Superior Intracellular Antioxidant Activity of an Astaxanthin-Containing Corynebacterial Extract

Jan Seeger and Nadja A. Henke *

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Article

Superior Intracellular Antioxidant Activity of an Astaxanthin-Containing Corynebacterial Extract

Jan Seeger [1] and Nadja A. Henke [2,*]

[1] Genetics of Prokaryotes, CeBiTec & Faculty of Biology, Bielefeld University, Bielefeld, Germany

[2] Institute of Process Engineering in Life Sciences, Karlsruhe Institute of Technology

* Correspondence: nadja.henke@kit.edu

Abstract

Astaxanthin can be derived from different sources such as petrochemical synthesis, natural sourcing from green algae or by microbial fermentation. As one of the strongest antioxidants known by nature, astaxanthin is rising attention as an active ingredient in cosmetical products to support the skin against oxidative stress. In contrast to widely performed chemical antioxidant activity assays, this study focuses on the comparison of synthetic, algal and corynebacterial astaxanthin in a physiological relevant test setting: the intracellular antioxidant activity in cultured human skin cells, keratinocytes. The astaxanthin-rich corynebacterial oleoresin demonstrated to be the superior antioxidant in that assay with a EC_{50} of 2.7 µM whereas the synthetic and algal-based variants showed no significant effect. In terms of an application of such raw materials, it is therefore tempting to speculate that astaxanthin-containing corynebacterial oleoresins could serve as a natural and superior active ingredient for skin health applications in the future.

Keywords: intracellular antioxidant activity; astaxanthin; keratinocytes; bioavailability

1. Introduction

Oxidative stress is closely linked to inflammatory processes and therefore also being associated with a series of health issues ranging from cardiovascular and neurodegenerative diseases, diabetes, cancer to skin diseases and aging [1–4]. Environmental triggers such as pollution and UV light creating ubiquitous oxidative stress to biological systems [5–8]. Oxidative stress can harm cells in multiple ways as different key processes, associated with different locations within the cell, can be impacted. At the plasma membrane/ cell envelope lipid peroxidation causes membrane alteration as well as interference with receptor signaling [9]. Moreover, intracellular oxidative stress can interfere with diverse cellular processes from DNA repair [10] to protein folding [11,12] as well as subcellular structures [13]. Antioxidants are therefore a dominating group of active ingredients [14] for different purposes ranging from nutrition to cosmetics and pharmaceutical scopes [15–17].

Astaxanthin, the queen of carotenoids, is one of the strongest antioxidants known by nature [18]. This excellent activity can be explained by the conjugated double bond system and further oxyfunctionalized groups at the β-ionone rings of the structure [18,19]. The astaxanthin market share is dominated by natural astaxanthin, with a share of > 600 Mio. USD in 2024 and a CAGR of 8.8% in the forecasted period till 2034 [20]. Natural variants on the markets are for example sources from the red yeast *Phaffia rhodozyma* [21–23], the bacterium *Paracoccus carotinifaciencs* [24] and the green microalgae *Haematococcus pluvialis* [25]. Besides this established production host, strain and bioprocess engineering allows to tackle the astaxanthin market with other microbial cell factories such as *Escherichia coli* [26,27] and *Yarrowia lipolytica* [28,29]. In the last years, *Corynebacterium glutamicum* was presented as an alternative microbial cell factory for astaxanthin as fermentative protocols [30–32] as well as downstream processes [33,34] were established and optimized.

Besides it different sources the synthesized astaxanthins differ in stereochemistry as well as esterification. Synthetic astaxanthin is present as a racemate (1:2:1 mixture of (3S,3'S), (3S,3'R) and

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(3R,3'R)) as free form astaxanthin [35]. Natural biosynthesis typically results in either 3S,3'S (dominantly algal, bacterial and plant sources) [36,37] or 3R,3'R (dominantly yeast sources) [38] enantiomers. Depending on the specific production host, astaxanthin can be present as free form or being esterified with fatty acids [39,40]. This means that a series of structurally different astaxanthin variants exists with potentially different functional properties. In vitro assays such as the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay allow the determination of antioxidant activities in an easy manner based on the color change during quenching of the free radical DPPH in the presence of a ROS-scavenger [41]. This assay is widely applied due to its easy, fast and cheap handling. However, the interpretation of such chemical assays for real-world application is limited. Active (pharmaceutical) ingredients have to be bioavailable [42–44] in order to fulfill their function at the desired cellular location.

As a promising active ingredient for skin care products it was shown that topical application of astaxanthin reduced wrinkles, improved elasticity and pigmentation [45], supported wound healing [46], and mitigated UV-induced skin damage [47]. Therefore, the scope of this work is on the investigation of the antioxidant activity of three different astaxanthin sources namely, synthetic, algal-based and corynebacterial astaxanthin (as present in a corynebacterial natural extract) in a more physiological relevant test setting using human keratinocytes.

2. Materials and Methods

2.1. Production and Extraction of Corynebacterial Astaxanthin

Astaxanthin-producing *C. glutamicum* strain ASTA** was cultivated for 48 h in shake flasks as described by [32] using the optimized trace element solution [31]. After cultivation, the biomass was harvested by centrifugation at 10,000 x g for 20 min. Subsequently, the cell pellet was extracted using 90% (v v^{-1}) ethanol, at a solvent-to-biomass ration of 15 mg_{CDW} mL^{-1} at 60 °C for 30 min in a 1 L stirred bottle reactor equipped with an anchor stirrer (DWK Life Sciences, Mainz, Germany) at 500 rpm. The astaxanthin oleoresin was prepared as described in [33] and was stored at -20 °C until further usage. The astaxanthin content in the oleoresin was 11.6 mg g^{-1} as determined by HPLC [32].

2.2. Intracellular In Vivo Antioxidant Assay

For the intracellular in vivo antioxidant assay (AOP1), the corynebacterial astaxanthin (as oleoresin) (CA) was compared to two other astaxanthin sources: synthetic astaxanthin (SA) (Sigma-Aldrich, St. Louis, MA, USA; catalog number 1044200) and esterified astaxanthin (as oleoresin; containing 100 mg g^{-1} astaxanthin (calculated as free astaxanthin); astaxanthin is present as 75% monoester, 20% diester, 5% free) from *Haematococcus pluvialis* (AA) (Sigma-Aldrich, St. Louis, MA, USA; catalog number 1044210). All samples were dissolved in DMSO to concentration of 3 mM (calculated as free astaxanthin) at 40 °C and 1000 rpm (Thermomixer comfort, Eppendorf, Germany). Stock solutions were shipped frozen to Anti Oxidant Power (Toulouse, France). The assay was performed as described by [48] using human keratinocytes (HaCaT).

2.3. AOP1 Assay with De-Esterified Algal Astaxanthin

In order to verify that the low activity of the algal astaxanthin is based on the esters, a second AOP1 assay was performed, comparing both the free and the esterified astaxanthin from algae. To obtain the free version of the esterified from *H. pluvialis*, an enzymatic cleavage based on [49] with slight alterations was performed. Per mL of acetone, 1.5 mg *H. pluvialis* oleoresin was dissolved. The enzyme solution consisted of 2 U mL^{-1} cholesterol esterase from *Pseudomonas fluorescencs* (Sigma-Aldrich, St. Louis, MA, USA; catalog number: C9281) in 0.05 M Tris HCl pH 7. The astaxanthin-containing acetone and the enzyme solution were mixed at a ratio of 3:2 and the reaction mixture was shaken at 37° C in the dark for 4 h. The cholesterol esterase was replaced by bovine serum albumin (BSA) as negative control. After incubation, 5 mL hexane was added to the reaction mixture and was

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mixed vigorously. The hexane layer was transferred into a new tube and subsequently evaporated (Concentrator Plus, Eppendorf, Germany). Successful de-esterification (>99%) was verified by HPLC (figure S1). BSA-treated samples (control) and the cholesterol esterase-treated samples (de-esterified astaxanthin) were dissolved in DMSO at concentrations of 2.5 mM and 2.9 mM (calculated as free astaxanthin), respectively.

2.4. Quantification of Astaxanthin by HPLC

Astaxanthin was quantified by using the Agilent 1200 series (Agilent Technologies, Santa Clara, CA, US) equipped with a reversed-phase precolumn (LiChrospher 100 RP18 EC-5, 40 × 4 mm) (CS-Chromatographie, Langerwehe, Germany), a reversed-phase main column (LiChrospher 100 RP18 EC-5, 125 × 4 mm) (CS-Chromatographie, Langerwehe, Germany), and a diode array detector (DAD) recording the absorption at λ = 470 nm. A defined amount of sample was dissolved in a 7:3 methanol:acetone mixture, of which 50 µL were injected. The mobile phases consisted of methanol:water (9:1) (A) and methanol (B). A gradient at a flow rate of 1.5 mL min^{-1} was used as the following: 0 min B: 0%, 10 min B: 100%, and 32.5 min B: 100%. Synthetic astaxanthin was used as a reference standard.

3. Results

3.1. Intracellular Antioxidant Activity Testing of Different Astaxanthins

The human keratinocyte cell line HaCaT is a widely used model of the epidermis and therefore is employed to investigate skin physiology and the evaluation of novel cosmetical ingredients [50,51]. As astaxanthin is a promising ingredient for skin applications [52], the assay was performed on human keratinocytes (HaCaT). The assay is based on the fluorescent dye thiazol orange, which is taken up by the cells and upon being illuminated by light, triggers the generation of ROS such as singlet oxygen and hydroxyl radicals. The ROS can be neutralized by the addition of an antioxidant [48]. The results of the AOP1 assay are shown in Figure 1. The half-maximal effective concentration (EC_{50}) of corynebacterial astaxanthin (CA) was determined to be 2.7 µM. In contrast, neither algal-based astaxanthin (AA) nor synthetic astaxanthin (SA) yielded a calculable EC_{50} as only partial activity was observed even with the highest examined concentrations of 120 µM.

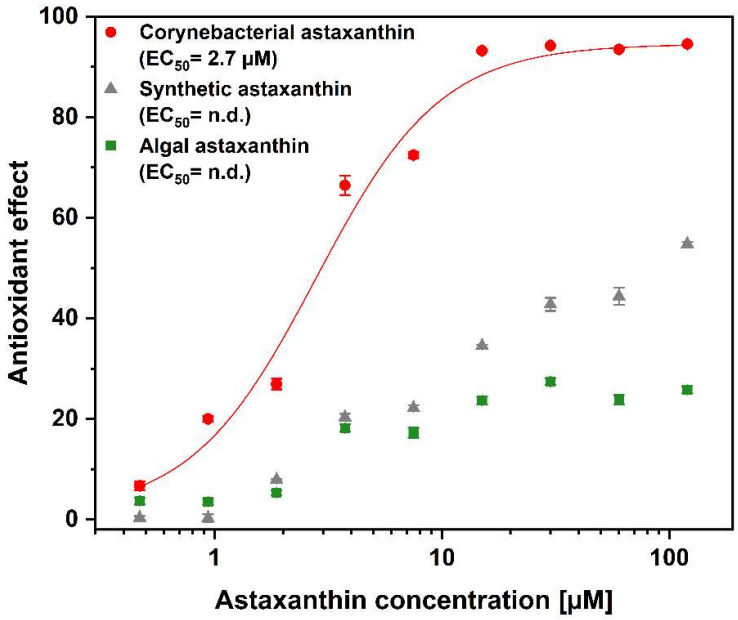

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Figure 1. Intracellular antioxidant activity of astaxanthin from different sources. Corynebacterial astaxanthin-containing oleoresin (CA), synthetic and algal (SA) astaxanthin-containing oleoresin (AA) are shown in red, grey and green, respectively.

3.2. De-Esterification Algal-Based Astaxanthin and Its Intracellular Activity

We aimed on de-esterifying the astaxanthin-(di)esters in the algal-based oleoresin in order to verify that the low activity of the algal astaxanthin is based on its esters. Therefore, astaxanthin derived from *H. pluvialis* was de-esterified by cholesterol esterase, which was confirmed by HPLC analysis (Figure S1). The AOP1 assay comparing the de-esterified and the esterified astaxanthin was performed as described previously and the results are depicted in Figure 2. No statistical differences were observed between the control and de-esterified samples. Notably, the samples containing the de-esterified astaxanthin exhibited cytotoxic effects at concentrations >15 µM (data not shown).

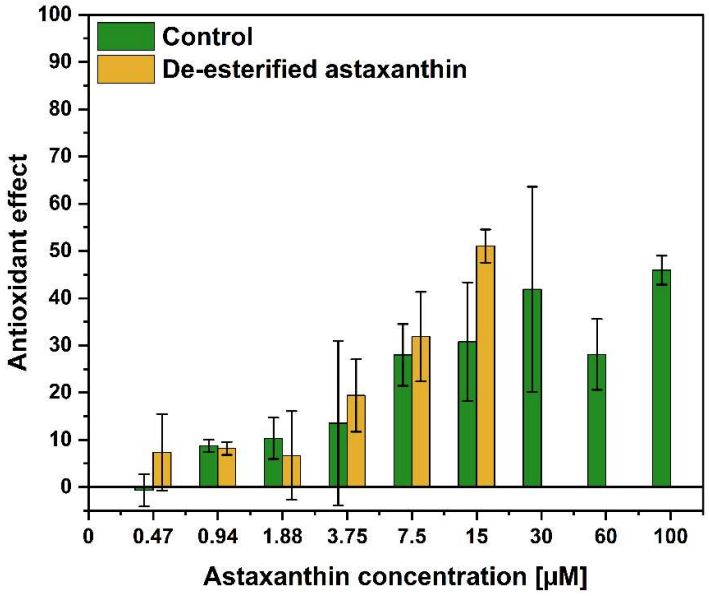

Figure 2. Intracellular antioxidant activity of de-esterified astaxanthin. Extract from *H. pluvialis* was treated with BSA (control; shown in green) or cholesterol esterase (de-esterified; shown in yellow).

4. Discussion

This work demonstrates that the source and molecular form of astaxanthin strongly influence its intracellular antioxidant activities in cultured human keratinocytes. Astaxanthin-containing corynebacterial oleoresin showed a pronounced intracellular antioxidant effect with an EC_{50} of 2.7 µM.

In a previous study we have shown that corynebacterial and algal astaxanthin exhibited comparable antioxidant activities as quantified with the widely used DPPH assay [33]. While the natural corynebacterial and algal astaxanthins showed comparable EC_{50} of 3.2-3.7 µg mL^{-1}, the racemic mixture of the synthetic astaxanthin possessed an approximately 10-fold lower antioxidant activity (correspondingly a higher EC_{50} of 42 µg mL^{-1}) [33]. These findings are in accordance with previous reports indicating a higher antioxidant activity of enantiomer pure (3S,3'S) astaxanthin compared to the (3R,3'R) enantiomer and the synthetic racemic mixture [53].

The discrepancy between DPPH-based antioxidant activity and the intracellular AOP1 assay highlights the importance to complement chemical assays for specific application evaluations [15]. Chemical antioxidant assays are strong in quantifying radical-scavenging capacity in cell-free solution, however, lack information on the bioavailability and cell uptake as practical hurdles that influence the antioxidant activity in cellular systems [14,54].

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Astaxanthin itself is a lipophilic compound [44], but the solubility and bioavailability of the esterified (algal) and free form (corynebacterial and synthetic) astaxanthins [55] as well as the enantiomere composition is considered to affect the stability and bioavailability [56,57]. In vitro and in vivo studies showed that free astaxanthin is absorbed faster as astaxanthin esters have to be hydrolyzed by digestive enzymes and fluids prior uptake [58–60].

As the conditions of the AOP1 assay do not favor ester hydrolysis, it is tempting to speculate that the algal-derived astaxanthin was not taken up properly by the HaCaT cells. This might be similar for the cosmetic usage of algal-derived astaxanthin: in contrast to free astaxanthin the esterified form of algal astaxanthin is less bioavailable to the skin due to the absence of hydrolyzing enzymes, disfavoring its usage for topical applications [39,57].

It was therefore questioned if the intracellular antioxidant activity of the algal-based astaxanthin could be elevated after de-esterification. Although the de-esterification was successfully validated by HPLC analysis (Figure S2), the intracellular antioxidant activity in cultured keratinocytes did not differ from the control algal-based oleoresin. Cytotoxic effects of the de-esterified astaxanthin were observed at concentrations >15 µM, leading to incomplete results. The cytotoxicity might be due to the release of free fatty acids during the de-esterification, which are known to exhibit cytotoxicity for in vitro cell culture [61,62].

Despite the clear activity differences observed some limitation should be considered. As the corynebacterial astaxanthin was tested as part of the oleoresin rather than as a purified compound synergistic effect of co-extracted compounds cannot be excluded. The oleoresin matrix itself might account for the superior activity as byproducts in the extract may promote the solubility and/or delivery of the lipophilic astaxanthin [63]. It is known that lipid-rich matrices and formulation strategies can improve the bioavailability of carotenoids as compared to purified compounds [52,58,64]. Additionally, microneedle-based delivery systems can bypass the outermost layer of the skin, thereby potentially facilitating transdermal absorption [65].

The corynebacterial oleoresin has been demonstrated to be the most potential astaxanthin resource for intracellular ROS-scavenging in keratinocytes in this work. The cell-based assay was developed by Gironde et al. 2020 who have also identified astaxanthin as a promising lipophilic compound in there screening, however with only a partial activity in the AOP1 assay [48]. Gironde et al. 2020 used synthetic astaxanthin in their study resulting in the same partial activity observed in this study. Although the synthetic astaxanthin was most likely taken up by the cells due to its free form, its less active enantiomers lead to the low activity. When being compared to the results of Gironde et al. 2020, it has to be highlighted that the astaxanthin-rich corynebacterial oleoresin belongs to the best performing antioxidants validated in the AOP1 assay with an EC_{50} of 2.7 µM in HaCaT cells (Figure 1).

As these results point out the significance of alternative antioxidant resources from microbial fermentation [26], its real-world applicability should be further evaluated in other cell lines and skin penetration assays to strengthen the conclusion [52]. From an application perspective *C. glutamicum* is a platform system that offers the opportunity to access other astaxanthin derivatives, such as astaxanthin-diglucoside [32] which may exhibit distinct bioactivities in comparison to the here tested astaxanthin variants.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Figure S1.

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Data Availability Statement: Dataset available on request from the authors.

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Abbreviations

The following abbreviations are used in this manuscript:

AOP	Antioxidant power test
AA	Algal astaxanthin
CA	Corynebacterial astaxanthin
CDW	Cell dry weight
SA	Synthetic astaxanthin

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