Review

Rhamnogalacturonan Endolyase Family 4 Enzymes: An Update on Their Importance in the Fruit Ripening Process

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Abstract: Fruit ripening is a process that produces fruit with top sensory qualities that are ideal for consumption. For the plant, the final objective is seed dispersal. One of the fruit characteristics observed by consumers is texture, which is related to the ripening and softening of the fruit. Controlled and orchestrated events occur to regulate the expression of genes involved in disassembling and solubilizing the cell wall. Studies have shown that changes in pectins are closely related to the loss of firmness and fruit softening. For this reason, studying the mechanisms and enzymes that act on pectins could help to elucidate the molecular events that occur in the fruit. This paper provides a review of the enzyme rhamnogalacturonan endolyase (RGL; EC 4.2.2.23), which is responsible for cleavage of the pectin rhamnogalacturonan I (RGL-I) between rhamnose (Rha) and galacturonic acid (GalA) through the mechanism of β-elimination during fruit ripening. RGL promotes the loosening and weakening of the cell wall and exposes the backbone of the polysaccharide to the action of other enzymes. Investigations into RGL and its relationship with fruit ripening have reliably demonstrated that this enzyme has an important role in this process.

Keywords: fruit ripening; rhamnogalacturonan-I; rhamnogalacturonan lyase enzyme; cell wall remodeling

1. Introduction

Ripe fruit has organoleptic qualities, such as flavor, aroma, and texture, that are optimal for consumption [1]. Texture influences the molecular mechanisms that result in changes to phytohormone levels, protein synthesis and volatile compounds, chlorophyll degradation, the accumulation of fat-soluble pigments, solubilization, and disassembly of the polysaccharides of the cell wall and middle lamella [2]. During the fruit ripening process, changes associated with texture lead to reduced firmness, a short shelf life, and susceptibility to attack by pathogens and other organisms [3,4].

To understand this phenomenon from the primary cell wall degradation perspective, studies have been performed on enzymes that cleave links between polysaccharides such as cellulose, hemicellulose, and pectins. The cell wall composition varies between species and tissues, and conditions such as biotic and abiotic stress can affect their composition [5–7]. Among the pectins, rhamnogalacturonan I polysaccharide (RG-I) is an interesting polymer to study, because an increase in the activity of the enzymes α-arabinofuranosidases and β-galactosidases has been reported to alter RG-I levels at different stages of fruit ripening. These enzymes cleave ramifications of arabinans and galactans; as a consequence, during ripening, an increase in solubilization and depolymerization occurs in this type of pectin,

as in the case of European and Chinese pear, banana, strawberry, and apple [8–11]. The rhamnogalacturonan endolyase enzyme (RGL), which is responsible for cleaving the back-bone of RG-I, appears to be an active element in fruit ripening [12,13]. Therefore, RGL has been a target of RNAi experiments and heterologous expression to understand its relevance in plant development and fruit ripening [14,15].

This review offers a panoramic view of our current knowledge about α-L-rhamnopyran osyl-(1→4)-α-D-galactopyranosyluronate endolyase, also known as rhamnogalacturonan endolyase or RGL, which belongs to polysaccharide lyase group four. Here, we describe their role in fruit ripening and present new perspectives for investigation into these enzymes.

2. Rhamnogalacturonan-I in the Fruit Ripening

Pectins are abundant polysaccharides involved in the growth and development of cells; they are mainly present in the middle lamella, which are soft tissues in the joint between the primary and secondary cell walls [16]. Seventeen different polysaccharides and twenty types of links compose three types of pectins described in plants: homogalacturonan (HG), rhamnogalacturonan-I (RG-I), and rhamnogalacturonan-II (RG-II) (Figure 1) [17]. These polysaccharides are found in average proportions of 65%, 20–35%, and 10% [18]. Kaczmarska et al. [19] presented an elaborate review of RG-I focused on the structure and functionality of this polysaccharide in the plant cell wall.
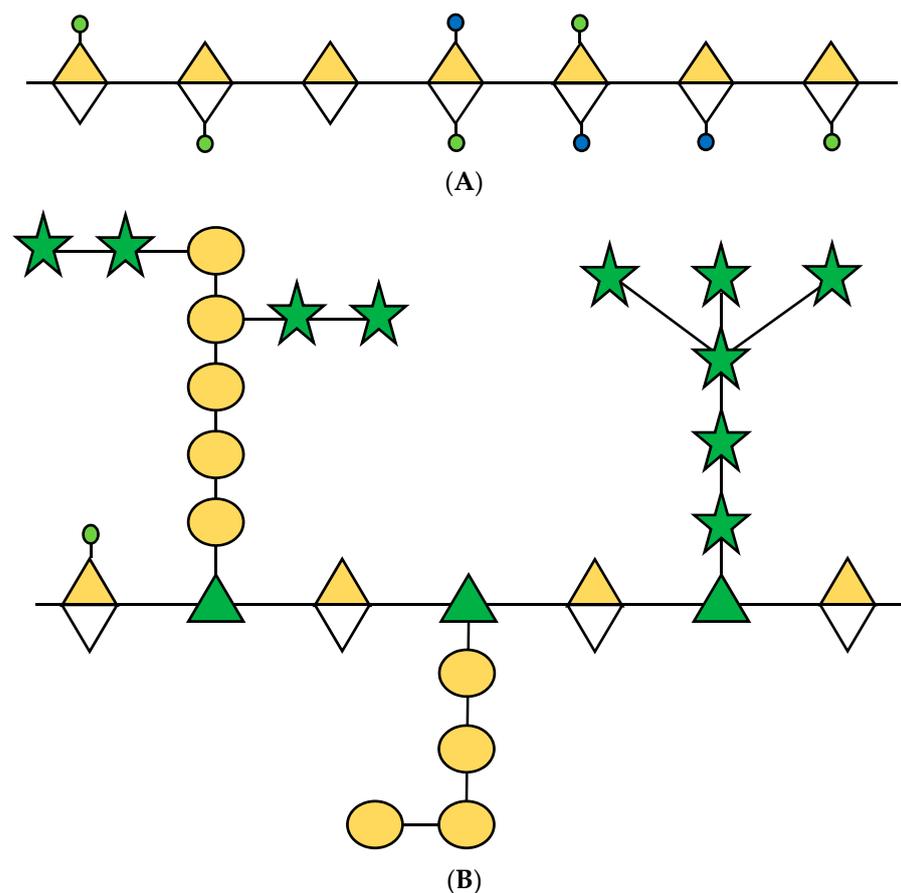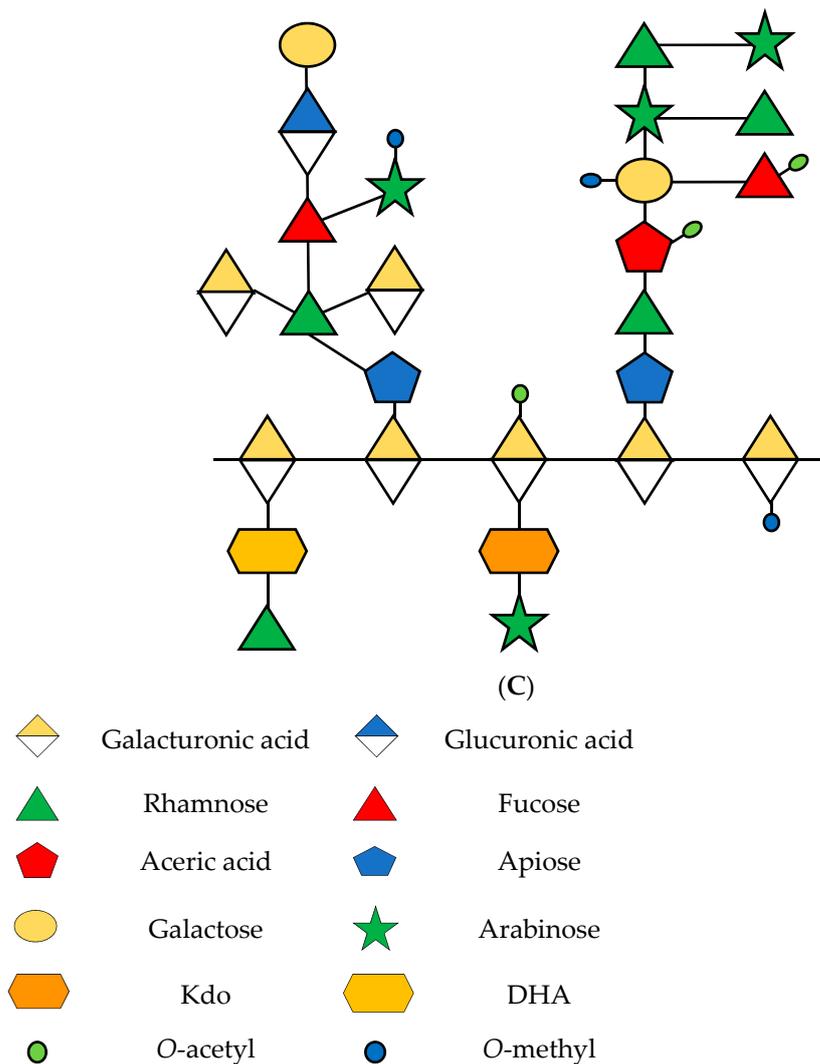

(A)

(B)

Figure 1. *Cont.*

Figure 1. Schematic representation of principal pectins in the primary cell wall of plants. (**A**) HG; (**B**) RG-I and (**C**) RG-II. Sugars are represented according to the Symbol Nomenclature for Glycans (SNFG) [20]. Based on [16,21,22].

The structure of RG-I is composed of alternating units of rhamnose (Rha) and galacturonic acid (GalA), linked to α-1,2 and α-1,4, respectively. In addition, Rha can be found in conjunction with a various proportions of galactose (Gal) and arabinose (Ara) [23].

The differences in fruit ripening and softening between different kinds of fruits depend on the cellular structure and composition, as well as enzymes present in the cell wall during this process [24].

Peña and Carpita [25] described RG-I debranching resulting in the loss of arabinans with high sugar units and correlated that loss with changes in the firmness and cell separation in the fruits of four cultivars of apple that had been stored for a long time. In olive fruit development, in the transition between the cherry and black stages during ripening, a release of the side chains of RG-I occurs [26]. In particular, the release of side chains of arabinans helps determine the ripening stage [27].

In *Dacryodes edulis*, a fruit from Africa, a 50% loss of the harvest has been described as a result of a rapid reduction in firmness. The analysis of the pectins that compose the fruit indicates that in the ripening stage, it is possible to find low molecular weight RG-I because of the modifications to this polysaccharide during the fruit ripening process [28].

3. Structural and Phylogenetic Classification of the Rhamnogalacturonan Endolyase Enzyme

The RGL enzyme is not only found in plants but has also been described in saprophytic fungi and bacteria of the genera *Aspergillus*, *Bacillus*, and *Dickeya* [29–32]. This species secretes enzymes into the extracellular environment that cleave the cell wall polysaccharides of plants for use as a carbon source [33]. Silva et al. [34] detailed a comprehensive review of endo- and exohydrolases that cleave RG-I. The authors built a phylogenetic tree in which RGL was classified into two groups: polysaccharide lyase 4 (PL4) and polysaccharide lyase 11 (PL11). This information is directly related to a report by Lombard et al. [35], which explains that enzymes in the same PL group have the same enzymatic activity but a different three-dimensional structure due to convergent evolution from different scaffolds. For this reason, the PL4 and PL11 enzyme groups cleave the same linkages, but their proteins differ structurally. In addition, PL4 is present in plants and fungi, and PL11 is present solely in bacteria. In particular, heterologous expression of two RGLs of the PL11 group from *Bacteroides ovatus* revealed a different preference for substrates: the Bo4416 clone prefers RG-I without substitutions in its side chains, while Bo3128 degrades polysaccharides with short substitutions in their side chains [36]. This result implies that different isoforms of the same enzyme have activity depending on the side chains of the substrate. This phenomenon has not been reported or studied in the RGLs of plants or fungi.

Garron et al. [37] described the structure of the PL4 group of enzymes as having three β-sandwich domains and that of the PL11 group as having eight bladed β-propellers. Furthermore, both groups depend on Ca^{2+} ions for enzymatic activity, where the β-elimination catalytic mechanism requires a Ca^{2+} ion coordinated between sites -1 and $+1$ [38]. Nevertheless, other reports indicate that Ca^{2+} is a hexacoordinated ion that interacts with five protein ligands and one water molecule and plays a structural role in promoting catalysis [39]. The enzymatic activity of RGL in plants is suppressed in the absence of Ca^{2+} ions. A 2 mM concentration of $CaCl_2$ recovered the activity to 100% [15].

In the Protein Data Bank (https://www.rcsb.org/, accessed on 25 January 2022), four crystallographic structures of RGL from the PL4 group exist in fungi and bacteria. All reported structures share Ca^{2+} ions as single molecules coordinated by protein-ligand residues [31,39] (Table 1). Although there are no crystallographic structures of RGL in plants, sequence alignment between RGL from *Fragaria chiloensis* and *Aspergillus aculeatus* shows a high percentage of identity in the carbohydrate binding module (CBM) [15].

Table 1. Crystallographic structures of RGL belonging to the PL4 group, deposited in the Protein Data Bank.

PDB ID	Specie	Reference
3NJX	*Aspergillus aculeatus*	[31]
2XHN	*Aspergillus aculeatus*	[31]
3NJV	*Aspergillus aculeatus*	[31]
1NKG	*Aspergillus aculeatus*	[39]

The PL4 group of RGL enzymes (EC 4.2.2.23) cleave the backbone of RG-I internally between L-α-rhamnopyranosyl-(1→4)-α-D-galactopyranosyluronic acid bonds. The result of this cleavage is the formation of L-rhamnopyranose at the reduced end and 4-deoxy-4,5-unsaturated D-galactopyranosyluronic acid at the nonreduced end [40]. Three functional domains have been reported in plant RGLs, similar to those in *A. aculeatus*: domain I or the catalytic domain; domain II or the fibronectin type III-like domain (FnIII); and domain III or the carbohydrate binding module (CBM) (Figure 2) [15,35].

To determine the phylogenetic classification of RGLs, Mokshina et al. [41] built a phylogenetic tree using 98 plant-derived sequences containing the three functional domains. Six classification groups were generated. Similar to dicotyledonous plants, monocotyledonous plants are classified into two clades tagged as Groups I and II. Vascular plants and primitive angiosperms are classified into two separate clades. In *F. chiloensis*, similar results have

been obtained, where dicot plants formed two clades, while monocots formed a different group [15]. Unfortunately, the authors of both reports did not establish which sequences or regions were key to the differences in clade classification. However, monocots could be classified into another group, due to their low level of pectins; for that reason, there could be a smaller number of isoforms.
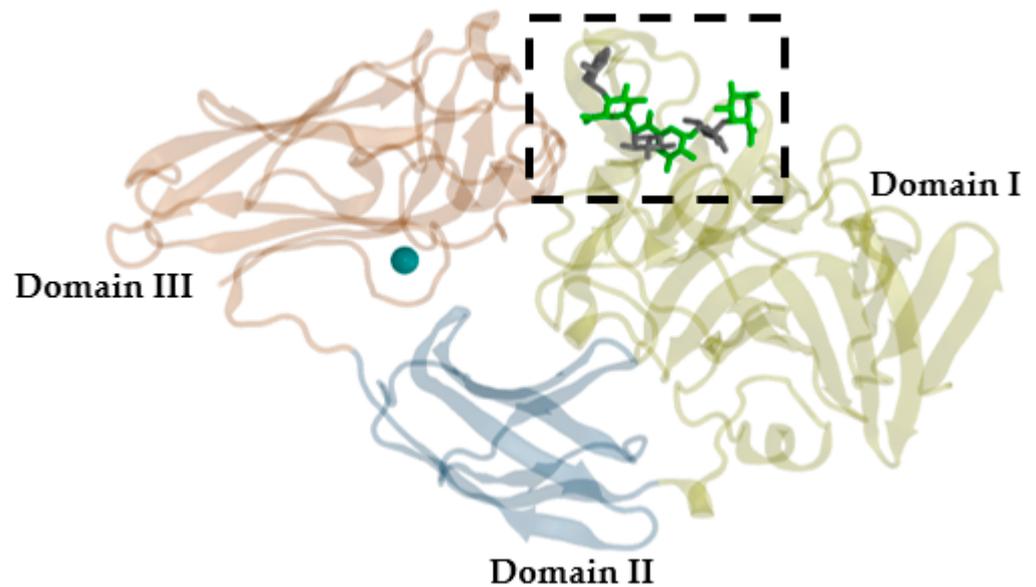
(**A**)
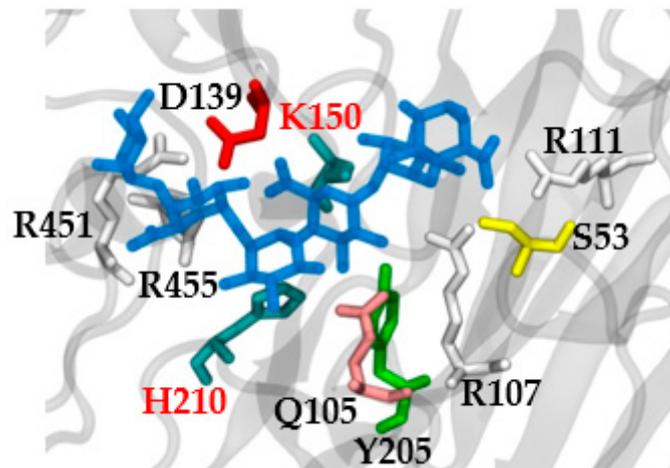
(**B**)

Figure 2. (**A**) Three-dimensional protein structure of RGL from *A. aculeatus* (PDB ID 1NKG) with a crystalized ligand from PDB ID 3NJV with the K150A mutation. Every domain is tagged, and the calcium ion is represented in VDW (green). The active site is shown in the dotted box. (**B**) Active site zoom (dotted box Figure 1A); the ligand is in blue, and the different amino acids are presented in different colors. H210 and K150 are key amino acids for enzyme activity [31]. The representation was created with Visual Molecular Dynamics [42].

4. Rhamnogalacturonan Endolyase in Fruit Ripening

As mentioned earlier, changes in fruit texture are directly associated with the molecular event that alters the cell wall composition, either by depolymerization of glycans and polysaccharides, pectin solubilization, or the loss of neutral sugars during pectin ramification [43] (Table 2). Wang et al. [44] summarized genes that code for enzymes that

participate in the fruit ripening process as those with activity on fruit pectins, where the publication by Molina-Hidalgo et al. [14] focused solely on an RGL of *F. x ananassa*. At the molecular level, the gene that encodes *FaRGlyase1* is located in a locus related to fruit hardness and firmness. The transcript levels of this gene increase as the fruit ripens, whereas silencing of this gene by agroinfiltration of RNAi into fruits indicates that no phenotypic differences are observed; however, at the histological level observed by pectin staining, the cell wall remains more intact with RGL inhibition. Similar experimental results were reported by Méndez-Yáñez et al. (2020) [15], who observed an increase in the transcript levels of the gene that codes for RGL during fruit ripening. Using the heterologous expression of RGL from *F. chiloensis* in *Pichia pastoris* yeast, the biochemical parameters and enzymatic characterization of the enzyme were obtained. An assay in seeds of *A. thaliana* dyed with ruthenium red demonstrated that the heterologously expressed enzyme was active and could cleave seed mucilage, which is rich in non-ramified pectins [45]. Promoter analysis of *Fragaria vesca* accounts for the increased regulation of response elements for phytohormones, such as abscisic acid (ABA), methyl jasmonate, gibberellins, salicylic acid, and auxin, and stress response, such as drought, defense, and low temperature [46].

RGL enzymes have also been studied in the fruit ripening process of tomato (Table 2). Unlike strawberry, tomato is a climacteric fruit, and for this reason, the crosstalk between phytohormones and transcription factors associated with the ripening process differs considerably [47]. Promoter analysis of the gene that encodes RGL in *S. lycopersicum* and *A. thaliana* demonstrated a response to ABA, dehydration, and light [48]. In another study, the expression of the RGL promoter *Solyc11g011300* was constitutively induced in fruits via the CaMV35S sequence and tagged with GUS as a reporter gene; the results showed that gene expression was first observed in the red ripe stage [49]. On the other hand, constitutive expression of three RGL isoenzymes during the development and ripening of *S. lycopersicum* fruit resulted in firmer tomatoes with a longer shelf life due to a cosuppression mechanism [12]. One of the genes evaluated displays a strong correlation with the increase in ethylene production and therefore could be important for the loss of firmness. Regarding the expression of the other genes, the authors suggest that they participate in the modification of RG-I at the initial stages of the ripening [13].

Table 2. Summary of results obtained in research on RGL, until the year 2022.

Specie	Results	Reference
F. x ananassa	Changes in the spatio-temporal expression of *FaRGlyase1* across the fruit ripening. Transient silencing of *FaRGlyase1* induces a high conservation of cell wall. *FaRGlyase1* expression could be induced by abscisic acid. *FaRGlyase1* gene is located in a QTL linked to firmness.	[14]
F. chiloensis	Structural characterization and biochemical parameters of RGL enzyme. Transcripts levels increase during fruit ripening. Phylogenetic tree with same results according to Mokshina et al. (2018) [37]. Calcium dependence on enzymatic activity.	[15]
F. chiloensis	Elements of response to phytohormones: ABA, methyl jasmonate, gibberellins, salicylic acid, and auxin; response elements to biotic and abiotic stress such as defense, low temperatures, and drought.	[46]
S. lycopersicum L.	*GUS* under the control of an RGL promoter induces expression in the red ripe stage of fruit ripening. Increased expression levels of *GUS* 40 days after anthesis.	[49]
S. lycopersicum L.	Constitutive expression of RGL promoter to produce tomatoes with greater firmness and a longer shelf life. Overexpression under CaMV35S of *Solyc11g011300* promoter reduces viability and pollen germination and decreases the number of seeds and fruits. Delay in fruit ripening of one week.	[12]

Table 2. *Cont.*

Specie	Results	Reference
S. lycopersicum L. and *A. thaliana*	RGL genes respond to abscisic acid, dehydration, and light.	[48]
S. lycopersicum L.	*Solyc11g011300* gene increases activity in fruit ripening, correlated with ethylene production increasing. *Solyc04g076630* and *Solyc04g076660* could participate in re-engineering on RG-I pectin at the beginning of fruit ripening.	[13]

5. New Perspectives and Challenges

A large battery of enzymes participate in the fruit ripening process, acting in a coordinated manner to disassemble the cell wall. Environmental and other signaling conditions, phytohormones, and transcription factors, among others, are responsible for regulating the protein level and activity of these enzymes. The matrix formed by cellulose, hemicellulose, and pectin constitutes one of the most important components of the plant cell wall and is a key target for enzymes and proteins during the developmental processes of fruit ripening and softening. In this matrix, RG-I accounts for almost 30% of the pectic fraction and is of paramount importance for understanding the structural dynamics during different physiological processes. The challenge for researchers is to identify the key enzymes that take part in these different processes with special attention given to fruit ripening and conditions needed for adequate enzyme performance. The integrative understanding of the participation of each of these members, specifically RGLs, in the changes in fruit texture could help to create strategies that delay their emergence or inhibit their activity, thus prolonging the shelf life of the fruit. Possible strategies will be generated depending on the role each enzyme plays in modulating the functional properties of the cell wall at a specific developmental stage.

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