Biocatalytic cross-linking of pectic polysaccharides for designed food functionality: Structures, mechanisms, and reactions

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Abstract

Recent research has demonstrated how cross-linking of pectic polysaccharides to obtain gel formation can be promoted by enzymatic catalysis reactions, and provide opportunities for functional upgrading of pectic polysaccharides present in agro-industrial sidestreams. This review highlights the mechanisms of formation of functional pectic polysaccharide cross-links, including covalent cross-links (notably phenolic esters and uronyl ester linkages) and non-covalent, ionic cross-links (which involve calcium and borate ester links). The treatise examines how such cross-links can be designed via specific enzymatic reactions, and highlights the most recent data concerning enzyme catalyzed engineering of cross-links for in situ structural design of functional properties of foods.

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1. Introduction

There is a large body of research data available that has aimed at unraveling the structures of plant cell wall polysaccharides, including pectin and various pectic polysaccharide elements, in order to understand their biosynthesis and cell wall functionality in planta.
(Cosgrove, 2000; Ridley et al., 2001; Caffall and Mohnen, 2009). Plant cell walls consist of complex matrices of polysaccharides, which (particularly in the primary cell walls) include cellulose, hemicelluloses, and pectin, and, depending on the tissue and type of cell wall, may include various levels of lignin. Each of these components have different structural complexities and play different physiological roles in plants (Caffall and Mohnen, 2009). In addition to the distinct significance of each type of polysaccharide in plant cell walls, various covalent and non-covalent cross-links exist between the polysaccharides within the cell wall matrices. These cross-links and conjunctions include ionic bridges, borate-diol ester bonds, hydrophobic interactions, di-ferulic acid structures (i.e. dehydrodiferulic acid conjugates), and presumably also some other types of bonds including other covalent bonds (Sila et al., 2009). These different types of pectic cross-links appear to exert crucially significant functions in the plant during cell wall growth and development, and they also have a distinct impact on the physical and macromolecular properties of plant materials and in turn on plant food functionality, processing, and quality (Parr et al., 1996; Waldron et al., 2003; Singh et al., 2010). Hence, the control of and/or the controlled manipulation of these polysaccharide cross-links can be used to design certain functionalities in foods and provide a way for utilization of pectic polysaccharide functionalities in various other applications. Pectins or pectic polysaccharides are already widely used as food ingredients in various applications, e.g. as gelling agents and emulsion stabilizers (Itoh et al., 2011; Munarin et al., 2011; Takei et al., 2011), and for food applications such as for biomedical and biopharmaceutical purposes (Ngouemezong et al., 2012a, b; Zaidel et al., 2011, 2012; Zeeb et al., 2012), and the most recent functionalities in various other applications. Pectins or pectic polysaccharides are already widely used as food ingredients in various applications, e.g. as gelling agents and emulsion stabilizers (Itoh et al., 2011; Munarin et al., 2011; Takei et al., 2011) or as new components in (bio)plastic manufacture (Liu et al., 2011).

Significant progress has recently been achieved within exploitation of enzymatic reactions for improving pectic polysaccharide functionality for these applications (Ngouemezong et al., 2012a, b; Zaidel et al., 2011, 2012; Zeeb et al., 2012), and the most recent direction of this research includes the use of enzyme catalyzed modifications for valorization of agro-industrial byproduct streams via controlled improvement of textural and macromolecular properties of specific structural elements of pectin (Zaidel et al., 2011, 2012; Min et al., 2011; Fissore et al., 2012). A crucially important prerequisite for the further development of these novel applications is the understanding of the chemical structures, reactions, and mechanisms of the different cross-links that determine the macromolecular properties of the cross-linked materials. The purpose of this review is (i) to highlight the different types of naturally occurring cross-links in various polysaccharide matrices, focusing mainly on pectin polysaccharides, and (ii) to examine the chemical structures and the mechanisms of cross-linking that can be induced directly or indirectly by enzyme catalyzed reactions, and in turn be used to design specific rheological or other functional traits. The treatise will also include an overview of the application of enzymatically cross-linked polysaccharides for structural design of food macromolecular properties, and discuss the functional assessment methodologies used to evaluate these properties.

2. Structures of pectic polysaccharides

Pectic polysaccharides can principally be divided into three main types of structures having different backbones of homogalacturonan (HG), rhamnogalacturonan I (RGI) and rhamnogalacturonan II (RGII), respectively, which are covalently linked to each other (Willats et al., 2006; Coenen et al., 2007; Holck et al., 2011) (Fig. 1A). HG mainly consists of linear α-(1,4)-linked-galacturonic acid residues, and the galacturonic acid (GalpA) moieties within this backbone may be methyl esterified at C-6 and/or O-acetylated at the C-2 and/or C-3 position, and certain HG stretches may be extensively substituted with xylose (β-(1,3)-Xylp substitutions) to form xylagalacturonan (Voragen et al., 2009). The RGI backbone is made up of consecutive repeating units of [−2-α-L-Rhap-(1,4)-α-D-GalpA–1→] and the rhamnose moieties of the RGI may be substituted at the O-4 position with different glycan side chains including α-(1,5)-linked-arabinans, β-(1,4)-linked-galactan (Oosterveld et al., 2000) and/or arabinogalactan I (AGI) or arabinogalactan II (AGII) (Lerouge et al., 1993) (Fig. 1A). AGI is composed of a β-(1,4)-linked-galactan backbone with Ara residues attached to O-3 of the galactosyl residues whereas AGII is substituted by short chains of α-(1,6)-linked-Ara–β-(1,6)-linked-GalpA-p (n = 1, 2 or 3) (Ridley et al., 2001), and the galactosyl residues of the side chains can be substituted with α-(1,3)-linked-Ara. On the RGI side chains, feruloyl groups, either as single ferulic acid (FA) moieties or in the form of ferulic acid dehydrodimers (diFAs), are esterified to the O-2 position of the Ara residues in the α-(1,5)-linked-arabian backbone, but may also be bound to the O-5 on the terminal arabinose (Levigne et al., 2004), or, to a much lesser extent, to the O-6 position of the galactopyranosyl (Galp) residues in the β-(1,4)-galactan chains (Colquhoun et al., 1994). The exact abundance and distribution of the FA substitutions vary among different plants and among different plant tissues. In for example sugar beet pectin the arabinan side chains on RGI contain about 0.7–0.8 wt% of FA, with approximately 0.1 wt% diFAs (Micard et al., 1997; Zaidel et al., 2011) whereas in e.g. potato RGI side chains there is less than 0.05 wt% FA and hardly any diFAs (Singh et al., 2011). Rhamnogalacturonan II (RGII) contains eleven different glycosyl residues which are attached as side chains (assigned A to D) to the HG backbone built of α-(1,4)-linked-GalpA residues, hence despite the name RGII there is no rhamnogalacturonan galactan backbone made up of repeating units of [−2-α-L-Rhap-(1,4)-α-D-GalpA–1→] and the side chains protrude directly from the C-2 and C-3 of the GalpA moieties (Whitcombe et al., 1995; Ridley et al., 2001). In spite of its complexity and low amount in the cell wall (typically ~1–4 wt%) (O’Neill et al., 2004), RGII is thought to have a highly conserved structure and to play an important role in the plant cell wall functionality (Willats et al., 2006).

3. Covalent cross-linking of pectic polysaccharides

The physico/chemical properties of pectins and notably their gelation ability rest on different kinds of cross-linking mechanisms involving different structural entities of the pectin molecule(s). In addition to the ongoing research concerning the biosynthesis, structure and functionality of pectic polysaccharides during plant growth and development, a relatively large number of studies have been published recently concerning modification, including enzymatic modification, of polysaccharide cross-links in situ for food applications (Table 1).

As detailed further below, two main types of linkages can be promoted either directly or indirectly via enzymatic catalysis on pectic polysaccharides; these types include: (i) ionic cross-linking of HG taking place via divalent cation bridges, and (ii) phenolic ester oxidative cross-linking between feruloyl groups on the side chains of RGI. As discussed further, below, RGII cross-links via borate ester bonds, but it is uncertain if this cross-linking can be promoted by enzyme catalysis, e.g. if shorter RGII moieties resulting from enzyme catalyzed cleavage of adjacent backbone stretches of the RGII/HG chains increase the cross-linking propensity (Table 1).

3.1. Ionic cross-links

3.1.1. Cross-linking mechanism and reaction conditions

Ionic cross-linking mainly refers to the interaction via divalent cations between two non-esterified GalpA moieties in
The divalent cations are very often Ca^{2+} ions that can interact with the negatively charged carboxylate ions on C-6 of non-esterified GalpA. These interactions are widely referred to as ionic junction zones once a number of consecutive GalpA moieties are aligned in the cross-linking (Kastner et al., 2012). The junctions connect two HG chains in an anti-parallel fashion at a time, and may form larger pectate networks (Fig. 1B). This type of ionic cross-linking and the properties of the gel

(low methylated) HG. The mixture of pectic polysaccharides backbone and side chains, and three types of covalent cross-links by (B) calcium bridges, (C) ferulic acid oxidation and (D) RGII-borate ester.
Table 1
Different types and mechanisms of cross-linking in polysaccharides in situ by direct or indirect enzymatic reaction/modification for designed functionality.

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produced from this cross-linking are the basis for the long standing use of pectin as a gelating agent in foods. The extent of cross-linking as well as the gelation are obviously influenced by (i) the pattern and degree of methoxylation (DE) of the HG (Willats et al., 2001), (ii) the substrate chain length (DP of HG), (iii) the Ca$^{2+}$ level, and (iv) the pH. Low methylated HG (DE < 50%) is easily cross-linked by Ca$^{2+}$, however for highly methylated HG (DE > 50%), demethoxylation of HG has been shown to increase the ability of the HG polysaccharides to be cross-linked by Ca$^{2+}$. Depending on the reaction conditions, and the type of substrate used, gelation occurs when 7 to 14 consecutive non-esterified Gal residues are coordinated in this way (Fraeye et al., 2010b) (Fig. 1B). Short chains (or low DP) can form less ionic junction zones per molecule which decreases the extent of cross-linking and reduces the overall gel strength, as e.g. evidenced by the finding that fragments from three different size-classes of DP 2–13 (low DP), DP 7–21 (medium DP) and DP 13–42 (high DP), respectively, exhibited different yield stress, with the high DP size cross-linked showing a higher yield point than the lower DP substrates, suggesting stronger binding of GalpAs by Ca$^{2+}$ with higher DP (Luzio and Cameron, 2008).

Demethoxylation of methylated GalpA can be catalyzed enzymatically by pectin methyl esterases (PMEs) (EC 3.1.1.11). PMEs thus catalyze the hydrolysis of the methyl esters in methylated GalpA with formation of carboxylic acid groups in HG during methanol release. PMEs from different sources, plant or fungi, produce different demethoxylation patterns. In general, PMEs from fungal sources, usually having an acidic pl, form relatively random patterns of carboxylic acids in HG, whereas PMEs from plants, which often have neutral or alkaline pl, catalyze the demethoxylation of longer consecutive stretches of the methoxylated GalpAs in HG before attacking the next chain, resulting in blockwise demethoxylation patterns in the HG (Fraeye et al., 2010a). Because of the blockwise demethoxylation pattern, plant derived PMEs generally produce Ca$^{2+}$ sensitive pectins (i.e. with a high gelation propensity), whereas fungally derived PMEs may also be used to design other types of macroscopic functionalities than gelation via PME catalyzed modification of the HG backbone (Willats et al., 2006). Some plant PMEs have been reported to exhibit different modes of action depending on the pH, hence at pH 7 these plant PMEs were shown to exhibit a typical single chain blockwise demethoxylation pattern but at pH 4.5 this same plant PME catalyzed more random cleavage of esterified carboxylic group on the HG backbone (Denes et al., 2000). This difference with pH may be related to changes of the specificity of the enzymes via enzyme-protein factors, or a result of changes in the charge of the partially demethoxylated HG substrate in response to pH (see below). In any case more specific and controlled patterns of demethoxylation can be designed by use of enzymes than via chemical (alkaline) reaction which generates more random demethoxylations in HG backbone (Fraeye et al., 2010a). Gelation of HG “pectin” may also take place directly via hydrophobic interactions between methylated GalpAs or via hydrogen bonding of undissociated carboxyl groups on neighboring HG chains (Kastner et al., 2012). Since the ionic bonds or bridges require dissociated carboxyl groups this type of gelation is favored at higher pH than the type of cross-linking taking place via hydrogen bonding between undissociated carboxyl groups.

In addition to the pH and reaction temperature, the activity of PMEs may be influenced by other reaction parameters. Notably, the activity of PME used to promote pectin gelation has been reported to be influenced by salt addition, hence the PME
deesterification with PME from orange peel was reported to increase by 14% when NaCl was added to the polysaccharide solution of citrus pectin (Yoo et al., 2003). Depending on the DE, the level of Ca\(^{2+}\) added into the low methylated HG pectin also influences the properties of the pectin gel; a higher concentration of Ca\(^{2+}\) generally promotes a stronger gel (Ngouémazong et al., 2012d), but excess Ca\(^{2+}\) has been shown to induce aggregation of the ionic binding via weak electrostatic interactions (Braccini and Pérez, 2001).

3.1.2. Enzymatic pectin modification to promote ionic cross-links and changed pectin functionality

Modification of pectin via enzymatic reaction using PMEs from various sources (plants or fungi) has been shown to improve the functionalities of pectin, particularly in food applications (Willats et al., 2006). These modified functionalities include the change in the properties of the Ca\(^{2+}\)-pectin gels by the difference in the degree and pattern of methoxylation of the pectin (Willats et al., 2001; Fraeye et al., 2010a; Ngouémazong et al., 2012b,c). In general, from this type of modification, the gels produced from a blockwise pattern of demethoxylation (using plant PME) are weaker or more brittle than those produced by random demethoxylation (using fungal PME) where the gels are stronger or more elastic. Combination of both plant and fungi PMEs for demethoxylation of GalPAs on HG has shown a synergistic effect on the Ca\(^{2+}\)-pectin gel: producing a more stable and stronger gel (Yoo et al., 2009a; Ngouémazong et al., 2012b). A tailored demethoxylation of GalPAs on HG by chemical (NaOH) reactions produced weaker Ca\(^{2+}\)-pectin gels compared to the enzymatic demethoxylation induced by plant PMEs (Thanathan-Nasser et al., 2011). The demethoxylation of high-methylated (HM) pectins catalyzed by PMEs produces (low methoxyl) calcium sensitive pectins which can then form a gel in the presence of calcium without addition of sucrose (Hotchkiss et al., 2002), however, addition of sucrose to the Ca\(^{2+}\)-pectin gel has been shown to support the gelation by binding of water and thus promoting close contact of neighboring molecules (Kastner et al., 2012). Furthermore, the gel formed from the calcium sensitive pectin was softer than a LM pectin control gel due to higher water retention, which is more desirable for dietetic food and other food applications. Enzymatic modification of pectin by PMEs can also influence the release of flavor in the pectin gels i.e. flavor release may increase when the DE of pectin decreases (Yoo et al., 2009b). Functionalization of pectin in stabilization of juices has been shown to slightly improve when PMEs are added since the PME catalysis induced cloud loss in the juice (Corredig et al., 2001; Wicker et al., 2003; Croak and Corredig, 2006). Modified pectins catalyzed by PMEs were also reported to have a better interaction with acidified caseins than non-PME treated controls (Kim and catalyzed by PMEs were also reported to have a better interaction with acidified caseins than non-PME treated controls (Kim and Croak and Corredig, 2006)).

3.1.3. Macromolecular functionality assessment of the ionic cross-links

The functional effects of this type of ionic cross-linking can be assessed by several different methods (Table 1). The measurement of the change in molecular size distribution has been widely used, since the gradual Ca\(^{2+}\)-pectin cross-links will form larger molecular sizes than the corresponding non-gelated pectic polysaccharides, however, other methods, including direct titration of the PME reaction (Hotchkiss et al., 2002), NMR analysis to determine the mode of action of PME catalysis (Denès et al., 2000; Hotchkiss et al., 2002), and a calcium sensitivity assay, have also been used to assess the enzymatic action and the functionality of the resulting pectin after treatment with PMEs (Hotchkiss et al., 2002). The calcium sensitivity is determined by measuring the increase in viscosity of the pectin solution in the presence of calcium ions relative to the non-cross-linked pectin solution.

Classically, rheological measurements are done to assess the functionality of the resulting gels, for example, the gelation and gel properties of PMEs treated pectins are often evaluated by compression tests (Willats et al., 2001; Fraeye et al., 2010a) or oscillatory rheometry measurements (Lee et al., 2008; Ngouémazong et al., 2012a,b,c) (Table 1).

In compression testing, the elasticity of the gel in the low strain region is calculated by measuring the force acting on the gel (Willats et al., 2001). Oscillatory tests provide measurement of elastic (\(G'\)) and viscous (\(G''\)) moduli indicating the behavior of the gel produced from the action of PMEs on the HG. In general, gelation of pectin with high DE (>70%) produces low values of \(G'\) and \(G''\), whereas gelation of pectin with low DE (<50%) produces maximum values of \(G'\) and \(G''\) (in the presence of sufficient Ca\(^{2+}\)) demonstrating the influence of DE on the gel properties (Lee et al., 2008; Ngouémazong et al., 2012a,b,c). When measuring the influence of PMEs treatment in fruit juices, a cloud stability test was performed by measuring the particle size distribution by laser diffraction and the turbidity of the centrifuged juice supernatant (Corredig et al., 2001).

3.2. Phenolic ester cross-links

3.2.1. Cross-linking mechanism and reaction conditions

The ferulic acid (FA) moieties esterified to the arabinan side chain at the backbone of RGI provides a way for enzyme catalyzed oxidative cross-linking of feruloylated pectic polysaccharides by chemical oxidizing agents e.g ammonium persulphate (Thibault and Rombouts, 1986) or oxidoreductase enzymes e.g. peroxidase or laccase catalysis to promote gelation (Zaidel et al., 2012; Micard and Thibault, 1999; Norsker et al., 2000). The cross-linking reaction of two feruloylated polysaccharide takes place via radical coupling of the FA resulting in the formation of intermediate products (Fig. 2B) which then leads to formation of different difAs: 5–5, 8–0–4, 8–5 and 8–8 (Fig. 1C; Fig. 2C) (Ralph et al., 1994; Saulnier and Thibault 1999). Recently, FA trimer and tetramer compounds were identified, which mainly involve the coupling of 8–0–4 and/or 5–5-difAs (Bunzel et al., 2005; Bunzel et al., 2006).

Oxidative cross-linking reaction of feruloylated polysaccharide varies with the types of enzymes or oxidizing agents used. The enzymatic reaction catalyzed by horseradish peroxidase (EC 1.11.1.7) (HRP) (with hydrogen peroxide (\(H_2O_2\))) has been shown to produce a more rapid increase in viscosity than the reaction catalyzed chemically by ammonium persulphate (Thibault and Rombouts, 1986). At high concentrations of ammonium persulphate the pectin molecules may be degraded resulting in a reduced viscosity of the gel at the end of reaction (Thibault and Rombouts, 1986). Furthermore, cross-linking of pectic polysaccharide with a high arabinose content using ammonium persulfate did not result in an increased viscosity, unless the arabinan side-chains had been removed (Guillon and Thibault, 1990).
The enzymatic cross-linking of feruloylated pectic polysaccharides, as typically catalyzed by laccase (EC 1.10.3.2) or horseradish peroxidase (EC 1.11.1.7), is significantly affected by the reaction conditions. The optimum pH for laccases varies between pH 4.5 and 6.5 and temperatures between 55 and 65 °C depending on the enzyme source, whereas horseradish peroxidase (HRP) works optimally at pH 6 and room temperature (Micard and Thibault, 1999). Laccases from various fungal sources such as Polyporus insitius, Mycekiophtora thermophili, Trametes versicolor, and Trametes hirsuta (Table 1) have been proven to be able to catalyze the cross-linking of feruloylated pectin from sugar beet without any addition of an oxidizing agent (except for the dissolved O₂ present in the reaction mixture). Laccases use molecular oxygen (O₂) to oxidize the substrate by a radical-catalyzed reaction mechanism (Thurston, 1994), a reaction which takes place via electron transfer in a cluster of four copper atoms forming the catalytic core of the enzyme. The laccase catalyzed reaction involves oxidation of four benzene-diol equivalents in the phenolic substrates, including ferulic acid, by concomitant reduction of one molecule of O₂ to two molecules of water (H₂O₂). HRP catalyzes the oxidation reactions using H₂O₂ as the acceptor and the esterified FA as the donor of hydrogen atoms; the enzyme catalyzed reaction progresses (rapidly) through formation of ferulate radicals and the dIFAs cross-links form as the reaction proceeds until the H₂O₂ or

![Diagram of the enzymatic cross-linking process](image-url)
the available FA are consumed (Figueroa-Espinoza and Rouau, 1998). No specific enzyme dosage has been reported for the cross-linking to occur, but the initial rates of gelation have been shown to correlate positively to increased enzyme dosage for both laccase and HRP (Zaidel et al., 2012). For HRP catalysis to proceed, a sufficient amount of H₂O₂ must be added to oxidize the cross-linking reaction. However, reaction with excess amount of H₂O₂ has been shown to cause a significantly decreased level of FA but no increase in diFAs (Zaidel et al., 2011), possibly because the H₂O₂ led to direct oxidation of the FA without cross-linking and/or partial inactivation of the enzyme (Bunzel et al., 2008).

3.2.2. Enzymatic pectin modification to promote phenolic ester cross-links and changed pectin functionality

Sugar beet pectin (SBP) has been amply used as a substrate in this type of cross-linking due to the abundant existence of FA moieties esterified to the arabinose side-chains of RGI (Table 1). Another reason is that SBP is composed of relatively short HG polymers, that are highly acetylated, which is why SBP is difficult to bring to cross-link and form gels via the ionic, (Ca²⁺) induced gelation mechanism. Modification of SBP by different extraction methods have been shown to change the gelling ability of SBP (Oosterveld et al., 2000; Guillon and Thibault, 1990; Micard and Thibault, 1999). Pectin extracted via systematic enzymatic hydrolysis have been reported to produce more conserved pectin structures having a higher gelling ability as compared to pectin extracted via hot acid hydrolysis, whereas more harsh extraction conditions were found to affect the composition of pectin by degrading the arabinose side-chains on RGI which (in SBP) contain the FA moieties (Guillon and Thibault, 1990; Micard and Thibault, 1999). In general the enzyme catalyzed oxidative gelation of SBP by different types of oxidoreductase enzymes, i.e. by HRP or laccase, has been shown to produce different gel properties (Norsker et al., 2000; Zaidel et al., 2012). Laccase catalyzed gels have generally been found to be firmer or stronger, i.e. having higher C′ values than the corresponding HRP catalyzed gels, even though the rates of enzyme catalyzed oxidative gelation of laccase catalysis were slower than the rates of gelation with HRP (Zaidel et al., 2012). Besides that, slower rates of gelation in laccase catalysis i.e. at lower enzyme activity level, has been shown to increase the gel strength (higher final C′). The rates of oxidative gelation of laccase catalysis has also been shown to be influenced by addition of Ca²⁺ into the laccase catalysis (Kuva et al., 2003), hence, Ca²⁺ addition retarded the laccase catalyzed rate of gelation of SBP but improved the texture of the gel at high enzyme activities (Kuva et al., 2003). Several reaction parameters that affect the enzyme catalyzed oxidative gelation of SBP e.g. pectin level, temperature, enzyme dosage, and, for HRP, the H₂O₂ concentration, can be tuned to adjust the rate of gelation and thus the properties of the gels produced (Zaidel et al., 2012).

Oxidative gelation of SBP catalyzed by laccase (Littoz and McClements 2008; Chen et al., 2010; Zeeb et al., 2012; Jung and Wicker, 2012) or HRP (Li et al., 2012) has also been carried out in emulsion systems (or multilayered emulsions systems). Laccase or HRP were added into the emulsion systems containing oil droplets coated by (multilayered) polysaccharide interfaces that were cross-linked by the enzyme. The emulsions coated by these cross-linked polysaccharide–protein interfaces had improved stability. These results demonstrate that (multilayered) emulsions with improved functional performance can be prepared by utilizing oxidoreductase enzymes to cross-link adsorbed biopolymer interfaces coating the oil droplets. Promising results have also been obtained in investigations of the gelling ability of enzymatically cross-linked SBP catalyzed by either peroxidase (with H₂O₂) or laccase when the SBP was incorporated into three different food products, i.e. black currant juice, milk and luncheon meat, and the enzymatic oxidation reactions were done in situ (Norsker et al., 2000). A cohesive SBP gel was thus formed in the luncheon meat which did bind the meat pieces together, making the meat sliceable, but undesirable side effects were observed in the black currant juice and milk, hence, besides oxidation of FA, the anthocyanins in the black currant juice and the milk lipids were also oxidized by the oxidoreductase enzyme catalysis (Norsker et al., 2000).

For the sake of completeness it should be mentioned that principally the same type of enzyme catalyzed cross-linking has been reported for feruloylated arabininoxylan (AX) substrates, including AX structures from wheat, rye, and maize (Table 2). Analogously to the effect with SBP, significant changes in rheological properties i.e. gelation, of the AX has resulted after treatment with either HRP or laccase. For these reactions a laccase from Pycnoporus cinnabarinus (Figueroa-Espinoza and Rouau 1998; Vansteenkiste et al., 2004) and Trametes versicolor (Berlanga-Reyes et al., 2009, 2011), respectively, were used (Table 2). The successful enzyme catalyzed gelation of AX substrates show that although the arabinose is bonded differently in SBP and AX – in AX the arabinose is substituted to the O-2 and/or O-3 position of the xylosyl units (Izydorczyk and Biliaderis, 1995), whereas the FA moieties is esterified to the O-5 position of the arabinose residues (Smith and Hartley 1983) – the oxidative enzymatic gelation is apparently able to override this difference. Also, laccase or HRP catalyzed oxidation of mixtures of caseins and different types of AX

### Table 2

Phenolic ester cross-linking in arabinoxylan in situ by enzymatic reaction/modification for designed functionality.

<table>
<thead>
<tr>
<th>Reaction mechanism</th>
<th>Substrate</th>
<th>Enzyme/catalyst</th>
<th>Functionality/modification/application</th>
<th>Assessment</th>
<th>References</th>
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<tbody>
<tr>
<td>Oxidative cross-linking of FA esterified to AX and formation of FA dimers</td>
<td>Wheat AX</td>
<td>Horseradish peroxidase and hydrogen peroxide; laccase from Pycnoporus cinnabarinus</td>
<td>Different enzyme catalysis</td>
<td>Capillary viscometric measurement</td>
<td>Figueroa-Espinoza and Rouau (1998)</td>
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<td></td>
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<td>Laccase from Pycnoporus cinnabarinus</td>
<td>Addition of bovine serum albumin to the oxidative cross-linking reaction</td>
<td>Small amplitude shear oscillatory measurement</td>
<td>Vansteenkiste et al. (2004)</td>
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<td></td>
<td></td>
<td>Laccase from Pycnoporus cinnabarinus</td>
<td>Stability of the gel; Structural of AX impact on gel properties</td>
<td>Small amplitude shear oscillatory measurement, swelling capacity</td>
<td>Carvajal-Millan et al. (2005a,b)</td>
</tr>
<tr>
<td></td>
<td>AX from wheat and rye, sugar beet pectin AX from maize; wheat bran</td>
<td>Horseradish peroxidase and hydrogen peroxide</td>
<td>Mechanical properties of gel from different substrates</td>
<td>Gelation visual observation, swelling capacity</td>
<td>Robertson et al. (2008)</td>
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<tr>
<td></td>
<td></td>
<td>Laccase from Trametes versicolor</td>
<td>Mechanical properties of AX gels at different AX concentrations; influenced by extraction methods</td>
<td>FA and diFA: RHEPLC; Gelation: small amplitude oscillatory shear by rheometer, swelling capacity</td>
<td>Berlanga-Reyes et al. (2009, 2011)</td>
</tr>
</tbody>
</table>
have been reported to improve the protein stability via catalysis of bond formation between tyrosine and feruloylated AX (Boeriu et al., 2004; Selinheimo et al., 2008). The available data cannot rule out, however, a mixed effect of diFAs cross-linking of feruloylated AX and the tyrosine-FA interaction in these systems.

3.2.3. Macromolecular functionality assessment of phenolic ester cross-links

The ability of FA to cross-link can be measured directly in a spectrophotometer by measuring the disappearance of FA with time at absorbance ranges from 316 to 375 nm (Oosterveld et al., 2000; Micard and Thibault, 1999; Waldrön et al., 1996). Both oxidoreductase enzymes and oxidative agents, respectively, have been shown to induce a rapid decrement of absorbance at the beginning of the cross-linking reaction indicating disappearance of FA due to oxidative cross-linking. The initial rapid decrease in absorbance is usually followed by a further gradual decrease over time to reach a pseudo-steady state, signifying Michaelis–Menten kinetics. Presumably, at this point some FA groups might still be left, either because they are unaccessible for cross-linking or the kinetics. The absorbance is usually followed by a further gradual decrease over time to reach a pseudo-steady state, signifying Michaelis–Menten kinetics. Presumably, at this point some FA groups might still be left, either because they are unaccessible for cross-linking or the reactions are slowed down by the increased viscosity, preventing the consumption of FA to form diFAs (Zaidel et al., 2011).

The levels of FA and diFAs can be analyzed using a GC-MS (Ralph et al., 1994) or HPLC after saponification (Waldrön et al., 1996). Decrement of FA and increment of diFAs after the addition of oxidoreductase enzymes demonstrate the formation of diFAs via oxidative cross-linking of FA (Zaidel et al., 2011). Gel formation through the oxidative cross-linking of feruloylated polysaccharides can be observed visually or by rheological measurements, such as compression test, swelling capacity, or dynamic oscillatory measurements (Table 1). For oscillatory measurement, using a rheometer (Oosterveld et al., 2000; Kuuva et al., 2003; Zaidel et al., 2012), each oxidative gelation reaction is initiated by adding the required amount of oxidoreductase enzyme to the polysaccharide solution and the G’ and G’’ are recorded for a required reaction time. The value of G’ increases with time demonstrating the increased number of cross-links formed as catalyzed by the oxidoreductase enzyme or oxidizing agent, and gel formation is indicated by the point when G’ > G’’.

A so-called swelling test provides an indication of the compactness of the polymeric structure of the polysaccharide after cross-linking, where lower swelling ratio indicates a more compact polymeric structure that limits the water absorption of the cross-linked polysaccharide (Micard and Thibault, 1999).

3.3. Rhamnogalacturonan II–borate ester cross-links

3.3.1. Cross-linking mechanism and reaction conditions

The formation of the RGII–borate ester cross-links involves the formation of 1:1 boronic acid or 1:1 borate-mRGII-intermediates. These intermediates react rapidly with RGII monomers (mRGII) to form the RGII-borate dimer (dRGII–B) (Fig. 3) (O’Neill et al., 1996). In general, the cross-linking is based on that two RGII monomers (mRGII) are covalently cross-linked by borate esters at the 3-linked-Apif residues of the 2-O-Me-Xyl-containing side chains (side chain A) of the RGII to form a RGII-borate dimer (dRGII–B) (Fig. 1D; Fig. 3). This formation of dRGII–B may lead to the development of a three-dimensional pectic network (Ishii and Matsunaga, 2001). The optimal pH for formation of dRGII–B has been reported to be between pH 2.2 and 4.8 with the maximum formation of dimers at pH 3.0 and 3.4 at room temperature (O’Neill et al., 1996; Ishii et al., 1999). In red wine, RGII has been found predominantly as dRGII–B since this form contains relatively high amounts of boronic acid and low pH (3.0–3.5) which is optimal for this type of cross-linking. The rate of cross-linking of two mRGII into dRGII–B has been reported to be more rapid in the presence of divalent cations mainly by Pb^{2+}, Ba^{2+}, Sr^{2+}, having ionic radii > 1.1 Å (O’Neill et al., 1996; Ishii et al., 1999). Other divalent cations such as Ca^{2+}, Ni^{2+}, Cd^{2+} and Zn^{2+} have been found to be less effective in increasing the rate of cross-linking, while Mg^{2+} and Cu^{2+} have been reported to cause a decrease in the amount of dRGII formed. These results suggest that steric factors may regulate dRGII–B formation since they have ionic radii of > 1.0 Å. O’Neill et al. (1996) hypothesized that the hydrolysis and formation of borate ester(s) are enzymatically catalyzed in vivo but it has not yet been unequivocally proven that enzymes are involved in the cross-linking of RGII by boronic acid. The ability of RGII to form a dimer but not a trimer or larger complexes suggests that RGII’s chemical structure and conformation are major factors that regulate its interaction with borate (O’Neill et al., 2004). The mechanism and reaction of the RGII–borate ester cross-linking are still being actively investigated and the focus has been mainly on its role in plant cell wall development.

3.3.2. Pectin modification to promote RGII–borate ester cross-links and changed pectin functionality

RGII has been found to be relatively abundant in red wine and other fermented beverages derived from fruits or vegetables.
The action of microbial glucanases and pectinases in the cell wall of fruits or vegetables during fermentation is thought to release the RGII, in turn increasing the levels of RGII in fermented fruits or vegetables. The RGII found in red wine readily cross-links to form a dimer because of the existence of boric acid and acidic pH (between 3.0 and 3.5) which, as mentioned above, favor dimer formation (O’Neill et al., 1996). Cross-linking of RGII with boric acid can lead to formation of a polymeric network which has been shown to improve berry juice clarification and processability (Hilz et al., 2006) (Table 1). Heavy metals which exist abundantly in wine e.g. lead, barium and strontium can form a complex with dRGII-B, and this functionality has been suggested to affect heavy metal absorption; hence, addition of dRGII-B has been shown to decrease the intestinal absorption and tissue retention of lead (Pb) in rats (Tahiri et al., 2000) (Table 1).

### 3.3.3. Macromolecular functionality assessment of RGII–borate ester cross-links

The mRGII and dRGII-B released from enzymatic reaction of HG degrading enzymes, e.g. endo-polygalacturonase, can be assessed by size exclusion chromatography due to their molecular size differences (Ishii and Matsunaga, 2001). Furthermore, the structure of mRGII and dRGII-B can be characterized and monitored using time-resolved $^{11}$B NMR spectroscopy, GLC-MS and MALDI-TOF-MS (Table 1). A spectrum of methylated dRGII-B from $^{11}$B NMR corresponds to the existence of 1:2 borate-diol-ester and structure of mRGII and dRGII-B have been determined by GLC-MS and MALDI-TOF-MS (O’Neill et al., 1996).

### 3.4. Other types of covalent cross-links

#### 3.4.1. Uronyl ester cross-linking mechanism, reaction and enzymatic pectin modification

HG may also be covalently cross-linked to a hydroxyl group of other components in polysaccharides via formation of 1:2 borate-diester (Brown and Fry, 1993). In addition to catalyzing the hydrolysis of the methyl ester linkages on methylated GalpA in the HG backbone, some PMEs may also catalyze transacylation, i.e. catalyze the transfer of the C-6 carboxyl group of a GalpA moiety from methanol to a hydroxyl group of a sugar moiety in the cell wall polysaccharides e.g. on another HG chain, via a “double displacement” mechanism (Fig. 4) (Hou and Chang, 1996). These ester linkages are more stable and resistant to the action of PME compared to the methyl ester linkages on the methylated GalpA in HG and have been proposed to contribute to the strength of the plant cell wall and to enhance the firmness of vegetables during pre-cooking (Hou and Chang, 1996). An investigation of such transacylation of citrus pectin catalyzed by PMEs from various sources such as jelly fig, tomato, citrus and tendril shoots of chayote have demonstrated both a remarkable increase in molecular size of the polysaccharides as measured by gel permeation chromatography (Jiang et al., 2001a,b) and an induced turbidity of the pectin solution (Hwang et al., 2003; Wu et al., 2004) (Table 1). Addition of NaCl was found to influence the transacylation reaction as shown by the increased in molecular size of pectin compared to the reaction without the NaCl (Jiang et al., 2001a). Viscosity and compression tests of such transacylated pectin showed that the gelling properties of the pectin were improved, corroborating the formation of cross-linked polysaccharides via the transacylation (Wu et al., 2004). Thus, cross-linking of polysaccharide through transacylation in situ could be used in the food industry to prepare reduced sugar jellies and jams.

#### 3.4.2. Pectic polysaccharide-XG cross-linking

Pectin has also been reported to be covalently cross-linked to xyloglucan (XG). XG consists of a β-(1→4)-bonded backbone of glucose molecules with short side chains of α-L-arabinose, β-D-galactose and α-L-fucose residues, and is usually considered to be a part of the hemicellulose fraction (Popper and Fry, 2008). Covalent cross-linking between XG and pectic polysaccharides has been proposed to occur between the XG reducing ends, i.e. via the C1 of the XG backbone glucose to the RGII side chains (Fig. 5). Popper and Fry (2008) proposed two models for this covalent cross-linking between XG and RGII. The first reaction involves an enzymatic transglycosylation reaction between XG–RGII by an endo-transglycosylase using XG as the donor substrate and

![Fig. 4](https://example.com/fig4.png) Proposed mechanism for the catalysis of PME on the methyl groups on HG (I) forming an intermediate (II) which then undergoes hydrolysis (III), and transacylation reaction between HG and other polysaccharide molecule as catalyzed by PME to form the uronyl ester cross-link (IV) (adapted from Hou and Chang (1996)).
RGI as the acceptor substrate. The second reaction suggests that the formation of XG–RGI linkages occurs through NDP-sugar-dependent chain elongation of XG. Evidence for this covalent XG–RGI cross-linking has been reported by several authors (Thompson and Fry, 2000; Popper and Fry, 2005), but the presence of the cross-linking has only been interpreted from co-elution profiles of the presumed cross-linked polymers in anion-exchange columns, and direct evidence for the existence and nature of the covalent cross-linking of XG-pectic polysaccharides is still lacking. As far as this review was constructed, no study was reported on the influence of the cross-linking of HG–XG in food texture or other functionality except in plant cell wall development and metabolism.

4. Concluding remarks

The exploitation of enzymatic reactions for improving pectic polysaccharide functionality has shown significant progress in recent years. Such modifications may have great potential for various applications particularly for use in food products. The understanding of the cross-linking mechanisms and the relationship between structure-functionality of the polysaccharide cross-links is an important prerequisite in determining the functional and macromolecular potential of the polysaccharides for use in food and non-food applications. In the near future, more focus should be given to enzymatic cross-linking of polysaccharides in situ and the macromolecular functionality of cross-linked polysaccharides as food ingredients. With the increasing demand for improved technical and nutritional functionality of food products, an understanding of the physiological effects of cross-linked polysaccharides which contains potentially bioactive components such as phenolic acids should also be emphasized.

Acknowledgment

The authors would like to acknowledge Universiti Teknologi Malaysia, Skudai and the Ministry of Higher Education, Malaysia for financial support.

References


