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Development of an accurate and high-throughput methodology for structural comprehension of chlorophylls derivatives. (II) Dephytylated derivatives

Kewei Chen, José Julián Ríos, María Roca, Antonio Pérez-Gálvez*

Food Phytochemistry Department, Instituto de la Grasa, Consejo Superior de Investigaciones Científicas (CSIC), University Campus Pablo de Olavide, Building 46, Carretera de Utrera km. 1, Sevilla 41013, Spain

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ABSTRACT

Dephytylated chlorophylls (chlorophyllides and pheophorbides) are the starting point of the chlorophyll catabolism in green tissues, components of the chlorophyll pattern in storage/processed food vegetables, as well as the favoured structural arrangement for chlorophyll absorption. In addition, dephytylated native chlorophylls are prone to several modifications of their structure yielding pyro-, 13^2-hydroxy- and 15^1-hydroxy-lactone derivatives. Despite of these outstanding remarks only few of them have been analysed by MS^n. Besides new protocols for obtaining standards, we have developed a new high throughput methodology able to determine the fragmentation pathway of 16 dephytylated chlorophyll derivatives, elucidating the structures of the new product ions and new mechanisms of fragmentation. The new methodology combines, by first time, high resolution time-of-flight mass spectrometry and powerful post-processing software. Native chlorophyllides and pheophorbides mainly exhibit product ions that involve the fragmentation of D ring, as well as additional exclusive product ions. The introduction of an oxygenated function at E ring enhances the progress of fragmentation reactions through the β-keto ester group, developing also exclusive product ions for 13^2-hydroxy derivatives and for 15^1-hydroxy-lactone ones. Consequently, while MS^2-based reactions of phytylated chlorophyll derivatives point to fragmentations at the phytyl and propionic chains, dephytylated chlorophyll derivatives behave different as the absence of phytyl makes β-keto ester group and E ring more prone to fragmentation. Proposals of the key reaction mechanisms underlying the origin of new product ions have been made.

1. Introduction

Chlorophylls are considered the most important pigments on Earth considering their essential role in photosynthesis during the light-harvesting process. The biosynthetic machinery of plant tissues is able to metabolise more than 10^9 tons of natural chlorophylls per year, a figure to note the wide distribution of this family of green pigments and the significance of the intermediates and metabolites derived from that process [1]. The first reaction steps of the chlorophyll breakdown pathway involve the enzymatic loss of the phytyl chain [2] being chlorophyllides and pheophorbides key catabolites during the ripening/senescence of green tissues, or during storage/processing of foods, and the starting point of successive reactions implied in the catabolic pathway of chlorophyll. In addition, one of the few evidences to date related with the intestinal assimilation of chlorophylls in mammals shows that the dephytylation process is necessary to allow absorption of chlorophylls [3,4]. Consequently, it is essential to have a deep knowledge of the structural features of different dephytylated chlorophyll derivatives, to get accurate procedures to isolate the corresponding standards, as well as methodologies for unequivocal identification.

In this sense, natives chlorophyllides (dephytylated chlorophylls) and pheophorbides (Mg-free chlorophyllides) (Fig. 1) are very sensitive to a diversity of oxidants, and consequently they form very easily two groups of oxidised compounds. One modification is the substitution of the hydrogen atom at $C13^2$ by a hydroxyl group, i.e. the 13^2-hydroxy derivatives (Fig. 1) and the second alteration implies the rearrangement of the isocyclic ring, setting up a lactone group, the 15^1-hydroxy-lactone derivatives (Fig. 1). Finally, pyro-derivatives (pyro-chlorophyllides and pyro-pheophorbides) are formed due to the decarbomethoxylation at $C13^2$ position (Fig. 1) in heated, canned or storage food materials. Chlorophyllide *a*/*b* and pheophorbide *a*/*b* are widely found in plant materials such as green vegetables or fruits [5–7], edible

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* Corresponding author. Tel.: +34 954 61 15 50; fax: +34 954 61 67 90.
E-mail address: aperez@cica.es (A. Pérez-Gálvez).

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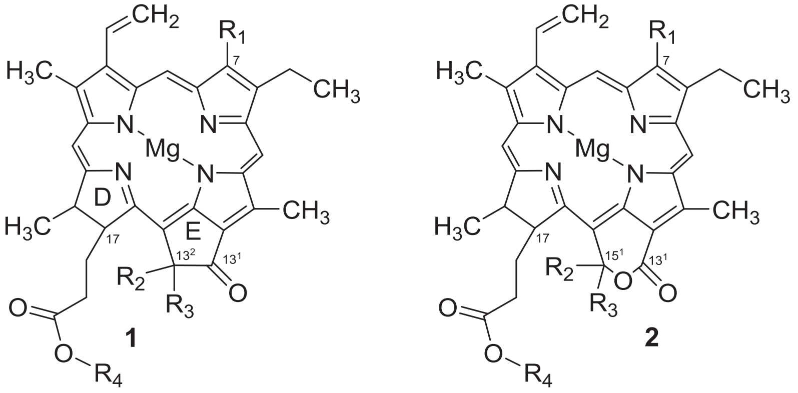

Compound	Structure	Mg[a]	R_1	R_2	R_3	R_4
Chlorophyllide *a*	1	+	CH_3	H	$COOCH_3$	hydrogen
13^2-Hydroxy-chlorophyllide *a*	1	+	CH_3	OH	$COOCH_3$	hydrogen
15^1-Hydroxy-lactone chlorophyllide *a*	2	+	CH_3	OH	$(15^2)COOCH_3$	hydrogen
Pyro-chlorophyllide *a*	1	+	CH_3	H	H	hydrogen
Chlorophyllide *b*	1	+	CHO	H	$COOCH_3$	hydrogen
13^2-Hydroxy chlorophyllide *b*	1	+	CHO	OH	$COOCH_3$	hydrogen
15^1-Hydroxy-lactone chlorophyllide *b*	2	+	CHO	OH	$(15^2)COOCH_3$	hydrogen
Pyro-chlorophyllide *b*	1	+	CHO	H	H	hydrogen
Pheophorbide *a*	1	-	CH_3	H	$COOCH_3$	hydrogen
13^2-Hydroxy pheophorbide *a*	1	-	CH_3	OH	$COOCH_3$	hydrogen
15^1-Hydroxy-lactone pheophorbide *a*	2	-	CH_3	OH	$(15^2)COOCH_3$	hydrogen
Pyro-pheophorbide *a*	1	-	CH_3	H	H	hydrogen
Pheophorbide *b*	1	-	CHO	H	$COOCH_3$	hydrogen
13^2-Hydroxy pheophorbide *b*	1	-	CHO	OH	$COOCH_3$	hydrogen
15^1-Hydroxy-lactone pheophorbide *b*	2	-	CHO	OH	$(15^2)COOCH_3$	hydrogen
Pyro-pheophorbide *b*	1	-	CHO	H	H	hydrogen

[a] for pheophorbide derivatives Mg atom is substituted by two hydrogen atoms

Fig. 1. Structural configuration, names and numbering system for parent chlorophyllide *a* and *b*, and their Mg-free derivatives originated by different protocols and analysed by HPLC–ESI–hrTOF-MS^n.

seaweeds [8], or natural herbs [9], the initial metabolites of chlorophyll degradation [2]. In heated, storage or canned food materials, pyro-pheophorbides are frequently present [9,10].

As their phytylated counterparts do, dephytylated chlorophylls show strong abilities of antioxidant activity towards free radicals, preventing lipid oxidation and oxidative damage [11,12] and potent anti-inflammatory activity *in vitro* [13]. Particularly, dephytylated chlorophylls (mainly pheophorbide *a*/*b* derivatives) are involved in the development of photodynamic therapy, where they behave as strong photosensitizers to catalyze the production of reactive oxygen species in cancer cells, when irradiated with a selected wavelength [14–16]. In addition to native pheophorbides, many *pheophorbide-like* compounds were identified in this research field, such as pyro-pheophorbides and derivatives [17,18], as well as oxidised pheophorbides including 13^2-hydroxy pheophorbide *a* and 15^1-hydroxy-lactone pheophorbide, arranged structurally as methyl ester [19].

Historically, the analysis of dephytylated chlorophylls has been limited to chlorophyllide, pheophorbide and pyro-pheophorbide. High performance liquid chromatography (HPLC) combined with MS have been applied to pigment separation and mass determination by means of different molecular ionisation methods and *m*/*z* analyzers, including fast atom bombardment mass spectrometry (FAB-MS) [20,21], atmospheric pressure chemical ionisation mass spectrometry (APCI-MS) [9,22,23] and matrix-assisted laser desorption/ionisation–time-of-flight mass spectrometry (MALDI–TOF-MS) [24]. However, data available of the product ions are limited to chlorophyllide and pheophorbide [21,25].

The objective of this work is the development of a new high-throughput methodology able of determining the fragmentation pathways of 16 dephytylated chlorophyll derivatives, and interpreting the possible structures of the new product ions and the novel mechanisms of fragmentation. To achieve this aim, it was necessary to develop new protocols to produce and isolate the standards of dephytylated chlorophyll derivatives, and furthermore to select more efficient ionisation technique taking into account the chemical behaviour of the standards analysed. The proposed methodology combines by first time, high resolution time-of-flight (hrTOF) mass spectrometry with a powerful post-processing software to the MS^n analysis of a complete set of dephytylated chlorophyll derivatives. The former measures the accurate mass of a selected molecular/product ion, while the latter allows prediction structural arrangement of new product ions not described so far. The blend of these useful tools helps to progress on the knowledge of the fragmentation pattern of dephytylated chlorophyll derivatives, compounds continuously present in our diet and, some of them intermediates of significant metabolic processes in plant tissues or arising from the absorption process in the gut of mammals.

2. Materials and methods

2.1. Reagents

Selenium dioxide (sublimed for synthesis, 98%), ammonium sulfate (99.5%), anhydrous sodium sulfate (99.0%) and silica gel 60 GF_{254} for thin layer chromatography were supplied by Merck (Darmstadt, Germany). Potassium phosphate (98%), ammonium acetate (98%), Tris–HCl, Triton X-100, sodium hydroxide, sodium chloride (99.5%), chlorophyll *a* and *b* were provided by Sigma–Aldrich (St. Louis, MO, USA). Pheophorbide *a* was purchased