains. Then, we induced the expression of both *lacZ* and *DmelGal* under the control of *dpp-Gal4*, which drives expression in the boundary of the anterior compartment and the posterior compartment in the larval imaginal discs [16–18]. We dissected wandering third instar larvae and stained leg imaginal discs with X-Gal using standard techniques [13]. We fixed the discs in a fixative containing 0.25% glutaraldehyde for 2 min, then incubated them in 0.2% X-Gal solution in Fe/NaP buffer (150 mM NaCl, 1 mM $MgCl_2$, 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 10 mM sodium phosphate buffer, pH 7.0) for 20 min. We performed experiments at 25°C, which is a physiologically optimal temperature for *Drosophila*, not at 37°C that is an optimal temperature of *E. coli* β-galactosidase. No staining was detected for discs with *dpp-Gal4* but no UAS construct (Fig. 1). In *dpp > lacZ* animals, the anterior/posterior compartment boundary was clearly stained. In *dpp > DmelGal* animals, the anterior/posterior compartment boundary was also stained, although the intensity of staining was relatively weak. These data indicated that the *UAS-DmelGal* transgene can be used for the X-Gal activity staining similarly to *UAS-lacZ*.

Next, we stained leg imaginal discs with X-Gal in Fe/NaP buffer at 25°C for 20 min without fixation. While no staining was observed in the *dpp>+* control discs, in the *dpp > lacZ* and *dpp > DmelGal* animals, the anterior/posterior compartment boundary was specifically stained (Fig. 1). The intensity of staining was relatively weak compared with the staining after fixation. These data suggested that both *E. coli* and *Drosophila* β-galactosidase show cell-type specific enzymatic activity without fixation. We also stained the same series of discs with X-Gal in PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4) and HL3 buffer (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). HL3 is a buffer used for preserving *Drosophila* tissues in physiological conditions, for example, for electrophysiological analyses [19]. However, diffuse and non-specific staining was observed for *dpp > lacZ* and *dpp > DmelGal* discs, possibly because of the diffusion of indoxyl monomers in the absence of ferri-ferrocyanide as previously reported (data not shown) [4].

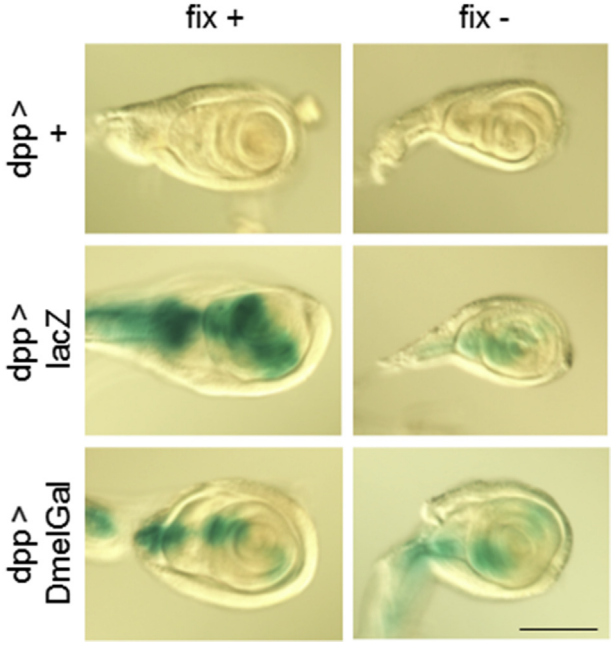

Fig. 1. **X-Gal activity staining of leg imaginal discs expressing *E. coli* and *Drosophila* genes encoding β-galactosidase driven under the control of *dpp-Gal4***. *dpp-Gal4* is specific to the anterior/posterior compartment boundary. Staining was performed at 25°C in Fe/NaP buffer with or without fixation. While no-UAS control showed no signal, both *E-coli lacZ* and *Drosophila DmelGal* showed specific staining both with and without fixation. Scale bar: 100 μm.

3.2. Quantification of X-Gal staining of imaginal discs

To quantify the intensity of the X-Gal staining, we next used the ubiquitous *actin5C-Gal4* driver. We dissected wandering third instar larvae and stained leg imaginal discs with X-Gal without fixation at 25°C for 10 min. In addition to Fe/NaP buffer, which is usually used for X-gal staining, we also tested PBS and HL3 buffer. While no staining was observed in *actin5C>+* control animals, whole leg discs were stained in all of the conditions tested in *actin5C > lacZ* and *actin5C > DmelGal* animals (Fig. 2). Next, we quantified the staining intensity by imaging analysis (Fig. 3). In Fe/NaP buffer, strong signals were observed, while the signals were relatively weak in PBS and HL3, which are less optimal for colorimetry from X-gal. In all of the buffers tested, there was no significant difference in staining intensity between *lacZ* and *DmelGal*.

3.3. Quantification of β-galactosidase activity using larval lysate

Next, we quantified β-galactosidase activity *in vitro* in the lysates of whole larvae using the substrate, chlorophenol red-β-D-galactopyranoside (CPRG) by a previously described method [14,15]. Wandering third instar larvae were collected and homogenized, and then the β-galactosidase activity was assayed by measuring the hydrolyzing activity of CPRG in Fe/NaP, PBS or HL3 buffers. Among the three genotypes tested,

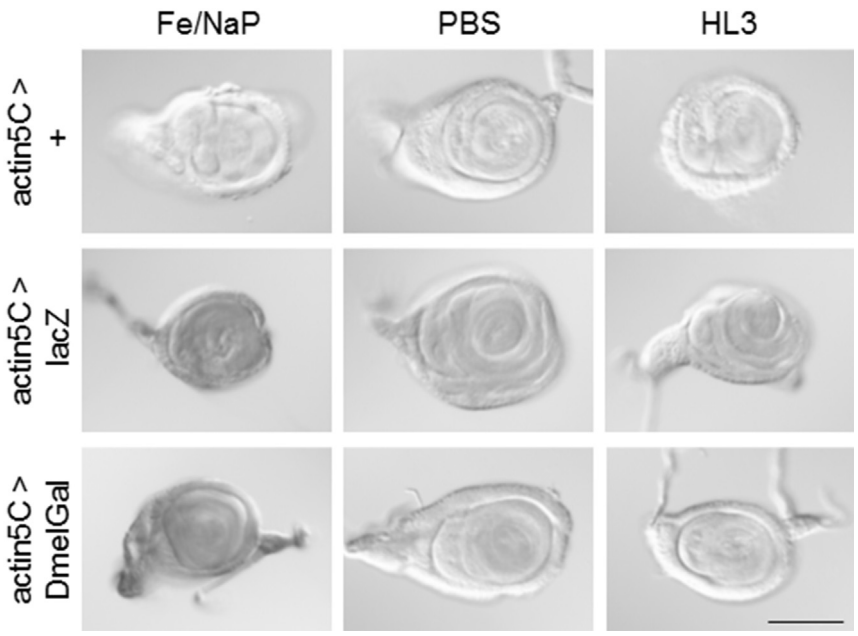

Fig. 2. **X-Gal activity staining of leg imaginal discs expressing *E. coli* and *Drosophila* genes encoding β-galactosidase driven under the control of ubiquitous *actin5C-Gal4***. Staining was performed at 25°C in Fe/NaP, PBS and HL3 buffers without fixation. While no UAS control showed any signal, both *E-coli lacZ* and *Drosophila DmelGal* showed staining throughout the discs. Scale bar: 100 μm.

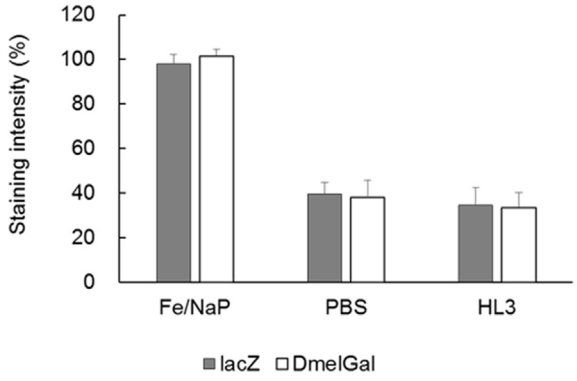

Fig. 3. **Quantification of the staining intensities of leg imaginal discs expressing *E. coli* and *Drosophila* genes encoding β-galactosidase driven under the control of the ubiquitous *actin5C-Gal4***. Error bars indicate s. e. m.

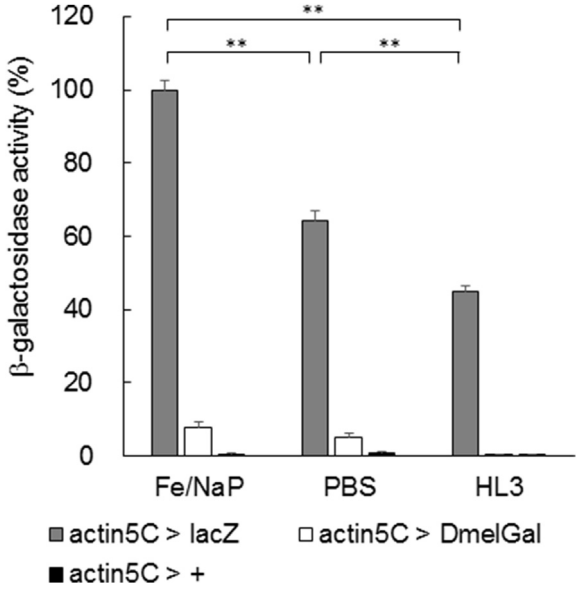

Fig. 4. **Quantification of the β-galactosidase activity of whole larval lysates expressing *E. coli* and *Drosophila* genes encoding β-galactosidase under the control of the ubiquitous *actin5C-Gal4***. Assays were performed at 25°C in Fe/NaP, PBS and HL3 buffers. Error bars indicate s. e. m. **: $p < 0.01$ (Games–Howell test followed by one way ANOVA).

actin5C > lacZ, *actin5C > DmelGal* and *actin5C>+*, *lacZ*-expressing animals showed the highest activity compared with the other two genotypes (Fig. 4). They showed the highest activity in Fe/NaP buffer, moderate activity in PBS and the lowest activity in HL3 (one way ANOVA, $p < 0.01$; Games–Howell test, $p < 0.01$). *DmelGal*-expressing animals showed lower activity than *lacZ*-expressing animals. Finally, almost no β-galactosidase activity was detected in larvae with no UAS transgene.

3.4. Effect of pH for β-galactosidase activity

A previous study reported that DmelGal protein purified biochemically from adult flies showed an optimal enzyme activity at pH 6.0 when assayed by the hydrolysis of *p*-nitrophenyl-β-D-galactopyranoside *in vitro* [10]. Therefore, we tested the activity of β-galactosidase from *E. coli* and *Drosophila* at pH 6.0 by the activity staining of imaginal discs using X-Gal and by an *in vitro* assay using CPRG without fixation in Fe/NaP buffer. In Fe/NaP buffer at pH 6.0, both *actin5C > lacZ* and *actin5C > DmalGal* leg imaginal discs showed staining by X-Gal (Fig. 5). The staining intensity of *actin5C > lacZ* at pH 6.0 was significantly lower than at pH

7.0, which is a standard experimental condition (Fig. 6; one way ANOVA, $p < 0.01$; Tukey test, $p < 0.01$). Next, we examined the *in vitro* β-galactosidase activity of the larval lysates. Compared with pH 7.0, *E. coli* β-galactosidase showed remarkably and significantly lower activity at pH 6.0 (Fig. 7; one way ANOVA, $p < 0.01$; Games–Howell test, $p < 0.01$). However, the activity of both exogenously expressed and endogenous *Drosophila* β-galactosidase showed no significant difference between pH 7.0 and pH 6.0.

4. Discussion

In this study, we report the characterization of the enzymatic activities of *E. coli* and *Drosophila* β-galactosidase that are ectopically expressed through genetic engineering in *Drosophila* tissues. We showed that the *UAS-DmelGal* transgenic fly, which we created, can be used for X-Gal activity staining that shows spatial and temporal specificity in combination with *Gal4* drivers that confer various specificities in fixed tissues using standard techniques.

We also examined their activities in non-fixed *Drosophila* tissues. When they were driven by ubiquitous *actin5C-Gal4*, the intensity of activity staining was not significantly different between *E. coli lacZ* and *Drosophila DmelGal* in the Fe/NaP (pH 7.0), PBS and HL3 buffers. Although both *lacZ* and *DmelGal* showed weaker staining in PBS and HL3 than in Fe/NaP buffer, this result may reflect the slow oxidation of indoxyl monomers, resulting in the less efficient deposition of blue-colored indoxyl dimers in the absence of an oxidation catalyst such as ferri-ferrocyanide [4]. We also performed *in vitro* β-galactosidase activity assay using whole larval lysates and the substrate, CPRG. In these assays, *E. coli* β-galactosidase showed remarkably higher activity than *Drosophila* β-galactosidase. The activity of *E. coli* β-galactosidase was dependent on the buffers used. There was a difference in the preferences of experimental conditions for the activity of β-galactosidase between two experimental

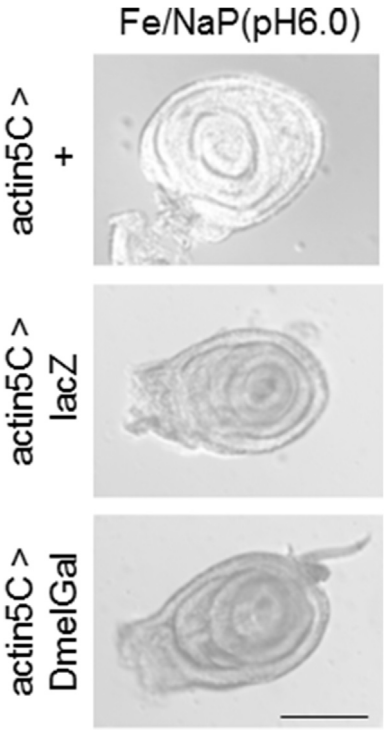

Fig. 5. **pH dependency of X-Gal activity staining of leg imaginal discs expressing *E. coli* and *Drosophila* genes encoding β-galactosidase driven under the control of the ubiquitous *actin5C-Gal4***. Staining was performed at 25°C in Fe/NaP buffer (pH 6.0) without fixation. While no-UAS control showed no signal, both *E-coli lacZ* and *Drosophila DmelGal* showed staining throughout the discs. Scale bar: 100 μm.

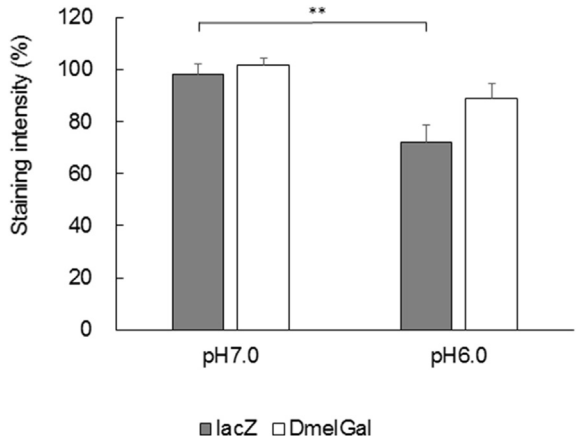

Fig. 6. **pH dependency of quantification of the staining intensities of leg imaginal discs expressing *E. coli* and *Drosophila* genes encoding β-galactosidase driven under the control of the ubiquitous *actin5C-Gal4***. Staining was performed at 25°C in Fe/NaP buffer (pH 7.0 and pH 6.0) without fixation. Error bars indicate s. e. m. **: $p < 0.01$ (Tukey test followed by one way ANOVA).

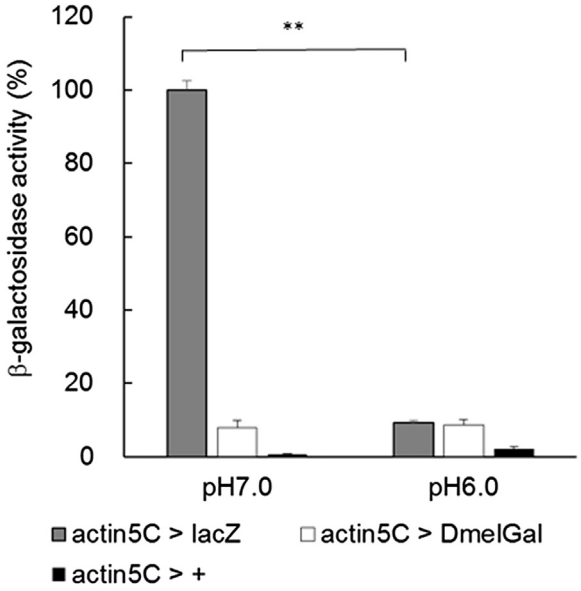

Fig. 7. **pH dependency of quantification of the β-galactosidase activity of whole larval lysates expressing *E. coli* and *Drosophila* genes encoding β-galactosidase driven under the control of the ubiquitous *actin5C-Gal4***. Assays were performed at 25°C in Fe/NaP buffer (pH 7.0 and pH 6.0). Error bars indicate s. e. m. **: $p < 0.01$ (Games–Howell test followed by one way ANOVA).

methods, in the activity staining of imaginal discs and in the *in vitro* assay using larval lysates. It may reflect the subcellular localization, dimerization or interaction with other molecules of ectopically expressed β-galactosidase. In an *in vitro* assay, the β-galactosidase molecule exists in the assay buffer that contains homogenously dissolved components of larval lysate. However, in the cells of *Drosophila* whole-mount imaginal discs, the β-galactosidase proteins are localized in specific subcellular sites that provide appropriate environments for enzymatic reactions, especially in the case of *Drosophila* β-galactosidase. Such subcellular environments may also enable the cooperative action with other proteins or cofactors. Because mammalian β-galactosidase is known to be localized in the lysosome that provides an acidic environment [20], the lysosome is the candidate subcellular site for the optimal activity of β-galactosidase. *Drosophila* β-galactosidase is also suggested to be localized in lysosomes [21]. It is noteworthy that there is a remarkable difference in the pH preferences between *E. coli* and *Drosophila* β-galactosidase both in the activity staining and the *in vitro* assay. Although *E. coli* β-galactosidase is less active at pH 6.0 than at pH 7.0 in both experiments, *Drosophila* β-galactosidase is as active at pH 6.0 as at pH 7.0. These results suggest that *E. coli* β-galactosidase prefers neutral pH environments to acidic pH when it was compared with *Drosophila* β-galactosidase in our experimental conditions.

Although *lacZ* is widely utilized in fixed tissues, its enzymatic activity has not been extensively examined in non-fixed tissues. In this study, we showed that β-galactosidase that was ectopically expressed from a transgene possesses an enzymatic activity in non-fixed tissues, which means that artificially expressed β-galactosidase can be used to modify chemical compounds that were ectopically introduced into living animals in a tissue or cell-type specific manner. In chemical biology, a variety of chemical compounds are introduced into cells, tissues or animals and their effects will be examined [22]. However, it is difficult to introduce chemical compounds into specific cells in complex multicellular animals such as *D. melanogaster*. One possible way to solve this problem is to introduce molecules non-specifically and then activate them only in specific cells by means of enzymatic conversion. For this purpose, the chemical compounds to be introduced need to be designed and synthesized to be modified chemically to be inactivated, and then they will be converted to active state by an enzymatic activity that resides in specific cells. In *Drosophila*, the activity of an enzyme can be triggered in distinct spatial and temporal patterns by sophisticated genetic methods, including the *Gal4-UAS* system. If the molecule is inactivated by the modification of galactose, it will be able to be activated by an enzymatic activity of β-galactosidase. *Drosophila* β-galactosidase shows its optimum activity in the lysosomes of living *Drosophila* cells, which is at 25°C and at acidic pH. It is expected not to be toxic compared with the *E. coli* molecule. Therefore, for the purpose of the application for the chemical biological experiments using living flies, utilization of *Drosophila* β-galactosidase transgene may be beneficial because of its properties on optimum temperature, optimum pH and potential toxicity in the living *Drosophila* cells.

5. Conclusion

Our data suggested that both *E. coli* and *Drosophila* β-galactosidase show enzymatic activity at the physiological conditions in living animals when they are ectopically expressed in a desired specific spatial and temporal pattern. Further investigation will be needed to reveal whether it can be applied to the purpose of the cell-specific activation of ectopically introduced chemical compounds.

Conflict of interest

We certify that there is no conflict of interest with any organization regarding the material discussed in the manuscript.

Acknowledgments

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