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Significant longevity-extending effects of a tetrapeptide from maize on *Caenorhabditis elegans* under stress

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ABSTRACT

A tetrapeptide (Leu-Asp-Tyr-Glu) from maize (TPM) is a bioactive peptide. Here we reported that TPM extended the lifespan of *Caenorhabditis elegans* under heat and oxidative stress. Specifically, TPM (10 mM) increased the average longevity of *C. elegans* by 36.9% and 27.6% under heat stress (35 °C) and oxidative stress, respectively. Further studies demonstrated that the significant longevity-extending effects of TPM on *C. elegans* could be attributed to its *in vitro* and *in vivo* free radical-scavenging effects and its up-regulation of stress-resistance-related proteins, including superoxide dismutase-3 (SOD-3) and heat shock protein-16.2 (HSP-16.2). Real-time PCR results showed that the up-regulation of ageing-associated genes such as daf-16, sod-3 and hsp-16.2, in addition to skn-1, ctl-1 and ctl-2, could also contribute to the stress-resistance effect of TPM. These results indicate that TPM can (or has the potential to) protect against external stress and extend lifespan under stress.

1. Introduction

Oxidative stress represents an imbalance between the production of reactive oxygen species (ROS) and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Disturbances in the normal redox state of tissues can cause toxic effects through the production of peroxides and free radicals that damage the components of the cell, including proteins, lipids, and DNA.

Extensive studies suggest that ROS, by-products of cellular respiration, play a role in normal ageing by causing random deleterious oxidative damage to a variety of tissue (Harman, 1956; Muller, Lustgarten, Jang, Richardson, & Van Remmen, 2007). Resistance to ROS, due to high activities of ROS-detoxifying enzymes and/or low intrinsic ROS-production levels, characterise commonly long-lived *Caenorhabditis elegans* mutants and these findings are taken to substantiate the free radical theory of ageing (Tanja, Kai, Hannelore, & Uwe, 2010). Longevity genes also mediate increased resistance to oxidative stress; long-lived dauer mutants show increased resistance to oxidative stress and up-regulation of antioxidant genes (Sang-Kyu, Patricia, & Thomas, 2009).

Maize is a plant whose food value and wide variety of uses make it one of the most important crops in the world. With the development of science and technology, as well as the need of using food for health promotion, the understanding of the significance of corn nutrition has constantly advanced for the past century.

A tetrapeptide from maize (TPM), characterised as Leu-Asp-Tyr-Glu, is a bioactive peptide (Xu et al., 2004). In this study, we investigated the anti-ageing effects of TPM in *C. elegans* and found that TPM could significantly improve the longevity of *C. elegans* under stress conditions. Our results suggest that TPM may provide strong protection against stress and extend the longevity of *C. elegans* by scavenging reactive oxygen species and up-regulating the expression of stress resistance-associated genes, such as daf-16, sod-3, skn-1, ctl-1, ctl-2 and hsp-16.2. We believe these findings will provide insight regarding anti-ageing research into bioactive peptides and corn.

2. Materials and methods

2.1. Reagents

FUDR (5-fluoro-2′-deoxyuridine), 98%, was bought from Sigma (St. Louis, MO). ABTS [2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonate)] (Sigma) and pyrogallol (Sigma) were used as free radical providers. H_2DCF-DA (2′,7′-dichlorodihydro fluorescein

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diacetate) (Sigma) was used as a fluorescent probe. Juglone (5-hydroxy-1,4-naphthoquinone), a reactive oxygen species-generating compound, was used to induce oxidative stress in worms.

TPM was prepared *via* protease hydrolysis of zein catalysed by Alcalase®, an alkaline protease, followed by isolation and purification (Xu et al., 2004).

2.2. Worm strains and maintenance

Standard nematode growth medium (NGM) was used for *C. elegans* growth and maintenance at 20 °C. Unless stated otherwise, plates were seeded with live *Escherichia coli* OP50 bacteria. Bristol N2 (Caenorhabditis Genetics Center; CGC) was used as the wild-type strain. The transgenic strain CF1553 (muIs84, CGC) containing the SOD-3::GFP-linked reporter was used to visualise SOD-3 expression. The CL2070 (dvIs70) strain, containing the HSP-16.2::GFP-linked reporter used to visualise HSP-16.2 expression, was a generous gift from Y. Luo of the University of Maryland (College Park, MD).

2.3. Stress resistance assay

Heat-shock assays were performed at 35 °C using 2-day-old adult worms. The worms were treated with TPM (10 mM) for 2 days and then transferred to an incubator at 35 °C. The number of dead worms was recorded every hour (Hansen, Hsu, Dillin, & Kenyon, 2005; Lithgow, White, Melov, & Johnson, 1995).

The expression of HSP-16.2::GFP in CL2070 worms was investigated by fluorescence microscopy. The worms were treated for 2 days with or without 10 mM TPM, followed by heat shock (treatment at 25 or 30 °C for half an hour and then 35 °C for an hour) and recovery for 24 h (Rea, Wu, Cypser, Vaupel, & Johnson, 2005).

Juglone sensitivity was assessed at 20 °C using 2-day-old adult worms. The worms were incubated with TPM (10 mM) for 2 days and then transferred to plates with 500 μM juglone. The number of dead worms was counted and recorded every hour.

For all life span assays, every experiment was repeated three times and conducted in a double-blind manner.

2.4. ABTS assay

ABTS (38.4 mg) and $K_2S_2O_8$ (6.6 mg) were dissolved in 5 mL water. The solution was kept at room temperature for 12–16 h to form $ABTS^+$ solution by oxidising ABTS with potassium persulfate. The $ABTS^+$ solution was diluted 1:100 in absolute ethanol to prepare the working solution. Subsequently, 1.8 mL of $ABTS^+$ working solution was mixed with 0.2 mL of TPM solution, and the absorbance at 734 nm was measured after a 20-min delay (Beckman UV–Vis spectrophotometer, Model DU640B; Beckman Coulter Inc., Fullerton, CA). The final concentrations of TPM used were 0.3125, 0.625, 1.25, 2.5, and 5 mM.

2.5. Pyrogallol self-oxidation assay

The *in vitro* superoxide anion-scavenging effects of TPM were measured by monitoring the chemiluminescence in the pyrogallol luminol system. All reagents were equilibrated in a water bath at a constant temperature (25 °C) and then added to a glass luminescence tube (1 × 5 cm) in a water bath in the following order: 10 μL 3 mM pyrogallol, 80 μL 4 mM NaOH, 10 μL TPM, and 900 μL 0.1 mM luminol (in sodium carbonate buffer, pH 10.2). The final concentrations of TPM were 0.001, 0.003125, 0.00625, 0.0125, 0.025, 0.05, 0.1 mM. Light emission was observed after a 15-s delay at 25 °C.

2.6. Fenton's reaction

The *in vitro* hydroxyl free radical-scavenging effects of TPM were measured by monitoring the chemiluminescence in the Fenton's reaction system. All reagents were equilibrated in a water bath at a constant temperature (25 °C) and then added to a glass luminescence tube (1 × 5 cm) in a water bath in the following order: 10 μL 3% H_2O_2, 10 μL 0.1 mM Fe^{2+}, 10 μL TPM, and 970 μL 0.1 mM luminol (in sodium carbonate buffer, pH 10.2). The final concentrations of TPM were 0.0925, 0.185, 0.925, 1.85, 3.7 mM. Light emission was observed after a 15-s delay at 25 °C.

2.7. Measurement of intracellular ROS in C. elegans

Intracellular ROS in *C. elegans* were measured using molecular probe H_2DCF-DA. For ROS detection under normal culture conditions, worms that had just reached adulthood were treated with or without TPM (10 mM) for 2 days. For the ROS test under oxidative stress, worms that had just reached adulthood were treated with 300 μM juglone for 1 h and then treated with or without TPM (10 mM) for 2 days. After 48 h of exposure to the respective compounds, animals were washed off the plates with cold M9 buffer. Bacteria were removed by three repeated washes and subsequent centrifugation at low speed. Animals were resuspended in M9 buffer, and a 50-μL volume of the suspension was pipetted in four replicates into the wells of a 96-well plate with opaque walls and transparent bottom and allowed to equilibrate to room temperature. In the meantime, a fresh 100 μM H_2DCF-DA solution from a 100 mM stock solution in DMSO was prepared in M9 buffer, and a volume of 50 μL was pipetted to the suspensions, resulting in a final concentration of 50 μM. On each plate, control wells containing nematodes from each treatment without H_2-DCF-DA and wells containing H_2-DCF-DA without animals were prepared in parallel (Schulz et al., 2007). Immediately after addition of H_2-DCF-DA, basal fluorescence was measured in a microplate reader at excitation/emission wavelengths of 485 and 520 nm. Plates were measured at 20 °C every 30 min for 2 h.

2.8. Fluorescence quantification and visualisation

Overall GFP fluorescence of GFP-expressing populations was assayed using a Thermo Labsystems Fluoroskan Ascent microplate reader (Thermo Fisher, Waltham, MA). Adult worms were treated with or without 10 mM TPM for 2 days. Twenty control or treated adult animals of the indicated age were transferred into 100 μL of M9 buffer to a well of a Costar 96-well microtitre plate (black, clear, flat-bottom wells), and total GFP fluorescence was measured using 485 nm excitation and 530 nm emission filters. Quadruple populations were used for each determination. For fluorescence microscopy, the worms were mounted with a drop of levamisole (10 mM) and placed on a cover slip covered with 3% agarose. The GFP pictures of transgenic worms were taken using an AXIO Imager M2 microscope system (Zeiss).

2.9. Quantitative real-time PCR

Adult worms were treated with or without 10 mM TPM for 2 days. Total RNA was extracted from adult worms with TRIzol reagent (Invitrogen, Carlsbad, CA), and cDNA was produced by oligo(dT) priming. The RT-PCR primers were as follows: daf-2 (NM_065249), 5′-GGCCGATGGACGTTATTTTG-3′ and 5′-TTCCACAGTGAAGAAGCCTGG-3′; daf-16 (NM_001026247), 5′-TTTCCG TCCCCGAACTCAA-3′ and 5′-ATTCGCCAACCCATGATGG-3′; sod-3 (NM_078363), 5′-AGCATCATGCCACCTACGTGA-3′ and 5′-CACCAC CATTGAATTTCAGCG-3′; skn-1 (NM_171347), 5′-AGTGTCGG CGTTCCAGATTTC-3′ and 5′-GTCGACGAATCTTGCGAATCA-3′;