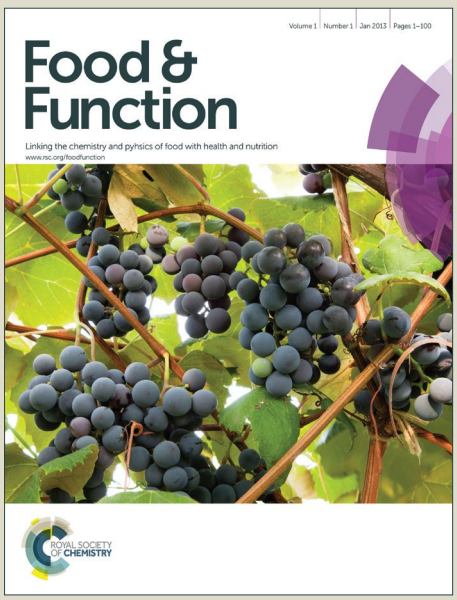
Food & Function

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/foodfunction

Food & Function

PAPER

The novel dipeptide Tyr-Ala (TA) significantly enhances the lifespan and healthspan of *Caenorhabditis elegans*

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Z. Zhang[a], Y. Zhao[b], X. Wang[d], R. Lin[a], Y. Zhang[a], H. Ma[a], Y. Guo[a*], L. Xu[a*], B. Zhao[c*]

Food-derived bioactive peptides may have various physiological modulatory and regulatory functions and are now being studied extensively. Recently, the novel dipeptide Tyr-Ala was isolated from hydrolyzed maize protein. Tyr-Ala significantly prolonged the lifespan of wild-type *Caenorhabditis elegans* and extended the nematode healthspan and lifespan during heat/oxidative stress. Compared with its constituent amino acids, Tyr-Ala was more efficient in enhancing stress resistance. Further studies demonstrated that the significant longevity-extending effects of Tyr-Ala on *Caenorhabditis elegans* were attributed to its *in vitro* and *in vivo* free radical-scavenging effects, in addition to its ability to up-regulate stress resistance-related proteins, such as SOD (Superoxide Dismutase)-3 and HSP (Heat Shock Protein)-16.2. Real-time PCR results showed that the up-regulation of aging-associated genes, such as *daf-16*, *sod-3*, *hsp-16.2* and *skn-1,* also contributed to the stress-resistance effect of Tyr-Ala. These results indicate that the novel dipeptide Tyr-Ala can protect against external stress and thus extend the lifespan and healthspan of *Caenorhabditis elegans*. Thereby, Tyr-Ala could be used as a potential medicine in anti-aging research.

1. Introduction

Geriatric diseases, such as Alzheimer's disease and Parkinson's disease, make up an increasing share of the global burden of disease (GBD). As an organism ages, it experiences a gradual deterioration of physical functions over time, with increasing susceptibility to environmental stimulation and increasing risk of disease and death.[1] Extensive research determined that reactive oxygen species (ROS) play an important role in aging.[2, 3] It is important to keep ROS at a proper level to maintain homeostasis in organisms.[4, 5] However, oxidative stress induced by excess peroxides and free radicals can damage cell components and trigger numerous human diseases, including diabetes, atherosclerosis, cancer, and aging.[6, 7, 8] These effects represent an imbalance between the production of ROS and the ability to degrade reactive intermediates or repair damage *in vivo*. To fight against oxidative damage *in vivo,* it is necessary to find an antioxidant that has a strong antioxidative capacity and a relatively well-defined mechanism.

Interest in the identification and characterization of oligopeptides derived from plants and animals has grown.[9] Exogenous food-derived bioactive peptides may have various physiological modulatory and regulatory activities that may supplement endogenous gut protein-derived bioactive peptides.[10] These peptides may also have additional functions, such as opiate-like[11], mineral binding[12], immunomodulatory[13], antimicrobial[14], antioxidative[15], antithrombotic[16], hypocholesterolemic[17] and antihypertensive functions[18]. Moreover, several peptides have been found to possess multiple properties that influence various physiological systems.[7] Maize is an important, popular, widely available and economically significant crop around the world and has been widely studied as a source of novel bio-activators.[19] Corn peptides, including some small peptides with molecular weights of less than 1,000 Da, and antioxidant amino acids, such as Tyr, Phe, Pro, Ala, His, and some branch amino acids, were prepared from corn gluten meal by proteolysis with Alcalase or other alkaline proteases[20]. These small peptides can be absorbed directly in the intestine at a rate 2 to 3 times faster than that observed for amino acids and proteins to exert biological functions.[21, 22, 23]

[a] *Key laboratory for Molecular Enzymology and Engineering, the Ministry of Education, National Engineering Laboratory for AIDS Vaccine, School of Life Sciences, Jilin University, Changchun 130012, China*
[b] *Department of Food Sciences and Technology, Harbin Institute of Technology at Weihai 264209, China*
[c] *State Key Laboratory of Brain and Cognitive Science, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China*
[d] *Jilin Province People's Hospital, Changchun 130021, China*
*Corresponding authors:
Yi Guo, Key laboratory for Molecular Enzymology and Engineering of Ministry of Education, National Engineering Laboratory for AIDS Vaccine, School of Life Sciences, Jilin University, Changchun 130012, China, Tel: +86-431-85155246, Email: guoyi@jlu.edu.cn
Li Xu, Key laboratory for Molecular Enzymology and Engineering of Ministry of Education, National Engineering Laboratory for AIDS Vaccine, School of Life Sciences, Jilin University, Changchun 130012, China, Tel and Fax: +86-431-85155226, Email: xuli@jlu.edu.cn
Baolu Zhao, State Key Laboratory of Brain and Cognitive Science, Institute of Biophysics, Chinese Academy of Sciences, Beijing 100101, China, Email: zhaobl@sun5.ibp.ac.cn

Current aging research aims to identify drugs that can reduce the morbidity of age-related disease and degeneration by improving youthful physiological function. The nematode *C. elegans* is a pseudo-coelomate nematode that has been used and studied widely as a model organism because these animals decline behaviorally and physiologically with age in a manner similar to that observed for higher mammals, such as humans.[3] As they age, the animals accumulate oxidized proteins and exhibit autofluorescence, which are hallmarks of aging that are common to many species.[24,25] In 2012, we reported that a small tetrapeptide (Leu-Asp-Tyr-Glu) derived from maize exerts life-prolonging effects in *C. elegans* under stress by diminishing reactive oxygen free radicals.[7] These results provoked our interest in a further study of the anti-aging effects of more bioactive peptides derived from maize. Moreover, as differences between the life-prolonging effects of peptides and their constituent amino acids are little studied in *C. elegans*, we want to investigate the advantages of peptides over their constituent amino acids.

In this study, the antioxidant effects of the novel bioactive dipeptide Tyr-Ala (TA), which was also isolated from hydrolyzed maize protein, is investigated. In addition, we revealed the protective effect of this novel food-derived dipeptide against stress and the anti-decrepit and life-prolonging effect of TA on *C. elegans*. The relationship among these effects is also discussed. The mechanism of action is also expounded based on the structure of this novel dipeptide, including the dipeptide's antioxidant activity and the effect of TA on stress-related proteins and genes.

2. Materials and methods

2.1. Reagents

5-fluoro-2'-deoxyuridine (FUDR), which had a purity of 98 %, was purchased from Sigma (St. Louis, MO, USA). 2', 7'-dichlorodihydrofluorescein-diacetate (H_2DCF-DA) (Sigma) was used as a fluorescent probe. 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS), which had a purity of 98 %, was purchased from Sigma (St. Louis, MO, USA). The ROS-generating compound 5-hydroxy-1, 4-naphthoquinone (juglone) was used to induce oxidative stress in worms (Sigma). Tyr-Ala (TA) was prepared via protease hydrolysis of zein, which was catalyzed by the alkaline protease Alcalase, followed by isolation, purification, and synthesis by China Peptides Co., Ltd. (Shanghai, China).

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, the plates were seeded with live *Escherichia coli* strain OP50 bacteria as the food source.[26] Bristol N2 (Caenorhabditis Genetics Center based at the University of Minnesota; CGC) was used as the wild-type strain. The transgenic strain CF1553 (muIs84, CGC) containing the SOD-3::GFP-linked reporter was used to visualize SOD-3 expression. The CL2070 (dvIs70) strain containing the HSP-16.2::GFP-linked reporter that is used to visualize HSP-16.2 expression was a generous gift from Y. Luo of the University of Maryland (College Park, MD).

2.3. Life span assay

Wild-type *C. elegans* N2 was used in the lifespan assays at 20 °C. The worms in the treatment groups were grown on dry NGM plates coated with the reproductive suppressant FUDR (80 μM) and TA (1 mM, 10 mM) diluted in a suspension of live *E. coli* OP50. The worms in the control group were grown on dry NGM plates coated with the reproductive suppressant FUDR only. To obtain a synchronous population,[25] gravid nematodes were placed on NGM plates with *E. coli* OP50 on the surface and allowed to lay eggs for approximately 3 h at 20 °C. The synchronized nematodes were raised and then transferred to treatment plates at the end stage of Level 4 immediately before they began to lay eggs, which was counted as day 1. Sixty nematodes were incubated on each treatment plate and transferred to fresh treatment plates every 2 days for the first 10 days. *C. elegans* individuals were considered to be dead when they had no response to prodding with the tip of a platinum wire.[27] For the lifespan assay, the experiment was repeated three times in a double-blind manner.

2.4. Healthspan Assay

In this study, the spontaneous fluorescence in the *C. elegans* gut is used as an age-related signal.[28] The animals (N=20 per group) were raised on NGM plates with *E. coli* OP50 from L1, as described for the life span assays. In the treatment groups, the worms were treated with 1 mM TA and 10 mM TA because the worms were grown to the end stage of Level 4. Randomly selected 10-day-old worms from each set of experiments were washed 5 times with M9 buffer and then anesthetized with levamisole. The worms were then mounted onto 3 % agarose pads, capped with cover slips and photographed via fluorescence microscopy (Olympus IX51, Japan). Fluorescence microscopic studies revealed that spontaneous fluorescence exists in the guts of nematodes. Autofluorescence images in the intestine were collected using a 525 nm bandpass filter, and the fluorescence intensities were measured using Image J Software (NIH). Twenty nematodes were examined per treatment, and three replicates were performed.

The overall fat levels in wild-type *C. elegans* were also measured using the Oil Red O stain, which has been used widely to stain lipid deposits.[29] Forty animals per group were raised from L4on NGM plates with *E. coli* OP50 in the presence or absence of 10 mM TA. Five-day-old worms from each set of experiments were washed 5 times with PBS buffer and fixed with 1 % paraformaldehyde for 20 min. The worms were subjected to three freeze–thaw cycles between -80 °C and 40 °C, followed by resuspension in 60 % isopropanol to dehydrate the worms. A 60 % Oil-Red-O staining solution was then added (prepared as follows: a stock solution (0.5 g/100 mL of isopropanol) was equilibrated for several days, freshly diluted with 40 % water and 60 % stock, allowed to sit for 10 min and filtered using a 0.2 to 0.4 mm filter). Thirty minutes later, the worms were washed 3 times with PBS buffer, and images were collected via fluorescence microscopy (Olympus IX51, Japan).

For the locomotion assays, the animals were raised from L1, as described for the lifespan assays. On adult day 5, 20 individuals from each set were placed independently onto a glass slide in a drop of M9 buffer and filmed for 30 seconds using a camera attached to a stereo microscope (ZSA302, Chongqing Optical & Electrical Instrument Co., Ltd, Chongqing, China). Body bends were counted by reviewing each frame of the 30-second film at a lower speed.

2.5. Stress resistance assay

To evaluate the potential longevity-extending effect of TA on wild-type *C. elegans* N2 during oxidative stress, the worms that had just reached adulthood were pretreated with various concentrations of TA (1 mM, 10 mM), as well as Tyr (1 mM) and Ala (10 mM) and a mixture of both amino acids, for 48 h and then exposed to juglone (500 µM) at 20 °C. The number of dead worms was counted and recorded every hour.

Heat-shock assays were performed using 2-day-old adult worms at 35 °C, which was considered to be a heat stressor. The worms were subjected to the same treatments described for the oxidative stress experiments for 2 days at 20 °C and then transferred to an incubator at 35 °C. The number of dead worms was recorded every hour.[27, 30] For all lifespan assays, every experiment was repeated three times and conducted in a double-blind manner.[31]

2.6. ABTS assay

ABTS (38.4 mg) and $K_2S_2O_8$ (6.6 mg) were dissolved in 5 mL of water. The solution was kept at room temperature for 12–16 h to form an $ABTS^+$ solution via the oxidation of ABTS with potassium persulfate. The $ABTS^+$ solution was diluted 1:100 in absolute ethanol to prepare the working solution. Subsequently, 1.8 mL of the $ABTS^+$ working solution were mixed with 0.2 mL of TA 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 TA used were 0.01, 0.05, 0.1, 0.2, 0.5, 1, 10 and 50 mM.

2.7. Measurement of intracellular ROS in *C. elegans*

Intracellular ROS in *C. elegans* were measured using the molecular probe H_2DCF-DA. For ROS detection under normal culture conditions, worms that had just reached adulthood were treated with or without TA (10 mM) for 2 days. For the ROS test under oxidative stress conditions, worms that had just reached adulthood were treated with 300 µM juglone for 1 h and then treated with or without TA (10 mM) for 2 days. After 48 h of exposure to the respective compounds, the animals were washed off of the plates with cold M9 buffer. Bacteria were removed by three repeated washes and subsequent centrifugation at low speed. The animals were resuspended in M9 buffer, and 50 µL volumes of the suspension were pipetted into the wells of a 96-well plate with opaque walls and a transparent bottom in four replicates and allowed to equilibrate to room temperature. In the meantime, a fresh 100 µM H_2DCF-DA solution in M9 buffer was prepared from a 100 mM stock solution in DMSO, and a volume of 50 µL was pipetted into the suspensions, resulting in a final concentration of 50 µM. On each plate, control wells containing nematodes from each treatment without H_2DCF-DA and wells containing H_2DCF-DA without animals were prepared in parallel.[31] After the addition of H_2DCF-DA, the basal fluorescence was measured immediately in a microplate reader at excitation/emission wavelengths of 485 and 538 nm. The plates were measured at 20 °C every 30 min for 2 h.

2.8. Fluorescence quantification and visualization

To study the expression of HSP-16.2::GFP in CL2070, the worms were treated for 2 days with or without 10 mM TA, followed by heat shock (treatment at 25 or 30 °C for half an hour and then at 35 °C for an hour), recovery for 24 h, and examination via fluorescence microscopy.[32] The CF1553 worms that were used to study the expression of SOD-3 were treated with or without TA after being stimulated with juglone (300 µM) for one hour at 20 °C. The overall GFP fluorescence of GFP-expressing populations was assayed using a Thermo Labsystems Fluoroskan Ascent microplate reader (Thermo Fisher, Waltham, MA). Twenty control or treated adult animals of the indicated age were transferred into 100 µL of M9 buffer in a well of a Costar 96-well microtiter plate (black, clear, flat-bottom wells), and the total GFP fluorescence was measured using 485 nm excitation and 530 nm emission filters. Four 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 images of transgenic worms were captured using an AXIO Imager M2 microscope system (Zeiss).

2.9. Quantitative real-time PCR

Adult worms were treated with or without 10 mM TA for 2 days. Total RNA was extracted from the adult worms with the TRIzol reagent (Invitrogen, Carlsbad, CA), and cDNA was produced by oligo (dT) priming. The RT-PCR primers were as follows: *daf-16* (NM_001026247), 5'-TTTCCGTCCCCGAACTCAA-3' and 5'-ATTC-GCCAACCCATGATGG-3'; *sod-3* (NM_078363), 5'-AGCATCATGC-CACCTACGTGA-3' and 5'-CACCACCATTGAATTTCAGCG-3'; *daf-2* (NM_065249), 5'-GGCCGTAGGACGTTTA-TTTG-3' and 5'-TTCCACAGTGAAGAAGCCTGG-3'; *hsp-16.2*, 5'-C-GTCGAAGAGAATACTGCTGAA-3' and 5'-TGCAGCGAACATAACT-GTATATTTAG-3'; *skn-1* (NM_171347), 5'-AGTGTCGGCGTTC-CAGATTTC-3' and 5'-GTCGACGAATCTTGCGAATCA-3'; and *cdc-42* (NM_063197), 5'-CTGCTGGACAGGAAGATTACG-3' and 5'-CTCGGACATTCTCGA ATGAAG -3'.

Cdc-42 was used as the internal control.[33] mRNA expression was assessed by quantitative real-time PCR on a Rotor-Gene 6000 real-time PCR detection system (Qiagen, Düsseldorf) using SYBR green. The gene expression data were analyzed using the comparative $2^{-\Delta\Delta t}$ method.[34]

2.10. Statistical tools

The data obtained from the lifespan assays were processed using the Kaplan–Meir survival analysis tool of Origin 8.0. For other types of data, one-way ANOVA was used to compare more than two groups; when the result was significant ($p < 0.05$), the Tukey HSD test was applied to test for differences between individual groups. $p<0.05$ was considered to be statistically significant. All of the data were analyzed with Origin 8.0 (Northampton, MA). The standard error bars of the averages are shown in the figures.

3. Experimental Results

3.1. TA can extend the lifespan of wild-type *C. elegans* N2 under normal culture conditions

To determine the life extension properties of TA, a lifespan assay was performed at 20 °C, which is an optimal temperature for wild-type *C. elegans*. Herein, we found that TA had a significant ability to extend the lifespan of N2 at both concentrations tested (1 mM and 10 mM), as shown in Figure 1.

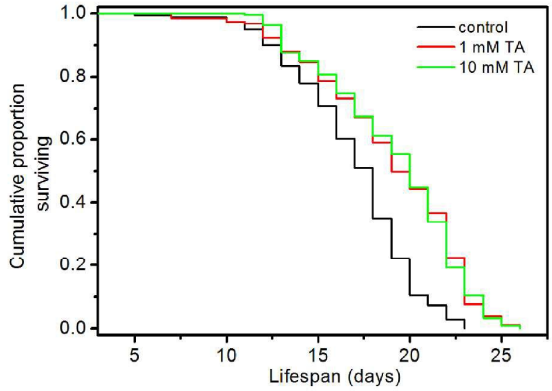


Fig. 1 TA (Tyr-Ala) significantly prolonged the lifespan of wild-type *C. elegans* N2 under normal culture conditions at 20 °C. Compared with the control group (N=181), TA at concentrations of 1 mM (N=196) and 10 mM (N=222) significantly extended the mean lifespan of wild-type *C. elegans* N2. The data were processed using the Kaplan–Meir survival analysis tool of Origin 8.0.

Compared with the controls, TA increased the mean lifespan of the wild-type worms (as shown in Table 1, 10.37 % at 1 mM TA, 12.43 % at 10 mM TA) in a statistically significant, dose-dependent manner. Statistical analysis revealed that the maximum lifespan of the worms was increased to 26 days by 1 mM and 10 mM TA (Table 1). Thus, TA demonstrated a significant lifespan-extending effect in *C. elegans*.

Table 1 Effects of TA on wild-type *C. elegans* N2 feeding on NGM plates covered with live *E. coli* OP50 at 20 °C.

	Genotype	Food	Treatment (20 ℃)	Total (N)	Mean lifespan	Mean SE	Maximum	Change in mean lifespan (%)	Log-rank test χ2	Log-rank test p
1	Wild-type	Live-OP50	control	65	17.05	0.36	23			
			1mMTA	56	19.05	0.53	26	11.73	18.35802	<0.0001
			10mMTA	76	18.76	0.43	25	10.03	16.18145	<0.0001
2	Wild-type	Live-OP50	control	63	16.97	0.43	23			
			1mMTA	65	19.17	0.5	25	12.96	18.66352	<0.0001
			10mMTA	71	19.23	0.47	26	13.32	19.62529	<0.0001
3	Wild-type	Live-OP50	control	53	16.92	0.48	23			
			1mMTA	64	18.03	0.6	26	6.56	10.27231	0.00135
			10mMTA	75	19.28	0.43	25	13.95	18.35207	<0.0001
Total	Wild-type	Live-OP50	control	181	16.98	0.24	23			
			1mMTA	196	18.74	0.32	26	10.37	45.66358	<0.0001
			10mMTA	222	19.09	0.26	26	12.43	54.87175	<0.0001

% indicates the percentage relative to the control.

3.2. TA lengthens the healthspan of *C. elegans*

To determine whether TA induces an extended healthspan in a whole-animal context, we examined overall fat levels in wild-type *C. elegans* using the fluorescent stain Oil Red O, which has been used widely to stain lipid deposits. As shown by fluorescence microscopy, nematodes treated with TA exhibited a lower fluorescence intensity than nematodes in the control group, which were treated with water (Figure 2A). Using the Image J software, we quantified the Oil Red O fluorescence *in vivo,* and the results are shown in Figure 2C. Animals raised in the presence of 10 mM TA showed lower levels of Oil Red O fluorescence (Figure 2A), and the quantified results showed that TA could significantly reduce the accumulation of lipid deposits by 39.68 % in N2 (Figure 2C).

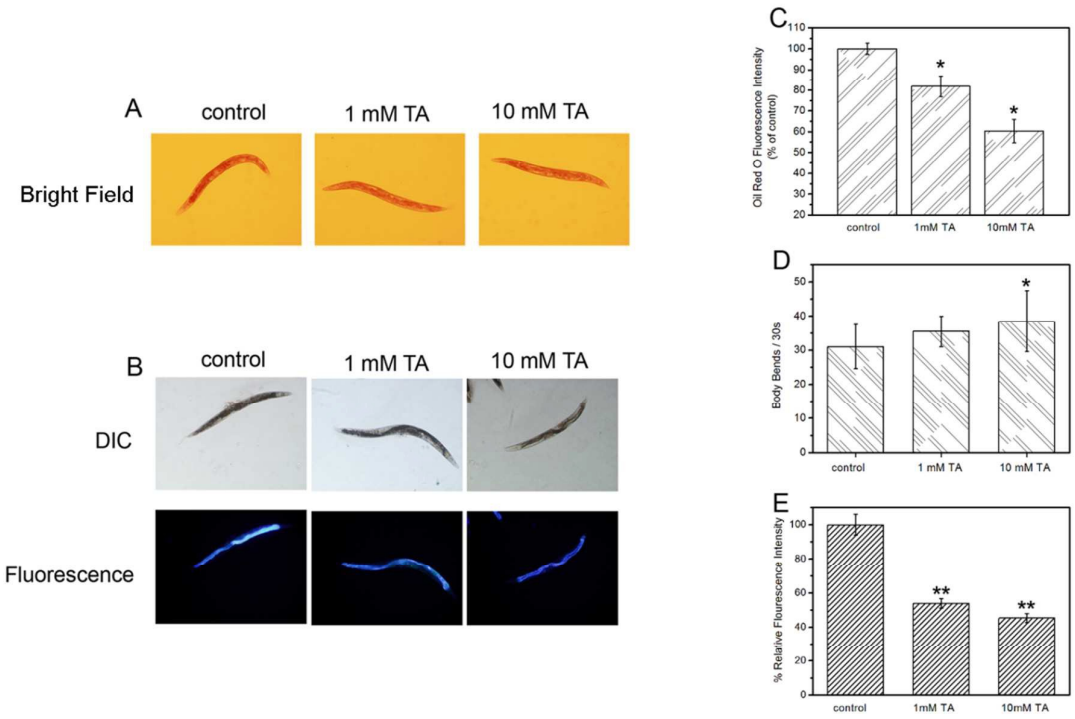


Fig. 2 Effects of TA on the healthspan of *C. elegans* N2. (A) Image of Oil Red O staining of lipid deposits in *C. elegans* N2. (B) Images of intestinal autofluorescence in the control group and the treated group (1 mM TA and 10 mM TA). (C) Quantitation of the fluorescence of Oil Red O staining of lipid deposits in *C. elegans* N2. The fluorescence level decreased significantly in the 10 mM TA-treated groups. (D) Swimming rates of wild-type animals raised on plates covered with 1 mM and 10 mM TA at 20 °C. (E) The comparison of the relative fluorescence intensity of intestinal autofluorescence was shown in E. Compared with the control group, the TA-treated group showed lower spontaneous fluorescence intensity. The bars represent the mean ± S.E.M. *P<0.05; **P<0.01.

Food & Function

PAPER

When compared with the control group, the groups treated with TA (1 mM and 10 mM) exhibited a lower fluorescence intensity, as shown by fluorescence microscopy (Figure 2B). The intensity of the fluorescence was then quantified, and the data showed that TA significantly reduced the spontaneous fluorescence intensity by 54.66 % in N2 (Figure 2E, $p < 0.01$ compared with the control) after 10 days.

The locomotion rates of *C. elegans* on solid media and in liquid, which are important signals of healthspan, decline as the animals age. To explore whether TA can extend locomotory healthspan and thus increase quality of life, we tested the effects of 1 mM and 10 mM TA on the swimming vigor of *C. elegans* (quantitated as body bends per 30s) in aging populations (Figure 2D). Compared to the non-treated control group, the TA-treated groups displayed significantly faster swimming rates. Taken together, we found that TA increased the nematode healthspan and prolonged locomotory ability in a dose-dependent manner.

3.3. TA improves the stress resistance of *C. elegans* under stress conditions

To detect the stress resistance effects of TA during environmental stress, assays were carried out under oxidative stress and thermal stress conditions. Juglone, which is a pro-oxidant that can be reduced by diaphoreses in the presence of NAD(P)H, converts oxygen to superoxide anions and consequently increases intracellular oxidative stress.[35, 36] N2 animals treated with TA showed increased resistance to juglone. When the worms were pre-treated with TA at doses of 1 mM and 10 mM, the mean survival times were increased significantly by 13.53 % and 40.58 % (Figure 3A), respectively. The effects of Ala, Tyr and a mixture of Ala and Tyr on the longevity of *C. elegans* under oxidative stress were also studied. The results showed that Tyr has the ability to extend the longevity of wild-type *C. elegans* during oxidative stress, while Ala has no significant effects (Figure 3C). However, Tyr is practically insoluble in water or ethanol but soluble in formic acid, and we cannot treat organisms with Tyr dissolved in formic acid, which means that we cannot view Tyr as a candidate antioxidant for use *in vivo*. One potential substitute, a disodium salt of Tyr that is generated by dissolving Tyr in a NaOH solution, can be used at higher concentrations (Fig. S2 A). However, that compound failed to extend the lifespan. The results described above indicated that TA improved the worms' resistance to juglone-induced oxidative stress and was more effective than the single amino acids Ala and Tyr and the mixture of Ala and Tyr *in vivo*.

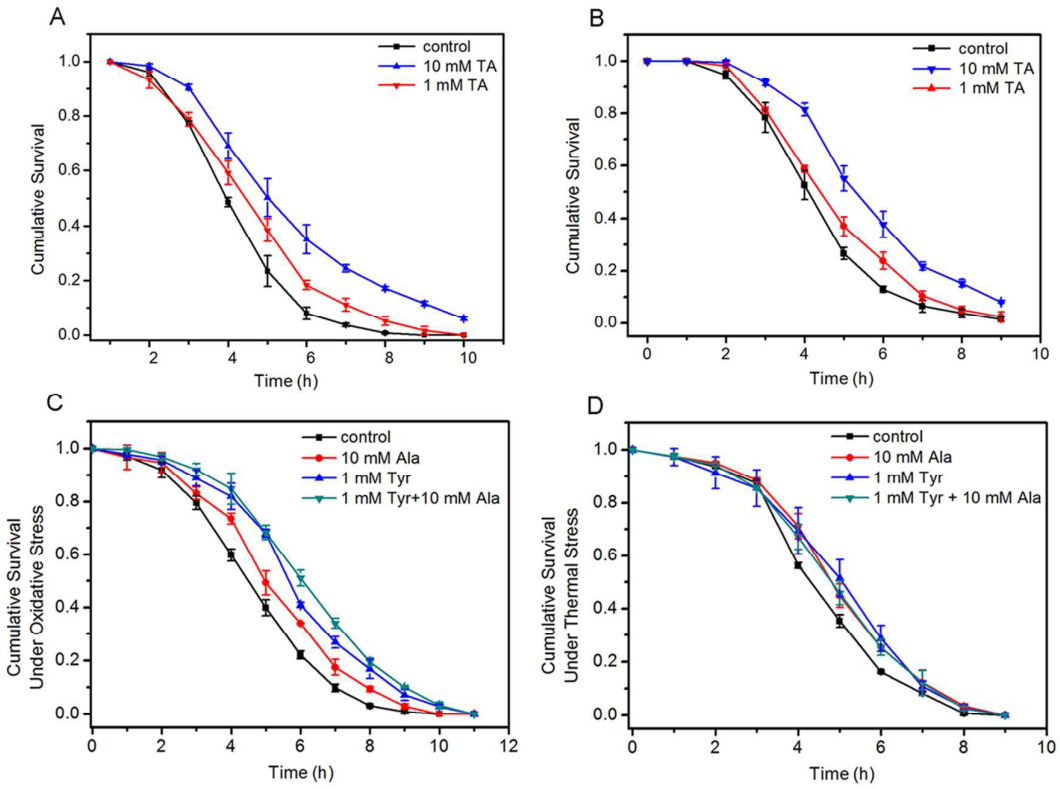


Fig. 3 TA (Tyr-Ala) improves the stress resistance of *C. elegans* under stress conditions. (A) Protective effects of TA (Tyr-Ala) on wild-type *C. elegans* N2 during oxidative stress. (B) Protective effects of TA (Tyr-Ala) on wild-type *C. elegans* N2 during heat stress. (C) The effects of 1 mM Tyr, 10 mM Ala and a mixture of 1 mM Tyr and 10 mM Ala on the lifespan of *C. elegans* during oxidative stress. (D) The effects of 1 mM Tyr, 10 mM Ala and a mixture of 1 mM Tyr and 10 mM Ala on the lifespan of *C. elegans* during heat stress. All survival curves are presented based on three individual experiments.

Next, we examined the effect of TA in a thermo-tolerance assay. Worms that had just reached adulthood were pre-treated with different concentrations of TA (1 mM and 10 mM) for 48 h before being exposed to heat shock at 35 °C. The data showed that TA treatment significantly increased the mean survival time of the worms by 35.49 % (Figure 3B). The results indicated that TA pre-treatment enhanced the worms' resistance to heat stress, thus generating an increased survival rate during heat shock. We also tested the effects of Ala and Tyr on the longevity of *C. elegans* during heat stress and found that the mean survival time was increased by pre-treatment with 1 mM Tyr, 10 mM Ala and 1 mM Tyr + 10 mM Ala (Figure 3D). However, the results showed that at the concentrations tested, the thermal resistance effects of the single amino acids could not match that of the dipeptide. A disodium salt of Tyr, which was formed by dissolving Tyr in a NaOH solution, was also used at a higher concentration during heat stress (Fig. S2 B). However, that compound failed to significantly extend the lifespan.

We conclude that in comparison to its constituent amino acids, TA is more efficient in enhancing the stress resistance of *C. elegans*. In addition, TA works more effectively during oxidative stress, suggesting that TA might exert this effect by scavenging free radicals.

3.4. TA decreases the intracellular ROS level in *C. elegans*

To explore the mechanisms by which TA prolonged the lifespan and enhanced the stress resistance of *C. elegans* during environmental stress, the free-radical scavenging abilities of TA were evaluated in subsequent experiments. First, the total antioxidant ability of TA was investigated *in vitro*. ABTS is frequently used to measure antioxidant capacities. TA effectively scavenged the ABTS radical cation ($ABTS^{+}$), and the IC50 (the concentration of antioxidant at which 50 % of the reaction was inhibited) of TA in this reaction system was 3.5 mM (Figure 4A). The results indicated that TA had a strong total antioxidant capacity *in vitro*.

Next, the ROS-scavenging ability of TA in *C. elegans* was also demonstrated. As shown in Figure 4B, at a concentration of 10 mM, TA significantly inhibited the production of ROS *in vivo* (compared with the control, $p < 0.01$) under regular culture conditions. Furthermore, TA (10 mM) pre-treatment

effectively reduced ROS accumulation in juglone (300 μM)-treated wild-type *C. elegans* (compared with the control, $p < 0.01$) (Figure 4B). These results showed that TA was a versatile free radical scavenger both *in vitro* and *in vivo*.

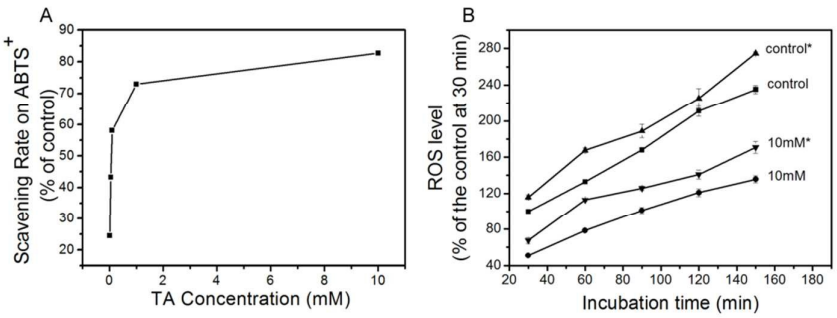


Fig. 4 Free radical-scavenging effect of TA (Tyr-Ala) *in vitro* and *in vivo*. (A) TA (Tyr-Ala) effectively scavenged $ABTS^+$ (IC50 = 3.5 mM). (B) TA (Tyr-Ala) at a concentration of 10 mM decreased ROS accumulation in *C. elegans* under regular culture conditions and in response to juglone-induced oxidative stress, assayed in a 150-min course. 10 mM* and control* were pre-treated with juglone.

3.5. TA up-regulates SOD-3::GFP expression in transgenic *C. elegans* CF1553

To further study the mechanisms that underlie the protective effects of TA in *C. elegans*, SOD-3::GFP reporter gene expression was investigated in transgenic CF1553 animals treated with or without TA. When compared with the control worms, the TA-treated worms demonstrated higher SOD-3::GFP intensity, as shown by confocal laser scanning microscopy (Figure 5A and B).

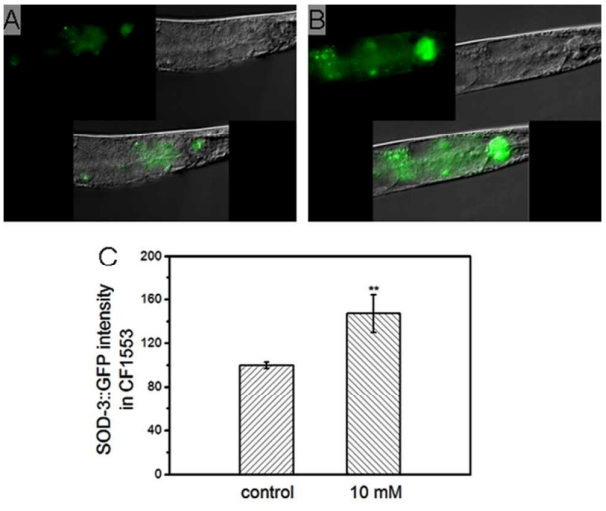


Fig. 5 TA (Tyr-Ala) up-regulates SOD-3GFP expression in transgenic *C. elegans* CF1553. (A) Image of SOD-3GFP expression in control worms. (B) Image of SOD-3GFP expression in 10 mM TA-treated worms. The SOD-3GFP expression in TA-treated worms (B) is higher than that observed in control worms (A). (C) Quantified GFP intensity (±SE) in CF1553 worms from four individual experiments, with 40 worms in each experiment (**p<0.01).

The intensity of the fluorescence was then quantified using a Thermo Labsystems Fluoroskan Ascent microplate reader. The results showed that TA at a concentration of 10 mM significantly up-regulated SOD-3::GFP expression by 45.2 % in transgenic CF1553 worms (Figure 5C), indicating that TA could increase the expression of SOD-3 in *C. elegans* during oxidative stress.

3.6. TA up-regulates the expression of the heat shock protein HSP-16.2 in transgenic *C. elegans* CL2070

HSP-16.2 can serve as a stress-sensitive reporter for predicting longevity in *C. elegans*. Higher levels of HSP-16.2::GFP predict a longer remaining life in *C. elegans*.[27] The effect of TA on the expression of HSP-16.2 was investigated to provide further details concerning the protective effects of TA on *C. elegans* during environmental stress. CL2070 worms containing the HSP-16.2::GFP reporter gene were placed in a 35 °C heat shock chamber for 1 h and allowed to recover at 20 °C for 12 h. When compared with the control group, the 10 mM TA-treated group showed higher HSP-16.2::GFP intensity, as shown by confocal laser scanning microscopy (Figure 6A and B). The intensity of the fluorescence was then quantified, and the data showed that TA significantly up-regulated HSP-16.2::GFP expression by 57.2 % in CL2070 (Figure 6C, $p < 0.01$ compared with the control), indicating that TA might increase the life expectancy of *C. elegans* during thermal stress by up-regulating the expression of HSP-16.2.

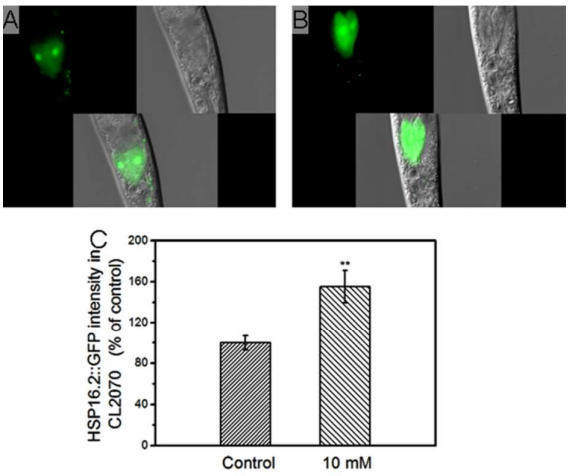


Fig. 6 Effects of TA (Tyr-Ala) on the expression of heat shock protein HSP-16.2 in CL2070. (A) Image of HSP-16.2::GFP expression in control worms. (B) Image of HSP- 16.2::GFP expression in the 10 mM TA-treated group. The HSP-16.2::GFP expression in TA-treated worms is higher than that observed in control worms. (C) The quantification of HSP-16.2::GFP intensity (±SE) in CL2070 was performed using a Thermo Labsystems Fluoroskan Ascent microplate reader (**p < 0.01), based on three individual experiments with 30 worms in each treatment.

3.7. TA regulates the mRNA expression of aging-associated genes in wild-type *C. elegans* N2

A variety of genes are involved in regulating the life expectancy of *C. elegans*. Among those genes, the DAF-16/forkhead transcription factor, which is the downstream target of insulin-like signaling in *C. elegans*, is indispensable for both lifespan regulation and stress resistance.[37] DAF-2 is an insulin/IGF-1 receptor that signals through a conservative PI3-kinase/AKT pathway, ultimately phosphorylates daf-16 and inhibits the entry of daf-16 into the nucleus.[38, 39] The downstream effectors of DAF-16 (SOD-3 and HSP-16.2) also act as important regulators of lifespan and stress resistance in *C. elegans*.[40, 41] SKN-1 is another transcription factor that can positively regulate lifespan and stress resistance in *C. elegans*.[6, 40 ,41] Quantitative real-time PCR experiments were therefore performed to investigate whether TA could regulate the expression of the aging-associated genes *daf-2*, *daf-16*, *sod-3*, *skn-1* and *hsp-16.2*. The results showed that TA significantly down-regulated the expression of *daf-2* ($p < 0.01$) and up-regulated the expression of *daf-16* ($p < 0.01$), *sod-3* ($p < 0.01$), *hsp-16.2* ($p < 0.01$) and *skn-1* ($p < 0.05$) (Figure 7). The up-regulation of *sod-3* expression by TA is consistent with the observed effect of TA on SOD-3::GFP expression. These results suggested that TA might improve the stress resistance of *C. elegans* and extend the life expectancy of the worms by regulating the above-mentioned aging-related genes.

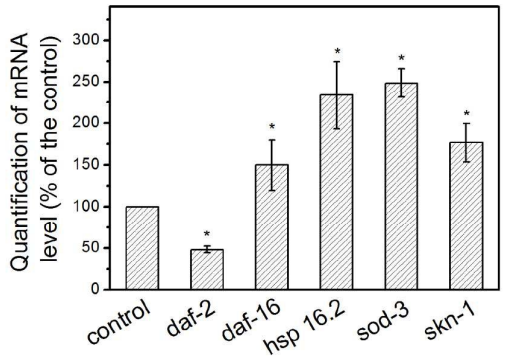


Fig. 7 Effects of TA (Tyr-Ala) on the expression of aging-associated genes in wild-type *C. elegans* N2. The worms were treated with or without 10 mM TA, and quantitative real-time PCR was performed to investigate the expression of the aging-associated genes *daf-2*, *daf-16*, *hsp-16.2*, *sod-3*, and *skn-1* in *C. elegans*. The error bars indicate the SE; *p < 0.05.

4. Discussion

Certain bioactive peptides derived from hydrolyzed food proteins have been shown to exhibit noteworthy anti-oxidative activities.[42] In this study, we have shown for the first time that TA (Tyr-Ala), which is a bioactive dipeptide isolated from hydrolyzed maize protein, significantly prolongs the life of *C. elegans*. The beneficial effects of TA on the healthspan of the animals were also detected experimentally. In the present study, the enterogenous autofluorescence levels in wild-type *C. elegans* decreased significantly after exposure to TA and showed a dose-dependent effect. Moreover, the fat level was decreased by TA, indicating that the lipid oxidation products that are detected as biomarkers of endogenous lipid peroxidation/oxidative stress may be reduced. The locomotory capacity, which represents the quality of life, was also enhanced by TA. These results indicated that TA could be a novel accessible and affordable candidate for use in anti-aging research.

Further study indicated that TA extended the survival of *C. elegans* during oxidative stress or heat stress. In fact, TA extends the lifespan of nematodes more effectively during stress than in normal conditions. We also treated wild-type *C. elegans* with heat-killed *E. coli* OP50 bacteria and 10 mM TA, and the stress resistance effect in that group was not significantly different from that of the group treated with live *E. coli* OP50 and 10 mM TA (Fig. S1). This result indicated that TA did not require bacterial metabolism for its activity. An interesting phenomenon was found in Figure 3 and Figure S2; at a concentration of 1 mM, Tyr had little ability to prolong the lifespan of *C. elegans* during oxidative stress and heat stress.

However, a substituted Tyr agent at a concentration of 10 mM demonstrated that Tyr had no ability to improve stress resistance at a higher concentration (Figure S2). In contrast, TA (Tyr-Ala) is easily soluble in water and possesses the functional groups of both Tyr and Ala. The resistance of the worms is significantly enhanced by 10 mM TA, indicating that TA as a biopeptide was more effective than the single amino acids Ala and Tyr and the mixture *in vivo*.

The anti-oxidative property of TA is probably related to its structure. An antioxidant will have a better ability to scavenge free radicals when it easily and stably forms a reaction intermediate with free radicals.[43] The catabolism and development of tyrosine is influenced by orthologous genes that are responsible for the metabolism of tyrosine in *C. elegans*, which may directly affect the activity of stress and longevity pathways, including daf-2/insulin signaling and daf-16/FOXO.[44] Our findings show that the supplementation of tyrosine in the culture medium did not extend the lifespan, which means that TA works as an active unit in prolonging the lifespan of *C. elegans*. Tyr contains a phenolic hydroxyl group, which plays a role as a proton donor that can quench free radicals. Meanwhile, the carboxyl group of Ala, which is in close proximity to Tyr in the dipeptide, provides an electron withdrawing effect, which diminishes the electron density around the oxygen atom in the Tyr phenolic hydroxyl and is more conducive to proton release, thus enhancing the effect of Tyr as a proton donor and its interaction with free radicals. The phenyl ring in the Tyr of TA is a conjugated system, which gives hydrogen to free radicals and turns into a stable intermediate. Therefore, the antioxidant activity of TA depends on the hydrogen donor capacity and the stability of the formed structure. By capturing radicals generated from the radical reaction chain, TA prevents or diminishes the reaction of the free radical chain. In addition, the hydrophobicity of a compound is important for its accessibility to hydrophobic targets. The N-terminal portion of TA is the hydrophobic amino acid Tyr, which could enhance its interaction with fatty acids and improve its ability to capture lipid free radicals. In addition, TA has good solubility in water, which means that this biopeptide can be utilized by organisms at higher doses. Moreover, as a dipeptide, TA can be absorbed faster than single amino acids.[23]

Excessive ROS may be generated when organisms are exposed to environmental stimuli, such as PM (particulate matter) 2.5. Excessive ROS could disturb homeostasis and lead to intracellular oxidative stress, thus accelerating cell apoptosis.[45] Our studies showed that TA effectively scavenged $ABTS^{+}$ and significantly down-regulated ROS levels *in vivo* under normal conditions. The ROS level in *C. elegans* is increased under oxidative stress induced by juglone, but TA could also significantly decrease the ROS level during oxidative stress. The ROS resistance of long-lived *C. elegans* mutants is due to high activities of enzymes that detoxify ROS and/or low ROS production levels; these discoveries are taken to confirm the free radical theory of aging.[44, 46] The ROS scavenging ability of TA could also contribute to its ability to prolong the lifespan. Furthermore, TA can not only scavenge free radicals *in vivo* but also significantly regulate the expression of a series of proteins that can protect the organism from stress, such as SOD, HSP, etc. SOD-3 and HSP-16.2 are the downstream effectors of DAF-16 and can serve as stress-sensitive reporters for predicting longevity in *C. elegans*. Both of these stress-sensitive reporters were up-regulated by TA in *C. elegans*. This finding may explain the antioxidative ability of TA and explain why TA could significantly extend the longevity of *C. elegans* during heat stress and oxidative stress. In addition, quantitative real-time PCR results suggested that TA significantly up-regulated the expression of aging-associated genes, such as skn-1. The longevity-improving ability of a bioactive compound is determined not only by its ability to directly scavenge free radicals but also by its ability to regulate anti-aging-associated genes, such as *daf-2*, *daf-16*, *sod-3*, *hsp-16.2*, and *skn-1*. Our results suggested that TA might prolong the life of *C. elegans* by scavenging ROS and by regulating these aging-associated genes.

In our study, we found that the longevity-improving and healthspan-extending effects of food-derived TA in the nematode *C. elegans* under normal conditions or during stress might be attributed to its direct ROS-scavenging activity and its indirect free radical-scavenging activity via the regulation of some anti-aging-associated genes, such as *daf-2*, *daf-16*, *sod-3*, *hsp-16.2*, and *skn-1,* in wild-type worms. In addition, we explored the differences in the longevity-improving effects of TA, Tyr, Ala and the mixture of Tyr and Ala by comparing the lifespan of transgenic *C. elegans* treated with these compounds under stress conditions.

Conclusions

We demonstrated for the first time *in vitro* and *in vivo* the anti-aging activity of the novel food-derived peptide TA, which was isolated from maize. TA reduced autofluorescence intensity in the gut, reduced fat levels and enhanced locomotivity in wild-type *C. elegans*, indicating that TA improves the healthspan. Nematodes pretreated with TA are more resistant to juglone-induced oxidative stress and heat stress, suggesting that cellular defense and immune system functions are also improved by TA. These interesting findings highlight the potential of TA to extend the average human life expectancy by providing protection against environmental stress.

Acknowledgements

This study was supported by grants from the National Natural Science Foundation of China (No. 81271697, 81571791, and 31571017), the Specialized Research Fund for the Doctoral Program of Higher Education (20100061120077), the Social Development Project of Science and Technology Department of Jilin Province and Changchun City, China (No. 20120967, YYZX2012, 20106031, and 09SF02), the China Postdoctoral

Science Foundation (No. 20100481048) and the "985 Project" of Jilin University, Key Laboratory of Mental Health, Institute of Psychology, Chinese Academy of Sciences.

The authors thank Dr. Y. Luo from the University of Maryland for providing research materials. Some nematode strains used in this work were provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources.

References

1 T. B. L. Kirkwood, Cell, 2005, 120, 437–447.
2 D. Harman, J. Gerontol., 1956, 11, 298–300.
3 F. L. Muller, M. S. Lustgarten, Y. Jang, A. Richardson and H. Van Remmen, Free Radic. Biol. Med., 2007, 43, 477–503.
4 B. D'Autréaux and M. B. Toledano, Nat. Rev. Mol. Cell Bio., 2007, 8, 813–824.
5 J. White, B. Dawson, G. Landers, K. Croft, P. Peeling, Eur. J. Appl. Physiol., 2013, 113,1059–1067.
6 J. H. An and T. K. Blackwell, Gene Dev., 2003, 17, 1882–1893.
7 H. Wu, Y. Zhao, Y. Guo, L. Xu and B. Zhao, Food Chem., 2012, 130, 254–260.
8 K. Cao, A. Zheng, J. Xu, H. Li, J. Liu, Y. Peng, J. Long, X. Zou, Y. Li, C. Chen, J. Liu and Z. Feng, Free Radic. Biol. Med., 2014, 75, 156–166.
9 B. H. Sarmadi and A. Ismail, Peptides, 2010, 31, 1949–1956.
10 P. J. Moughan, S. M. Rutherfurd, C. a Montoya and L. a Dave, Nutr. Res. Rev., 2013, 1–5.
11 E. Sienkiewicz-Szłapka, B. Jarmołowska, S. Krawczuk, E. Kostyra, H. Kostyra and M. Iwan, Int. Dairy J., 2009, 19, 258–263.
12 K. J. Cross, N. L. Huq, J. E. Palamara, J. W. Perich and E. C. Reynolds, J. Biol. Chem., 2005, 280, 15362–15369.
13 S. F. Gauthier, Y. Pouliot and D. Saint-Sauveur, Int. Dairy J., 2006, 16, 1315–1323.1
14 K. B. McCann, B. J. Shiell, W. P. Michalski, A. Lee, J. Wan, H. Roginski and M. J. Coventry, Int. Dairy J., 2006, 16, 316–323.
15 E. Mendis, N. Rajapakse, H. G. Byun and S. K. Kim, Life Sci., 2005, 77, 2166–2178.
16 M. Shimizu, N. Sawashita, F. Morimatsu, J. Ichikawa, Y. Taguchi, Y. Ijiri and J. Yamamoto, Thrombo. Res., 2009, 123, 753–7.
17 F. Zhong, J. Liu, J. Ma and C. F. Shoemaker, Food Res. Int., 2007, 40, 661–667.
18 H. Jia, Junqiang; Ma and R. Zhao, Weirui ; Wang, Zhenbin ; Tian, Wanmin ; Luo, Lin ; He, Food Chem., 2010, 119, 336–342.
19 W. Wang, H. Fang, L. Groom, A. Cheng, W. Zhang, J. Liu, X. Wang, K. Li, P. Han, M. Zheng, J. Yin, W. Wang, M. P. Mattson, J.
20 H.-M. Li, X. Hu, P. Guo, P. Fu, L. Xu and X.-Z. Zhang, J. Food Biochem., 2010, 34, 44–60.
21 C. Collin-Vidal, M. Cayol, C. Obled, F. Ziegler, G. Bommelaer, B. Beaufrere, Am. J .Physiol., 1994;267.
22 H. Hara, R. Funabiki, M. Iwata and K. Yamazaki, J. Nutr., 1984, 114, 1122–9.
23 L. Xu, X.L. Li, X.X. Wu, H. Wang, R.Z. Hou, Chem. J. Chinese U., 2004;25(3): 466-469.
24 T. E. Johnson, Exp. Gerontol., 2003, 38, 1329–1332.
25 M. R. Klass, Mech. Ageing Dev., 1977, 6, 413–429.
26 S. Brenner, Genetics, 1974, 77, 71–94.
27 G. J. Lithgow, T. M. White, S. Melov and T. E. Johnson, P. Natl. Acad. Sci. USA, 1995, 92, 7540–7544.
28 C. Coburn, E. Allman, P. Mahanti, A. Benedetto, F. Cabreiro, Z. Pincus, F. Matthijssens, C. Araiz, A. Mandel, M. Vlachos, S. A. Edwards, G. Fischer, A. Davidson, R. E. Pryor, A. Stevens, F. J. Slack, N. Tavernarakis, B. P. Braeckman, F. C. Schroeder, K. Nehrke and D. Gems, PLoS Biology, 2013, **11**, e1001613.
29 A. a Soukas, E. a Kane, C. E. Carr, J. a Melo and G. Ruvkun, Genes Dev., 2009, **23**, 496–511.
30 M. Hansen, A. L. Hsu, A. Dillin and C. Kenyon, PLoS Genetics, 2005, 1, 0119–0128.
31 A. Strayer, Z. Wu, Y. Christen, C. D. Link and Y. Luo, Faseb. J., 2003, 17, 2305–2307.
32 S. L. Rea, D. Wu, J. R. Cypser, J. W. Vaupel and T. E. Johnson, Nat. Genet., 2005, 37, 894–898.
33 D. Hoogewijs, K. Houthoofd, F. Matthijssens, J. Vandesompele and J. R. Vanfleteren, BMC molecular biology, 2008, **9**, 9.
34 K. J. Livak and T. D. Schmittgen, Methods, 2001, 25, 402–8.
35 C. Burmeister, K. Lüersen, A. Heinick, A. Hussein, M. Domagalski, R. D. Walter and E. Liebau, FASEB. J., 2008, 22, 343–354.
36 A. Kampkotter, C. Gombitang Nkwonkam, R. F. Zurawski, C. Timpel, Y. Chovolou, W. Watjen and R. Kahl, Arch Toxicol, 2007, 81, 849–858.
37 K. Lin, H. Hsin, N. Libina and C. Kenyon, Nat. Genet., 2001, 28, 139–145.
38 H. J. D. Dorman, S. G. Deans, R. C. Noble and P. Surai, J. Essent. Oil Res., 1995, 7, 645–651.
39 C. Kenyon, J. Chang, E. Gensch, a Rudner and R. Tabtiang, Nature, 1993, 366, 461–464.
40 B. P. Braeckman and J. R. Vanfleteren, Exp. Gerontol., 2007, 42, 90–8.
41 J. B. Dorman, B. Albinder, T. Shroyer and C. Kenyon, Genetics, 1995, 141, 1399–1406.
42 D. Garigan, A. L. Hsu, A. G. Fraser, R. S. Kamath, J. Abringet and C. Kenyon, Genetics, 2002, 161, 1101–1112.
43 H. M. Chen, K. Muramoto, F. Yamauchi and K. Nokihara, J. Agr. Food Chem., 1996, 44, 2619–2623.
44 A. a Ferguson, S. Roy, K. N. Kormanik, Y. Kim, K. J. Dumas, V. B. Ritov, D. Matern, P. J. Hu and A. L. Fisher, PLoS genetics, 2013, **9**, e1004020.
45 T. Finkel and N. J. Holbrook, Nature, 2000, 408, 239–247.
46 T. Heidler, K. Hartwig, H. Daniel and U. Wenzel, Biogerontology, 2010, 11, 183–195.