Lifespan Extending and Stress Resistant Properties of Vitexin from Vigna angularis in Caenorhabditis elegans

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Abstract

Several theories emphasize that aging is closely related to oxidative stress and disease. The formation of excess ROS can lead to DNA damage and the acceleration of aging. Vigna angularis is one of the important medicinal plants in Korea. We isolated vitexin from V. angularis and elucidated the lifespan-extending effect of vitexin using the Caenorhabditis elegans model system. Vitexin showed potent lifespan extending activity and it elevated the survival rates of nematodes against the stressful environments including heat and oxidative conditions. In addition, our results showed that vitexin was able to elevate antioxidant enzyme activities of worms and reduce intracellular ROS accumulation in a dose-dependent manner. These studies demonstrated that the increased stress tolerance of vitexin-mediated nematode could be attributed to increased expressions of stress resistance proteins such as superoxide dismutase (SOD-3) and heat shock protein (HSP-16.2). In this work, we also studied whether vitexin-mediated longevity activity was associated with aging-related factors such as progeny, food intake, growth and movement. The data revealed that these factors were not affected by vitexin treatment except movement. Vitexin treatment improved the body movement of aged nematode, suggesting vitexin affects healthspan as well as lifespan of nematode. These results suggest that vitexin might be a probable candidate which could extend the human lifespan.

Key Words: Vigna angularis, Vitexin, Caenorhabditis elegans, Lifespan extension, Stress tolerance

INTRODUCTION

Aging is an inevitable natural process accompanied by a progressive accumulation of damage in all constituent macromolecules such as nucleic acids, lipids and proteins (Chondrogianni et al., 2015). Although the determined mechanisms of aging process are not completely identified, increasing new evidences suggest that aging is considerably associated with reactive oxygen species (ROS) (Si and Liu, 2014). ROS including superoxide radical, hydrogen peroxide and hydroxyl free radical, cause oxidative damage to DNA and other macromolecules in the cell (Chen et al., 2013). Oxidative stress caused by ROS can lead to oxidation of biomolecules such as protein, DNA and biomembranes which is assumed to be the major cause factor of aging. There are so many previous works dealt with the correlation between antioxidants and aging (Kimoto-Kinoshita et al., 1999; Sinha et al., 2010; Gruber et al., 2013). Therefore, drugs with antioxidant properties can be promising candidates for the various aging-related diseases.

Caenorhabditis elegans is a small soil nematode which offers several advantages to study aging and the related pathways, because it has a rapid reproduction rate and a short life span, furthermore the major signaling pathways that regulate longevity and stress resistance in mammals are well conserved in the nematode (Feng et al., 2015; Su and Wink, 2015). And its genome is completely sequenced, displaying great homology to human (Chondrogianni et al., 2015). So, the worm C. elegans has become a popular model organism to explore the potential anti-aging and stress resistance properties of natural compounds due to its features of ease of maintenance, short life cycle, and the availability of various transgenic strains (Feng et al., 2015). In the course of searching for compounds which are likely to prolong the life of human from plants by using the C. elegans...
model system, a methanolic extract of *Vigna angularis* (Leguminosae) was found to show significant longevity activity. This plant has been used as one of the traditional medicines for its diuretic and detoxification activities to treat edema, constipation and diabetes (Yao et al., 2011; Jiang et al., 2014). Previous phytochemical reports on this plant dealt with the several phenolic compounds, saponins and furanylmethyl glycosides (Ariga and Asao, 1981; Kitagawa et al., 1983; Jiang et al., 2014). Earlier pharmacological studies on this plan showed that it has various bioactivities such as anti-inflammatory, anti-oxidant, anti-hypotensive, hypoglycemic and hepatoprotective effects (Han et al., 2004; Itoh et al., 2009; Mukai and Sato, 2009; Yumiko et al., 2009; Jiang et al., 2014). Subsequent activity-guided chromatography of the methanolic extract of *V. angularis* led to the isolation of compound 1, vitexin (Fig. 1).

The aim of this work was to study the effect of vitexin on the lifespan and stress tolerance including thermal and oxidative stress in *C. elegans*. Moreover, antioxidant capacities of vitexin were analyzed by measuring intracellular ROS level and antioxidant enzyme activities of nematodes. Then, to investigate whether vitexin-mediated increased stress tolerance was due to the regulation of stress-response genes, we quantified SOD-3 and HSP-16.2 expressions using transgenic model system, a methanolic extract of *Vigna angularis* (Leguminosae) was found to show significant longevity activity. This plant has been used as one of the traditional medicines for its diuretic and detoxification activities to treat edema, constipation and diabetes (Yao et al., 2011; Jiang et al., 2014). Previous phytochemical reports on this plant dealt with the several phenolic compounds, saponins and furanylmethyl glycosides (Ariga and Asao, 1981; Kitagawa et al., 1983; Jiang et al., 2014). Earlier pharmacological studies on this plan showed that it has various bioactivities such as anti-inflammatory, anti-oxidant, anti-hypotensive, hypoglycemic and hepatoprotective effects (Han et al., 2004; Itoh et al., 2009; Mukai and Sato, 2009; Yumiko et al., 2009; Jiang et al., 2014). Subsequent activity-guided chromatography of the methanolic extract of *V. angularis* led to the isolation of compound 1, vitexin (Fig. 1).

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The age-synchronized N2 worms were bred on NGM agar plates (data are shown). Sephadex LH-20 column of ethyl acetate soluble extract gave five fractions (EA1-EA5) using methanol as a mobile phase. Fraction EA3 (120 mg) was chromatographed by RP Lobar-A column (MeOH-H2O, 40:60) to give five subfractions (EA31-EA33). Subfraction EA31 (71 mg) was chromatographed by silica gel column (CHCl3-MeOH-H2O, 35:10:1) and purified by Sephadex LH-20 column (MeOH) to give compound 1 (31.1 mg).

**Vitexin (1)**

^1H-NMR (400 MHz, DMSO-d6) δ : 8.02 (2H, d, J=8.1 Hz, H-2’, 6’), 6.89 (2H, d, J=8.1 Hz, H-3’, 5’), 6.77 (1H, s, H-3), 6.26 (1H, s, H-6), 4.70 (1H, d, J=9.4 Hz, H-1’). ^13C-NMR (100 MHz, DMSO-d6) δ : 181.8 (C-4), 163.9 (C-2’, 6’), 161.0 (C-4’), 160.2 (C-5), 155.9 (C-9), 128.7 (C-2’, 6’), 121.6 (C-1’), 115.8 (C-3’, 5’), 104.5 (C-8), 103.5 (C-10), 102.4 (C-3), 98.3 (C-6), 81.8 (C-5’), 78.6 (C-3’), 73.6 (C-1’), 70.9 (C-2’), 70.4 (C-4’), 61.2 (C-6’). Structure characterization of compound 1 was carried out by interpretation of their spectral data comparison with the data reported in the literature.

**C. elegans strains and maintenance**

Bristol N2 (wild-type) and *Escherichia coli* OP50 were kindly provided by Dr. Myon-Hee Lee (East Carolina University, NC, USA). The worms were grown at 20°C on nematode growth medium (NGM) agar plate with *E. coli* OP50 as described previously (Brenner. 1974). To prepare plates supplemented with compound 1, the stock solution in DMSO was inserted into autoclaved NGM plates (at 50°C). A final DMSO concentration of 0.2% (v/v) was maintained under all conditions.

**Lifespan assay**

The lifespan assays were performed using wild-type at least 3 times independently at 20°C. To obtain age-synchronized nematodes, eggs were transferred to NGM plate in the absence or presence of sample after embryo isolation. Test worms were considered dead when they failed to respond to prodding with the tip of a platinum wire (Lithgow et al. 1995). The worms were transferred to fresh NGM plate every 2 days.

**Assessment of stress resistance**

The age-synchronized N2 worms were bred on NGM agar plates with or without various concentrations of sample. For the heat tolerance assay the adult day 4 worms were transferred to fresh plates and then incubated at 36°C. The survival rate was scored over 20 h as previously described (Lee et al. 2005). Oxidative stress tolerance was assessed as described previously with minor modification (Mekheimer et al. 2012). In brief, the adult day 7 worms were subjected to plate containing 1 mM of juglone liquid culture and then survivals were recorded over 16 h.
Table 1. Effects of vitexin on the lifespan of C. elegans

| Treatment | Mean Lifespan (day) | Maximum lifespan (day) | Change in mean lifespan (%) | Log-rank test |
|-----------|---------------------|------------------------|----------------------------|---------------|
| Control   | 13.5 ± 0.5          | 19                     | -                          | -             |
| 50 µM     | 14.6 ± 0.4          | 20                     | 8.0                        | p<0.05*       |
| 100 µM    | 15.8 ± 0.5          | 21                     | 17.2                       | p<0.001***    |

Mean lifespan presented as mean ± S.E.M data. Change in mean lifespan compared with control group (%). Statistical significance of the difference between survival curves was determined by log-rank test using the Kaplan-Meier survival analysis. Differences compared to the control were considered significant at *p<0.05 and ***p<0.001.

Measurement of antioxidant enzyme activities

To assess enzymatic activity, the worm homogenates were prepared. Briefly, the wild-type worms were harvested from plate with M9 buffer on the adult day 5 and washed 3 times. Then, the collected worms were suspended in homogenization buffer (10 mM Tris·HCl, 150 mM NaCl, 0.1 mM EDTA, pH 7.5) and homogenized on ice. SOD activity was measured spectrophotometrically analyzing the decolorization of formazan using enzymatic reaction between xanthine and xanthine oxidase. The reaction mixture contained 5 µL of worm homogenates and 120 µL of 1.6 mM xanthine, 0.48 mM nitroblue tetrazolium (NBT) in 10 mM phosphate buffer (pH 8.0). After pre-incubation at room temperature for 5 minutes, the reaction was initiated by adding 100 µL of xanthine oxidase (0.05 U/ml) and incubation at 37°C for 20 min. The reaction was stopped by adding 275 µL of 69 mM SDS, and the absorbance at 570 nm was measured. Catalase activity was calculated by spectrophotometry as previously described (Aebi, 1984). Briefly, the prepared homogenates were mixed with the 25 mM H₂O₂ and after 5 min incubation, absorbance was determined at 240 nm. Catalase activity was expressed in U/mg protein (1 unit will decompose 1.0 µM of H₂O₂ per min at pH 7.0 at 25°C). The enzymes activities were expressed as a percentage of the scavenged amount per control.

Analysis of intracellular ROS

Intracellular ROS in the nematodes was measured using molecular probe 2’,7’-dichlorodihydrofluorescein diacetate (H₂DCF-DA). Equal number of wild-type worms was incubated in the absence or presence of sample. On the 4th day of adulthood, animals were exposed to 96-well plate containing 50 µM juglone liquid culture for 2 h. Subsequently, 4 worms were transferred into the wells of a 96-well plate containing 50 µL of M9 buffer. Immediately after addition of 50 µL of 25 µM H₂DCF-DA solution resulting in a final concentration 12.5 µM, basal fluorescence was quantified in a microplate fluorescence reader at excitation 485 nm and emission 535 nm.

Fluorescence microscopy and visualization

The age-synchronized transgenic nematodes including CF1553 containing a SOD-3::GFP reporter and CL2070 containing HSP-16.2::GFP reporter were maintained in the presence or absence of sample. Prior to microscopy observation, CL2070 mutants were received heat shock at 36°C for 2 h and allowed to recover at 20°C for 2 h. On the 3rd days of adulthood, both transgenic worms were anesthetized with sodium azide (2%) and mounted on 2% agarose pad. The GFP fluorescence of GFP-expressing populations was directly observed under a fluorescence microscope (Nikon Eclipse Ni-u, Japan). To determine the protein expression levels, photographs of the transgenic worms were taken and assayed using Image J software. All experiments were done in triplicate.
Measurement of aging-related factors and locomotion

The age-synchronized N2 worms were bred on NGM agar plates with or without sample. On the 4th day of adulthood, single worms were transferred to fresh plate followed by pharynx contractions and body movements of animals were counted under an inverted microscope for 1 min. For the growth alteration assay, photographs were taken of adult day 4 worms, and the body length of each animal was analyzed by the Nikon software (Nikon, Japan). Reproduction assay was conducted as follows. N2 worms were raised from embryo as in the lifespan assay. L4 larvae were individually transferred to the fresh plate every day to distinguish the parent from the progeny. The progeny was counted at the L2 or L3 stage. On the 7th day of adulthood, single worms were transferred to fresh plate followed by body movements were recorded under an inverted microscope for 10 seconds. The body movements of animals were analyzed by Nikon image software. All tests was completed in triplicate.

Data analysis

The data from the lifespan assay and stress resistance assays were plotted using Kaplan-Meier analysis and statistical significance was analyzed by log-rank test. Other data were presented as mean ± or standard error of the mean, as indicated. Statistical significance of differences between the control and treated groups were analyzed by one-way analysis of variance (ANOVA).

RESULTS

Effects of vitexin on the lifespan of C. elegans

In order to determine the lifespan extension properties of vitexin, lifespan assays were performed with wild-type N2 worms. We found a concentration-dependent effect of vitexin on longevity (Fig. 2A). In addition, there was a significant increase (17.2% at 100 \( \mu \text{M} \) of vitexin, \( p<0.001 \)) in the estimated mean life of vitexin-treated worms compared to control worms (Fig. 2B, Table 1). The mean life duration was 13.5±0.5 days for control worms, and the mean life duration of vitexin for the worms fed at 100 \( \mu \text{M} \) were 15.8±0.5 days.

Effects of vitexin on the stress tolerance of C. elegans

We determined the effect of vitexin on two different kinds of stress conditions including thermal and oxidative stress using wild-type N2 worms. We found a concentration-dependent effect of vitexin on longevity (Fig. 2A). In addition, there was a significant increase (17.2% at 100 \( \mu \text{M} \) of vitexin, \( p<0.001 \)) in the estimated mean life of vitexin-treated worms compared to control worms (Fig. 2B, Table 1). The mean life duration was 13.5±0.5 days for control worms, and the mean life duration of vitexin for the worms fed at 100 \( \mu \text{M} \) were 15.8±0.5 days.

Table 2. Effects of vitexin on the stress tolerance of C. elegans

| Stress condition     | Treatment | Mean lifespan (h) | Maximum lifespan (h) | Change in mean lifespan (%) | Log-rank test |
|----------------------|-----------|------------------|----------------------|-----------------------------|--------------|
| 36°C thermal         | Control   | 9.0 ± 0.6        | 15                   | -                           | -            |
| thermal tolerance    | 50 \( \mu \text{M} \) | 10.6 ± 0.7       | 17                   | 17.4                        | \( p<0.001 \)** |
|                      | 100 \( \mu \text{M} \) | 13.2 ± 0.8       | 20                   | 45.8                        | \( p<0.001 \)** |
| 1 mM Juglone         | Control   | 7.3 ± 0.4        | 11                   | -                           | -            |
|                      | 50 \( \mu \text{M} \) | 8.8 ± 0.5        | 14                   | 20.5                        | \( p<0.05 \)* |
|                      | 100 \( \mu \text{M} \) | 11.5 ± 0.6       | 16                   | 56.2                        | \( p<0.001 \)** |

Mean lifespan presented as mean ± S.E.M data. Change in mean lifespan compared with control group (%). Statistical significance of the difference between survival curves was determined by log-rank test using the Kaplan-Meier survival analysis. Differences compared to the control were considered significant at \( *p<0.05 \) and \( ***p<0.001 \).
over, it was found that vitexin-treated N2 worms lived longer than control worms under oxidative stress conditions induced by 1 mM juglone in a concentration-dependent manner (100 μM, p<0.001, Fig. 3B, Table 2).

Effects of vitexin on the antioxidant enzyme activities and intracellular ROS levels

To verify the possible mechanism of vitexin-mediated lifespan extension and elevated stress resistance of nematodes, activities of stress resistance proteins were investigated. The superoxide dismutase (SOD) and catalase enzymatic activities were measured spectrophotometrically using prepared worm homogenates. Our results showed that vitexin was able to elevate SOD and catalase activities of worms significantly (p<0.05 and p<0.001; Fig. 4A, 4B). We further quantified intracellular ROS levels of vitexin-treated worms compared with untreated controls. Fig. 4C shows that vitexin-fed worms effectively reduced the production of ROS by 14.6% (100 μM, p<0.01) compared with solvent-treated control worms.

Effects of vitexin on the SOD-3 and HSP-16.2 expressions in transgenic nematodes

To investigate whether vitexin-mediated increased stress tolerance was due to regulation of stress-response genes, we quantified SOD-3 and HSP-16.2 expressions using transgenic strains including CF1553 and CL2070, respectively. Our data shows that vitexin-treated CF1553 worms exhibited significantly higher SOD-3::GFP intensity (25.4% at 100 μM, p<0.001), compared with untreated control worms (Fig. 5A, 5C). The CL2070 worms containing HSP-16.2::GFP reporter gene were received heat shock at 36°C for 2 h and allowed to recover at 20°C for 2 h, followed by quantifying fluorescence intensity. This heat shock-induced HSP-16.2::GFP expression was further up-regulated by 100 μM of vitexin about 23.5% (p<0.001, Fig. 5B, 5D).

Effects of vitexin on the aging-related factors of C. elegans

In order to verify the possible mechanism of vitexin on the lifespan of nematodes, we observed vitexin-induced change in parameters of aging-related factors such as progeny, pharyngeal pumping, and body length. We did not find any significant statistical changes between vitexin-fed worms and control worms in the reproduction rate, food intake, and body length (Fig. 6A, 6B, 6C). These results demonstrate that the alteration of those aging-related factors is not responsible for vitexin-mediated lifespan extension in C. elegans.

DISCUSSION

The average human lifespan has increased with the development of economy and medicine. Naturally, people are interested in lifespan extension. In this work, we isolated vitexin from the seed of *V. angularis* and elucidated its longevity activity in *C. elegans*. Vitexin was reported by the isolation from several plants (Quercia *et al*., 1978, Palme *et al*., 1994, Khole *et al*., 2014). Several early studies have revealed that vitexin has been shown to various pharmacological properties including antiviral (Li *et al*., 2002), anti-depressant-like (Can *et al*., 2013), α-glucosidase inhibitory (Choo *et al*., 2012), antinociceptive (Özkay and Can, 2013) and anti-diabetic activities (Choi *et al*., 2014).

To verify the effect of vitexin on the longevity, we carried out lifespan assay with wild-type N2 worm, *C. elegans* under normal culture condition. The *C. elegans* model system offers various useful methods for aging-related research because it has diverse excellences such as short lifespan, ease of handling, rapid generation and a large number of mutant strains.
We found that vitexin treatment considerably enhanced the lifespan of nematode in a concentration-dependent manner. In addition, vitexin considerably increased the survival rate of N2 worms under both of heat stress and juglone-induced oxidative stress conditions. Since there is a clear correlation between lifespan-extension and the stress tolerance (Kenyon, 2010), resistant ability against stress condition may positively affect vitexin-mediated prolonged lifespan. In this study, vitexin-treated worms exhibited significant increase in survival rate under thermal stress condition, compared to control worms, suggesting that the vitexin-treatment enhanced thermo-tolerance. Furthermore, the result of juglone-induced oxidative stress assay showed that vitexin-treated worms survived longer than the control as well. These results indicate that the lifespan extending ability of vitexin is quite possibly associated with increased stress tolerance. Heat shock proteins (HSPs) are expressed under heat stress condition (Swindell, 2009), so we tried to reveal possible involvement of HSP-16.2 in the vitexin-mediated stress resistance. The HSP-16.2 family of C. elegans is expressed under thermal stress conditions, so they can role as stress-sensitive reporters to assess longevity (Strayer et al., 2003). In this study, GFP-fused transgenic strain CL2070 was used to measure HSP-16.2 expression level. We found that HSP-16.2 expression induced by heat shock was significantly elevated in the worms treated with vitexin, compared with control worms, suggesting the vitexin-mediated longevity and enhanced stress resistance against thermal stress of vitexin may be explained by this property.

The ROS accumulation is largely regulated by a complicated antioxidant defense system such as SOD, catalase and glutathione peroxidase (Finkel and Holbrook, 2000). To know

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**Fig. 5.** Effects of vitexin on the expression of SOD-3 and HSP-16.2 was determined using transgenic nematodes. Mean GFP intensity of CF1553 (A) and CL2070 (B) mutants were represented as mean ± S.E.M. of values from 18 to 26 animals per each experiment. The GFP intensity was quantified using Image software by determining average pixel intensity. Images of SOD-3::GFP (C) and HSP-16.2::GFP (D) expressions of CF1553 worms in the presence or absence of vitexin. Data are expressed as the mean ± standard deviation of three independent experiments (N=3). Differences compared to the control were considered significant at *p*<0.05, **p**<0.01 and ***p***<0.001 by one-way ANOVA.
how vitexin controls oxidative stress, we analyzed the antioxidant enzymes such as SOD and catalase activities using nematode homogenates. Our results showed that both enzyme activities were significantly up-regulated by vitexin, suggesting attenuation of hydroxyl radical levels resulted in diminished oxidative stress. As mentioned above, vitexin treatment provided lifespan extension under oxidative stress condition induced by juglone. It was also found that SOD activity was considerably up-regulated in the vitexin-treated worms compared with the control worms. To verify whether this enhanced enzyme activity was due to direct scavenging of ROS or altered protein expression, further quantification of SOD expression was performed using GFP-fused transgenic strain CF1553. The results exhibited that vitexin-fed worms showed higher GFP intensity compared with the control worms, indicated that vitexin treatment increased SOD expression. In addition, intracellular ROS levels were also considerably reduced by vitexin treatment. These results suggest that antioxidant properties of vitexin might be partially attributed to extended lifespan and increased survival rate of worms under stress conditions.

Reductions in aging-related factors like reproduction, food intake and body size are closely related to longevity (Mörck and Pilon, 2006; Surco-Laos et al., 2012), we further investigated whether vitexin affects aging-related factors. The results revealed that there were no significant variation in the number of progeny, body length and pharyngeal pumping between vitexin-fed worms and control worms. These results present evidence that vitexin extends lifespan in C. elegans independent of altering aging-related factors. Furthermore, to know vitexin’s influence on the functional aging, locomotion assay was conducted. Vitexin slightly up-regulated the body movement of N2 worms suggesting that vitexin might provide a beneficial effects on healthspan to some extent as well as lifespan.

Several flavonoid compounds like quercetin, kaempferol, naringenin and myricetin were reported their lifespan extending properties in C. elegans (Grünz et al., 2012). As a flavone C-glycoside, vitexin has been isolated from several medicinal and other plants. Plant extracts containing vitexin were reported to possess antioxidant, anti-inflammatory and antino-ciceptive activities (Borqhi et al., 2013). Previous study also revealed that vitexin has several aging-related effects such as antioxidant, antitumor and inflammatory pain inhibitory activities as well as protecting effect against ischemia/reperfusion injury via modulating mitogen-activated protein kinase and apoptosis signaling in mice (Kim et al., 2005).

In conclusion, in this work, vitexin showed lifespan extension of C. elegans through its antioxidant potential and regulating stress resistance protein. Therefore, the seed of V. angularis and vitexin could be useful for longevity in human, but yet, since these are the preliminary data, the question about if vitexin provide positive or negative effect against aging in human is still unknown and it should be studied furthermore.

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