Cloning, Expression, and Characteristic Analysis of the Novel β -Galactosidase from Silkworm, *Bombyx Mori*

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Research Article

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Abstract

β -galactosidase is a critical exoglycosidase involved in the hydrolysis of lactose, the modification and degradation of glycoprotein *in vivo*. In this study, the β -galactosidase gene of silkworm (*BmGal*), whose cDNA comprises 11 exons and contains an intact ORF of 1821bp, was cloned. The protein sequence of BmGal showed high similarity with other known insect β -galactosidases. No activity of the BmGal expressed in *Escherichia coli* or *Pichia pastoris* was detected while it was successfully expressed with high enzyme activity in baculovirus–silkworm expression system, and the electrophoresis result revealed that the BmGal showed activity in oligomer mode. Enzyme activity assay showed that its optimum pH was 8.4 and its optimum temperature was 40°C. What's more, we found that iron ions can stimulate the activity of the enzyme while cobalt, nickel or lead ions can inhibit its activity significantly. Besides, the temporal-spatial expression pattern of the *BmGal* mRNA level was analyzed, which showed that *BmGal* was expressed at the highest level in the fifth larval instar but relatively low level in the pupal and adult stage, and the highest expression level of *BmGal* was found in testis among all the tissues concerned.

Introduction

β -galactosidase (β -D-galactosidase, galactosidase, EC 3.2.1.23), also called lactase, is a kind of glycoside hydrolase (GH), which catalyses the hydrolysis of β -D-galactopyranosides or transgalactosylation. For its significant role in biological metabolism process, it was universally expressed in microorganisms (bacteria, yeasts, fungi), plants and animals [1]. Based on sequence homology and evolutionary relationships, β -galactosidase can be classified into 4 distinct glycoside hydrolase families (GH): GH1, GH2, GH35, and GH42 [2]. The β -galactosidases of GH1, GH2, and GH42 groups have been found mainly in bacteria and fungi, whereas the β -galactosidases from GH35 family have been found not only in bacteria, but also in fungi, plants, and animals as well [3]. Most research of β -galactosidase is limited to that encoded by the *Escherichia coli* lacZ gene—which is widely used as a reporter molecule in molecular biology in a wide variety of animals. This enzyme is composed of four homologous monomers, each of which contains 1023 amino residue with a molecular weight of 116483 Da [4]. About 70% of adults worldwide suffer from lactose indigestion or lactose intolerance [5]. The natural substrate for most β -galactosidases is lactose; hence they are widely used in food processing and pharmaceutical industry, especially in the dairy industry [6]. In addition, β -galactosidase can produce galactose-oligosaccharide(GOS), a probiotic, by transgalactosylation. Since GOSs can improve the intestinal flora to influence the health of the body, they have been widely used in medical and health products [7].

Studies on β -galactosidase in insects were limited and only a few paper reported in this area, mainly in *Drosophila melanogaster*. The structural gene, β -Gal-1 encoding β -galactosidase of *Drosophila melanogaster*, has been mapped by two independent genetic approaches [8]. With the help of the X-gal staining, Schnetzer et al. comprehensively analyzed the activity of β -galactosidase of *Drosophila melanogaster* in different tissues and development stages [9]. It was reported that *Drosophila* β -galactosidase showed enzymatic activity in the physiological conditions of living animals when they were ectopically expressed in a desired specific spatial and temporal pattern [10]. Ferreira et al. purified β -

galactosidase(β -Gly4) from the intestine of Yellow mealworm (*Tenebrio molitor*) [11] and this enzyme had a very high activity for the β -galactose glycoside and could hydrolyze fucose but not glucose. Furthermore, it was found that the expression of β -galactosidase can be induced by ecdysterone in *Drosophila melanogaster* cell cultured *in vitro* [12].

Silkworm (*Bombyx mori*) which has been domesticated over five thousand years as a kind of excellent economic insect, belongs to *Lepidoptera*. With the completion of genome sequencing program, silkworm received widespread attention as an insect model organism [13]. In silkworm, the β -glucosidase and the amylase have been reported [14, 15], but there were no reports on the β -galactosidase of silkworm so far. In this study, the β -galactosidase of silkworm (*BmGal*) was cloned, and expressed in baculovirus–silkworm expression system. Enzyme activity assay revealed that BmGal showed high activity in oligomer mode. Its optimum pH was 8.4 and the optimum temperature was 40°C. In addition, the temporal-spatial expression pattern of the BmGal was also analyzed.

Materials And Methods

Materials

Escherichia coli strains Top 10, BL21(DE3) and pET-28a, *Pichia* receptor strains GS115 and *Pichiapastoris* expression vector pPIC9- γ , baculovirus transfer vector pVL1393, Bmbacpak-6 virus containing the β -galactosidase gene of *Escherichia coli*, *Bombyx mori* cell line, parental virus BmBacmid made from *Bombyx mori* nucleopolyhedrovirus genome which lacks the ORF1629 gene [16, 17], Bm-N cell and silkworm variety JY-1 were all kept in our own laboratory. Chemical reagents were purchased from Sigma. Restriction endonucleases were purchased from Promega. Vector pMD18-T, LA Taq polymerase, DEPC, DNA quick purification and recovery kit, plasmid rapid extraction kit were purchased from TaKaRa. Insect cell culture medium and FBS were purchased from Invitrogen.

Methods

Gene clone and sequence analysis

The β -galactosidase amino acid sequences of *Drosophila* and *Anopheles* were retrieved in NCBI and highly conserved sequences of which were blasted in the silkworm EST database. cDNA fragments of FS799235.1–FS793759.1–FS792709.1 and FS792597.1 were obtained for probes to retrieve in the silkworm EST database once again. Assembling all the EST fragments obtained, we got three long cDNA sequences that cannot be incorporate with each other. And then, they were retrieved in the silkworm genome database (<http://silkworm.genomics.org.cn/>) [18] and transcriptome database (<http://124.17.27.136/gbrowse2/>) [19] and a cDNA fragment, which contains a complete ORF, was obtained. Sequence alignment with β -galactosidase amino acid sequences of *Drosophila* and *Anopheles* showed that the fragment encoded an intact silkworm β -galactosidase.

To confirm the assembled sequence, specific primers *BmGal*-F and *BmGal*-R (Table S1) were designed to amplify the β -galactosidase cDNA sequence from the cDNA of silkworm according to the assembled cDNA sequence next to the 3' ending of galactosidase gene. Meanwhile, the 3'-UTR was determined by 3'-RACE and the full-length cDNA sequence, which contains a ORF, was obtained. Tools from EBI (<http://www.ebi.ac.uk/Tools/>) were used for the analysis of the signal peptide and conserved domains of the protein, as well as the prediction of molecular weight and isoelectric point. Phylogenetic tree was constructed by Mega 7.0 [20].

Construction of expression vector and recombinant baculovirus expression of the enzyme

RNA was extracted according to the standard protocol by Trizol instruction. The total silkworm cDNA was obtained through reverse transcription according to the specification of Promega Kit (Cat No. K1005S). The following primers: *BmGal*-OF (containing *Bam*H $\square$ site) and *BmGal*-OR (containing *Hind* $\square$ site) (Table S1) were designed to clone the whole ORF to obtain pMD-18T-*BmGal*. pMD-18T-*BmGal* was verified by sequencing and the correct fragments were cloned to pET-28a to construct the recombinant plasmid pET-28a-*BmGal*.

As the *Pichia Pastoris* expression vector pPIC9- γ lacked the common cloning sites, we designed two new primers Ps (containing *Spe* $\square$ site) and Px (containing *Not* $\square$ site) (Table S1). The primers were used to introduce the new restriction enzyme sites; the amplified fragment was confirmed by DNA sequencing. The expression procedures in *Pichia Pastoris* were done referring to the methods of Hildebrandt [21].

*Bam*H $\square$ and *Pst* $\square$ were used to digest pMD-18T-*BmGal*. The obtained fragment was ligated to pVL1393 to construct pVL1393-*BmGal*. The pVL1393-*BmGal* was co-transfected with ORF1629 defaulted DNA of BmBacmid [16, 17] into *Bm*-N cell. Obvious cytopathic effect (CPE) occurred after 4 days' cultivation at 27°C. Recombinant virus rBmNPV-*gal*, which was obtained from the supernatant through two rounds of plaque isolation, was used as stock virus after molecular detection.

Real-time PCR for quantification of BmGal mRNA level in different tissues

According to the cDNA sequence of *BmGal*, the forward primer *BmGal*-QF was designed basing on the ninth exon and the reverse primer *BmGal*-QR basing on the tenth exon (Table S1). The target fragment was cloned to pMD18-T. The recombinant pMD18-T which were diluted to 10^6 – 10^2 copy per microliter were used as standard samples for real-time PCR after its concentration was measured. The calibration curve and linear equation ($y=ax+b$) were derived from the graph constructed according to the Ct values and the corresponding logarithms of copy number. 1 μ g RNA extracted from different tissues of the fifth instar larva, pupa (5th day) and adult silkworm were used for reverse transcription. Real-time PCR was performed using 1 μ l reverse transcription products (equal to 10ng RNA) as templates. Absolute quantification was used. X value was calculated based on the calibration curve and the copy number of the target gene was calculated by the formula $Y=10^X$ [22].

Enzyme activity assay

Enzyme activity was determined by the modified ONPG method [23]. The enzyme activity was determined according to the formula $U = 1000 \times (OD_{420} - 1.75 \times OD_{550}) / (T \times V \times OD_{595})$. In this formula, U represents the activity of β -galactosidase, T represents the reaction time (min) after ONPG was added, V represents the volume (volume in control was subtracted in the final calculation). Expression in baculovirus–silkworm expression system was measured using properly diluted hemolymph (after ultrasonic treatment and centrifugation) of the fifth instar larvae, by comparing to the silkworm blood infected by wild virus.

Non-denaturing gel electrophoresis and staining

SDS was omitted when preparing the PAGE gel and electrophoretic buffer. Meanwhile, β -mercaptoethanol was also omitted from loading buffer in order to ensure that protein could be isolated under a non-denaturing condition. Gel was packed in self-sealing bag and incubated with 5ml 1g/L X-*gal* solution (dissolved in PBS) at room temperature for 2h in the dark. Expression production from BmBacPAK6 which contained β -galactosidase gene of *Escherichia Coli* was used as positive control.

Results

Clone of silkworm β -galactosidase

According to the β -galactosidase amino acid sequences of *Drosophila* and *Anopheles* in NCBI, we got a 2154bp sequence of complete *BmGal* cDNA (GenBank: FJ571146.1), comprising 11 exons and containing a whole ORF of 1821bp. A protein containing 606 amino acid residues (GenBank:NP_001157373.1) was obtained by the translation of the complete ORF. The result of conserved domain analysis in NCBI indicated that this protein contained three conserved domains: a β -galactosidase domain, a carbohydrate binding domain and a β -galactosidase-like domain. As the result of the phylogenetic tree, we can find that β -galactosidase in vertebrates and invertebrates obviously differs from each other. The evolution relationship between silkworm *BmGal* and β -galactosidase gene in *Plutella xylostella* is the closes (Fig. 1).

*Expression profile of silkworm *BmGal* mRNA in different stages, different tissues*

The RT-PCR results of different tissues extracted from 5th instar 3rd day's larva showed that *BmGal* was expressed in all tissues of the fifth instar larva. However, the expression level of *BmGal*, varies widely in different tissues, which were very low in silk gland and hemolymph but were high in testis, epidermis and middle silk gland (Fig. S1). More expression information of *BmGal* in different stages and tissues was obtained through Real-Time Quantitative PCR (qPCR) (Fig. 2). The results obtained by qPCR were basically consistent with those obtained by RT-PCR. In all tissues of the fifth instar larva, the highest expression level was detected in testis and the lowest was in terminal silk gland (Fig. 2A). *BmGal* was expressed unequally in different stages, and the expression level in larva was the highest. The result just accorded with the fact that the silkworm grew rapidly in the larval stage, in which the gene was highly expressed in most tissues. However, the highest expression level in pupa and moth were in head and Malpighian tubule, respectively (Fig. 2B, Fig. 2C).

Time-course expression analysis of silkworm BmGal mRNA level in testis

According to the results of *BmGal* expression level from qPCR in different tissues and different stages, testis was selected for more in-depth analysis because expression level in it was the highest in the fifth instar larva and relatively high in the other two periods. From the first day of fifth instar, the total RNA in testis was extracted every day or every two days for qPCR analysis after reverse transcription. As shown in Fig. 3, the expression of *BmGal* did not vary smoothly in the continuous phase process of tasis. The expression level in larva varied slightly from the first day of the fifth instar to the seventh day of the fifth instar. However, big decline occurred during the wandering stage and the early spinning stage, especially the first day of the spinning, on which the expression of *BmGal* was the lowest. Then it gradually increased until the maximum value was reached in the pre-pupa stage. Afterwards, the mRNA level showed a tendency of gradual decline. In the end, when it came to the ninth day of pupation, the dramatic decrease process appeared.

Expression of BmGal in Escherichia Coli and Pichia Pastoris

The precipitate contained most of the recombinant protein expressed by the recombinant *Escherichia Coli* strains, indicating that most expressed galactosidase existed in inclusion bodies. The results of the enzyme activity assay showed that recombinant protein expressed in the prokaryotic expression system was of no biological activity, which indicated that higher structure was essential for the activity of silkworm β -galactosidase. In consideration of the possible existence of post-translational modification or higher structure in eukaryote, five recombinant strains of *Pichia pastoris* with higher β -galactosidase activity were screened out from the detection of 100 strains. However, the activity in those were not significantly higher than that of the control, which made the analysis difficult to carry on.

Expression of BmGal in silkworm

As the expression result in *Pichia pastoris* was dissatisfactory, silkworm expression system was chosen to analyze the expression activity of BmGal. It was found that there was about 800 IU of enzyme activity per milliliter of hemolymph, but almost no enzyme activity was detected in the control group. The optimum pH and the optimum temperature of the enzyme activity were measured. As shown in Fig. 4, the optimum pH was 8.4 (Fig. 4A) and the optimum temperature was 40°C (Fig. 4B). In fact, the activity was all along high when the pH was between 8 and 9.

The enzyme activity is closely related to the reaction buffer. The activity of BmGal in different solutions containing different kinds of metal ions or anionic detergents were measured, and the results revealed that the enzyme activity was the highest in iron ion solution but reduced significantly in cobalt, nickel, or lead ion solution (Fig. 4C).

Structure analysis of BmGal

Non-degeneration gel electrophoresis was used to determine the quaternary structure of BmGal in silkworm. According to the structure analysis of *Escherichia Coli* β -galactosidase (Matthews 2005), the

enzyme is a tetramer composed of four identical amino acid chains and the molecular weight of one monomer is about 116kDa. The predicted molecular weight of BmGal monomer was about 68.1kDa. As shown in Fig. 5, *BmGal* may function in multimer mode using *Escherichia Coli* β -galactosidase expressed in silkworm as the control.

Discussion

It is reported that β -galactosidase is universally expressed in animals, plants, and microorganisms. However, studies on β -galactosidase in insects are limited, and specifically, it has not been reported in the silkworm, *Bombyx mori*. Through comparison on NCBI, silkworm EST database, silkworm genome database, and transcriptome database, etc. [18, 19], we found that the complete cDNA sequence of *BmGal* gene was 2154bp in length, comprising 11 exons, containing an ORF of 1821bp, and encoding a protein of 606 amino acid residues. The protein had an N-terminal signal peptide of 24 residues according to the analysis by SignalP 4.0 Server (<http://www.cbs.dtu.dk/services/SignalP/>) [24]. SWISS-MODEL prediction showed that the protein can form a basket-shaped structure comprising three conserved domains. The largest domain had a TIM barrel constructed by α helices and β sheets.

Homology analysis of β -galactosidase amino acid sequences from different species revealed that β -galactosidase in various insects shared a high identity at about 40% in conserved regions and identity in other regions was rather low. Phylogenetic tree constructed using β -galactosidase amino acid sequences in 26 species (Neighbor-Joining method) showed that β -galactosidase in vertebrates and invertebrates significantly differed from each other. What's more, the evolutionary relationship between BmGal and β -galactosidase in *Plutella xylostella* was the closest. However, there is no clear report on β -galactosidase in invertebrates. Therefore, the report on *BmGal* in this paper can provide a theoretical basis for the study of β -galactosidase in invertebrates.

The endogenous β -galactosidase activity was found in various organs at larval, prepupal, pupal and adult stages of *Drosophila melanogaster* reared under axenic condition by applying X-gal staining methods, such as larval intestine, pupal cellular epidermis, adult pericardial cells and so on [9]. In this study, the *BmGal* gene was widely expressed in all organs no matter at larval stage, pupa stage, or adult stage according to the mRNA distribution in silkworm tissues. Overall, the expression level of *BmGal* in the pupal stage was higher than that in the moth stage and higher in the larval stage. In the larval stage, the expression level of *BmGal* in testis was the highest and the lowest was in the terminal silk gland; in the pupal stage, the highest expression level was detected in the head and the lowest was in the midgut; The expression level of *BmGal* in the moth stage was relatively less, expression in the Malpighian tubule became the highest and the lowest expression level occurred in the antenna. In conclusion, *BmGal* was a widely distributed gene and its mRNA level in all organs at larval, pupal, and adult stages of silkworm was quite high.

Testis was chosen for *BmGal* gene Time-course expression analysis because its expression level of the *BmGal* gene was the highest among the organs in the fifth instar larva. The expression level increased

gradually from the newly molted larva to the seventh day at the fifth instar. However, from the seventh day of the fifth instar to the day before the spinning stage, the mRNA level emerged a rapid decrease of 100 fold in 48 hours. And in the 48 hours from the spinning stage to the day before pupation, the mRNA level increased by 120 fold and reached the peak. In recent years, β -galactosidase has been found to play an essential role in various biological processes. For example, Senescence-associated β -galactosidase staining has now been employed to identify the presence of senescent cells [25]. Su et al. found that the level of β -galactosidase in B cells and serum of rheumatoid arthritis (RA) patients is higher and revealed that altered β -galactosidase may be implicated in the progression of inflammation [26].

Moreover, *Streptococcus thermophilus* can inhibits colorectal tumorigenesis through secreting β -galactosidase [27]. Interestingly, the results showed that the high expression level and dramatic variation in testis may relate to its morphology changes in the metamorphosis period. And we inferred that the *BmGal* gene may participate in the development of testis and the procedure of spermatogenesis.

The expression experiments showed that BmGal expressed in the prokaryotic system had no activity, either did it in *Pichia pastoris*. Therefore, we expressed it in the silkworm expression system, and high enzyme activity was detected. The optimal pH and temperature for β -galactosidase of many bacteria and fungi were found in the range of 6.5-4.5 and 40-60 °C, respectively [3, 7, 28, 29]. However, the optimum pH and temperature of some β -galactosidase are not in this range, for example, β -galactosidase from *Aspergillus lacticoffeatus* [30]. In this study, we found that the optimum pH and temperature of BmGal were, respectively, 8.4 and 40°C. Effects of metal ions and organic reagents play important roles in the activity of β -galactosidase. Ba^{2+} , Fe^{2+} , Li^{+} , and K^{+} can inhibit the activity of *Aspergillus lacticoffeatus* β -galactosidase, while additives (except ascorbic acid) and detergents can promote its activity [30]. Most metal ions promoted the activity of β -Galactosidase from *Alteromonas sp.* QD01, especially Mn^{2+} and Mg^{2+} [7]. According to the analysis of influences caused by metal ions to BmGal, we found that iron ions can stimulate the activity of the enzyme while cobalt, nickel, or lead ions can inhibit its activity significantly. At present, many β -galactosidases are found to function in the form of oligomers. For example, *Thermotoga maritima* β -galactosidase is arranged in the form of a peculiar octamer [31]. Structural characterization revealed that β -galactosidase from *Bacillus subtilis* was a homotrimer in solution [28]. β -galactosidase from the Deep-sea bacterium *Alteromonas sp.* ML52 is active as a homotetrameric protein [32]. The active phospho- β -galactosidase from *Bacillus velezensis* was identified to be a homodimer [33]. According to non-degeneration gel electrophoresis of BmGal and *Escherichia Coli* β -galactosidase, we found that the molecular weight of active BmGal was even larger than that of *Escherichia Coli* β -galactosidase. As *Escherichia Coli* β -galactosidase functioned in tetramer mode, we inferred that the enzyme showed activity in oligomer mode, even at least in tetramer mode. This well explained why the expression products in *Escherichia Coli* or *Pichia pastoris* didn't show enzyme activity and indicated that baculovirus–silkworm expression system can express a foreign gene that functioned in multimer mode.

Declarations

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Conflicts of interest/Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data transparency

All data and materials as well as software application or custom code support our published claims and comply with field standards.

Code availability

Not applicable.

Authors' contributions

Yongzhu Yi: Methodology, Investigation, Writing-Original Draft. **Jialei Li:** Methodology, Investigation, Writing-Original Draft. **Zhipeng Zong:** Investigation, Formal analysis. **Xingjian Liu:** Visualization. **Haozhi Song:** Investigation. **Haining Wang:** Investigation. **Zhifang Zhang:** Project administration. **Huan Zhang:** Validation. **Yinü Li:** Conceptualization, Supervision, Writing-Review & Editing.

Ethics approval

We state that the contents in this manuscript have not been published or submitted for publication elsewhere. All authors have seen and approved the manuscript, and the authors and the institutes agreed to submit to "Molecular Biology Reports". We also state that our experiment is ethically and legally acceptable.

Consent to participate

Not applicable.

Consent for publication

The authors declare that we agree with the content and give explicit consent to submit and we obtain consent from the responsible authorities at the institute/organization where the work has been carried out, before the work is submitted.

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Figures

Figure 1

Evolutionary relationship between BmGal and β -galactosidase in other insects and vertebrates (Neighbor-Joining method). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) were shown next to the branches. Evolutionary analyses, which involved 26 amino acid sequences, were conducted in MEGA7.

Figure 2

Distribution of BmGal mRNA in different tissues, different stages. The mRNA level of BmGal in each tissue with 10ng total RNA was detected by qPCR. (A) The mRNA level of BmGal in each tissue at the fifth instar larva. (B) The mRNA level of BmGal in each tissue at pupal stage. (C) The mRNA level of BmGal in each tissue at moth stage. C, Cuticle; WD, wing disc; T, testis; O, ovary; MT, Malpighian tubule; FB, fat body; TC, tracheal cluster; MG, midgut; A-SG, anterior silk gland; M-SG, middle silk gland; P-SG, posterior silk gland; H, head; He, hemocyte; OM, ommateum; A, antenna.

Figure 3

Time-course expression of silkworm BmGal mRNA level in testis. The mRNA level of BmGal in testis at different stages was detected by qPCR. 5th, the fifth instar larva; d, days; S, spinning; P, pupa stage.

Figure 4

Effect of pH, temperature, and different ion solution buffers on the activity of BmGal. (A) The activity of BmGal under different pH; (B) The activity of BmGal under different temperature; (C) The activity of BmGal in different ion solution buffers. The concentration of all metal ion solutions was 1.0mM. The activity of the BmGal in pure water was used as the control, and taking the ratio of enzyme activity of BmGal in various solutions to that in pure water as the ordinate.

Figure 5

Non-degeneration gel electrophoresis of BmGal. Lane M, protein Marker; Lan1, Escherichia Coli β -galactosidase expressed in silkworm; Lane2, BmGal expressed in silkworm.

Supplementary Files

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