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Cyanobacteria produce high levels of ergothioneine

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ABSTRACT

Ergothioneine (ET) is a unique natural antioxidant. We have examined the origin of ET in zebrafish. There was virtually no ET, measured by LC–MS, in most tank vegetation (plant, green and red alga). However, ET was detected in a *Phormidium* sample, a cyanobacterium. In commercial fish feed preparations, ET content increased with the content of cyanobacteria *Arthrospira platensis* or *Arthrospira maxima* (Spirulina). High levels of ET (up to 0.8 mg per g dry mass) were measured in cyanobacteria preparations sold as dietary supplements for humans and in fresh *Scytonema* and *Oscillatoria* cultures. Cyanobacteria contained as much ET as King Oyster mushrooms (*Pleurotus eryngii*). All samples with substantial ET content also contained the biosynthesis intermediate hercynine; this strongly suggests that cyanobacteria synthesise ET de novo. In conclusion, our data establish that cyanobacteria can produce high levels of ergothioneine. Spirulina is a novel, safe, accessible, and affordable source of ergothioneine for humans.

1. Introduction

Ergothioneine (ET) is a derivative of thiourea, a sulphur atom attached by a double bond to the imidazole ring of histidine betaine. As a consequence of the prevailing thione tautomer, ET is a very stable antioxidant with unique properties (Hartman, 1990). By contrast to GSH and ascorbate, ET may scavenge oxidising species that are not free radicals (Chaudiere & Ferrari-Iliou, 1999).

ET is a natural compound which humans and other vertebrates cannot synthesise themselves; it must be absorbed from food in which it is distributed very unevenly. The best known dietary source of ET are mushrooms (0.1–1 mg/g dried material). After ingestion, ET is rapidly cleared from the circulation and then avidly retained with minimal metabolism. The content of ET varies greatly among human tissues (Hartman, 1990). The ability to absorb, distribute, and retain ET depends entirely on a specific transporter (ETT; human gene symbol *SLC22A4*) (Bacher et al., 2009; Grigat et al., 2007) which in humans is strongly expressed in small intestine, kidney, erythrocyte progenitor cells in bone marrow, and monocytes. Cells lacking ETT do not accumulate ET, since the plasma membrane is virtually impermeable for this compound. ETT has high affinity for ET (K_m = 21 μmol/l) and catalyses cotransport of ET with Na^+ (Gründemann et al., 2005). The existence of a specific transporter suggests a beneficial role for ET. However, the precise physiological purpose of ET and the consequences of ET deficiency are still unclear.

Much interest in ETT has been generated by case-control studies that suggest an association of a polymorphism in the *SLC22A4* gene with susceptibility to chronic inflammatory diseases, such as Crohn's disease (Babusukumar, Wang, McGuire, Broeckel, & Kugathasan, 2006; Fisher et al., 2006; Leung et al., 2006; Martinez et al., 2006; Peltekova et al., 2004; Törkvist et al., 2007), ulcerative colitis (Waller et al., 2006) and Type I diabetes (Santiago et al., 2006). It is presently unknown how the mutation in the transporter gene promotes disease.

To further investigate the function of ET, we have started to examine zebrafish. We have observed organ-specific accumulation of ET, and have cloned and functionally verified ETT from zebrafish (Bach & Gründemann, unpublished). Since only fungi and mycobacteria are known to biosynthesise ET (Melville, 1958), the natural origin of ET for the fish was unclear.

Thus, the aim of the present study was to search for a novel source of ET, accessible to fish.

2. Materials and methods

2.1. Analyte quantitation by LC–MS

Cultured cyanobacteria and algae were washed twice with water and then immediately freeze dried with an ALPHA 1-4 LSC machine (Martin Christ Gefriertrocknungsanlagen, Osterode, Germany) before lysis. Mushroom powder was lysed with 4 mM perchloric acid ($HClO_4$), since methanol (99.9%, Rotisolv®, HPLC

Abbreviations: ET, ergothioneine; ETT, ergothioneine transporter; LC, liquid chromatography; MS, mass spectrometry.

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gradient grade, Roth, Karlsruhe, Germany) lysis yielded less ET. All other materials shown in Figs. 1 and 2 were lysed with methanol (same ET yield as perchloric acid lysis), homogenised with a metal pestle in a centrifuge tube, and centrifuged (30 min, 16,000*g*). Supernatants were filtered (Minisart RC 25, Sartorius Stedim Biotech, Göttingen, Germany).

Fishes ($n = 4$ for each group) were euthanised in ice water, weighed and then individually homogenised in 1 ml 4 mM perchloric acid with a Potter S homogeniser (Sartorius Stedim Biotech) on ice. One hundred microlitre of the homogenate was mixed with 400 µl methanol and centrifuged (30 min, 16,000*g*).

Twenty microlitre samples were analysed by LC–MS/MS on a triple quadrupole mass spectrometer (4000 Q TRAP, AB Sciex, Darmstadt, Germany). The following LC conditions (SLC-20AD Prominance Liquid Chromatography, Shimadzu, Kyoto, Japan; precolumn: 20 × 4.6 mm) were used: ET, Atlantis HILIC Silica column (particle size 5 µm, diameter × length = 3.0 × 100 mm; Waters, Eschborn, Germany); (A) 0.1% formic acid, (B) 0.1% formic acid in acetonitrile; gradient: 0.4 ml/min, 5% B at 0 min, 5% B at 2 min, 60% B at 5 min, 5% B at 7 min, stop at 10 min; hercynine, Atlantis HILIC Silica column (5 µm, 3.0 × 50 mm; Waters); (A) 10 mM ammonium acetate pH 4.5, (B) methanol; gradient: 0.3 ml/min, 90% B at 0 min, 10% B at 4 min, 10% B at 5 min, 90% B at 8 min, stop at 9 min. Atmospheric pressure ionisation with positive electrospray was used. For quantification (scan time 150 ms), the optimal collision energy for nitrogen-induced fragmentation in the second quadrupole was determined for each analyte. From the product ion spectra, the following fragmentations were selected for selected reaction monitoring (m/z parent, m/z fragment, collision energy (V)): ET: 230, 127, 27; hercynine: 198, 95, 29.

For each analyte, the area of the intensity versus time peak was integrated. Weighted ($1/y^2$) linear calibration curves were constructed from at least six standards (25–400 ng/ml), which were prepared using methanol as solvent. The sample analyte content was calculated from the analyte peak area and the slope of the calibration curve.

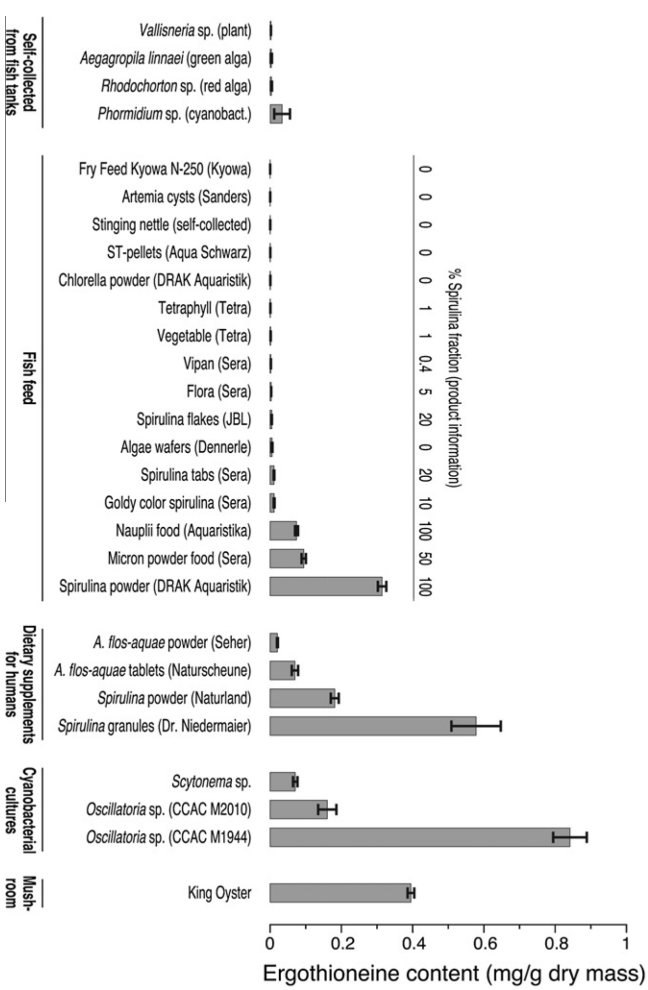

Fig. 1. Ergothioneine content of fish feed, dietary supplements, and cultured cyanobacteria. Sample sources and measurement of ET by LC–MS are described in Section 2. The Spirulina fraction values of fish feed samples are taken from container declaration or personal correspondence with distributor.

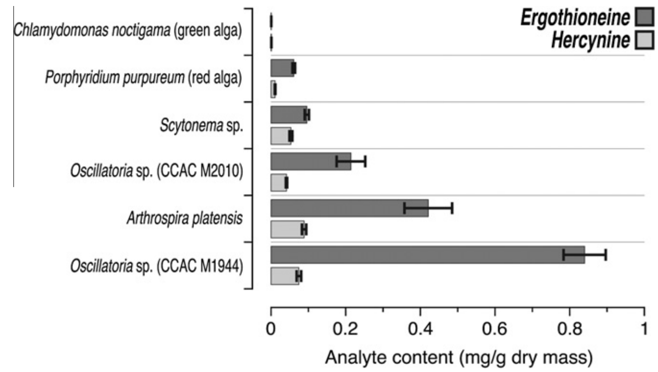

Fig. 2. Hercynine and ergothioneine content of fresh cultures of algae and cyanobacteria. All cultures were washed and immediately freeze-dried as described in Section 2.

2.2. Culture of cyanobacteria and algae

The cultures were from the Culture Collection of Algae at the University of Cologne (CCAC, www.ccac.uni-koeln.de; *Oscillatoria* sp. M1944, by courtesy of Algenion GmbH & Co. KG, Dietzenbach, Germany; *Oscillatoria* sp. M2010; *Scytonema* sp. M3193); or purchased from the Sammlung von Algenkulturen Göttingen, University of Göttingen, Göttingen, Germany (SAG, www.uni-goettingen.de/en/45175.html; *Arthrospira platensis* SAG 85.79; *Chlamydomonas noctigama* SAG 36.72; *Porphyridium purpureum* SAG 1380-1c).

The cultures were grown (23 °C, light intensity 10–20 µmol photons/m^2/s, light/dark cycle 14:10 h) in Erlenmeyer flasks with the following media, specified on the respective homepages: Waris-H (M1944, M2010, M3193, SAG 36.72); Spirulina medium (SAG 85.79); Jones medium (SAG 1380-1c).

2.3. Calculations and statistics

All results are given as arithmetic mean ± SEM with at least $n = 3$. The unpaired t-test was used to test for significance; two-tailed P values are given.

2.4. Materials

Chemicals: L-Ergothioneine (F-3455, Bachem, Bubendorf, Switzerland). Hercynine was synthesised as described previously (Grigat et al., 2007). All other chemicals were at least of analytical grade.

Fish feed: Spirulina powder and Chlorella powder (DRAK Aquaristik, www.drak.de, Dr. Andreas Kremser, Schönaich, Germany); Micron, Goldy color spirulina, Spirulina tabs, Vipan, and Flora (Sera, www.sera.de, Heinsberg, Germany); Nauplii food (Aquaristika Wirbellosen Shop, www.aquaristika.de, Solingen, Germany); Spirulina flakes (JBL, www.jbl.de, Neuhofen, Germany); Vegetable and

Tetraphyll (Tetra, www.tetra.de, Melle, Germany); algae wafers (Dennerle, dennerle.com/de/, Vinningen, Germany); ST pellets (Aqua Schwarz, www.aquaschwarz.com, Göttingen, Germany); Fry Feed Kyowa N-250 (Kyowa Hakko Bio Co.); Sanders Great Salt Lake Artemia Cysts (REBIE, www.rebie-bielefeld.de, Bielefeld, Germany).

Dietary supplements: *Aphanizomenon flos-aquae* tablets (Naturscheune, www.naturscheune.de, Autenhausen, Germany); *A. flos-aquae* powder (Seher's Uralgenshop, www.uralgenshop.de, Eisingen, Germany); Naturland Spirulina powder (GSE Vertrieb, www.gse-vertrieb.de, Saarbrücken, Germany); Spirulina Base granules (Dr. Niedermaier Pharma, www.niedermaier-pharma.de, Hohenbrunn, Germany).

3. Results

3.1. Cyanobacteria amass ET

We have measured by LC–MS/MS that zebrafish from a local large scale facility (no intentional addition of plants or algae to the tanks) contained almost 30-fold less ET (0.16 ± 0.03 μg/g wet mass; whole fish homogenate) than from a native habitat (Suthimari river, West Bengal, India; 4.4 ± 0.8 μg/g wet mass). Interestingly, animals fed like the facility fish, but kept in a tank together with several plants and algae had wild-life ET levels (5.5 ± 0.8 μg/g wet mass). There was virtually no ET in the fish feed components (Kyowa, ST pellets, and artemia) and most tank vegetation (a plant, a green alga, and a red alga; see Fig. 1). However, a sample consisting mainly of *Phormidium* sp. (identified by macroscopic appearance and development, and microscopic structure), a cyanobacterium, contained noteworthy amounts. We then screened a number of commercial fish feed preparations for the presence of ET (Fig. 1). The data suggest a positive correlation between ET content and the fraction of Spirulina; the latter term refers to cyanobacteria *Arthrospira platensis* (older name *Spirulina platensis*) and *Arthrospira maxima*. In samples of cyanobacteria preparations (*Aphanizomenon flos-aquae* and *S. platensis*) sold as dietary supplements for humans, ET was measured at up to 0.6 mg per g dry mass (Fig. 1). High levels of ET (up to 0.8 mg per g dry mass) were also measured in fresh *Scytonema* and *Oscillatoria* cultures (Fig. 1). Cyanobacteria can contain as much as or even more ET than King Oyster mushrooms (*Pleurotus eryngii*) which we measured at 0.4 mg per g dry mass (Fig. 1). No ET was detected (not shown) in seawater algae *Fucus* sp. (brown alga) and *Ulva lactuca* (green alga).

3.2. Cyanobacteria synthesise ET

To determine whether cyanobacteria accumulate ET from extracellular space or whether they synthesise it themselves, we scanned by LC–MS/MS analysis for known intermediates of ET biosynthesis (Melville, 1958; Seebeck, 2010) in freeze-dried culture samples. Hercynine, which was available from a previous study (Grigat et al., 2007), was clearly detected by SRM (selected reaction monitoring) in all samples with substantial ET content (Fig. 2). A sample of γ-glutamyl S-hercynyl cysteine sulfoxide (compound **3** in the paper by Seebeck (2010); $m/z = 462$) generated major fragments at $m/z = 444$, 333, 246, and 202 (ESI, positive ionisation). However, we could not detect this compound by SRM quantification in culture samples. Likewise, we could not detect by SRM S-hercynyl cysteine sulfoxide (compound **4**; $m/z = 333$) which, by analogy to compound **3**, we expected to generate fragments of $m/z = 246$ and 202 (ESI, positive ionisation). In full agreement, in mushroom powder (*P. eryngii*, King Oyster) only hercynine was detected, too.

3.3. Zebrafish accumulate ET from Spirulina

Adult fishes from the large scale facility were fed exclusively with Spirulina flakes (made from DRAK Aquaristik powder). The content of ET in whole fish homogenate increased from 0.07 ± 0.03 up to 0.94 ± 0.14 μg/g wet mass (Fig. 3; $P = 0.0007$ for the day 3 sample compared with control).

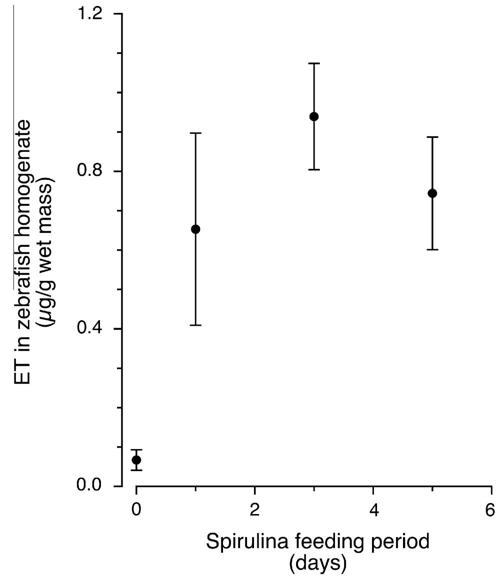

Fig. 3. ET content in whole fish homogenate increases during feeding of Spirulina. Fishes ($n = 4$ for each time point) were weighed (range: 0.09–0.24 g) and then homogenised as described in Section 2. To allow intestinal absorption of ET, fishes were not analysed at the end of the feeding period, but 1 day later. The tank plants contained no ET.

4. Discussion

Until now, the only documented sources of ET biosynthesis have been fungi (including edible mushrooms) and mycobacteria (Hartman, 1990; Melville, 1958). Our data establish that several species of cyanobacteria can also produce large amounts of ET (Fig. 1). Fishes and other aquatic life forms that feed to some extent on cyanobacteria are thus provided with plenty of the antioxidant (Fig. 3). By contrast, aquatic photosynthetic eukaryotes contained no (*Chlorophyta*, *Phaeophyceae*) or little (*Rhodophyta*) ET (Fig. 2).

Synthesis of ET by cyanobacteria themselves is supported by the detection of the intermediate hercynine (Fig. 2). Two further postulated intermediates (Seebeck, 2010) could not be used as marker of biosynthesis here, since they could be detected neither in cyanobacteria nor in mushroom material. However, further, indirect evidence comes from the presence of 2 genes (*EgtB*, *EgtD*) for key enzymes of ET synthesis within the genomes of cyanobacteria (Seebeck, 2010).

The highest ET content of cyanobacteria in our samples was close to 1 mg per g dry mass (Fig. 1). This is the same level as the top values (1–2 mg per g dry mass) reported previously for several mushrooms *e.g.* Shiitake (*Lentinus edodes*), Oyster (*Pleurotus ostreatus*), Maitake (*Grifola frondosa*), King Oyster (*P. eryngii*), and *Agaricus bisporus* (Dubost, Beelman, Peterson, & Royse, 2006; Dubost, Ou, & Beelman, 2007; Melville, 1958). Thus, cyanobacteria are a "high density" source of ET. Of course, the ET content of samples may vary with species and conditions of growth and harvest.

Specific cyanobacteria are sold as dietary supplements for human use. Spirulina is produced at large scale and is relatively inexpensive. It is considered non-toxic for humans (Park et al., 2008) since it lacks the contamination risk of *A. flos-aquae* (Gilroy, Kauffman, Hall, Huang, & Chu, 2000; Saker, Welker, & Vasconcelos, 2007). Spirulina has been used as traditional food in equatorial countries *e.g.* by the Aztecs. It has been lauded as the "best food for the future" (United Nations World Food Conference, 1974) and proposed by NASA and ESA as one of the primary foods to be cultivated during long-term space missions. Thus, Spirulina is a safe, accessible, and affordable alternative source of ET for humans.

There are clues that Spirulina promotes health in many aspects (Khan, Bhadouria, & Bisen, 2005; Lu, Hsieh, Hsu, Yang, & Chou, 2006). A major feature are potent antioxidant effects *in vitro* and *in vivo* (Deng & Chow, 2010; Miranda, Cintra, Barros, & Mancini Filho, 1998; Ponce-Canchihuaman, Perez-Mendez, Hernandez-Munoz, Torres-Duran, & Juarez-Oropeza, 2010; Ray, Roy, & Sengupta, 2007; Viswanadha, Sivan, & Rajendra Shenoi, 2011). However, so far only few controlled Spirulina supplementation studies with humans have been reported. The plasma markers of elderly males and females were improved (TBARS assay and others; Spirulina supplementation for 16 weeks, 8 g per day) (Park et al., 2008), and there was less lipid peroxidation after exercise in young males (TBARS assay with serum; Spirulina supplementation for 4 weeks, 6 g per day) (Kalafati et al., 2010) or a mixed-gender group of college students (malondialdehyde assay with plasma; Spirulina supplementation for 3 weeks, 7.5 g per day) (Lu et al., 2006). It is unclear from these studies what ingredients in Spirulina actually cause the benefit. In this context, we consider cysteine and methioneine (the increments from 6 to 8 g Spirulina are negligible compared to other food sources (Kalafati et al., 2010), phycocyanin (as a protein, it can not be absorbed in the gut), and ascorbate and vitamin E (these are contained in Spirulina at 0.1–1 and 0.15 mg per g dry mass, respectively (Babadzhanov et al., 2004; Clement, Giddey, & Menzi, 1967), *i.e.* at similar or lower levels than ET) as unimportant. By contrast, ET, which is scarce in most other food sources, efficiently absorbed in the intestine via ETT, not metabolised, and distributed to cells and tissues in contact with blood, may be an important if not the critical factor for the above described antioxidant effects of ingested Spirulina.

The exact benefit that cyanobacteria, mycobacteria, and fungi obtain from the synthesis of ET is still unresolved. Cyanobacteria represent a new simple model to explore this question.

In conclusion, our data establish that cyanobacteria can produce high levels of ergothioneine. Spirulina is a novel, safe, accessible, and affordable source of ergothioneine for humans.

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