

Research Article

In Vivo Bioassay of Recombinant Human Growth Hormone Synthesized in *B. mori* Pupae

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The human growth hormone (hGH) has been expressed in prokaryotic expression system with low bioactivity previously. Then the effective *B. mori* baculovirus system was employed to express hGH identical to mature hGH successfully in larvae, but the expression level was still limited. In this work, the hGH was expressed in *B. mori* pupae by baculovirus system. Quantification of recombinant hGH protein (BmrhGH) showed that the expression of BmrhGH reached the level of approximately 890 $\mu\text{g}/\text{mL}$ pupae supernatant solution, which was five times more than the level using larvae. Furthermore, Animals were gavaged with BmrhGH at the dose of 4.5 mg/rat.day, and the body weight gain (BWG) of treated group had a significant difference ($P < .01$) compared with the control group. The other two parameters of liver weight and epiphyseal width were also found to be different between the two groups ($P < .05$). The results suggested that BmrhGH might be used as a protein drug by oral administration.

1. Introduction

The human growth hormone (hGH) is a ~22 kDa polypeptide synthesized and stored by somatotroph cells within the anterior pituitary gland. It acts either directly or indirectly on various tissues and physiological systems such as longitudinal bone, skeletal muscle, liver, total body nitrogen balance, and so on [1]. Expression of hGH polypeptide has been performed in *Escherichia coli* [2]. Since the signal peptides of proteins from eukaryotes are not recognized in prokaryotes, fusion proteins such as maltose binding protein *mal E* [3] or thioredoxin [4] have been used to produce active rhGH protein. A major disadvantage of this approach is that the fusion partner contributing the majority of the total mass of the fusion protein could be highly immunogenic. The hGH has also been expressed in *sf9* cells [5], eukaryotic alga [6], and other eukaryotic system. On the whole, the expression level has been limited. Then the effective *Bombyx mori* (*B. mori*) baculovirus system was employed to express 654bp hGH cDNA encoding signal and mature peptide; as

a result, 160 μg rhGH /mL larvae hemolymph identical to nature hGH was purified [7]. But the expression level was still limited and it was not easy to purify the rhGH expressed by larvae. Therefore, it was very difficult that the recombinant hGH could be used as an injectable drug.

Oral administration was a good way for protein drugs. Zhang et al. proved that protein expressed by silkworm pupae bioreactor could bring into effect as active cytokine through oral administration [8]. In this study, we inserted hGH cDNA encoding mature peptide into BmNPV by baculovirus system. Constructed recombinant virus infected diapausing *B. mori* pupae instead of larvae. Furthermore, hypophysectomized male rats were used to evaluate the biological activity of BmrhGH by oral administration.

2. Materials and Methods

2.1. Cell Lines and Virus. The *B. mori*-derived cell line BmN (conserved by our lab) was maintained at 27°C in

TC-100 medium (GIBCO) supplemented with 10% fetal bovine serum. Wild type BmNPV (WT BmNPV, conserved by our lab) were propagated on BmN cells. The human erythroleukemia K562 cell was the gift from the Cancer Institute of Zhejiang University. Cell medium PRMI1640 and fetal bovine serum were purchased from GIBCOL.

2.2. Construction of Recombinant Virus Harboring hGH Sequence. Construction and identification of recombinant virus vBmrhGH containing hGH were performed as previously [7]. Briefly, the hGH cDNA was amplified from the plasmid PWR-hGH by PCR using the following primers: forward: 5'GGGGATCCAGTTTCCTACTATAACCAC3' and reverse: 5'TTGAATTCCTAGAAGCCACAGCTGCC3'. Enzyme sites of *Bam*H I and *Eco*R I were introduced at 5'-terminus and 3'-terminus, respectively, by designing primers. PCR products were digested by the two enzymes and ligated into the same cloning site of pBacPAK8 using T4 DNA ligase. The recombinant transfer vector was designated as pBacPAK-hGH. The sequence of the inserted hGH DNA synthesized by PCR was confirmed by nucleotide sequencing.

The purified DNAs of pBacPAK-hGH and linearized WT BmNPV were used for cotransfection of BmN cells (1×10^6 cells) with Lipofectin (Roche Applied Science) as described previously [9]. Six days after the transfection, the culture medium was removed and the recombinant virus was isolated by the end point dilution method in 96-well plates. The recombinant virus isolates, showing cytopathic effect but no polyhedral inclusion body production, were purified by three cycles of plaque assay [10]. The purified virus was designated as vBmrhGH. It propagated by infecting a great amount of BmN cells. The titer of the vBmrhGH preparation determined by TCID₅₀ was about 6×10^8 PFU/mL.

Total cellular genomic DNA was isolated from BmN cells infected by vBmrhGH or WT BmNPV and used for Southern blotting to confirm the authenticity of inserted fragment. They were probed by enzyme-digested hGH cDNA from PWR-hGH labeled by DIG (DIG DNA labeling and detection kit, Roche Applied Science).

2.3. BmrhGH Sample Preparation. *B. mori* pupae (Qingsong×Haoyue, purchased from Zhejiang Chinagene Biomedical Co., Ltd.) were released from cocoons and inoculated by vBmrhGH or WT BmNPV in body cavity through a syringe at a multiplicity of 10^6 PFU/pupa. *B. mori* pupae were collected each day and "crashed". The pupae mash was centrifuged at 15,000 g for 30 minutes at 4°C to remove most of the top lipids and the bottom depositions. The upper solution was centrifuged for two more times as described to remove the remaining lipids and depositions. The fraction of pupae supernatant solution was stored at -20°C as protein sample. Protein samples were electrophoresed through 15% SDS-PAGE and stained with Coomassie brilliant blue (CBB).

2.4. Western Blotting. The specificity of the recombinant protein BmrhGH was identified by Western blotting. Proteins in polyacrylamide gel were transferred onto PVDF membrane

(Millipore). The blots were blocked with 1% BSA in 20 mM PBS (pH 7.4) for 1h at room temperature and incubated with 1 : 2000 dilution of anti-hGH antiserum (Sigma) in blocking reagent for 2 hours at room temperature. The membrane was washed three times in PBS having 0.2% Tween-20 (PBST) for 10 minutes and incubated in goat antirabbit IgG antibody conjugated with horseradish peroxidase (Vector lab, with a dilution of 1:2000 in blocking reagent) for 1 hour at room temperature. After washed with PBST for two times and PBS for another one time, the blots were monitored for color development in 10 mL 100 mM Tris-Cl (pH 7.4) including 0.05% diaminobenzidine and 0.2% hydrogen peroxide.

2.5. ELISA. The amount of recombinant BmrhGH expressed in silkworm pupae was assayed by ELISA according to the protocol of Ausubel et al. [11]. The ELISA standard curve was constructed using a series of hGH standard (from Zhejiang Shao Yifu Hospital) from 39 ng/mL to 1.2 μg/mL according to the manual of Wang and Fan [12]. Protein samples (hGH standard protein and pupae supernatant solution) were coated onto 96-well polystyrene plate overnight at 4°C. The plate was blocked with 1% BSA in PBS for 2 hours at room temperature. Then it was incubated with 1 : 3000 dilution of anti-hGH antiserum in PBST for 2 hours at 37°C. The plate was washed and incubated with 1 : 2000 dilution of goat antirabbit IgG conjugated with peroxidase for 45 minutes at 37°C. Wells were washed and incubated with detecting buffer in dark for 20 minutes at room temperature. 2 M H₂SO₄ was used to stop developing reaction. Values of OD₄₉₀ were read in Elx800 Universal Microplate Reader (Bio-Tek instruments, INC, USA). Standard curve was gained based on the OD₄₉₀ value and corresponding concentrate of standard protein. The amounts of rhGH synthesized in pupae were determined by its OD₄₉₀ value from the standard curve.

2.6. In Vitro Assay for Bioactivity of BmrhGH. The bioactivity of BmrhGH was identified by colony-formation analysis of K562 cells. BmrhGH expressed by silkworm pupa was diluted with 1 M Tris (pH9.0) to a series of concentrations (40 ng/mL, 80 ng/mL, 100 ng/mL, and 200 ng/mL). 1.8% methyl cellulose was added into the 24-well plastic culture plate for 1 mL/well, and then 2 × (PRM1640 + BSA) medium was also added for 1 mL/well. 50 μl K562 cell culture medium (containing approximately 2000 cells) and 50 μl BmrhGH with different concentrations were mixed, transferred into the 24-well plastic culture plate, and cultured at the conditions of 37°C, 5% CO₂. Each sample was assayed in triplicate. The cells treated with hGH standard protein were defined as positive control, and the Tris (pH9.0) solution-treated cells were defined as negative control. After cultured for 4 days, the number of colonies was counted under invert microscope (NIKON, Japan). A cluster of cells (over 40) was defined as a colony. The colony forming rate was calculated as follows: colony forming rate = the number of cell colonies in treatment group/the number of cell colonies in negative control group × 100%. The colony forming rate can identify the ability of rhGH for promoting cell colony-forming.

2.7. Bioassay for Oral Administration Bioactivity of BmrhGH on Hypophysectomized Rats. Male Wistar rats, 26~28d, provided by National Institute for the Control of Pharmaceutical and Biological Products (China), were hypophysectomized according to Zhu [13]. Beginning two weeks after hypophysectomy, the body weight (BW) of hypox rats was measured daily for 14 days. The rats whose body weight gain (BWG) was greater than 1g/day during the 14-day period were considered incompletely hypox and excluded from the bioassay. At the end of the 14-day period, the remaining hypox rats were assigned to treatment groups of eight rats each according to the BW. Mean BW of hypox rats was 100~110 g.

Silkworm pupae supernatant solution from insects infected by vBmrhGH or WT BmNPV was dried frozenly into powder. When treating animals, protein samples were prepared by dissolving powders in 0.9% NaCl solution. Hypox rats were gavaged with protein samples once per day. One group of Hypox rats was gavaged with the protein sample of BmrhGH from pupae infected by vBmrhGH. The dose of treatment was 4.5 mg BmrhGH/rat.day. The other group of animals as control was gavaged with the protein sample from pupae infected with WT BmNPV at the same dose. The third group of animals as blank was dealt with the same volume of 0.9% NaCl solution. The BW of each rat was measured daily throughout the entire period of bioassay, beginning on the first day of gavage. BWG was used to analysis.

Rats were killed by cervical dislocation in the morning of the 14th day. Liver weight of each rat was obtained. Tibias were anatomized and stored in 10% formaldehyde. After sliced from the top in an arrowy side, tibias were stained in 2% AgNO₃ for 2 minutes and washed in water. Then tibias were exposed in strong light until they were brown. 10% Na₂S₂O₃ was used to fasten the color. The stained tibias were stored in 80% ethanol solution to be observed. The width of tibias epiphyseal was measured in inverted microscope (zoomed in 10 × 10). With all statistical analysis by *t*-test, differences were considered statistically significant for *P* < .05.

3. Results

3.1. Construction of vBmrhGH and Protein Expression in Silkworm Pupa. A recombinant BmNPV harboring correct hGH cDNA was generated by recombinant between plasmid pBacPAK-hGH DNA and WT BmNPV genome lipofected into BmN cells. By three cycles of plaque assays, the recombinant virus was purified. Southern blotting confirmed that the hGH cDNA was inserted into BmNPV genome successfully.

The *B. mori* pupae were infected with purified vBmrhGH, and pupae supernatant solution was extracted as protein sample. The SDS-PAGE analysis of pupae protein sample showed a specific 22-kDa band corresponding to the size of native hGH, which was obviously absent from the protein samples of both pupae infected by WT BmNPV and normal pupae (Figure 1(a)). The identity of the protein to hGH was confirmed by its specific reaction with anti-hGH antiserum in a Western blotting (Figure 1(b)).

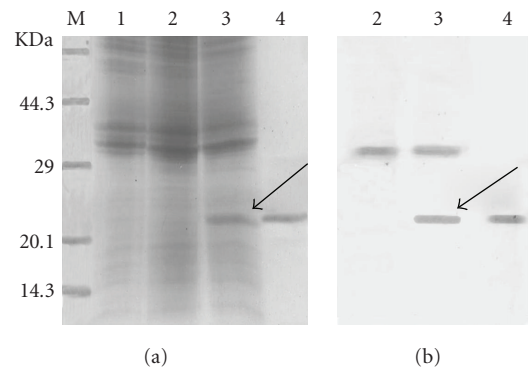


FIGURE 1: SDS-PAGE and Western blotting analysis of BmrhGH expressed in *B. mori* pupae. The silkworm pupae were infected with the recombinant virus vBmrhGH or wild type BmNPV. The supernatant solution of pupae homogenate was prepared at 120 hours after infection and electrophoresed through 15% SDS-PAGE (a) and identified by Western blotting analysis with anti-hGH antiserum (b). Lanes: M, Low molecular weight marker; 1, supernatant solution of normal pupae; 2, supernatant solution of pupae infected by wild type BmNPV; 3, supernatant solution of pupae infected by recombinant virus vBmrhGH; 4, standard hGH. Arrow indicates BmrhGH protein.

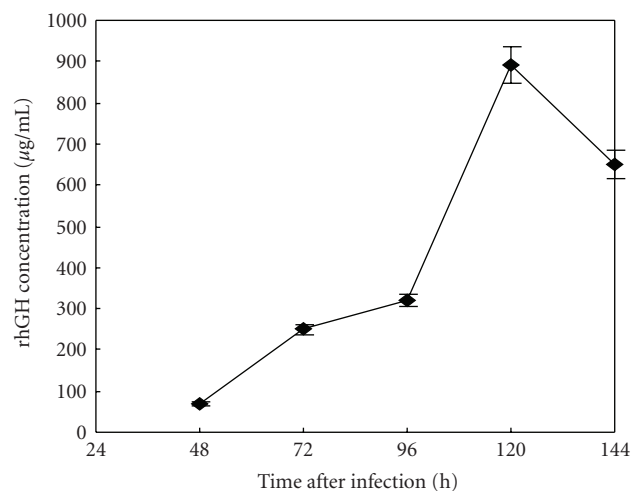


FIGURE 2: Time course of the BmrhGH expression in silkworm pupae. Pupae were collected each day from 48 hours postinfection and protein samples were prepared by centrifuging at the speed of 1,5000 g. BmrhGH in silkworm pupae extracts was determined by ELISA. Each value represents the mean of triplicate experiments.

In silkworm pupae, symptoms of infection were observed at 48 hours after inoculation. Pupae began to be soft and discoloring and dark skin areas began to appear. Almost all of pupae died at 120 hours. The expression of BmrhGH in *B. mori* pupae was quantified by ELISA (Figure 2). The result demonstrated that the pupae “bioreactor” could produce recombinant hGH effectively. At 48 hours after infection, the amount of BmrhGH was approximately 70 µg/ mL pupae supernatant solution. Then the amount increased day by day until 120 hours postinfection. The peak level of BmrhGH was

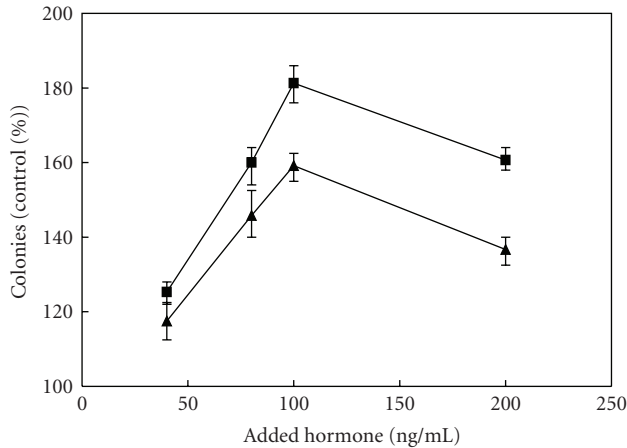


FIGURE 3: Effects of rhGH on erythroleukemia cell K562 colony formation. Most colonies were found at the dose of 100 ng/mL hGH and BmrhGH, respectively. ■, standard human growth hormone protein; ▲, BmrhGH purified from silkworm pupae.

approximately 890 μ g/mL supernatant solution and then it decreased.

3.2. Bioactivity of BmrhGH In Vitro. The bioactivity of BmrhGH in vitro was determined by the effect of it on colony formation of human erythroleukemia K562 cells. The result showed that BmrhGH could stimulate the colony formation of K562 cells (Figure 3). The ability of K562 cells colony formation gradually increased along with the increase of hGH concentration (<100 ng/mL). Most cell colonies could be found when the concentration of hGH reached 100 ng/mL and then cell colony-forming ability declined when the hGH concentration continued to increase. The effect of BmrhGH on the colony formation in K562 cells was similar to hGH standard protein and the dose-dependent was clear too; the result was in accordance with the report by Gauwerky [14].

3.3. Biological Activity of BmrhGH on Hypox Rats. The growth curves of hypox rats' groups in our laboratory are presented in Figure 4. Values of BWG ($BW_{14}-BW_1$) were calculated and used for *t*-test. The BWG of the group treated with BmrhGH was 12.50 ± 0.831 g and that of the control group was 1.78 ± 0.343 g. While in blank group no BWG was observed. By statistic analysis, it was interesting to note that there was a significant difference between the group treated with BmrhGH and the control group ($P < .01$).

To evaluate the difference of liver weight and epiphyseal width between different groups, rats were killed at the 14th day, and the two parameters were measured (Table 1). The result showed that the mean liver weight of the group treated with BmrhGH was 4.973 ± 0.1633 g. While the mean value of the control group was 4.424 ± 0.0928 g. The result of *t*-test showed that there was a significant difference between them ($P < .05$). Comparing the liver weight between treated group and blank group, we also found the significant difference ($P < .05$).

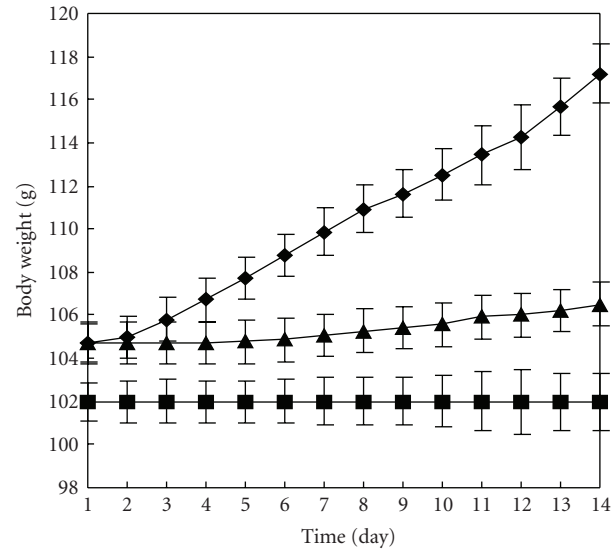


FIGURE 4: Growth curves of hypox rats. Hypox rats were gavaged with BmrhGH at the dose of 4.5 mg/rat.day in silkworm pupae protein sample (◆). In the control group, hypox rats were gavaged with protein sample from silkworm pupae infected by WT BmNPV at the same dose (▲). The hypox rats of third group were gavaged with 0.9% NaCl solution as blank (■).

TABLE 1: The liver weight and epiphyseal width of hypox rats gavaged with BmrhGH (mean \pm SD).

Group	<i>n</i>	Liver weight \pm SD (g)	Epiphyseal width \pm SD (μ m)
BmrhGH protein preparation*	8	$4.973 \pm 0.1633^{\Delta}$	$44.777 \pm 0.9867^{\Delta}$
Control	8	4.424 ± 0.0928	39.453 ± 0.9216
Blank	3	4.404 ± 0.0805	40.200 ± 0.9473

*BmrhGH protein preparation was obtained from pupae infected by vBmrhGH, and the dosage of BmrhGH was approximately 4.5 mg/rat.day. Control protein extracts were from pupae infected by WT baculovirus. Blank group was treated with 0.9% NaCl solution. " Δ " means $P < .05$.

At the same time, the values of epiphyseal width were compared. We observed that the mean epiphyseal width of the group treated with BmrhGH was approximately $44.777 \pm 0.9867 \mu$ m, while that of the control group was $39.453 \pm 0.9216 \mu$ m. Result of *t*-test showed that there was a similar significant difference between them ($P < .05$).

4. Discussion

The baculovirus expression system was first reported to produce human interferon α in *B. mori* larvae [15]. Because of its ability to efficiently synthesize the highly active products, in the following decades it has been successfully used to produce various recombinant protein. For example, the *B. mori* has synthesized hepatitis B virus surface antigen, human insulin-like growth factor II, human β -interferon, human interleukin-2, human lactoferrin, and so forth. Therefore,

BmN cells and *B. mori* were the preferred bioreactors in most work [16]. In our present work, the diapausing pupae of silkworm were used to produce recombinant hGH. We found that this bioreactor was more efficient than other systems. In our work, the peak expression of BmrhGH was approximately 890 $\mu\text{g}/\text{mL}$ pupae supernatant solution (Figure 2), which was corresponded to an average yield of 200~300 μg per pupa. We also detected that the BmrhGH expressed in silkworm larvae and the peak production was 200 $\mu\text{g}/\text{mL}$ larvae hemolymph (data not shown) and approximately 60~70 μg per insect, which is similar to previous work [17]. It is interesting to find that the expression of BmrhGH is about 4~6 times more in diapausing silkworm pupae than that in larvae. This difference might be the result of the different protein metabolism of silkworm in different developing stages.

From the present work, we found that the synthesis of BmrhGH increased at first day until reaching a peak, and then it decreased rapidly. The decrease of BmrhGH might be the result of a cysteine protease encoded by the BmNPV, which causes the degradation of the recombinant proteins and destruction of tissues during the later stages of the infection. A mutated BmNPV lacking the cysteine proteinase gene was constructed to express recombinant protein and the stability of recombinant protein could be enhanced in later postinfection stage [18].

The physiological role of hGH is achieved by stimulating the synthesis of SM factor in liver. At the same time, many reports have proven that hGH itself can also act directly on multiple tissues and organs, promoting synthetic metabolism and growth [19]. The results of tests in vitro showed that ng-level hGH can promote the physiological activity of many cells, including thymus cells [20], rat epithelial cells [21], and islet cells [22]. Gauwerky et al. showed that hGH can promote the colony formation of human erythroleukemia K562 cells [14]. In present study, effects of BmrhGH on colony formation of K562 cells at different dose were investigated. The most colonies were found at the dose of 100 ng/mL hGH and BmrhGH, respectively. The result indicated that rhGH expressed by *B. mori* pupa showed bioactivity similar to hGH. Additionally, FBS is composed of many complex compositions which might contain some hormone-like factors and could affect the colony formation of K562 cells. Therefore, the FBS was replaced by BSA in the cell culture medium, and thus the effect on colony formation of K562 was only caused by BmrhGH.

Biological activities of hGH preparations have been determined mostly by the body weight gain (BWG) assay [23], or the tibia assay test [8] by the way of injection, both in hypox rats. Hypox rats were also used in our study to evaluate the biological activity of expressed BmrhGH. For thousands of years, pupa was nutritious food, and we tried the way of oral administration instead of injection. We are interested to find that the rat's body weight increased significantly (Figure 4, $P < .01$), and we also discovered the improving activity of BmrhGH in pupae extracts to both the parameters of tibia epiphysial growth plate width and liver weight. It could be concluded that the BmrhGH in pupae extracts had biological activity by oral administration in hypox rats. We

deduced that there were some factors in pupae that could protect functional proteins from digesting in stomach, or BmrhGH was digested into functional peptides that could be absorbed by body. More work is needed to illustrate it. The previous studies in our lab proved that BmrhGM-CSF, a 29 kDa recombinant hGM-CSF protein expressed by silkworm pupae bioreactor, could bring into effect as active cytokine through oral administration [24]. The clinical trial for BmrhGM-CSF has been carried out and demonstrated that the oral administered BmrhGM-CSF was absorbed into the blood [25]. We also expressed many useful proteins by silkworm pupae bioreactor, such as cholera toxin B subunit-insulin fusion protein, Osteoprotegerin, Human lactoferrin, and so forth, and also found the bioactivities of them by oral administration [16, 26–28]. Due to the oral administration activity, BmrhGH might be a potential choice for short stature dementia or Alzheimer's disease.

More over, another interesting result in our work is that there are some other proteins in silkworm pupae that are reactive to anti-hGH antiserum (Figure 1(b)). Interestingly, the band was also found in the protein sample from silkworm pupae infected by WT BmNPV. BWG bioassay of hypox rats indicated that the protein sample from WT virus infected pupae is effective compared with the blank group ($P < .05$). The result indicated that there might be some proteins in *B. mori* pupae with similar characteristic and activity to hGH. More studies on this deduction are doing in our laboratory.

5. Conclusions

Taken together, the recombinant hGH protein BmrhGH was synthesized with a level of approximately 890 $\mu\text{g}/\text{mL}$ pupae supernatant solution, which was five times more than the level using larvae. The animal experiments proved that BmrhGH has a high oral administration bioactivity. Due to the high activity and oral administration, BmrhGH might be used as a potential drug for short stature dementia or Alzheimer's disease and would be a low-cost choice.

Acknowledgments

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