Catabolism and bioactive properties of chlorophylls

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Abstract

Chlorophylls are fundamental molecules for life and probably the most important of all natural pigments. Although chlorophyll breakdown is inevitably associated with the loss of green color during ripening or senescence, the truth is that chlorophyll molecules continuously degrade through so-called chlorophyll turnover (lifetime of hours). In addition to their primary function, chlorophylls are in demand due to their bioactive properties. Recently, several chlorophyll derivatives have shown health promoting activities. This review also provides an updated outline of the most recent advances that have occurred in the deciphering mechanism of chlorophyll catabolism; it will also describe the novel biological properties associated with chlorophyll derivatives.

Introduction:

Besides their essential role during photosynthesis, chlorophylls are highly valuable in foods as green color is a valued attribute for consumers, being a universal practice the addition of authorized green food coloring [1]. In nature there are more than 100 different chlorophyll structures [2] mainly in green bacteria, but strictly speaking there are five structurally characterized species of “chlorophyll” *a*, *b*, *c*, *d* and *f* [3] (Figure 1). In nature, chlorophyll *a* and *b* predominate in photosynthetic organisms while chlorophyll *c*, *d* and *f* are pigments found exclusively in certain microalgae, algae and several classes of photosynthetic bacteria (Table 1). The polar chlorophyll *c* differs from chlorophylls *a*, *b*, *d*, and *f* since it is Mg-phytylporphyrins rather than Mg-chlorins [4] due to the unsaturation at ring D. Since 1990, the number of members of the chlorophyll *c* family has increased from seven (*c1*, *c2*, *c3*, *cCS-170* (actually [7-methoxycarbonyl-8-vinyl]-Pchl_{ide}), *c2*-like pigment from *Pavlova gyra*ns, DV-P- chlorophyllides and a nonpolar chlorophyll *c*-like pigment) to include galactolipid esters [4], recently reviewed [5]. Chlorophyll *d*, that constitutes 95% of the photosynthetic apparatus of *Acaryochloris marina*, is formed from the oxidation of the vinyl group at C3 in chlorophyll *a* to form a formyl group in chlorophyll *d* (Table 1). This structural characteristic provides a potential selective advantage because the chromophore extension is responsible for an increase in the light absorption spectrum, in the infrared light (700–750 nm), that is not absorbed by chlorophyll *a* [6]. Recently, an even more red-shifted chlorophyll termed chlorophyll *f* has been isolated from microalgae [6]. Chlorophyll *f* differs from chlorophyll *a*, in the presence of a formyl group substitution at the C2 position (Table 1). The chlorophyll absorption properties depend on their structure, as the number of conjugated double bonds and the use of different substituents on the pyrrole rings strongly influence the energy levels, change of colors and bioactive potential [7]. In spite of the existence of different chlorophylls, this review deals only with the fate of chlorophyll *a* and *b* (Table 1) and the main chlorophyll compounds present in foods, as up to now the scientific evidences related to the catabolism or functional properties of the other chlorophyll structures are scarce.

Catabolism of chlorophylls:

Chlorophyll degradation occurs not only during senescence and ripening, but also in response to different biotic and abiotic stresses. The chlorophyll breakdown pathway, now called “PAO/phyllobilin” has been biochemically almost completely determined and recently reviewed [8]. The pathway has been studied mainly during leaf senescence and fruit ripening and partially in algae. In any case, in the biochemical route, except for one exception, all chlorophyll catabolites that are currently known derive from chlorophyll *a*. Consequently, it is assumed that chlorophyll *b* is transformed into chlorophyll *a* before degradation. Those

reactions are part of the so-called “chlorophyll cycle” where chlorophyll *a* and *b* could interconvert in accordance with the physiological demands. The formyl group at C7 in chlorophyll *b* results in a methyl group (in chlorophyll *a*, Figure 1) through a two-step reaction, with the first reaction catalyzed by NONYELLOW COLORING 1 (NYC1) and NYC1-LIKE (NOL) [9-10], and the second reaction by HYDROXYMETHYL CHL *a* REDUCTASE (HCAR) [11]. From chlorophyll *a* two consecutive reactions that includes loss of the phytol chain and Mg dechelation, results in pheophorbide *a* (Figure 1). How those reactions proceed have been a matter for debate over recent years. Historically, it has been assumed that chlorophyllase (CHL) was the first enzyme in the chlorophyll degradation pathway [12] hydrolyzing the phytol chain from chlorophyll *a* and forming chlorophyllide *a*, followed by a Mg dechelation that results in pheophorbide. However, since CHL was cloned in 1999 [13-14] several evidence has generated doubts about its physiological role in the senescence of leaves [15], although additional evidence has also confirmed the involvement of CHL in the ripening of fruits [16]. Meanwhile, an alternative route was proposed, pheophytinase (PPH) [17] was described as the responsible for chlorophyll dephytilation, forming pheophytin in senescent leaves (Figure 1), although in fruits, pheophytinase seems not to be essential [18]. To complete the scenario, recently, the so-called stay-green (SGR) (also identified as non-yellowing, NYE), responsible for the green color in Mendel’s experiments and with several hypothetic functions for years, have been identified as the overlooked enzyme that catalyze the magnesium dechelation [19] (Figure 1). Interestingly, authors identified three SGR in Arabidopsis, SGR 1/2 with high affinity for chlorophyll *a*, but SGRL (SGR-LIKE) showed higher affinity for chlorophyllide *a*, suggesting a new chlorophyll degradation pathway via chlorophyllide. From the different pathways showed to form pheophorbide *a*, probably the combination of SGR and PPH is actually, the main and more widespread route in plants. However, green tissue plausibly has developed alternative dephytlase to degrade chlorophyll during senescence/ripening. In this regard, a new dephytlase CLD1 (CHLOROPHYLL DEPHYTYLASE1) has been recently identified as the long-sought enzyme for removing the phytol chain from chlorophyll in the continuous chlorophyll turnover in a steady state [20]. This function is essential for the repair of D1 and PSII and it has been proposed that presumably it is performed in conjunction with SGRL (Figure 1).

Independently of the enzymes involved in the formation of pheophorbide *a*, PHEOPHORBIDE *a* OXYGENASE (PAO), the main enzyme of the route which gives its name to the route, opens oxygenolytically the macrocycle forming a new set of chlorophyll compounds named phyllobilins [21] (Figure 2). This term includes all the chlorophyll breakdown products with a linear tetrapyrrole structure and chemically they are defined as formyl-oxobilin-type or

dioxobilin-type tetrapyrroles due to the structural relationship with the heme-derived bilins [22]. As a consequence, phyllobilins follow a different numbering system showed in Figure 2. Anyhow, RCC (red chlorophyll catabolite) is the first phyllobilin to be formed, which is rapidly transformed into pFCC (primary fluorescent chlorophyll catabolite) due to RCCR (RCC reductase) that specifically reduces the C15/C16 double bond of RCC. This primary FCC could be hydroxylated at C3² by TIC55 [23], but hydroxylated or not, FCCs are exported from the chloroplast through an active transporter, not identified at the moment, to the cytosol. In this new environment, FCCs are in a species-specific manner modified in concrete positions: they could be deformylated in C1 forming DFCCs (by a cytochrome P450 mono-oxygenase CYP89A9 [24], demethylated at C8² by methylesterase MES16 [25], hydroxylated at C18 and different functional groups could react at C3². These different structures are finally imported to the vacuole through an ATP-binding cassette where they are stored. The acidic pH of the vacuole isomerizes the different FCCs at C10 to NCCs (no fluorescent chlorophyll catabolites) [26], which are considered to be, at present, the final chlorophyll catabolites. At present, over 40 structurally different phyllobilins have been identified [8].

As in other biochemical pathways, once the main reactions are described and the enzymes identified, the study of the regulation mechanism begins. This is the actual situation of the chlorophyll catabolism, and in the recent years the transcriptional regulation of the PAO/phyllobilin has been explored, with interesting results [27-28].

However, it is important to highlight the existence of other chlorophyll compounds not considered in the PAO/phyllobilin pathway. Actually, these chlorophyll structures deriving from chlorophyll *a* and *b*, formed through active metabolism (probably through an alternative enzymatic system as peroxidase) or during food processing or storage (Figure 1). In this regard, chlorophylls can be oxidized at C13² resulting in the 13²-hydroxy-chlorophylls, a lactone group that can be formed at C15¹ forming the corresponding C15¹-hydroxylactone-chlorophylls or can even lose the carboxymethoxy group at C13² to form pyro-derivatives. The interrelation between this metabolism and the PAO/phyllobilin pathway is unclear. For example, in the oxidation route different enzymes have been implicated as peroxidase, while pheophorbidease have been proposed as the responsible of the formation of pyro-derivatives. However, the physiological meaning of these reactions, the significance in the metabolism or the existence of a connection with the PAO/phyllobilin pathway are at present unknown. In addition, more severe chemical treatments induce the opening of the isocyclic ring constituting chlorin (it derives from chlorophyll *a*) or rhodin (from chlorophyll *b*). Directed chemical modifications

over these opened molecules generate a complex and diverse set of structures employed as photosensitizer in the photodynamic therapy of cancer [3].

Bioactive properties:

Independently of the primary function of chlorophylls, the application as a food additive, the monitoring of agricultural production and ocean primary productivity are currently the commercial uses of these molecules. Although the natural color of chlorophyll is the main economic attribute, these compounds have drawn attention in recent years, not because of their chromatic properties, but due to their potential health benefits [29]. The bioactive properties of chlorophylls are closely related to their chemical structure outlined previously. For example, the substitution of the central magnesium ion by other metals (zinc or copper) modified the ability to bind proteins, affecting to the xenobiotic capacity to chlorophylls [3] and also affect to the chlorophyll stability and kinetic during the transports through tissues [30]. In other side, the presence of the phytol chain increases the hydrophobicity of the molecule and consequently the aggregative properties [3]. Additional rearrangements in peripheral positions as in different chlorophylls or at the isocyclic ring in oxidized chlorophylls (Table 1), can modify the properties of the π -electron system, the absorption properties and consequently specific bioactive properties. Such biological activities attributed to chlorophylls and their catabolites consist of cancer prevention, antioxidant and antimutagenic activities, mutagen trapping, modulation of xenobiotic metabolism, induction of apoptosis, antimicrobial properties and anti-inflammation activity (Table 2).

The most research has focused on chlorophyll *a*, chlorophyll *b* and catabolites of chlorophylls, and several contributions support the antioxidant properties of these compounds. However, literature data show conflicting results. Formerly, the antioxidant capacity of chlorophyll *a* derivatives were found to be more effective as radical quenchers than those of chlorophyll *b*. And, metal-free catabolites such as pheophytins, and pyropheophytins exhibited significantly lower antiradical capacity than metal derivatives such as Mg-chlorophylls [31-32]. However, other authors suggest that while the basic porphyrin structure is crucial for free radical reduction, magnesium chelation strengthened apparent antioxidant activity of chlorophylls [33]. Hoshina et al. [34] also report the porphyrin structure as well as the presence and nature of the central metal, which are considered important for the antioxidant activity of chlorophyll derivatives. On the other hand, other research related chlorophyll *a* showed the weakest antioxidant activity among all greenish pigments over the

whole range of concentration studied [35]. Pheophorbide *b* and pheophytin *b* were the strongest natural antioxidant compounds. The high antioxidant activity found in pheophorbide *b*, in comparison to pheophorbide *a*, demonstrated the importance of the aldehyde group for functionality. These authors [35] mention that the mechanism of action involved might not be related to the capacity of hydrogen donation but, to the protection of linoleic acid against oxidation and/or prevention of the decomposition of hydroperoxides.

As shown in Table 2 different chlorophyll compounds have shown to have chemopreventive properties. However, the majority of the *in vitro* studies on antimutagenic activity have been developed with chlorophyllins or with the so-called sodium copper chlorophyllins (SCC) [3, 36]. In any case, the mechanism seems to be related with the formation of a complex between the tetrapyrrole and the mutagen, facilitating the elimination of the mutagen through the digestive tract [37]. This mechanism could be for example, the responsible in the attenuation of the intestinal inflammation (through the modulation of several signaling pathways) in mice with liver fibrosis after chlorophyllin administration. At *in vivo* level, different studies on animals, including humans demonstrate the possibilities of several chlorophyll derivatives to inhibit cancer initiation or progression by multiple mechanisms [38]. However, probably the most promising bioactivity of chlorophyll compounds is their role as “drugs” during the photodynamic therapy (PDT), as their photophysical properties makes them an ideal photosensitizer. Since the development of the first approved photosensitizer Photofrin®, the aim is to improve structurally the chlorophyll configuration through the progress of different generations [39]. In this sense, the most recent advances include the microencapsulation to reduce the efflux and improve the efficacy [40-41]. In addition, the photosensitizing properties of chlorophylls are used also in the cancer detection [42] through fluorescence, magnetic resonance or nuclear imaging, for an early and exact diagnosis. Chlorophylls, are even strategically designed as bifunctional agents [43]. Alternatively, a secondary effect of the PDT as the overproduction of ROS is used as an effective approach to treat microbial infection [44]. In this sense, a recent review [45] have detailed all the applications of chlorophyllin compounds in food safety as inactivating agent of microorganisms due to their photosensitizer properties. For example, *in vitro* studies have shown the capacity of chlorophyllins against different cultures of foodborne bacteria and fungi and even as decontaminating agents in fruits and vegetables, elongating the corresponding shelf lives.

Finally, the first evidences that support the role of chlorophyll derivatives as prebiotics has been obtained [37]. Authors demonstrated that chlorophyllin directly impact the gut

microbiota in mice, reducing the population of Firmicutes and restoring the Bacteroidetes phylum. The influence of chlorophylls in the rebalance of the gut microbiota is a promising research line.

Conclusions

Although the chlorophyll degradation pathway has been almost deciphered, different questions are still open: the exact reactions implied in the chlorophyll turnover, the physiological role of chlorophyllase or the nature of the chlorophyll transporters. On the other hand, recent advances have been produced in the knowledge of the regulatory mechanism of the chlorophyll breakdown and more new phyllobilins are continuously identified. The possibility of discovering new molecules of natural chlorophylls and the knowledge about the relationship between the structure and the health benefits is still challenged. Although certain taxonomic groups are a good source of new chlorophylls, the full exploitation of the antioxidant, antimutagenic and anti-inflammatory effects of these pigments in these groups are rarely observed. Great progresses have been done, however the study of the influence of the chlorophyll structure on the bioactivity of the molecules deserve future advances with important applications.

Conflict of interest statement

Nothing declared.

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Figure 1: Catabolism of chlorophylls. Upper figure: first reactions of the PAO/phyllobilin pathway during the senescence/ripening: including the chlorophyll cycle (from chlorophyll *b* to chlorophyll *a*) and the formation of pheophorbide *a* from chlorophyll *a* (showing the two alternative routes). Dotted arrows and green color show the plausible route for chlorophyll turnover. Enzymes implied in the pathway: NYC1: NONYELLOW COLORING 1; NOL: NYC1-LIKE; HCAR: HYDROXYMETHYL CHL *a* REDUCTASE; CHL: chlorophyllase; PPH: pheophytinase; SGR: stay-green; SGRL: SGR-LIKE; CLD1: CHLOROPHYLL DEPHYTYLASE1. Lower figure: oxidative reactions from chlorophyll *a*. The color of the nomenclature of the chlorophyll compounds is indicative of their approximate pigmentation.

Table 1 Distribution of chlorophylls types in major bioresources

Bioresources	Chl <i>a</i> (a)	Chl <i>b</i> (b)	Chl <i>c</i> (c)	Chl <i>d</i> (d)	Chl <i>f</i> (e)	BChl <i>a</i> (f)	BChl <i>b</i> (g)	BChl <i>c</i> (h)	BChl <i>d</i> (i)
Cyanophyta	+	(+)	(+)	(+)	(+)				
Rhodophyta	+			(+)					
Chlorophyta	+	+							
Charophyta	+	+							
Miozoa	+		+						
Haptophyta	+		+						
Euglenozona	+		+						
Glaucophyta	+								
Cryptophyta	+		+						
Cercozoa	+	+							
Ochromytha	+		+						
Bacillariophyta	+		+						
Photosynthetic bacteria						+	(+)	+	+
Bryophytes	+	+							
Pteridophytes	+	+							
Higher plants	+	+							

Chl *a*: chlorophyll *a*; (b) chl *b*: chlorophyll *b*; (c) chl *c*: chlorophyll *c*; (d) chl *d*: chlorophyll *d*; (e) chl *f*: chlorophyll *f*; (f) BChl *a*: bacteriochlorophyll *a*; (g) BChl *b*: bacteriochlorophyll *b*; (h) BChl *c*: bacteriochlorophyll *c*; (i) BChl *d*: bacteriochlorophyll *d*. Symbol between brackets means low presence.

Figure 2: Catabolism of chlorophylls: second part of the PAO/phyllobilin pathway during the senescence/ripening. The reactions comprised, in the chloroplast, the opening of pheophorbide *a* to form pFCC/FCC (primary or not, fluorescent chlorophyll catabolite) with RCC (red chlorophyll catabolite) as intermediate. Both compounds are exported from chloroplast to cytosol where FCC can be deformylated to DFCC (dioxobilin-FCC). Next, both compounds are imported in the vacuole where they are tautomerized to NCCs (no fluorescent chlorophyll catabolites) or DNCCs (dioxobilin-NCC). Enzymes implied in the pathway: PAO: PHEOPHORBIDE *a* OXYGENASE; RCCR: RCC reductase; TIC55: TRANSLOCON AT THE INNER CHLOROPLAST ENVELOPE55; CYP89A9: cytochrome P450 mono-oxygenase.

Table 2 Bioactive properties of different types of chlorophylls and their catabolites

Chlorophylls/catabolites	Bioactive properties	Reference
Chlorophyll <i>a</i>	antimutagenic, chemopreventive and antioxidant	[30, 33, 47]
Pheophytin <i>a</i>	antimutagenic, chemopreventive and anti-inflammatory	[30, 46, 47]
Pheophorbide <i>a</i>	antioxidant and chemopreventive	[47]
Chlorophyll <i>b</i>	antimutagenic and antioxidant	[46, 47]
Pheophytin <i>b</i>	antimutagenic and anti-inflammatory	[30, 47]
Pheophorbide <i>b</i>	antioxidant	[33]