N. sphaeroides phycocyanin subunit Ns-α and Ns-β improve *C. elegans* antioxidative capacity via ROS-related regulation

Xiaoyu Wu[1,2,a], Caiyun Zhang[1,2,a], Shuwen Zhou[2], Chao Cheng [1,2] and Qing Fang[1,2,*]

[a]Hubei Key Laboratory of Biological Resources Protection and Utilization, Hubei Minzu University, Enshi, China

[b]College of Biological and Food Engineering, Hubei Minzu University, Enshi, China

[*]Address correspondence to fangqing2005@126.com

[a]These authors contributed equally to this work.

Abstract: Oxidative stress and damage to macromolecules due to free radicals are commonly considered factors that can impair health. Phycocyanin, a natural pigment-apoprotein complex composed of protein α- and β-subunits with attached linear tetrapyrrole chromophores, has health benefits such as reducing the impact of reactive oxygen species (ROS). However, the potential functions of the subunit proteins in vitro remain unexplored. In this study, bacterial expression vectors were separately constructed to induce two engineering subunit proteins, Ns-α and Ns-β, with genes derived from *Nostoc sphaeroides* (Gexianmi), a valuable resource with both medicinal and edible virtues. These engineering proteins were then examined for their potential to enhance antioxidative capacity in *C. elegans*. A proper concentration of the proteins Ns-α and Ns-β in vitro exhibited 2, 2-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging activity. While there were no other observed effects on the nematodes, those treated with the proteins showed significant improvements in motility and reduced levels of lipofuscin compared to the control group. Furthermore, the treated nematodes demonstrated increased resistance to oxidation, as evidenced by the higher survivals under oxidative conditions induced by 5 mM H_2O_2. Notably, the treated nematodes exhibited decline in endogenous ROS levels, and the redox-related genes, such as *SOD-3* and *CAT-1*, were down-regulated following consumption of the engineering proteins. These findings suggest that engineering proteins Ns-α and Ns-β improve the antioxidative capacity of *C. elegans* by modulating ROS-related regulation, making them potential modulators in responding to oxidative stressors.

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1. Introduction

Individuals are exposed to various environments throughout their life, and environmental conditions generally modulate physiological and mental states and can even have substantial influence on health via multifaced channels (Finkel and Holbrook 2000; Monzani et al. 2019; Romero et al. 2015). Hence short- or long-term stresses are aroused by exposure to unfavorable environmental conditions. There are specific stressors in vivo that potentially cause significant harm to the body through the activity of oxidative molecules, particularly, reactive oxygen species (ROS) (Romero et al. 2015; Labunskyy and Gladyshev 2013). ROS are a group of chemically molecules with high reactivity derived from partial reduction of molecular oxygen in cells (Labunskyy and Gladyshev 2013). Because they are specific by-products of metabolism, the endogenous redox mechanism is somewhat dependent on the presence of ROS-related regulation. These ROS are produced by organelles and enzymes, with mitochondria acting as the primary source, contributing to over 90% of the ROS generation within cells (Labunskyy and Gladyshev 2013). Excessive levels of these molecules, including hydrogen peroxide (H_2O_2) and other oxygen-containing free radicals can be harmful to cellular health, resulting in oxidative stress and subsequent damage. For instance, ROS can trigger nucleic acid fragmentation, enzyme deactivation, polysaccharide depolymerization, lipid peroxidation, and other destructive biochemical processes that ultimately lead to cell aging and death (Labunskyy and Gladyshev 2013; Finkel and Holbrook 2000).

Persistent or long-term oxidative stresses are strongly associated with the initiation and progression of aging and various diseases, such as Parkinson's disease (PD), Alzheimer’s disease (AD), and malignant tumors, which are becoming increasingly significant health

concerns (Hemmati-Dinarvand et al. 2019; Monzani et al. 2019; Trist et al. 2019; Aramouni et al. 2023). Aging is associated with an increased production of ROS and decrease of antioxidants in cells (Power et al. 2013; Trist et al. 2019). Under unfavorable conditions and without effective treatment, prevention of such diseases is hard to achieve during aging. PD is a neurodegenerative disease characterized with the loss of dopamine-producing neurons in the mid-brain that causes incapacitating symptoms including bradykinesia and muscular rigidity (Khan and Ali 2018). Oxidative stress is found to be one critical factor responsible for the initiation and progression of PD, and patients with PD have high oxidative stress and lower antioxidant activity as evidenced by the biomarker activities of superoxide dismutase (SOD) and catalase (CTL) enzymes (Khan and Ali 2018). In AD patients, biomarkers of oxidative stress have also been documented, including markers of protein, lipid, DNA and RNA oxidation (Butterfield et al. 2007). Oxidative stress occurs early in the course of AD, which supports its significant role in AD pathogenesis (Cheignon et al. 2018). Therefore, inhibition of excessive oxidation during aging for individuals is required via effective channels or treatment and is critical to prevent diseases in the aging population.

The effects of antioxidants in health promotion and disease prevention have been widely recognized, and findings suggest that antioxidant supplementation can slow the aging process and alleviate diseases linked to oxidative stress (Wang et al. 2017). Therefore, the integration of convenient and effective antioxidants into healthcare practices is crucial. Extensive research on natural antioxidant compounds, including polysaccharides and peptides, has been conducted in order to understand their mechanisms of action at the cellular and animal levels, as well as to enhance efficient extraction and large-scale production methods (Wang et al.

2017; Wu et al. 2015). These efforts are aimed at facilitating the incorporation of antioxidant products into pharmaceuticals, healthcare products, and diverse industrial applications. Of particular interest is the pigment-protein complex phycocyanin (PC) (Bannu et al. 2019; Liu et al. 2022).

The cyanobacteria PC belongs to a class of light-harvesting proteins, referred as phycobiliproteins, which are multi-chain holo-proteins composed of apo-proteins (protein subunits) with covalently-bound phycobilins, open chain tetrapyrrole chromophores (Eriksen 2008; Guo et al. 2022; Liu et al. 2022). Previous evidence has indicated that PC from cyanobacteria has various pharmacological activities, such as antioxidant properties, decreased inflammation, and boosting immunity (Bannu et al. 2019; Liu et al. 2022). A kind of cyanobacteria source with medicinal and edible properties, *Nostoc sphaeroides* (Gexianmi), is rich in amino acids (aa), vitamins, polysaccharides, and other antioxidant compounds such as PC (Zhu et al. 2023; Xu et al. 2018). Along with changes in climate and environmental conditions, this natural resource is under threat and becoming rare (Chen et al. 2021). In addition, the isolation of active compounds including PC from *N. sphaeroides* generally involves complex procedures and is expensive. Hence, modern biotechnology must be leveraged for the comprehensive exploration and utilization of valuable resources.

In the context of the development of bioscience and biotechnology, there is great interest in antioxidant-rich proteins derived from specific edible species. However, to date, relevant research on *N. sphaeroides* is limited. In this study, two subunit protein genes, *Ns-α* and *Ns-β*, were respectively expressed in engineered *Escherichia coli*, and the derived proteins were subjected to the model organism *C. elegans*. Treated nematodes by the two engineering

proteins both had significantly enhanced anti-oxidative capacity, and the functions were tightly linked to endogenous ROS modulation. The results introduce a novel basis for the efficient utilization of PC subunit protein resources and may potentially promote individuals' health.

2. Materials and methods

2.1. C. elegans, bacterial strain, and other reagents

Wild type *C. elegans* Bristol N2 and *E. coli* var strain, OP50, which initially originated from the Caenorhabditis Genetics Center (CGC), were provided by Professor Zhao's laboratory, in the Key Laboratory of High Magnetic Field and Ion Beam Physical Biology, Chinese Academy of Sciences (Hefei, China). Fresh *N. sphaeroides* was initially collected from Zouma Town (29°49'N, 110°25'E) in the southwest part of Hubei province, Enshi City. The empty expression vector pCold I (simply referred as pC) was purchased from TaKaRa-Bio (Dalian, China). *E.coli* expression strain BL21 (DE3) harboring the constructed vectors pC-*Ns*-*α* (BL21-Nc-*α*) and pC-*Ns*-*β* (BL21-Nc-*β*) were used in this study, while the strain harboring the empty vector pC (BL21-C) was used as control bacteria in the treatments. The reagents (agar, peptone, and yeast extract) were purchased from Yuanye Biotechnology Company (Shanghai, China); the 5-Fluoro-2-deoxyuridine (FUDR) and 2,7-dichlorofluorescein diacetate (H_2DCF-DA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). All reagents were of analytical grade.

2.2. Vector construction

The full-length ORF sequences from the two genes, *Ns-α* and *Ns-β*, were cloned from *N. sphaeroides* (maintained in our laboratory) by primer-specific PCR using total DNA as the template. Two restriction sites (NdeI and EcoR1) were designed for the recombinant plasmid pC-*Ns-α* and pC-*Ns-β* construction, leading to the gene being under the control of *cspA* promoter (*PcspA*). The PCR system was as follows: 1 μL of template DNA, 5 μL of GoTaq G2 Green Master Mix, and 1 μL of the primer mixture, supplemented with ddH_2O to a total volume of 10 μL. The reaction procedure consisted of an initial denaturation at 95 ℃ for 5 min, followed by denaturation at 94 ℃ for 30 s, annealing at 50 ℃ for 30 s, extension at 72℃ for 40 s, final extension at 72 ℃ for 5 min, and a final hold at 16 ℃ for 3 min. Thirty cycles were performed for the PCR. The primers used for the Ns-α and Ns-β genes were: Ns-α-F, GGCATATGACAAAAACACCTTTAACG, Ns-α-R, GAATTCACTCTAGCTTAGAGTAT TGA; Ns-β-F, GGCATATGGTTTTAGATGCATTTG, Ns-β-R, GAATTCTTAAGCTACTGC TGAAGCA.

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2.3. Ns-α and Ns-β protein expression

Two gene-expression vectors, pC-*Ns-α* and pC-*Ns-β,* were both transformed into *E. coli* BL21 (DE3) cells and screened on the Luria-Bertani (LB)-Ampicillin (Amp, 100 μg/mL) solid medium. A single colony was inoculated into LB-Amp liquid medium and grown with shaking at 37 °C for up to 14~16 h. The culture was then inoculated at a 1:50 dilution into fresh LB-Amp medium and grown for about 4 h at 37 °C to reach an optical cell density of approximately 0.5~0.6 at A600. For the target engineering protein induction, after 1.0 mM (final concentration) inducer isopropylthio-β-D-galactoside (IPTG) was added, the cultures

were induced at 16 °C for up to 4 h. Cells were collected by centrifugation at 5,000 rpm for 10 min and washed twice with washed buffer (20 mM NaCl, 50 mM Tris-HCl, pH 7.3). The bacteria that were resuspended in 5 mL of the homogenization buffer (3-4 mL/g wet cells) were homogenized by by ultra-sonication. After centrifugation (12,000 rpm, 15 min), supernatant was used as a crude extract for further isolation by His-tag affinity resin (Takara-Bio, Kyoto, Japan). The details of the method for this procedure were based on the manufacturer's instructions (Spriestersbach et al. 2015). Samples were subjected to SDS-PAGE and demineralization. Before further assays, the concentrations of the proteins were assessed using the Bradford method.

Commenté [H3]: Please remove by (repetition)

Commenté [H4]: Reference?

2.4. In vitro activity evaluation by ABTS assay

2, 2-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay was carried out referring to previous work from Wolosiak *et al*. (Wolosiak et al. 2021) with a little modulation. We mixed 7 mM ABTS and 2.45 mM $K_2S_2O_8$ with equal volumes to generate radical ABTS•+. In dark incubation at least for 12 h, the solution was further diluted with buffers of pH 3.6 and 7.4 to at Abs734. The protein solutions (40 µL) and radical solution (4,000 µL) were mixed together for 6 min, after which the Abs734 was again tested. BSA was used as a control and three replications were performed for each sample. Assays for each sample were carried out in triplicate to determine the mean level.

Commenté [H5]: Could the authors clarify the rationale for using BSA as a standard in the ABTS assay? BSA may not be the most appropriate reference.

2.5. C. elegans culture and treatment

C. elegans N2 strain were maintained at 20 ℃ on nematode growth medium (NGM) agar

177 plates seeded with *E. coli* var strain OP50, as previously described (Buchter et al. 2020). To
178 obtain synchronous nematodes, the gravid adults were treated using a bleaching solution
179 (NaOH (5M), NaClO (13%); 1:1) followed by three washing steps in liquid NGM. The
180 remaining eggs were allowed to hatch either on fresh NGM agar plates seeded with OP50 for
181 three days (synchronous L4 larvae) or in 1.5 ml S-medium for 12 h (synchronous L1 larvae).
182 For the following treatments, the larvae were fed on the suspensions containing heat
183 inactivated bacterial mixture, such as the OP50/BL21-Ns-α or OP50/BL21-Ns-β or
184 OP50/BL21-C, and each suspension supplemented FUDR (50 μM) to prevent nematode
185 spawning. In the suspensions, OP50 vs BL21- cells were controlled at ratios of 1:1 or 1:3, but
186 the whole concentration of bacteria was referred to 1.0×10^9 cfu/mL. Following a three-day
187 treatment period, the nematodes matured into adults for subsequent assays.
188
189 *2.6. Testing the body size of nematodes*
190 To address whether the growth is affected by the two-protein treatment, the body size and
191 stage-specific morphological characteristics were also tested. At depicted time points the
192 nematodes in each group were transferred in 10 μL medium to a microscope slide and mixed
193 with 10 μL of levamisole (20 mM). Images of individual nematodes were taken and analyzed
194 to determine the length of each nematode using ImageJ (NIH, Bethesda, MD, USA).
195
196 *2.7. H_2O_2 tolerance assay*
197 For the H_2O_2 tolerance assay, the treated nematodes in each group ($n\geq20$) were respectively
198 collected using M9 buffer and subjected to subsequent assays. Subsequently, the nematodes

were exposed to 5 mM H_2O_2 in a 24-well plate, and their survival was scored at 20 min intervals under a dissecting microscopy. When treated nematodes did not respond to poking with a metal wire, they were judged to be dead. Consequently, survival was calculated as the surviving nematode ratio in the population. Each treatment was carried out in triplicate.

2.8. Endogenous ROS level

A relative fluorescence quantification method was referred to test the level of endogenous ROS of nematodes (Buchter et al. 2020). The nematodes from different groups were fed on each bacterial lawn for 24 h and collected with M9 buffer. Bacteria were removed by washing three times, and nematodes were resuspended in M9 buffer. Each 50 µL aliquot of the suspension was mixed with H_2DCF-DA (50 µL) in a 48-well plate, leading to the ROS-specific fluorescent probe H_2DCF-DA (2', 7'-dichlorodihydrofluorescein diacetate) a final concentration of 0.1 mM. Two control wells, such as that containing nematodes from each bacterial mixture lawn without H_2DCF-DA or containing H_2DCF-DA without nematodes were designed. Incubation was carried out at at 25 °C for 1 h, and the nematodes were then transferred to slides, mounted with glycerol, and examined using a fluorescence microscope. ROS signal was subsequently imaged and quantified under a fluorescent microscope. The fluorescence was determined by subtracting the initial value from the final value for each well. Three independent assays were performed and for each replicate, ≥15 nematodes were assessed for the analysis.

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2.9. Motility

To test the motility, L4 stage *C. elegans* for each treatment were cultured in S liquid medium supplemented by different bacterial suspension mixture at 20 °C shock conditions for 3 days. Subsequently, the nematodes were collected in a dish with M9 solution, and their body bends were counted every 10 seconds for three consecutive observations under light microscopy. Each group nematode analysis was replicated at least three times (n≥20).

2.10. Lipofuscin accumulation

Treated and control group L4 stage nematodes were collected, washed with M9 buffer, anesthetized with 10 mM levamisole hydrochloride, and placed on a microscope slide. Spontaneous fluorescence was determined using fluorescence microscope (Eclipse Ni, Nikon; Tokyo, Japan; ImageJ, NIH; Bethesda, USA). The treatment experiment for each sample type was repeated three times (n ≥10).

2.11. Total RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

The nematodes fed different bacterial mixtures (the control group were the nematodes treated with OP50:BL-C, while the treatment groups were the nematodes treated with OP50:BL-Ns-α or Ns-β, and the ratio of the bacteria was all set as 1:1). Those sustained in the L3 stage were washed and collected in M9 buffer, and then the liquid nitrogen-treated nematodes were ground into powder for further total RNA isolation using Trizol reagent (Invitrogen, Waltham, MA, USA). Agarose gel electrophoresis was quickly performed to evaluate the total RNA quality. For each sample, after elimination of potential genomic DNA using gDNA eraser, 1.0 μg total RNA were subjected to RT-PCR using the First Strand cDNA Synthesis kit (TakaRa,

PrimeScript™ FAST RT reagent Kit with gDNA Eraser, RR092A) according to the manufacturer's instructions. For the first strand cDNA synthesis, a system of 20 μL containing each essential reagent such as Random 6-mers, dNTP Mixture, and RNA was prepared on ice. The cDNA was stored at -20 ℃ or evaluated subsequently by analyzed *Actin* expression using agarose gel electrophoresis. The qRT-PCR using the TB Green kit (TaKaRa, TB Green® Premix Ex Taq™ II (Tli RNaseH Plus), RR420L) and StepOne Plus real-time PCR system (Applied Biosystems) were then performed in our lab. Reactions were initiated at 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s, 58 °C for 15 s, and 72 °C for 30 s to complete target fragments smaller than 350 bp in length, followed by melt curve analysis. At least three repeats were performed for each sample and each gene to characterize the mean level in the assay. Relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. The control gene *Actin* was used as an internal reference to normalize gene expression data. The gene-specific primers were checked to test availability using BLAST and the detail sequences are shown in Table S2.

2.12. Nucleotide sequence

The referred genome was based on the sequence of *N. sphaeroides* ZMS-1 submitted to GenBank and can be accessed using the species name and accession number NZ_CP031941. The genes of *C. elegans* described in this study can be retrieved online from NCBI (https://www.ncbi.nlm.nih.gov/) or https://wormbase.org/.

2.13. Data statistics and analysis

Statistical analyses were based on the data given as mean ± SD using PASW Statistics (SPSSInc., Chicago, IL, USA). Statistical significance was determined by one-way ANOVA. Differences were considered to be significant at a level of $P < 0.05$ ("*") or $P < 0.01$("**").

3. Results and analysis

3.1. Engineering expression of Ns-α and Ns-β protein

Phycocyanin, a crucial component of the phycobilisome, consists of two primary subunit proteins, α and β, to which phycocyanobilins (PCB) are attached (Minato et al. 2021; Guo et al. 2022; Eisenberg et al. 2017). Practical applications of these compounds from various species have sparked significant interest. Notably, *N. sphaeroides* is commonly used as a food and in traditional Chinese medicine, but the functions of subunit proteins Ns-α and Ns-β remain unknown (Chen et al. 2012; Chen et al. 2020b). In the context of the reported genome, the coding sequences (CDS) of the *Ns-α* and *Ns-β* genes were identified, with lengths of 492 and 522 bp and resulting in proteins containing 163 and 173 aa residues, respectively (Table S1 and Figure S1). The cysteine residues in these proteins, particularly 85Cys in Ns-α and 85Cys and 154Cys in Ns-β, are probably crucial for binding PCB (Figure S1), and might be conferred some potential. Despite low aa sequence identities, the two subunit proteins, Ns-α and Ns-β, are each comprised of seven typical α helices and exhibit structural similarities (Figure 1A and Table S1).

To assess their activity, the recombinant expression vectors of *Ns-α* and *Ns-β* were constructed in the context of pC (Figure S2A and B). Sequenced plasmids pC-*Ns-α* and

Commenté [H7]: The author has already provided this information before, so it is unnecessary to repeat it here. I recommend removing the repetition to maintain the flow of the text.

287 pC-*Ns-β* were respectively transformed into *E. coli* BL21 (DE3) cells (the engineered strains
288 were named BL21-α and BL21-β, and the control stain harboring the empty vector pC was
289 called BL21-C). The target strains were treated with 1.0 mM IPTG inducer, and the
290 engineering proteins were isolated using a metal Ni affinity method targeting the 6xHis-tag in
291 the subunits (Figure 1B). Subsequently, the two engineering proteins were subjected to
292 oxidation assay. In vitro experiments demonstrated that, at concentrations of 0.60 mg/mL for
293 Ns-α and Ns-β, the scavenging ratio of ABTS▪— reached approximately 4.6% and 6.0%,
294 respectively. After increasing the engineering protein concentrations to 1.2 mg/mL, higher
295 inhibition levels occurred, indicating a dose-dependent manner in the testing assays (Figure
296 1C). Consequently, the engineering proteins Ns-α and Ns-β exhibited anti-oxidative
297 properties.

Commenté [H8]: Could the author clarify what is meant by ABTS▪— ?

298

299 *3.2. Ns-α and Ns-β improved nematode movement*

300 Although phycocyanin has health benefits (Yu et al. 2017; Eriksen 2008), further evidence is
301 needed to determine the specific roles of the subunit proteins α and β. For this, *C. elegans* N2
302 was treated with bacterial mixture suspension of OP50 and engineered bacteria expressing
303 Ns-α and Ns-β (OP50/BL21-α, OP50/BL21-β), while control nematodes were treated with the
304 suspension including the same OP50 and bacteria harboring empty vector (OP50/BL21-C).
305 The ratio of OP50 vs BL21-α and BL21-β or BL21-C in the mixture suspensions were
306 controlled by testing the bacteria concentration. During the treatment periods, no matter
307 whether the ratio was 1:1 or 1:3, there were no significant differences in the body length of
308 the synchronized nematodes compared to those in the control groups (Figure S3, $P > 0.05$),

indicating that other potential effects were present. Notably, the motility of nematodes treated with Ns-α and Ns-β was significantly improved. For example, when the ratio was 1:1, the mean number of body bends per unit time (10 seconds) increased by 12% and 22%, respectively, compared to the control group (Figure 2). After we increased the ratio of engineered bacteria in the suspensions, the motility of the nematode was slightly enhanced by 15% and 26% compared to the control, respectively. If the target bacteria were not induced by IPTG, the motilities of the nematodes from different groups displayed similarities (Figure S4), highlighting the role of engineering Ns-α and Ns-β.

Reproduction is a particular period in a lifespan, and a precious reflection of individual physiology. The treated groups produced much fewer nematode posterities than the control group in the same period, suggesting that the intake of the two engineering proteins delayed the reproductive period of the nematodes (Figure 3A). Under normal conditions, individual motility typically improves over time, while a decline in motility is often associated with aging. Lipofuscin, known as an aging pigment, can produce blue spontaneous fluorescence in related tissues (Di Guardo 2015; Hohn and Grune 2013). We found that the treated nematodes exhibited significantly lower levels of lipofuscin compared to the control group (Figure 3B and Figure S5). The physiological alterations in the nematodes supported the observed motility improvements during the testing periods. Therefore, it is reasonable to conclude that nematodes fed on the engineering proteins Ns-α and Ns-β were conferred enhanced motility.

3.3. Ns-α and Ns-β enhance nematode antioxidant capacity

Environmental stressors, such as oxidative stress, have significant effects on individuals and

impact the aging process and disease development (Finkel and Holbrook 2000; Venkataraman et al. 2013). Elevated oxidation is closely associated with aging and diseases. To directly evaluate the antioxidative property of Ns-α and Ns-β, nematodes were treated with bacterial mixture suspension (ratio of OP50/BL21-α or -β or -C was 1:1) for up to 3 days, and then collected and subjected to 5 mM H_2O_2 to simulate oxidative stress conditions. During the testing period, nematodes that fed on the two engineering proteins demonstrated more resistance to H_2O_2-induced oxidation compared to the control group. The survival rate of nematodes treated with Ns-α and Ns-β was approximately 2.0 and 4.0 times, respectively, of that of the control group at 240 minutes. Even after 320 minutes, a significant percentage of nematodes in the treated groups remained alive, while all nematodes in the control group had perished (Figure 4A).

ROS, which are natural byproducts of oxidative metabolism, can cause cellular damage, contributing to aging and senescence (Labunskyy and Gladyshev 2013). Nematodes that fed on Ns-α and Ns-β exhibited increased tolerance to H_2O_2 compared to the control group, suggesting a potential modulation of endogenous oxidation levels within the nematodes. To assess ROS levels in *C. elegans,* the fluorescent probe H_2DCF-DA was used. Subsequently, the treated nematodes showed significant reductions in ROS levels compared to the control group (Figure 4B and 4C), indicating that Ns-α and Ns-β facilitated the physiological modulation of ROS and enhanced the nematodes' tolerance to H_2O_2-induced oxidation.

3.4. Nematode oxidative stress-related genes expression

The consumption of Ns-α and Ns-β has been shown to enhance tolerance to oxidation in *C.*

353 *elegans* and their involvement in complex mechanisms. On the nutritional scale, the aa in the
354 Ns-α and Ns-β proteins should not be ignored as any protein has no nutritional value unless it
355 is hydrolyzed by proteases and peptidases to aa, dipeptides, or tripeptides in the lumen of the
356 small intestine (Wu 2016; Power et al. 2013). There were one or two Cys residues in the
357 subunit proteins, which may support their antioxidative role via potential polypeptides intake
358 or assimilation by the nematodes. The polypeptides interact with the ROS or other regulators
359 via multi-channels, e.g., by modulating gene expression, hence leading to the transforming of
360 nematodes (Figure 5).

361 To further investigate the antioxidative stress mechanism, total RNA was extracted from
362 nematodes and analyzed using qRT-PCR. Genes related to oxidative stress and longevity in
363 nematodes, such as *SOD-3* (Hermeling et al. 2022; Wong et al. 2023), *CAT-1* (Duerr et al.
364 1999), and *DAF-2* (Kimura et al. 1997), were examined. The results revealed down-regulation
365 of *SOD-3*, *CAT-1,* and *DAF-16* in treated nematodes compared to the control, while *SKN-1*, a
366 gene encoding a transcriptional regulator involved in oxidative stress and lifespan modulation
367 (Kimura et al. 1997; Roy et al. 2022; Deng et al. 2020), was clearly up-regulated in the treated
368 nematodes (Figure 6). The expression of other genes, for instance, *DAF-2,* was specifically
369 and largely changed in the Ns-α treatment group but remained relatively stable in the Ns-β
370 treatment group. *CTL-1* showed little alteration in both protein treatment groups compared
371 with the control group (Song et al. 2020; Song et al. 2014; Roy et al. 2022) (Figure S6). These
372 observed alterations in gene expression provide an initial molecular-level explanation for the
373 antioxidant properties associated with Ns-α and Ns-β.
374

4. Discussion

N. sphaeroides (Gexianmi) is well known for its medicinal and edible properties (Chen et al. 2012; Chen et al. 2020b). Extraction of natural and beneficial components usually involves intricate procedures and relatively high costs. Changes in climate and environmental conditions are also an increasing threat. Most crucial compounds from the species require further exploration by novel channels. In this study, gene-specific expression vectors were constructed to derive the *N. sphaeroides* phycocyanin engineering α- and β-subunit proteins in bacteria (Figure 1B). The engineering proteins Ns-α and Ns-β were fed on *C. elegans* via bacteria suspensions and demonstrated anti-oxidative stress properties, as the treated nematodes exhibited significantly improved motility and survival (Figure 2 & Figure 4). The recipient intake-assimilation-transformation of each exogenous nutrient is an essential cycle that involves a complex in vivo procedure. The alterations are closely linked to the nematode physiological status, highlighting the substantial benefits provided by these two engineering proteins. The clear decrease in lipofuscin and endogenous ROS levels in the treated nematodes (Figure 3B & Figure 4C) offers a plausible explanation for the observed differences in their responses.

Individuals encounter various and challenging environmental conditions through their life. When cells experience excessive oxidation or disruptions in redox regulation, aging can be accelerated and even lead to the development of diseases (Cheignon et al. 2018; Khan and Ali 2018; Labunskyy and Gladyshev 2013). Age-related chronic diseases associated with oxidative imbalances present significant challenges for individuals and can impose considerable burdens on both economic and social progress (Hemmati-Dinarvand et al. 2019;

Monzani et al. 2019). Therefore, it is crucial to maintain cellular oxidation equilibrium to delay aging and enhance overall well-being. In addition, physiological functions undergo various factors such as exercise capacity, metabolism, and levels of adipose tissue during aging (Hohn and Grune 2013; Monzani et al. 2019). Accumulating evidence has indicated that adequate and appropriate supplementation of beneficial proteins is generally essential for maintaining health (Finkel and Holbrook 2000; Liu et al. 2022; Song et al. 2014). The engineering protein Ns-α and Ns-β play a vital role in antioxidative stress, suggesting that the engineered components hold potential for practical applications.

In our assays, feeding on *C. elegans* with these engineering proteins via bacteria suspensions resulted in noticeable positive effects, such as increased motility and enhanced tolerance to oxidation compared to control nematodes (Figure 2 & Figure 4A). If the target protein were not supplemented via the previous bacteria strain without induction, the effects were not evident for the nematodes (Figure S3 & Figure S4), highlighting there were close association between the engineering proteins and alteration of the nematodes. The effects were significant for the nematodes. Most chances including the involved competition and defense from other stresses are conferred by motility (Roy et al. 2022). Motility is closely linked to aging, and the improved motility observed in the treated nematodes suggests profound physiological alteration. Lipofuscin, an age-related pigment and aging marker, can hinder proteasome function, contributing to the aging process (Di Guardo 2015). Interestingly, the levels of lipofuscin were clearly inhibited compared to the control group (Figure 3B & Figure S5), providing insights into the physiological status of the nematodes following treatment with the engineered proteins. Reproductive capacity means a fresh stage of life for

419 an individual. We also found that the reproductive posterities of treated nematodes by the two
420 proteins were reduced (Figure 3A), suggesting that the aging of the nematodes was actually
421 delayed. Proteins are the most fundamental component of tissues in animals. Dietary protein
422 has no nutritional value unless it is hydrolyzed by proteases and peptidases to aa, dipeptides,
423 or tripeptides in the lumen of the small intestine (Wu 2016). In this context, it is reasonable to
424 understand the enhancement of motility in the nematodes at same periods.

425 Environmental stressors can lead to the accumulation of endogenous free radicals and
426 excessive production of ROS, which can be significantly harmful to cellular macromolecules
427 (Hemmati-Dinarvand et al. 2019; Power et al. 2013). Antioxidant supplementation helps
428 protect health by inhibiting ROS damage. In vivo H_2O_2 can be generated as a byproduct of
429 oxidative protein folding in the endoplasmic reticulum, and homolytically cleaved in the
430 presence of redox-active Fe(II) iron (Fenton reaction) to form highly reactive hydroxyl
431 radicals (Labunskyy and Gladyshev 2013; Tu and Weissman 2002; Chen et al. 2020a).
432 Nematodes treated with the two subunit proteins exhibited increased tolerance to oxidative
433 environments (Figure 4A), such as exposure to 5 mM H_2O_2, simulating unfavorable
434 conditions. The results suggest a beneficial effect of nematode by consumption of the two
435 engineering proteins. The special Cys residues in Ns-α and -β protein might facilitate the
436 antioxidation role in vitro, because sulfhydryl (R-SH) in Cys takes on unique antioxidative
437 stress activity and generally interacts with the radical species by hydrogen donation from the
438 SH group (Power et al. 2013). However, nematodes' greater tolerance of H_2O_2 should not
439 only result from the special Cys residues. There should be multifaced regulation or alteration
440 in the treated nematodes, as in the hypothesized channel in Figure 5. Polypeptides from the

two engineering proteins' digestion or the proteins themselves are probably involved in ROS modulation. Changes in endogenous ROS levels in the treated nematodes further support physiological status compared to the control group (Figure 4).

Therefore, it can be concluded that the consumption of Ns-α and Ns-β aids in enhancing nematode tolerance to oxidative stress. Balance of ROS or redox in living organisms involves complex mechanisms. The expression of several genes associated with oxidation and aging in the treated nematodes, such as *SOD-3*, *CAT-1*, and *DAF-2*, showed differences compared to the control group (Figure 6). Relatively, superoxide radicals are usually unstable and could be dismutated to H_2O_2 and molecular oxygen in very short-term by superoxide dismutases (SODs) (Labunskyy and Gladyshev 2013). CAT-1 is the homolog of the mammalian vesicular monoamine transporter VMAT2 and plays a key role in dopamine release and packaging in *C. elegans* (Duerr et al. 1999). The down-regulation of *SOD-3* and *CAT-1* expression appeared to have fewer threats from ROS to treat *in vivo*, which was consistent with the reduced ROS levels in the treated nematodes. By contrast, the expressions of *SKN-1* were both largely up-regulated in the nematodes treated by the two engineering proteins. SKN-1, the C. elegans ortholog of mammalian Nrf, is a well-known transcription factor in longevity studies, and its activation is observed in several long-lived models. Evidence has indicated that *DAF-16* could be inhibited by the *SKN-1* in *C. elegans* stress responses (Deng et al. 2020). In this context, it is reasonable to understand that the two genes displayed a contrary expression pattern in the treatment groups, which implies the actual effect of both engineering proteins.

It was also notable that some genes displayed differentially in the treatments. *DAF-2* encodes the sole ortholog of the mammalian insulin and IGF-1 receptors (IIRc), a single

463 mutation in the gene can double the lifespan of *C. elegans* (Roy et al. 2022; Kimura et al.
464 1997). *DAF-2* transcripts were sharply down-regulated by Ns-α treatment while little was
465 affected by Ns-β treatment (Figure S6). Because the intake of the engineering protein Ns-α
466 and Ns-β leads to enhanced motility and higher tolerance to H_2O_2 in the nematodes, it may be
467 associated with DAF-2-involved regulation, because a previous study suggested that muscle
468 DAF-2/IIRc plays a major role in the loss of worm motility observed in *daf-2* mutants (Roy et
469 al. 2022). Although it was difficult to distinguish roles of Ns-α and Ns-β in vivo, for the Ns-α
470 and Ns-β treatment nematodes, the expressions of *CTL-1* were slightly inhibited in the treated
471 nematodes (Figure S6). The result appeared to be consistent with the evidence that *CTL-1*
472 encodes a catalase downstream of superoxide dismutase in the detoxification pathway of ROS
473 (Blaise et al. 2007). The comparable gene expression is largely due to the engineered proteins'
474 properties in the context of aa residues and specific advanced structure, which are believed to
475 play a crucial role in the reaction or alteration in nematodes. To further understand the roles
476 requires further exploration and assays, but our conclusion is that the consumption of Ns-α
477 and Ns-β facilitates oxidative stress tolerance in nematodes.
478
479 **5. Conclusion**
480 Oxidative stress and the aging process can have detrimental effects on individual health.
481 When these two factors are combined over a long period, significant effects such as
482 age-related diseases may arise. Therefore, preventing excessive oxidation and delaying the
483 aging process could be an effective approach to promoting healthy aging in a population. *N.*
484 *sphaeroides* is a valuable resource in both food and medicine. In this study, two engineered

proteins from *N. sphaeroides*, Ns-α and Ns-β, were expressed and demonstrated significant roles in antioxidation in vitro. This was evidenced by the fact that nematodes treated with these proteins showed enhanced tolerance to 5 mM H_2O_2, reduced levels of endogenous ROS and changed in expression of some ROS-related genes. The roles of these engineered proteins suggest that, if further developed, they could serve as potential regulators for responding to oxidative stressors and maintaining health.

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