

Anti-aging effect of polysaccharide from *Bletilla striata* on nematode *Caenorhabditis elegans*

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ABSTRACT

Background: Polysaccharide isolated from *Bletilla striata*, a well-known traditional Chinese medicine (*Bletilla striata* polysaccharide [BSP]) has been found to play important roles in endothelial cells proliferation, inducible nitric oxide stimulation, wound healing acceleration and other processes. Recent studies found that *B. striata* has anti-oxidative properties, however, potential anti-aging effects of BSP in whole organisms has not been characterized. **Objective:** To investigate whether BSP has anti-aging effects on *Caenorhabditis elegans*. **Materials and Methods:** After treatment with BSP, the lifespan, locomotion ability, and stress resistance of *C. elegans* was determined. To provide insight into the underlying mechanism for the anti-aging effect of BSP, we measured its effect on bacterial growth, brood size of *C. elegans*, and the insulin/insulin-like growth factor (IGF) signaling pathway. **Results:** After BSP treatment, the lifespan of *C. elegans* was extended, and its locomotion ability and stress resistance were increased. BSP was found to have no effect on bacterial growth or on reproduction of *C. elegans*, However, mRNA levels of *age-1* and *hcf-1* were reduced after BSP treatment. Additionally, we observed that BSP did not extend the lifespan of *daf-16* mutant animals. **Conclusion:** BSP produces an anti-aging effect on *C. elegans* through the insulin/IGF signaling pathway and holds promise for future development as a functional food.

Key words: Anti-aging, *Bletilla striata* polysaccharide, *Caenorhabditis elegans*, lifespan, stress resistance

INTRODUCTION

Bletilla striata is a famous traditional Chinese medicine that is widely used in the treatment of ulcers, bone injuries, acne and many other diseases in China. Many compounds have been isolated from *B. striata* such as bibenzyls, phenanthrenes, phenolic acid, and polysaccharide.^[1,2] Among these compounds, polysaccharide isolated from *B. striata* has been found to have many functions. *Bletilla striata* polysaccharide (BSP) has been found to induce endothelial cells proliferation and vascular endothelial growth-factor expression.^[3] BSP has also been found to stimulate inducible nitric oxide synthase and proinflammatory cytokine expression in macrophages.^[4] Hydrogel prepared from BSP has potential wound healing effects and has

been demonstrated to accelerate wound closure in a full-thickness trauma mouse model.^[5] In addition to its direct medicinal functions, BSP has been successfully used in the delivery of drugs such as oligonucleotide and antibiotics.^[6,7]

Recent studies found that *B. striata* has anti-oxidative effect. This anti-oxidative ability was first demonstrated through DPPH radical-scavenging activity assay and ferric-reducing antioxidant power assay.^[2] The anti-oxidative ability was further confirmed by measuring reactive oxygen species levels in response to H₂O₂ in the HepG2 cell line.^[2] This property has been demonstrated for both the *B. striata* extract as well as polysaccharide isolated from *B. striata*.^[8] Many drugs with the anti-oxidative ability have been found to have anti-aging effects in organisms.^[9-12] Therefore, we wanted to test if BSP similarly exhibits anti-aging effects. For these studies, we used the model organism *Caenorhabditis elegans*, widely used in aging mechanism research and anti-aging drug discovery due to its relatively short lifespan and conserved mechanisms for regulation of aging.^[13-18]

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MATERIALS AND METHODS

Preparation of *Bletilla striata* polysaccharide

Bletilla striata polysaccharide was prepared and stored in our lab using a protocol described previously.^[19] Briefly, homogenized, dry *B. striata* was dispersed in hot distilled water for 4 h and then filtered. Polysaccharide was then precipitated using 3 volume of 95% ethanol as crude extract. Proteins were removed from this extract using the Sevag method. The crude extract was then purified by DEAE-Cellulose column and Sephadex G-100 column to result in a single peak corresponding to purified BSP extract.

Worm strain maintenance

The strains used in this study were wild-type N2 and mutant strain *daf-16* (obtained from the Caenorhabditis Genetics Center). Nematodes were maintained on nematode growth medium (NGM) plates seeded with *Escherichia coli* OP50 at 20°C as described.^[20] Age synchronous populations of *C. elegans* were obtained as described previously.^[21] BSP was added to the NGM plates just before plating.

Lifespan assay and reproduction

The lifespan assay was performed as described.^[22] BSP treatment was performed throughout the lifespan from the L4-larvae stage. During the lifespan assay, the worms were transferred daily for the first several days of adulthood. The surviving nematodes were measured, and recorded every day and worms were scored as “dead” when they did not respond to the stimulation of a platinum wire. The results showed are representative of at least three trials.

Reproduction was assessed by the brood size, which was determined as the number of offspring at all stages beyond the egg. Ten replicates were performed.

Locomotion behavior

For the locomotion behavior assay, BSP treatment was performed throughout the lifespan from L4-larvae. Head thrash and body bend were used as endpoints for locomotion behavior. Head thrashes are defined as a change in the direction of bending at the mid body. Body bends are defined as a change in the direction of the part of nematodes corresponding to the posterior bulb of the pharynx along the y-axis, assuming that nematode was traveling along the x-axis.

During the locomotion behavior assay, the examined nematodes were transferred into the assay plate containing K medium on top of the agar. After a recovery time of 1 min, head thrashes, and body bends were counted for 1 min and 20 s respectively. Twenty replicates were performed for each experiment.

Stress resistance assays

To assay thermal stress resistance, worms pre-treated with BSP for 48-hr were transferred to the 35°C condition, and then the lifespan was measured and analyzed as described above. To assay oxidative stress resistance, worms pre-treated with BSP for 48-hr were transferred to medium containing paraquat, and then the lifespan was measured and analyzed as described above.

Bacterial growth assay

Escherichia coli OP50 was seeded into sterilized liquid medium with or without 50 µg/ml BSP, and initial OD595 values were measured against sterile medium. Samples were transferred to rocking shaker at 37°C, and OD595 values were measured for 10 h.

RNA isolation and Quantitative real-time-polymerase chain reaction

Total RNA was isolated using RNAiso Plus (Takara) from worms treated with or without 50 µg/ml of BSP for 48 h. Total RNA was then reverse-transcribed using PrimeScript 1st strand cDNA synthesis kit (Takara). Quantitative real-time-polymerase chain reaction (RT-PCR) was used to determine the relative quantification of the targeted genes in comparison to the reference *act-1* gene, and the results were expressed as the relative expression ratio (between targeted gene and internal control *act-1*). The primers used in this study were as follows: *Daf-2*, forward 5'-CCAACCGAACGGAGACCT-3', reverse 5'-CGATAGCCGAACACGAAC-3'; *age-1*, forward 5'-AATGGCAAAGGATCGCTG-3', reverse 5'-GGAGTTTCGTTCCGGATTG-3'; *daf-16*, forward 5'-CGTTTCCTTCGGATTTC-3', reverse 5'-ATTCCTTCCTGGCTTTGTC-3'; *hcf-1*, forward 5'-CGGAAGGCTTGAGTAAC-3', reverse 5'-ATGGTTGCTTGCGAGGAG-3'; *act-1*, forward 5'-TGACGGACAAGTCATCACC-3', reverse 5'-CATGGTGGTTCCTCCGAAA-3'.

Statistical analysis

Data are presented as means ± standard error of the mean. Graphs were generated using Microsoft Excel (Microsoft Corp., Redmond, WA). Statistical analysis was performed using SPSS 12.0 (SPSS Inc., Chicago, USA). Differences between groups were determined using analysis of variance. Probability levels of 0.05 and 0.01 were considered statistically significant. The lifespan data were statistically analyzed using a 2-tailed, 2 sample t-test (Minitab Ltd., Coventry, UK).

RESULTS

Bletilla striata polysaccharide extends lifespan of *Caenorhabditis elegans*

Nematodes were treated with different doses of BSP from L4-larvae stage in order to investigate BSP's effect on

lifespan of *C. elegans*. As shown in Figure 1a and b, all three concentrations of BSP tested showed extended lifespan for *C. elegans* and the best lifespan-extending effect was observed in worms treated with 50 $\mu\text{g}/\text{ml}$ of BSP.

Bletilla striata polysaccharide improves the locomotion behavior of *Caenorhabditis elegans*

Caenorhabditis elegans shows gradually impaired locomotion ability during its aging process especially at the last stage. We next investigated the effect of BSP on the locomotion ability of *C. elegans*. Two important endpoints for locomotion ability—head thrash and body bend of *C. elegans* were recorded every 4 days during its lifespan. As shown in Figure 2a and b, the *C. elegans* treated with BSP showed significantly enhanced locomotion ability during its aging process compared with untreated animals, indicating that BSP treatment enhances the locomotion behavior of *C. elegans* and it can improve the life quality of aged nematodes.

Bletilla striata polysaccharide improves the stress resistance of *Caenorhabditis elegans*

We next investigated the effect of BSP on the stress resistance of *C. elegans* as many drugs with anti-aging effect affects the survival of animals under stress conditions. Nematodes pretreated with BSP for 48 h were moved to 35°C or exposed to paraquat and their survival was recorded in order to determine the effect of BSP on stress resistance of *C. elegans*. As shown in Figure 3a and b, *C. elegans* pretreated with 50 $\mu\text{g}/\text{ml}$ of BSP showed significantly increased lifespan after both thermal and oxidative stress, indicating that BSP improves the stress resistance of *C. elegans*.

The effect of *Bletilla striata* polysaccharide on bacterial growth and reproduction of *Caenorhabditis elegans*

Previous work showed that the inhibition of *E. coli* OP50, present in these assays as the food source for the nematodes, can lead to the extended lifespan of *C. elegans*.^[23] In order to investigate whether the anti-aging of BSP is dependent on its anti-microbial effect, we studied the effect of BSP on *E. coli* growth. As shown in Figure 4, BSP has no effect on growth of *E. coli* OP50 at the doses effective to extend lifespan of *C. elegans*, indicating that the anti-aging effect of BSP is not through inhibition of bacterial growth.

The aging process of *C. elegans* can be delayed when its reproduction is reduced.^[24,25] To investigate whether the anti-aging effect of BSP is due to reproduction reduction, we measured and compared the brood size of nematodes with or without BSP treatment. As shown in Figure 5, there is no significant difference on brood size of *C. elegans*, indicating that the anti-aging effect of BSP does not occur by altering the reproductive system of *C. elegans*.

Bletilla striata polysaccharide extends lifespan of *Caenorhabditis elegans* through insulin/insulin-like growth factor signaling pathway

The aging process of *C. elegans* is regulated by several signaling pathways.^[26] Among all the pathways regulating *C. elegans* aging, the insulin/insulin-like growth factor (IGF) signaling pathway might be the most well-studied. Mutations of *Daf-2* and *age-1*, key components of the insulin/IGF signaling pathway, have been found to extend the lifespan of *C. elegans* and transcription factor *Daf-16* extends lifespan through regulation of longevity genes.^[27,28] In addition to

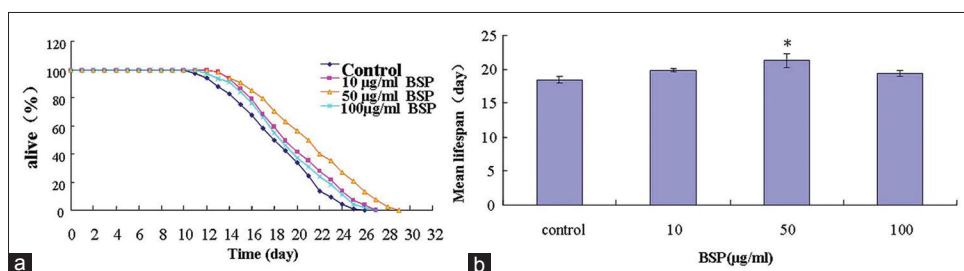


Figure 1: *Bletilla striata* polysaccharide (BSP) extends lifespan of *Caenorhabditis elegans*. (a) Survival curves of *C. elegans* treated with different dose of BSP. (b) Comparison of mean lifespans in nematodes treated with different dose of BSP (* $P < 0.05$)

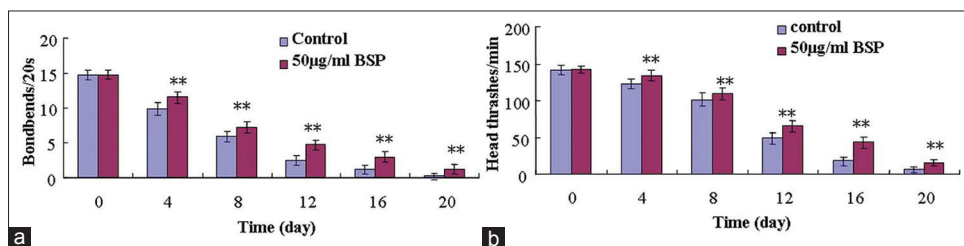


Figure 2: *Bletilla striata* polysaccharide (BSP) improves the locomotion behavior of *Caenorhabditis elegans*. (a) Body bends of *C. elegans* treated with 50 $\mu\text{g}/\text{ml}$ of BSP during its lifespan. (b) Head thrashes of *C. elegans* treated with 50 $\mu\text{g}/\text{ml}$ of BSP during its lifespan (** $P < 0.01$)

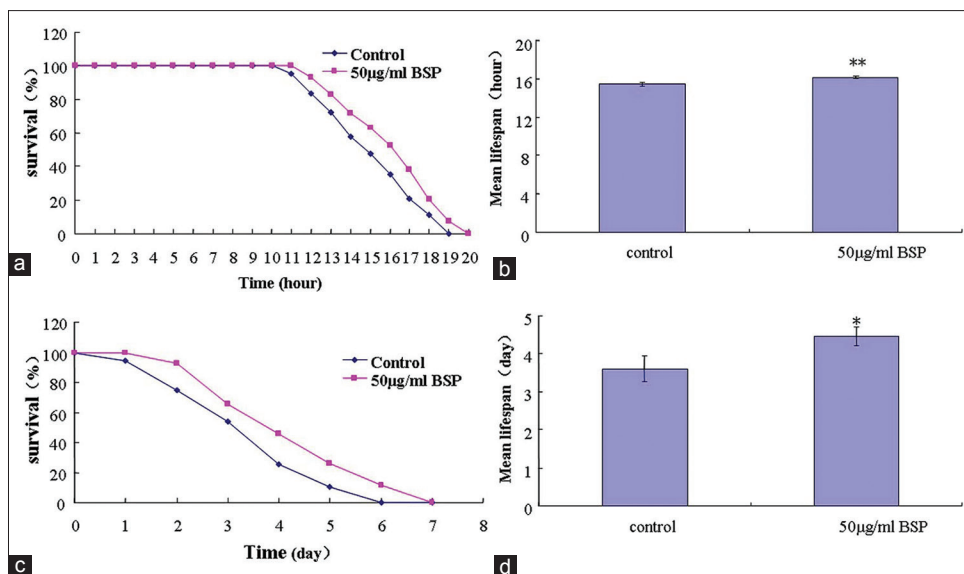


Figure 3: *Bletilla striata* polysaccharide (BSP) improves the stress resistance of *Caenorhabditis elegans*. (a) Survival curves of *C. elegans* pretreated with 50 µg/ml of BSP in 35°C. (b) Mean lifespans of *C. elegans* pretreated with or without BSP in 35°C. (c) Survival curves of *C. elegans* pretreated with 50 µg/ml of BSP in paraquat-containing medium. (d) Mean lifespans of *C. elegans* pretreated with BSP in paraquat-containing medium (* $P < 0.05$, ** $P < 0.01$)

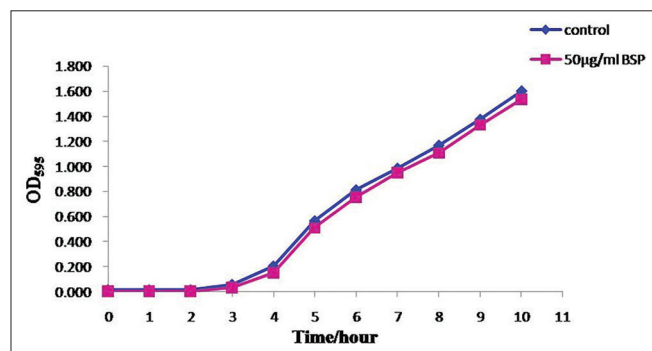


Figure 4: The effect of *Bletilla striata* polysaccharide (BSP) on bacterial growth. *Escherichia coli* OP50 was seeded to medium with or without BSP and then transferred to rocking shaker at 37°C. OD₅₉₅ values were measured for 10 h and growth curves of *Escherichia coli* OP50 in both mediums were prepared. Data are representative of three experiments

its roles in lifespan extension, the insulin/IGF signaling pathway also regulates resistance to thermal stress, oxidative stress, and infections.^[29-32]

It is interesting to know whether BSP extends lifespan of *C. elegans* through insulin/IGF signaling pathway considering that BSP extends the lifespan of *C. elegans* and enhances the stress resistance of *C. elegans*. We first analyzed the expression change of *daf-2*, *age-1*, *daf-16*, and *bcl-1*, which are key components of the insulin/IGF signaling pathway, after BSP treatment through RT-PCR. As shown in Figure 6a, the expression of *age-1* and *bcl-1* were down-regulated after BSP treatment. The expression of other genes, however, was not significantly changed. We next examined whether BSP can extend the lifespan

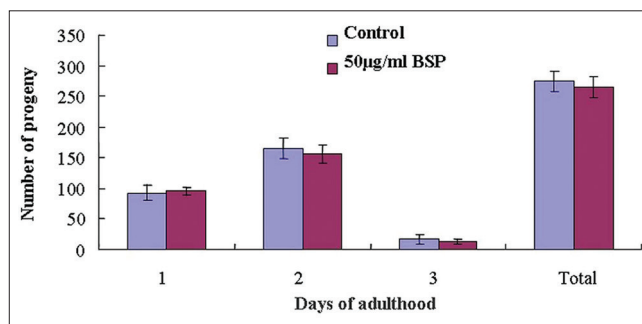


Figure 5: The effect of *Bletilla striata* polysaccharide (BSP) on reproduction of *Caenorhabditis elegans*. The reproduction of *C. elegans* was assessed by its brood size. worms were treated with BSP during their egg-laying period, and the endpoint was measured when worms developed into the adults

of *daf-16* mutant animals. As shown in Figure 6b, the lifespan of *daf-16* mutant animals was not extended after BSP treatment. These results indicate that the insulin/IGF signaling pathway is the target of BSP involved in the lifespan extension of nematodes.

DISCUSSION

In the present study, we demonstrated that polysaccharide isolated from *B. striata* significantly extended the lifespan of *C. elegans* and it had best lifespan-extending effect at the concentration of 50 µg/ml [Figure 1]. Moreover, worms treated with BSP showed greatly improved locomotion behavior during the aging process compared with control animals, especially in the latter stage of life [Figure 2]. These results showed that BSP has potential anti-aging effects.

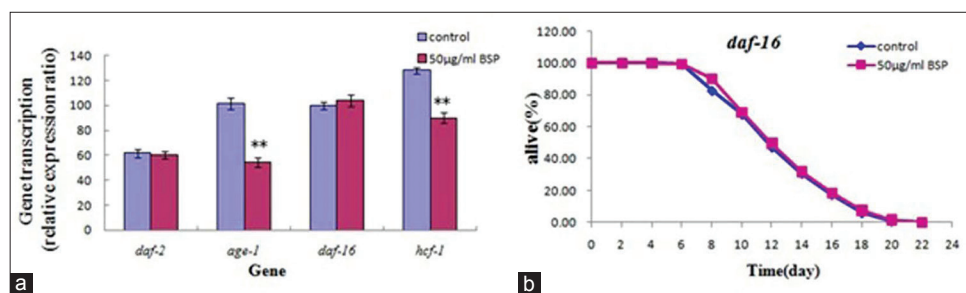


Figure 6: *Bletilla striata* polysaccharide (BSP) extends lifespan of *Caenorhabditis elegans* through insulin/insulin-like growth factor (IGF) signaling pathway. (a) Effects of BSP on expression patterns of key members in insulin/IGF signaling pathway. The nematodes were treated with 50 ug/ml of BSP for 48 h and then subjected to real-time-polymerase chain reaction. The expression levels of target genes were normalized to *act-1* (** $P < 0.01$). (b) Survival curves of mutant nematodes *daf-16* treated with 50 ug/ml of BSP

Worms pre-treated with BSP exhibited increased survival rate compared to control animals under both thermal stress and oxidative stress conditions [Figure 3]. These results indicated that BSP has beneficial effects on *C. elegans* under stress conditions in addition to its lifespan-extending effects under normal conditions.

In an effort to investigate the possible mechanisms for its anti-aging effect, we found that BSP has no effect on *E. coli* growth or brood size of *C. elegans* [Figures 4 and 5], indicating that the anti-aging effect of BSP is neither mediated by effects on bacterial growth nor effects on the reproductive system of *C. elegans*.

The aging process of *C. elegans* is regulated by several signaling pathways such as the insulin/IGF signaling pathway, the target of rapamycin (TOR) signaling pathway, and the germline signaling pathway.^[26] In the insulin/IGF signaling pathway, the receptor *DAF-2* activates a kinase cascade consisting of phosphatidylinositol 3-kinase (PI3K/*AGE-1*), 3-phosphoinositide-dependent kinase 1, and serine/threonine-protein kinase (SGK-1).^[26] SGK-1 then inactivates FOXO transcription factor *DAF-16* resulting in the blocking of expression of *DAF-16*-regulated genes.^[33]

We studied the mRNA levels of key genes from the insulin/IGF signaling pathway after BSP treatment and found the mRNA levels of *age-1* and *hcf-1* but not *daf-16*, were significantly reduced [Figure 6a]. We next studied the lifespan-extending effect of BSP on *daf-16* mutant animals and found that BSP could not extend the lifespan of *daf-16* mutant animals [Figure 6b]. These results indicate that the anti-aging effect of BSP is through its interaction with insulin/IGF signaling pathway. Because BSP does not change the mRNA level of *daf-16*, its lifespan-extending effect is not through the direct control of *daf-16* expression. However, our findings do not exclude the possibility that BSP may exert additional effects to extend the lifespan of *C. elegans* through other mechanisms such as by altering the nuclear translocation of *DAF-16* protein.

CONCLUSION

Our results demonstrated that BSP has lifespan-extending effects on nematode *C. elegans*. In addition, BSP was found to have the ability to improve the locomotion behavior and stress resistance of *C. elegans*. Moreover, our data revealed that the lifespan-extending effect of BSP is dependent on insulin/IGF signaling pathway and is not via effects on either bacterial growth or reproduction of *C. elegans*.

Our current study provides the first evidence that BSP has anti-aging properties. Future studies are warranted to examine the anti-aging effects of BSP in mammals and to develop BSP as a functional food.

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