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Kinetics and rheology

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Enzyme catalyzed oxidative cross-linking of feruloylated pectic polysaccharides from sugar beet – Kinetics and rheology

PhD Thesis

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Enzyme catalyzed oxidative cross-linking of feruloylated pectic polysaccharides from sugar beet – Kinetics and rheology

This thesis was prepared by
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January 2012

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PREFACE

This thesis is submitted in fulfillment of the requirements for the Doctorate (PhD) degree at the Technical University of Denmark (DTU). The work presents in this thesis was conducted during my PhD study at the Department of Chemical and Biochemical Engineering, DTU from 1st August 2008 until 31st January 2012. The work was accomplished under the supervision of Professor Anne S. Meyer. The PhD study was financed by a scholarship from the Ministry of Higher Education Malaysia (MOHE) and Universiti Teknologi Malaysia (UTM).

Most analytical work was performed at the Center for Bioprocess Engineering (BioEng) whereas macromolecular investigation was carried out at the DTU Food. We acknowledge Danisco A/S for supplying the materials for this study and Southern Danish University for performing the MALDI-TOF analysis.

For completing this study, I am most grateful to Anne Meyer, for the thorough supervision, valuable comments and continuous support throughout this study. I have enjoyed the "boot camps" session the most. I would like to express my appreciation to Ioannis Chronakis who has assisted me a lot in the rheological part of the work.

It has been an amazing three-and-a-half-year journey of my life, working and living in Denmark. I would like to thank all the people who have helped me during this study, for the analytical work, sharing of ideas, and inspirational discussion, especially my present and former colleagues in BioEng. My gratitude also goes to all my friends for sharing the happy moments, supporting me during my ups and downs, and for being who you are.

Last but not least, thanks to my parents, for your love and encouragement.

"Adat periuk berkerak,
adat lesung berdedak"

Dayang Norulfairuz Abang Zaidel
Lyngby, January 2012

ABSTRACT

Sugar beet pulp is a byproduct from sugar production consisting mainly of cellulose and pectic polysaccharide. Its utilization has been mostly as feedstock due to its high content of energy and fiber. This study emphasizes on the utilization of the pectin and arabinan fractions extracted from sugar beet pulp as a potential starting material for production of pectin derived products which could help maintain the competitiveness of the sugar beet based industry. The overall objective of this study has been focusing on understanding the kinetics of enzyme catalyzed oxidative cross-linking of feruloylated polysaccharide from sugar beet and relating the kinetics of this cross-linking to the properties of the cross-linked products. Several hypotheses have been formulated in order to accomplish our objective.

The first part of the study utilized arabinan-oligosaccharide fraction from sugar beet pulp byproduct. In this study we investigated the effect of arabinans backbone length on the kinetics of horseradish peroxidase (EC 1.11.1.7) (HRP) catalyzed oxidative cross-linking of ferulic acid (FA) moieties esterified to α-(1,5)-linked arabinans; taking into account that FA can be oxidatively cross-linked by HRP catalysis in the presence of hydrogen peroxide (H_2O_2) to form ferulic acid dehydrodimers (diFAs). The composition of the substrate was analyzed by HPAEC, HPLC and MALDI-TOF, confirming the structural make up of the arabinan-oligosaccharide (Arabinose: 2.9-3.4 mmol·g^{-1} DM; FA: 2.5-7.0 mg·g^{-1} DM) and verifying the formation of diFAs as a result of the enzyme catalyzed cross-linking reaction. The result demonstrates the influence of arabinans backbone length on the rates of FA cross-linking; longer arabinans exhibit a slower cross-linking rate than shorter, all other things being equal.

It has been our intention to study the rheological properties of cross-linked feruloylated arabinan-oligosaccharide, however the attempt has not been fully achieved. It might be due to small molecular weight of the arabinan (~1.3 kDa) which prevented the measurement of the rheological properties since the change in viscosity resulting from the cross-linking was insignificant. Therefore, the next part of the work presented in this thesis utilized sugar beet pectin (SBP) solid fraction extracted from sugar beet pulp which has molecular weight >100 kDa. The compositional analysis of the substrate shows abundant amount of FA (7.3 mg·g^{-1} DM) in SBP which can be oxidatively cross-linked *via* enzyme catalyzed reaction by oxidoreductase enzymes. We hypothesized that different mechanisms of two oxidoreductase enzymes, *i.e.* HRP and laccase (EC 1.10.3.2), might influence the kinetics of the oxidative cross-linking and consequently the

properties of the gels formed. The kinetics of oxidative gelation of SBP, taking place *via* enzyme catalyzed cross-linking of FA, was evaluated by small angle oscillatory measurements. The result indicates a significant difference between the SBP gels produced from the catalysis of HRP and laccase, that is, laccase catalysis produced stronger SBP gels albeit slower rates of gelation than the HRP catalysis. Statistically design experiment has been constructed to investigate the effect of several reaction factors which might influence the rates of gelation of SBP catalyzed by HRP or laccase, particularly the pectin level, temperature, enzyme dosage, pH and, for HRP, the H_2O_2 concentration. The result reveals that these reaction factors could be tuned in order to adjust the enzyme catalyzed gelation and the properties of the gels produced. Moreover, positive correlation between the rates of gelation and gel strengths was obtained for laccase catalyzed gels, but no such correlation exists for HRP catalyzed gels. Chemical analysis confirmed the formation of diFAs in the cross-linked products by both enzymes catalysis supporting that the gelation was a result of oxidative cross-linking of FA.

It is uncertain how the kinetics of enzyme catalyzed oxidative cross-linking of SBP and the gels properties are affected in emulsion systems. Thus, investigation on the enzyme catalyzed oxidative gelation of SBP was further performed on the SBP in emulsion systems. In this study, we have formulated two separate, identically composed, oil-in-water emulsion systems to study the effect of different methods of emulsion preparation on the emulsion stability in the presence of SBP and the kinetics of enzyme catalyzed oxidative gelation of SBP. The result shows that the different methods of emulsion preparation affect the emulsion stability and the rates of gelation of SBP in emulsion systems, and stronger gels were produced in the SBP containing emulsions as compared to the SBP without emulsions.

From this study, we have shown that arabinan-oligosaccharide and SBP solid fractions extracted from sugar beet pulp byproduct could undergo oxidative cross-linking of the feruloyl group, which abundantly esterified to the arabinan side-chains, through enzyme catalyzed reaction. Our study provides the insight into the relationship between the kinetics of the oxidative cross-linking of FA with the structural characteristic of the oligosaccharide, and the correlation between the rates of enzyme catalyzed oxidative gelation of feruloylated polysaccharide and the rheological properties of the gels produced. This knowledge could be useful for designing application of sugar beet pectin in food technology or similar application.

DANSK SAMMENFATNING

Sukkerroepulp produceres som et biprodukt ved fremstilling af sukker fra sukkerroer og består hovedsagelig af cellulose og pektin, og indeholder herunder forskellige pektiske polysakkarider. Sukkerroepulp har hidtil hovedsagelig været anvendt som foder på grund af dets høje indhold af energi og fibre. Dette PhD studie har haft fokus på at vurdere mulighederne for at udnytte pektin og arabinanfraktionerne ekstraheret fra sukkerroepulp som potentielle udgangsmaterialer til produktion af pektinafledte produkter med henblik på at opretholde konkurrencedygtigheden af den sukkerroebaserede sukkerproduktion. Det overordnede formål med dette studie har været at søge at beskrive og forstå kinetikken af enzymkatalyseret oxidativ krydsbinding af feruloylerede polysakkarider fra sukkerroer og at relatere denne kinetik til de krydsbundne produkters egenskaber. Adskillige hypoteser er blevet formuleret med henblik på at opnå ny viden og opfylde studiets overordnede formål.

I den første del af studiet blev arabinan-oligosakkarider fra sukkerroepulp udnyttet. I denne del af studiet blev effekten af kædelængden af arabinan på kinetikken af hesteradise peroxidase (EC 1.11.1.7) (HRP) katalyseret oxidativ krydsbinding af ferulasyre (FA) enheder esterificeret til α-(1,5)-bundne arabinaner undersøgt; undersøgelserne baserede sig på, at FA kan krydsbindes oxidativt ved hjælp af HRP katalyse under tilstedeværelse af hydrogen peroxid (H_2O_2) of danne ferulasyre dehydrodimere (diFAs). Kompositionen af substratet blev analyseret ved hjælp af HPAEC, HPLC og MALDI-TOF, og de opnåede resultater bekræftede den strukturelle opbygning af arabinan-oligosakkariderne (som havde følgende komposition: Arabinose: 2.9-3.4 $mmol \cdot g^{-1}$ DM; FA: 2.5-7.0 $mg \cdot g^{-1}$ DM) og verificerede dannelse af diFAs som et resultat af den enzymkatalyserede krydsbindingsreaktion. Resultaterne demonstrerede, at kædelængden af arabinan havde indflydelse på hastigheden af den enzymkatalyserede krydsbinding; længere arabinaner udviste en langsommere krydsbindingshastighed end kortere, alt andet lige.

Det var tillige vores intention at studere de reologiske egenskaber af de krydsbundne feruloylerede arabinan-oligosakkarider, men på grund af den meget lave viskositet af de krydsbundne produkter, som ikke kunne måles som en ændring af viskositeten i forhold til udgangsmaterialet, blev denne intention kun delvist opfyldt. Den lave viskositet, og den deraf følgende mangel på målbar gel-dannelse eller ændret viskositet, kan skyldes den lave molekylære vægt af arabinanen (~1.3 kDa). Af denne grund blev det valgt at den næste del af PhD arbejdet skulle vurdere mulighederne for at udnytte uopløselige, faste pektin fraktion (SBP) ekstraheret fra sukkerroepulp, som har

en molvægt >100 kDa. Kompositionsanalysen af dette substrat viste, at det indeholdt relativt store mængder FA (7.3 $mg \cdot g^{-1}$ DM), som kan krydsbindes oxidativt *via* enzym katalyseret reaktion med oxidoreduktaser. Vi opstillede den hypotese, at de forskellige mekanismer af to typer oxidoreduktaseenzymer, *i.e.* HRP og lakkase (EC 1.10.3.2), kunne have indflydelse på kinetikken af den oxidative krydsbinding og som en konsekvens heraf også påvirke egenskaberne af de dannede geler. Kinetikken for den oxidative gelering af SBP, som fandt sted *via* enzym katalyseret krydsbinding af FA, blev evalueret ved hjælp af oscillatoriske målinger. De opnåede resultater viste, at der var signifikant forskel mellem SBP geler dannet via katalyse med henholdsvis HRP og lakkase, således at lakkase katalyse producerede stærkere SBP geler på trods af en lavere geleringshastighed end den korresponderende HRP katalyserede reaktion. Statistisk designede forsøg blev udarbejdet med henblik på at undersøge effekten af en række reaktionsfaktorer som kunne have indflydelse på hastigheden af geleringen af SBP katalyseret ved hjælp af henholdsvis HRP og lakkase, herunder pektin niveauet, temperaturen, enzymdoseringen, pH og, for HRP's vedkommende, også H_2O_2 koncentrationen. Resultaterne viste, at disse reaktionsfaktorer kunne tilrettelægges, eller "tunes", således at hastigheden af den enzymkatalyserede gelering kunne justeres og at egenskaberne af de producerede geler dermed kunne styres. Desuden blev det vist, at der for lakkase katalyseret gel-dannelse er en positiv korrelation mellem gel-dannelseshastigheden og gel-styrken, mens en sådan korrelation ikke eksisterer for HRP katalyserede geler. Kemiske analyser af de krydsbundne produkter viste at der dannedes diFAs ved katalyse ved hvert af enzymerne og det blev dermed bekræftet, at gel-dannelsen var et resultat af oxidativ krydsbinding af FA.

Det viste sig at være uklart, hvorledes kinetikken af enzymkatalyseret oxidativ krydsbinding af SBP og egenskaberne af de resulterende geler blev påvirket i emulsions-systemer. Derfor blev der foretaget systematiske undersøgelser af enzym katalyseret oxidativ gelering af SBP i emulsioner. I studiet af emulsioners påvirkning af gel-dannelsen blev to, identisk sammensatte, olie-i-vand emulsions-systemer med SBP fremstillet på forskellig måde og effekten af forskellige emulsions-fremstillingsmetoder på emulsionsstabiliteten i relation til tilstedeværelsen af SBP, og kinetikken af den enzymkatalyserede oxidation af SBP blev undersøgt. Resultaterne viste, at de forskellige emulsions-fremstillingsmetoder påvirkede emulsionsstabiliteten med SBP foruden hastigheden af den enzymkatalyserede gel-dannelse og gelstyrken af de dannede SBP-geler i emulsionssystemerne.

I dette studie er det blevet vist, at både arabinan-oligosakkarider og SBP fraktioner ekstraheret fra sukkerroepulp kan bringes til at undergå enzymkatalyseret oxidativ krydsbinding via oxidation af feruloyl-grupperne, som findes esterificeret på arabinan-sidekæderne. Studiet har desuden

tilvejebragt en indsigt i forholdet mellem kinetikken af den oxidative krydsbinding af FA og de strukturelle karakteristika af oligosakkariderne, foruden at have vist korrelationen mellem hastigheden af den enzymkatalyserede oxidative gelering af de feruloylerede polysakkarider og de reologiske egenskaber af de producerede geler. Denne viden kan bruges til at designe anvendelser af sukkerroepektin og pektiske polysaccharider i fødevarer eller andre produkter.

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Abbreviations

AG	arabinogalactan
diFA	dehydrodimer of ferulic acid
DA	degree of acetylation
DE	degree of methoxylation
DM	dry matter
DP	degree of polymerization
EDTA	ethylenediaminetetraacetic acid
FA	ferulic acid
FAO	Food and Agriculture Organization of the United Nations
Gal*p*A	galacturonic acid
HILIC	hydrophilic interaction chromatography
HG	homogalacturonan
HM	high molecular
HPAEC	high-performance anion exchange chromatography
HPLC	high-performance liquid chromatography
HPSEC	high-performance size exclusion chromatography
HRP	horseradish peroxidase
H_2O_2	hydrogen peroxide
LM	low molecular
MWCO	molecular weight cut-off
PAD	pulsed amperometric detection
RG	rhamnogalacturonan
SBP	sugar beet pectin

Nomenclatures

G'	elastic modulus
G''	viscous modulus
G^*	complex modulus
G'_{10}	elastic modulus at 10min
η^*	complex viscosity
δ	phase shift angle

LIST OF PUBLICATIONS

This PhD thesis is based on the following papers, which will be referred to by their roman numerals in the text:

I Dayang Norulfairuz Abang Zaidel, & Anne S. Meyer
Biocatalytic cross-linking of pectic polysaccharide for designed food functionality: structures, mechanisms and reactions
Manuscript submitted to Biocatalysis and Agricultural Biotechnology. Jan 27, 2012

II Dayang Norulfairuz Abang Zaidel, Anis Arnous, Jesper Holck, & Anne S. Meyer
Kinetics of enzyme-catalyzed cross-linking of feruloylated arabinan from sugar beet
Journal of Agricultural and Food Chemistry, 2011, 59, 11598 – 11607.

III Dayang Norulfairuz Abang Zaidel, Ioannis S. Chronakis, & Anne S. Meyer
Enzyme catalyzed oxidative gelation of sugar beet pectin: Kinetics and rheology
Food Hydrocolloids, 2012, 28, 130 – 140.

IV Dayang Norulfairuz Abang Zaidel, Ioannis S. Chronakis, & Anne S. Meyer
Stabilization of oil-in-water emulsions by enzyme catalyzed oxidative gelation of sugar beet pectin
Manuscript submitted to Food Hydrocolloids. Jan 19, 2012

CHAPTER 1

Introduction

Sugar beet (*Beta vulgaris*) is used primarily for production of sugar (sucrose), mainly in Europe and America, which accounts for 30-35% of the world's sugar production. In 2009, approximately 227 million tons of sugar beets were produced worldwide with Europe being the biggest producer in the world (~77.5%) (Figure 1.1) (FAO, 2011). Sugar beet pulp is a byproduct from this sugar production and due to its low commercial value and high fiber it is widely used as feed supplements for livestock. However, the utilization of the pectin, pectin associated arabinan and arabinan-oligosaccharide extracted from the sugar beet pulp for production of pectin derived products could help maintain the competitiveness of the sugar beet based industry. Approximately 67% dry matter of sugar beet pulp consists of mainly polysaccharides, which consist of pectin (19-20%), pectin associated arabinan (21-22%), and cellulose (24-25%) (Oosterveld *et al*., 1996). For the sake of clarification, in the early chapter of this thesis, pectin is referred to the homogalacturonan (HG) fraction, whereas pectin associated arabinan is referred to the rhamnogalacturonan I (RGI) fraction and arabinan-oligosaccharide is referred to the arabinan side-chain of RGI, and most commonly in this thesis, sugar beet pectin (noted as SBP) is referred to the total pectin (sum of HG, RGI, RGI side-chains and RGII).

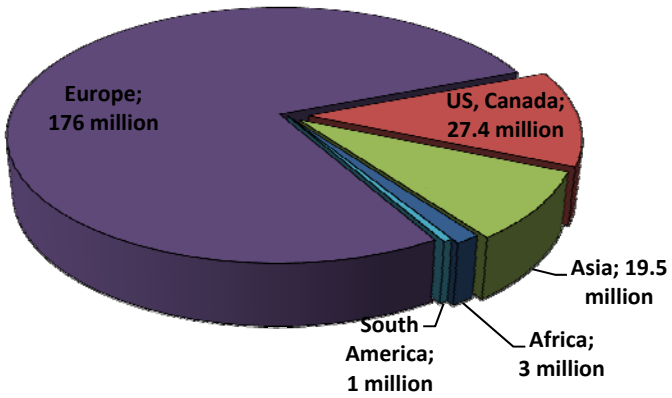

Figure 1.1: World annual sugar beet production in 2009 (FAO, 2011). The figures shown are in tons.

Most of the pectin used as food ingredients originates from apple or citrus and is primarily a HG (Voragen *et al*., 2009). This type of pectin mainly serves as gelling, thickening, and stabilizing agent in diverse food application such as jam, yogurt drinks, fruity milk drinks and ice cream (Laurent & Boulenguer, 2003). Pectin from these sources is also used in the production of a variety of specialty products including cosmetics, and adhesives (Mohnen, 2008), and also for use

in biomedical and biopharmaceutical (Itoh *et al*., 2011; Munarin *et al*., 2011). In the past, several attempts to commercialize pectin from sugar beet for use as gelling agent and stabilizer have failed since sugar beet pectin has less significant gelling properties due to a high acetylation and relatively small molecular size (or short HG stretches) compared to that of citrus pectin (Rombouts & Thibaults, 1986). However, continuous interest in finding new applications for beet pulp left from sugar industry has enhanced the possibility of utilizing this material. Hence, the existence of feruloyl groups esterified to the arabinose side-chain at the backbone of RGI provides a way for enzymatic modification of pectic polysaccharide to promote gelation of pectin from sugar beet (Micard & Thibault, 1999). Nowadays, pectic polysaccharide from sugar beet as an alternative source for gelling agent in food has shown equal or superior properties to citrus or apple pectin. Food applications of pectin from sugar beet include, for example, stabilization of flavored oil emulsions in juice concentrates, water-soluble pectin fibers, and stabilization of acidified yoghurt drink (Buchholt *et al*., 2004), and recently, pectins from sugar beet has been shown to have a potential suitability for use in non-food application such as for biomedical and biopharmaceutical purposes (Takei *et al*., 2011) or as new components in (bio)plastic manufacture (Liu *et al*., 2011).

1.1 Food gels

Gels are defined as polymeric 3-dimensional networks of connected molecules or particles entrapping a large volume of continuous liquid phase, which swell on contact with water but do not dissolve (BeMiller & Whistler, 1996). In many food products, the gel network can be joined in junction zones by hydrogen bonding, hydrophobic associations (van der Waals attractions), ionic cross bridges, entanglements, or covalent bonds, and the liquid phase is an aqueous solution of low molecular weight solutes and portions of the polymer chains (BeMiller & Whistler, 1996). Several types of food gels (Figure 1.2) are prepared according to their functional properties and purpose, either to obtain a certain consistency or to provide physical stability in food. Polysaccharide gel is one of the regularly used gels in food product, categorized by its fine texture and transparency. In general, it can be formed by heating and cooling, pH adjustment or specific ion addition. Particle gels may form through aggregation, induced by ionic strength, a change in pH, or solvent quality (Figure 1.2A). Polysaccharide gel cross-links consist of microcrystalline regions (Figure 1.2B), and the length of strands between cross-links is not very long (Walstra, 1996). Pectin, from sugar beet in particular, contains highly of pectin associated arabinan, can form gel *via* oxidative cross-linking of ferulic acid esterified to the arabinose moieties in the RGI side-chains. This type of gel from sugar beet pectin, illustrated as covalent cross-links polymer gel (Figure 1.2C), can be an alternative for gel formation in an acid-sugar system, or by calcium complexation.

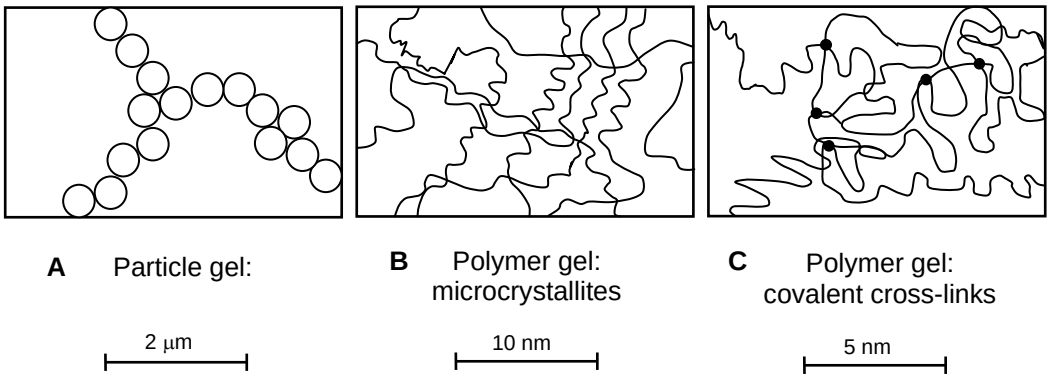

Figure 1.2: Schematic illustrations of three main types of gel. The dots in (C) denote cross-links. Note the difference in approximate scale (adapted from Walstra, 1996).

Oxidative gelation of sugar beet pectin

Gelation of sugar beet pectin (SBP) can be accomplished *via* oxidative cross-linking of ferulic acid esterified to the arabinan side-chains of RGI, catalyzed either by chemical oxidant such as ammonium persulphate (Thibault & Rombouts, 1986) or oxidoreductase enzymes (Micard & Thibault, 1999). During the reaction, FA was enzymatically oxidized into free radicals and 5,5', 8-O-4', 8,5' and 8,8' diFAs were formed (Oosterveld *et al*., 1997; Waldron *et al*., 1997). However, it has been reported that ammonium persulphate may degrade the pectin molecules during the reaction and hence reducing the viscosity of the gel at the end of the reaction (Thibault & Rombouts, 1986). Furthermore, cross-linking of SBP containing high arabinose level by ammonium persulfate catalysis has not shown an increase in viscosity of the SBP gel, unless the arabinan side-chains have been removed (Guillon & Thibault, 1990). In recent years, the prospect of producing SBP gel *via* enzyme catalyzed oxidative cross-linking of feruloylated polysaccharide *in situ* by using oxidoreductase enzymes has been explored intensively (Norsker *et al*., 2000; Kuuva *et al*., 2003; Berlanga-Reyes *et al*., 2009). The oxidative gelation of SBP has shown to produce a relatively good quality gel *via* controlled enzymatic reaction (Norsker *et al*., 2000; Kuuva *et al*., 2003). However, to-date, the quantitative kinetic aspects of such enzyme catalyzed cross-linking have been less explored. Similarly, scant data have been reported that allow more generic understanding of the enzymatic reaction factors, including the use of H_2O_2 dependent enzymatic oxidation such as with peroxidase versus oxygen dependent enzymatic oxidation as promoted by laccase, as well as the significance of the substrate sizes, the reaction pH and temperature. The availability of such knowledge is considered a prerequisite for knowledge based utilization of sugar beet pectic polysaccharides as a substrate for enzyme catalyzed oxidative gelation applications.

1.2 Hypotheses and objectives

This PhD study was built on that agro-industrial byproduct side-streams from sugar beet pulp are rich sources of pectic polysaccharide and abundantly available as starting materials for production of pectin derived products. The existence of feruloyl groups esterified to the arabinan side-chains of RGI in pectin from sugar beet is an advantage for this material to be explored *via* enzyme catalyzed treatment to produce cross-linked feruloylated polysaccharide.

This PhD study was built up based on these hypotheses:

- the size of the backbone of the feruloylated arabinan substrate affects the rate of the enzyme catalyzed cross-linking reaction, that is, the longer the backbone length, the slower the reaction
- the rate of the enzyme catalyzed cross-linking reaction affects the macromolecular properties of the polysaccharide gel
- enzyme catalyzed cross-linked sugar beet pectin can stabilize an emulsion in a gel
- addition of an emulsion system influences the rate of enzyme catalyzed oxidative gelation of feruloylated sugar beet pectin and the properties of the gel produced

The main objective of this study was to test the above hypotheses. These specific objectives were constructed to achieve this goal:

- to describe and relate the kinetics of the enzyme catalyzed oxidative cross-linking reaction with the substrate properties of sugar beet arabinan
- to assess the gelation rate of feruloylated sugar beet pectin in response to the enzyme dosage
- to characterize the rheological properties of the enzyme catalyzed cross-linked feruloylated sugar beet pectin gels
- to establish the correlation between macromolecular rheological properties of enzyme catalyzed cross-linked feruloylated sugar beet pectin gels and their rate of gelation
- to examine the effects of enzymatic reaction parameters for enzyme catalyzed oxidative gelation using response surface methodology
- to investigate the emulsion stability of oil-in-water emulsion in response to oxidative gelation
- to investigate the cross-linking reaction of the emulsion and sugar beet pectin mixture and the macromolecular properties of the gel produced from this cross-linking

1.3 Structure of pectic polysaccharide

Pectin is a term used for a family of structures, having different backbones and side-chains. In commerce the word "pectin" is mainly used to designate homogalacturonan, which, as discussed below, may also represent different types of chemical structures and molecular sizes. In this thesis, the term pectic polysaccharide will be used to refer to different types of structures, including the side-chains of RGI, which are sometimes also referred to as "neutral side-chains" (see Table 1.1). Pectic polysaccharide exists abundantly in plant primary cell walls, and in the middle lamella between plant cells where it helps to bind cells together. Pectic polysaccharide from various sources of plant, even within a plant over time and in various parts of a plant, differs in the amount, structure and chemical composition. Varies depending on the sources, the amount of pectic polysaccharide presents approximately 35% in dicotyledonous plant and 2-10% in grasses. Diverse functionalities of pectic polysaccharide in the plant include the growth, morphology, development and plant defense responses (Voragen *et al*., 2009).

Pectic polysaccharides can be divided into three main types of structures having different backbones of homogalacturonan (HG), rhamnogalacturonan I (RGI) and rhamnogalacturonan II (RGII) which has been reported to be covalently linked to each other (Coenen *et al*., 2007). The same structural elements occur in various quantities in different plant sources (Table 1.1), but relatively long, coherent stretches of HG seem to be particularly prevalent in fruits such as citrus (orange, lime, lemon), black currants, and grapes, whereas extensive side chains exist abundantly in *e.g.* apple, soybean, potato, and sugar beets (Zhan *et al.* 1998; Hilz, 2007; Voragen *et al*., 2009).

Table 1.1: Proportions of various structural elements in different plant sources

	Citrus[a]	Black currant[b]	Grape[b]	Soybean[c]	Apple[c]	Sugar beet[c]
Total polysaccharides (% DM)	81	19	11	16	20	67
Pectic substances (% of total PS)	80	61	56	59	42	40
Structural elements (% of pectic substances)						
HG	77	68	65	0	36	29
RGI	5	5	10	15	1	4
Neutral side-chains	4	24	23	60	47	48
RGII	0.3	3	2	4	10	4

DM dry matter; PS polysaccharide; HG homogalacturonan; RG rhamnogalacturonan
[a] from Zhan *et al.* (1998); [b] from Hilz (2007); [c] from Voragen *et al*. (2009)

1.3.1 Homogalacturonan

Homogalacturonan (HG) consists of mainly linear α-(1,4)-linked-galacturonic acid residues (Figure 1.3), and the galacturonic acid (Gal*p*A) moieties within this backbone can be methyl-esterified at C-6 and/or *O*-acetylated at the C-2 and/or C-3 (Voragen *et al*., 2009). The degree of methoxylation (DE) or acetylation (DA) in pectin from sugar beet can be approximately 50-62% (Buchholt *et al*., 2004) or 15-45% (Levigne *et al*., 2002). These substitutions are considered as key parameters for the characteristic of pectin within the cell wall and gelling properties of pectin as food additives. Due to high DA in SBP, divalent-cation (Ca^{2+}) promoted pectin gelation is less significant for pectin from sugar beet than for example citrus pectin.

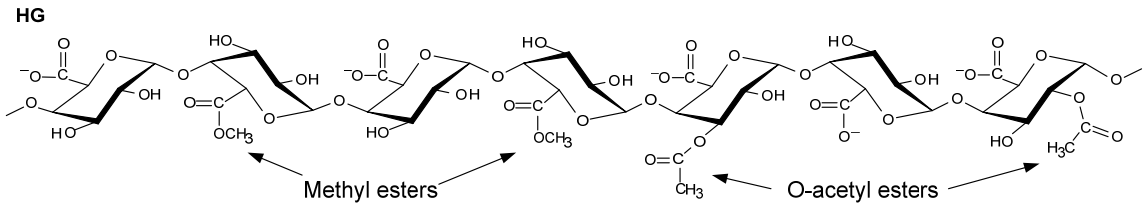

Figure 1.3: Structure of HG with methyl and O-acetyl esterifications.

1.3.2 Rhamnogalacturonan I

Rhamnogalacturon I (RGI) typically represents 20-35% of pectic polysaccharide (The sum of RGI and neutral side-chains, Table 1.1). Its backbone is made up of repeating units of [→2)-α-L-Rha*p*–(1,4)-α-D-Gal*p*A-(1→] (Figure 1.4), and the rhamnose moieties of the RGI in sugar beet pectin may be substituted at the *O*-4 position with α-(1,5)-linked-arabinans and with minor amounts of β-(1,4)-linked-galactan (Oosterveld *et al*., 2000a) (Figure 1.5) and/or arabinogalactan (as detailed below in the section *RGI side-chains*) (Lerouge *et al*., 1993). The proportion of branched rhamnose residues varies from 20 to 80% depending on the source of the polysaccharide (Voragen *et al*., 2009).

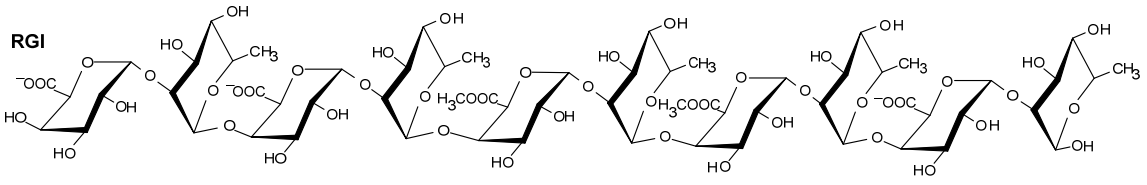

Figure 1.4: Structure of RGI backbone made up of repeating units of [→2)-α-L-Rha*p*–(1,4)-α-D-Gal*p*A-(1→].

RGI side-chains

The side-chains of RGI include arabinans that contain an α-(1,5)-linked-arabinan backbone having a high degree of α-(1,2)- and α-(1,3)-Ara*f* substitutions; oligosaccharides of α-(1,5)-linked-arabinose may also be present as side chains (Lerouge *et al*., 1993; Westphal *et al*., 2010) (Figure 1.5A), and in beet pectin short β-(1,4)-linked-galactan chains (Figure 1.5B) with low degrees of polymerization (DP) have also been reported (Guillon & Thibault, 1989), whereas longer chains of up to DP 47 have been found in soybean polysaccharide (Nakamura *et al*., 2002), arabinogalactan I (AGI) and/or arabinogalactan II (AGII).

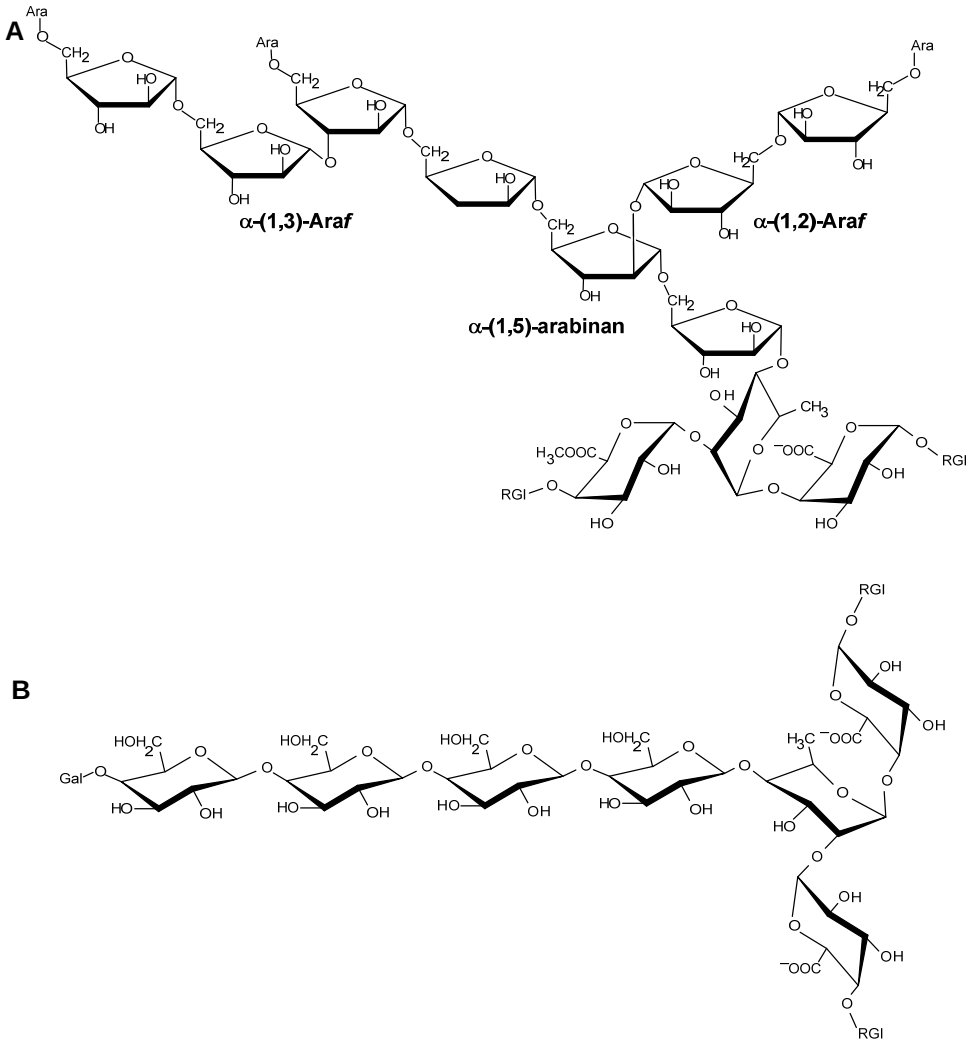

Figure 1.5: RGI side-chains (A) α-(1,5)-linked-arabinan backbone having a high degree of α-(1,2)- and α-(1,3)-Ara*f* substitutions; (B) β-(1,4)-linked-galactan chains

AGI is composed of a β-(1,4)-linked-galactan backbone with Ara*f* residues, either as single residues or short side-chains, attached to *O*-3 of the galactosyl residues. *O*-6 substitutions with β-galactose may also be found on the galactan backbone (Voragen *et al*., 2009). AGII is composed of β-(1,4)-linked-galactan backbone containing short chains of α-(1,6)-linked-Ara*f*-[β-(1,6)-linked-Gal*p*]$_n$ (*n* = 1, 2 or 3) (Ridley *et al*., 2001), and the galactosyl residues of the side-chains can also be substituted with α-(1,3)-linked-Ara*f*. Although AGII has mainly been believed to be a component of gymnosperm cell walls, the presence of both AGI and AGII side-chains has been detected in sugar beet, even though the exact amount has not been reported (Guillon & Thibault, 1989; Sakamoto & Sakai, 1995).

Feruloyl group

Ferulic acid (FA) is one of the major phenolic acids in ester linkages of pectic polysaccharides notably in spinach (Fry, 1983) and sugar beet (Rombouts & Thibaults, 1986). In beet pectin, feruloyl group, as single FA moieties or in the form of ferulic acid dehydrodimers (diFAs), is esterified to the *O*-2 position of the Ara*f* residues in the α-(1,5)-linked-arabinan backbone, but may also be bound to the *O*-5 on the terminal arabinose (Levigne *et al*., 2004), or, to a much lesser extent, at the *O*-6 position of the galactopyranosyl (Gal*p*) residues in the β-(1,4)-galactan side-chains (Colquhoun *et al*., 1994) (Figure 1.6). Four main naturally occurring diFAs have been detected in sugar beet pectin: 5,5', 8-O-4', 8,5' and 8,8' (Saulnier & Thibault, 1999) (Figure 1.7). Sugar beet pulp contains approximately 0.7-0.8% (w/w) of FA, of which approximately 0.1% (w/w) is the diFAs (Micard *et al*., 1997; Zaidel *et al*., 2011). Existence of FA has been shown to enhance the rigidity and strength of plant cell walls by cross-linking of the polysaccharide chains through dimerization reaction (Fry, 1983).

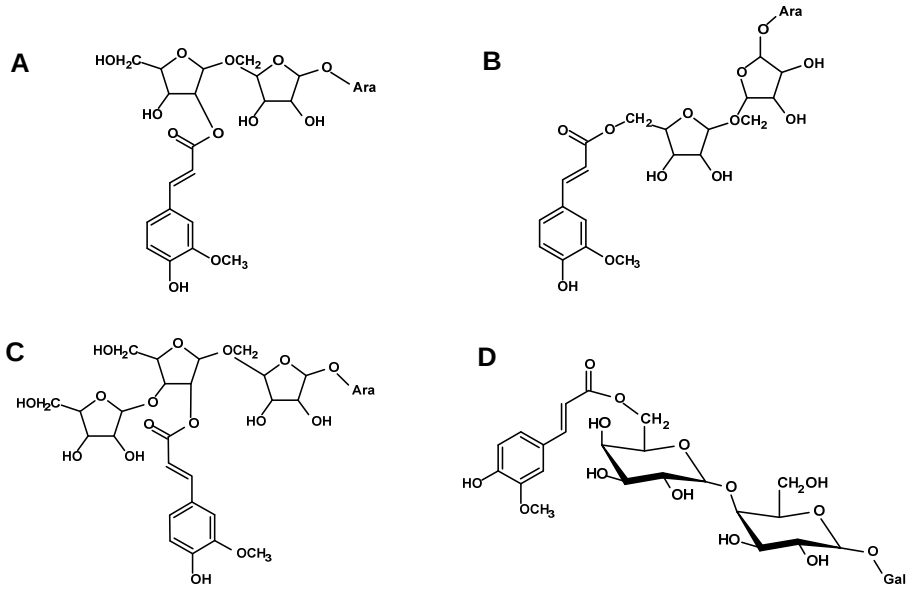

Figure 1.6: Structure of feruloylated oligosaccharides (A) (FA (*O*-2)-Ara)-(1,5)-Ara (B) (FA (*O*-5)-Ara)-(1,5)-Ara (C) Ara-(1,3)-(FA-Ara)-(1,5)-Ara (D) (FA-Gal)-(1,4)-Gal (Ishii, 1997).

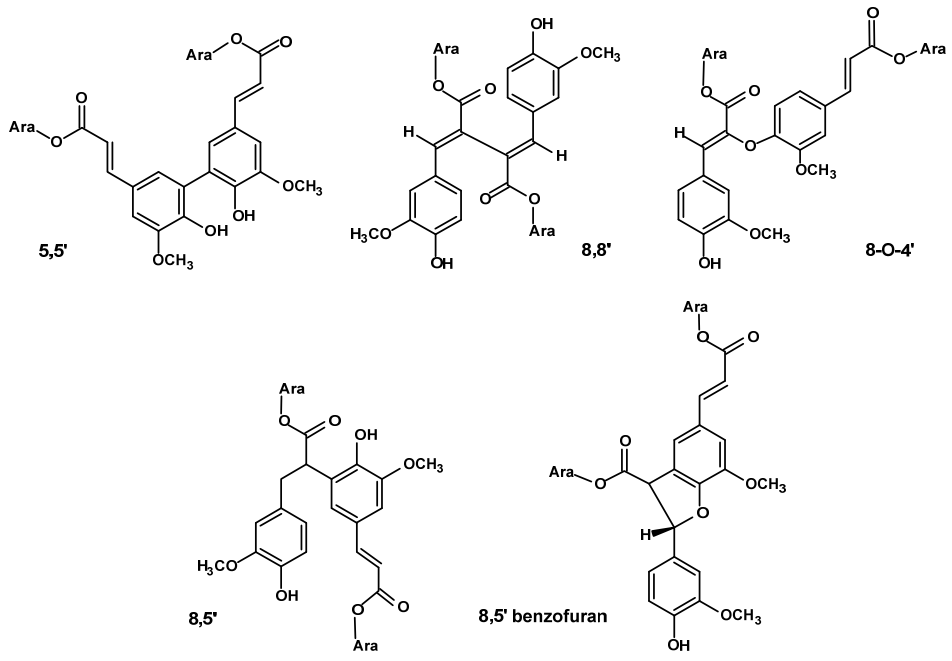

Figure 1.7: Structure of main dehydrodimers of ferulic acid esterified to arabinan backbone; 5,5', 8,8', 8-O-4' and 8,5' -diFA. The three most prevalent in sugar beet are 5,5', 8,5' and 8-O-4'-diFA.

1.1.3 Rhamnogalacturonan II

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-Gal*p*A residues, hence despite the name RGII there is no rhamnogalacturonan backbone made up of repeating units of [→2)-α-L-Rha*p*–(1,4)-α-D-Gal*p*A-(1→] in RGII and the side chains protrude directly from the C-2 and C-3 of the Gal*p*A moieties (Whitcombe *et al*., 1995; Ridley *et al*., 2001) (Figure 1.8). Despite of its complexity, RGII is thought to have a highly conserved structure and to provide an important role in cell wall function (Willats *et al*., 2006). RGII can form a borate-diol ester, together with Boron, which cross-link two RGII monomers at the 3-linked-Apiofuranosyl (Api*f*) residues of the 2-O-Me-Xyl-containing side-chains (side-chain A).

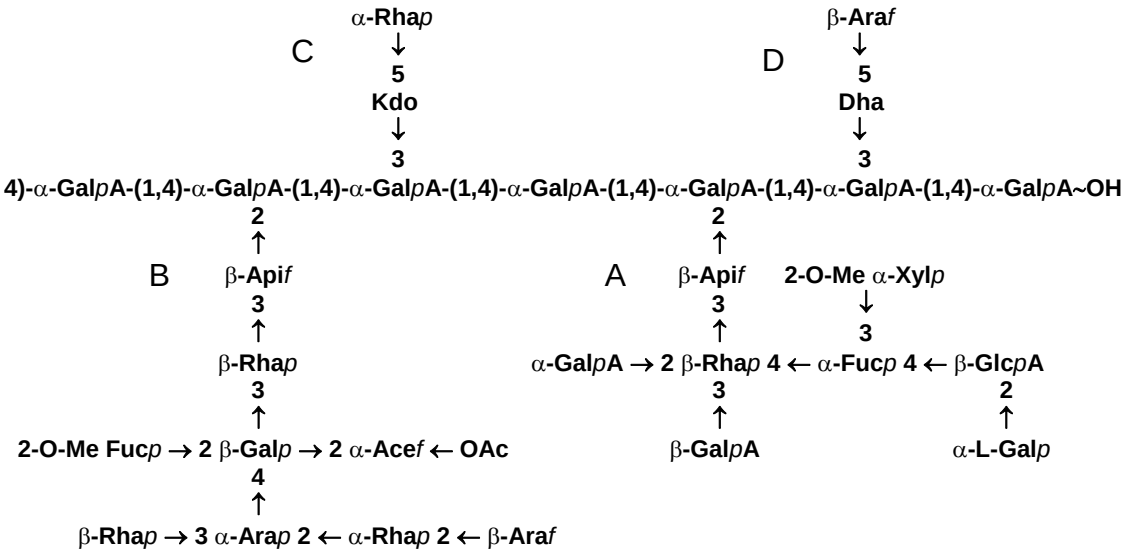

Figure 1.8: Primary structure of rhamnogalacturonan II (RGII) with four side-chains (assigned A to D) linked to the C-2 and C-3 of the Gal*p*A moieties (Ridley *et al*., 2001).

CHAPTER 2

Characterization of sugar beet pectic substrates

In order to assess the characteristic of the substrates used for this study, the first requirement in this thesis was to prepare and characterize the low molecular weight arabinan liquid and high molecular weight sugar beet pectin solid fractions obtained from the extraction of sugar beet pulp, which later is referred to as arabinan and sugar beet pectin (SBP), respectively. The analyses were a prerequisite in order to separate the pectin from the pulp and analyze both the arabinan and SBP fractions prior to the enzyme catalyzed oxidative cross-linking and gelation experiments.

2.1 Experimental approach

Low molecular (LM) arabinan rich liquid fraction and high molecular (HM) SBP solid fractions were obtained from the preparation of pectin from sugar beet pulp *via* nitric acid hydrolysis involving removal of insoluble cellulose, ultrafiltration, and diafiltration with a 50 kDa cut-off (Figure 2.1A) which was performed by Danisco A/S. A flow diagram of experimental approach is presented to provide the overall flow of the work performed in this thesis (Figure 2.1B). Both the arabinan and SBP fractions were characterized by performing several analyses for monosaccharides, oligosaccharides, molecular sizes, phenolic content and ferulic acid distribution. Measurement of the kinetics of cross-linking of feruloylated arabinan oligosaccharides will be discussed mainly in chapter 4 as presented in paper II, whereas the measurement of rates of SBP gelation and rheological properties of the gels obtained from the enzyme catalyzed cross-linking reaction will be discussed in chapters 5 and 6 as presented in paper III and IV. Detailed methods of the analysis will not be described in this chapter as they have been described in the respective paper II, III and IV.

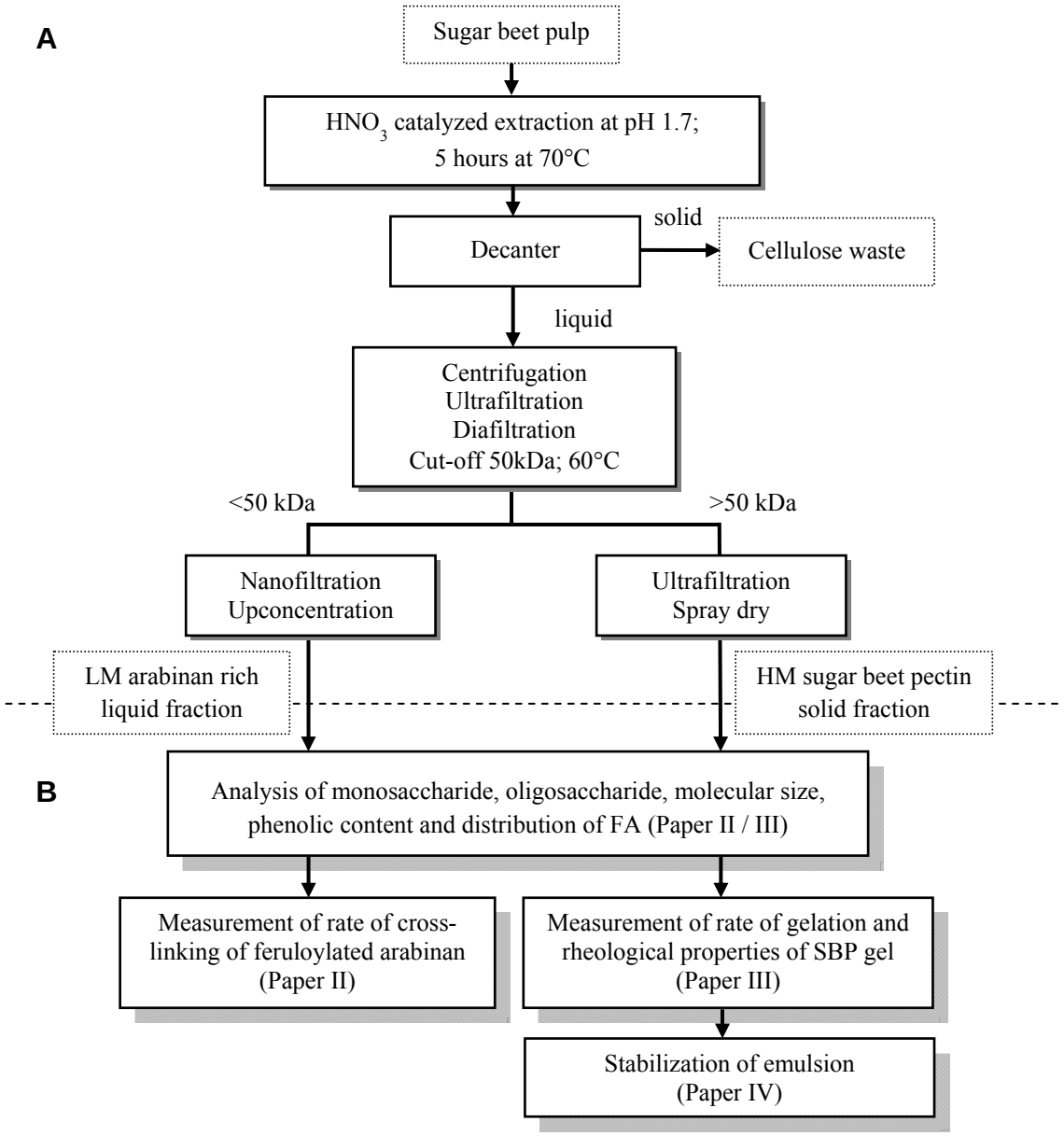

Figure 2.1: Flow diagram of the experimental approach to characterize and analyze the substrates and investigate the kinetics of enzyme catalyzed cross-linking of feruloylated oligosaccharide from sugar beet derived substrates; presenting (A) the extraction process performed by Danisco; (B) the work performed in this study.

2.2 Characterization of the arabinans and sugar beet pectin fractions

The arabinans used in this study were from two different permeate side-streams which will be referred as Ara1 and Ara2 whereas the HM SBP fraction will be referred as SBP. Both arabinans and SBP fractions were analyzed for the monosaccharide composition (Table 2.1), oligosaccharide

profiles, molecular size distribution (Figure 2.2), phenolic content (Table 2.2) and feruloyl group distribution (Figure 2.5).

Monosaccharide composition

Monosaccharide composition of the substrates were determined by High Performance Anion Exchange Chromatography with pulsed amperometric detection (HPAEC-PAD) on a Dionex BioLC system (Dionex Corp., Sunnyvale, CA, USA), after performing acid hydrolysis on the arabinans (as described in paper II- Zaidel *et al.*, 2011) or combined chemical and enzymatic hydrolysis method on the SBP (as described in paper III- Zaidel *et al.*, 2012). Combined chemical and enzymatic hydrolysis was employed for SBP for more accurate determination of galacturonic acid. The data obtained showed that arabinans consist mainly of arabinose (Ara1: 88 mol%; Ara2: 95 mol%) and traces of less than 1 mol% of rhamnose, galactose and galacturonic acid, whereas SBP consists mainly galacturonic acid (52.5 mol%), arabinose (22.8 mol%), galactose (17.9 mol%) and rhamnose (6.1 mol%) (Table 2.1).

Table 2.1: Monosaccharide composition in LM and HM fractions.

Component	Ara1 (mmol·g^{-1} DM)	Ara2 (mmol·g^{-1} DM)	SBP (mmol·g^{-1} DM)
Arabinose	3.4	2.9	1.35
Rhamnose	< 0.05	< 0.05	0.36
Glucose	0.27	< 0.05	0.04
Galactose	< 0.05	0.08	1.06
Galacturonic acid	< 0.05	< 0.05	3.11

Oligosaccharide profiles

The oligosaccharide analysis was performed only for the arabinans fraction using a Dionex BioLC system equipped with a CarboPac^{TM} PA1 analytical column (4 x 250 mm) controlled via Chromeleon 6.80 Sp8 Build 2623 software (Dionex Corp., Sunnyvale, CA), as described in paper II (Zaidel *et al.*, 2011). The profiles indicated short DP arabino-oligomers, including mainly monomers (notably arabinose) and α-(1,5)-arabino-oligosaccharides up to DP5 (arabinopentaose), with the Ara2 being relatively richer in all of the detected short oligomers, including the monosaccharide peak when evaluated at the same dry matter concentration (as presented in paper II, Fig. 2).

Molecular size distribution

The results obtained from size exclusion chromatography (HPSEC) signified the difference in molecular sizes between the two arabinans and SBP fractions (Figure 2.2). The peak for SBP was broadly distributed from 18–22 min corresponding to large molecular size (>110 kDa), whereas the arabinans presented dual peaks with one peak at ~24.5 min corresponding to monomers and an earlier peak at ~23.5 min, which correspond to molecular size ~1.3 kDa (Figure 2.2).

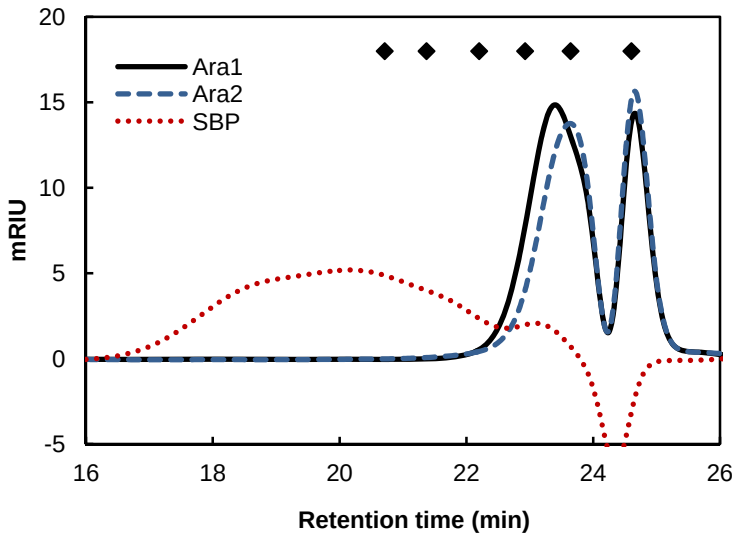

Figure 2.2: HPSEC chromatogram for arabinans (Ara1 and Ara2) and sugar beet pectin fractions. Standards (♦) from left to right 500 kDa, 110 kDa, 40 kDa, 10 kDa, 1.3 kDa and 150 Da (arabinose).

Phenolic content

Phenolic content was determined by reverse phase high-performance liquid chromatography (HPLC) after alkaline saponification (as described in paper II). FA was detected and quantified at 316 nm using an authentic external standard for retention time and spectral recognition; quantification by area linear regression. diFAs were also detected at 316 nm but quantified at 280 nm according to response factors as described in Waldron *et al.* (1996). For example, the chromatogram obtained in HPLC for arabinan fraction from sugar beet pulp (Figure 2.3) showed the identification of FA and at least four diFAs (5,5', 8-O-4', 8,5 benzofuran and 8,8') but only three diFAs were quantifiable, that is, a minimal peak was observed at the retention time equivalent to 8,8'-diFA but the level was too low to be quantified. The chromatogram for SBP fraction also showed peaks at similar retention times as the arabinan. Arabinan fractions showed relatively high diFAs compared to the pectin fraction (Table 2.2).

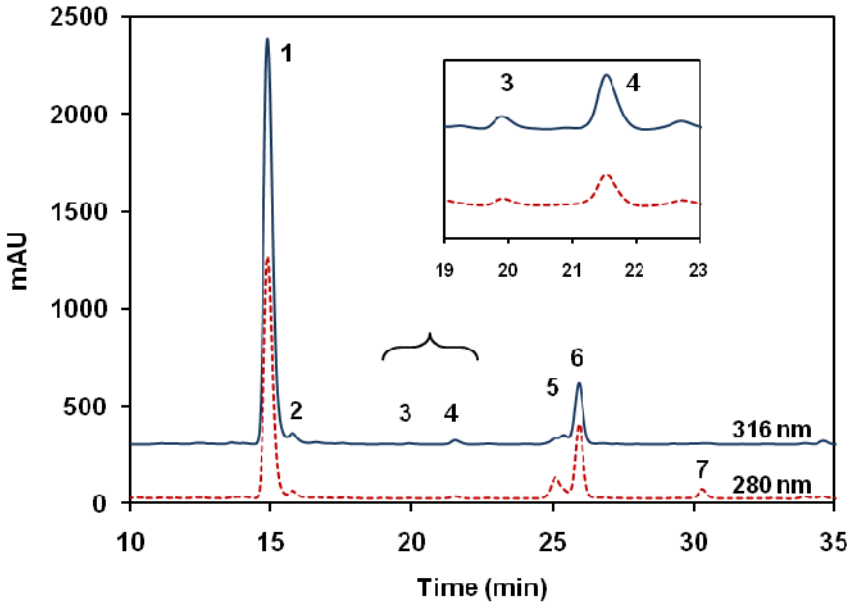

Figure 2.3: Chromatogram for arabinan fraction (Ara1) sample at 280 nm and 316 nm. (1) trans-ferulic acid; (2) cis-ferulic acid; (3) 8,8'-diFA; (4) 5,5'-diFA; (5) 8-O-4'-diFA; (6) 8,5' benzofuran-diFA; (7) trans-cinnamic acid.

Table 2.2: Phenolic content in LM and HM fractions obtained from sugar beet pulp extraction.

Phenolic	**Ara1 (mg·g^{-1} DM)**	**Ara2 (mg·g^{-1} DM)**	**SBP (mg·g^{-1} DM)**
Ferulic acid	7.0 ± 0.5	2.5 ± 0.6	7.3 ± 0.3
[a]Dehydrodimers of FA	[a]2.4 ± 0.6	[a]2.32 ± 0.03	[a]0.36 ± 0.01
5,5'	*0.26 ± 0.08*	*0.21 ± 0.02*	*0.072 ± 0.004*
8-O-4'	*1.1 ± 0.6*	*0.34 ± 0.01*	*0.15 ± 0.01*
8,5'(B)	*1.0 ± 0.3*	*1.77 ± 0.01*	*0.14 ± 0.02*
[b]*8,8'*	-	-	-

[a] total diFAs = 5,5' + 8-O-4' + 8,5'benzofuran-diFA
[b] a minimal peak was observed at the retention time equivalent to 8,8'-diFA but the level was too low to be quantified

The spectra (UV-Vis) for the compounds **1** to **7** were detected from 200 nm to 400 nm (Figure 2.4). A few unidentified peaks did occur on the chromatograms of the sample after cross-linking (not shown). The spectra of these peaks were similar to the spectra of trimers (Figure 2.4H;I) as reported by Bunzel *et al*. (2005; 2006), but exact identification was not performed in this study due to lack of external standards. Possible cross-linking and structure of trimers and tetramers of FA are illustrated in chapter 3.

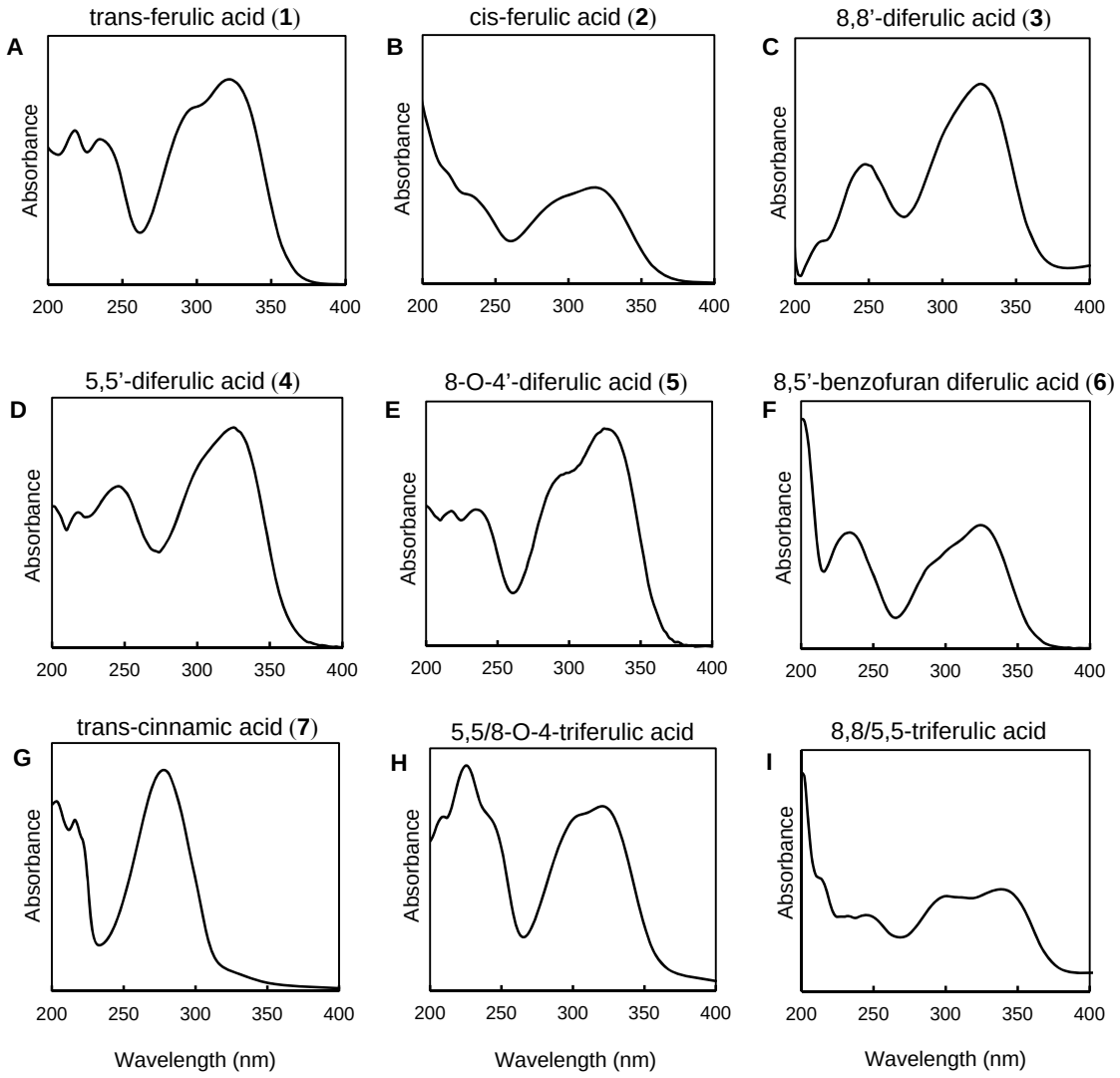

Figure 2.4: Spectra (UV-Vis) (A) to (G) for compounds 1 to 7 in Figure 2.3 (Waldron *et al*., 1996); (H) 5,5/8-O-4-triFA; (I) 8,8/5,5-triFA (Bunzel *et al*., 2005; 2006).

Feruloyl group distribution

The distribution of ferulic acid on the arabinan fractions was analyzed using hydrophilic interaction chromatography (HILIC) and the structural make-up of the LM fractions was confirmed by a 4800 *Plus* MALDI TOF/TOF^{TM} (AB SCIEX) mass spectrometer (as described in paper II).

The amide stationary phase provided a unique selectivity for the arabino-oligosaccharides under the hydrophilic interaction mode of chromatography (HILIC) as the hydrogen bonding retention mechanism between hydroxyl groups of the oligosaccharides and the carbamoyl group in the

stationary phase made it possible to elute different chains lengths based on the oligosaccharides chains' polarity and degree of solvation.

Additional from the MALDI-TOF analysis on Ara1 and Ara2 as reported in paper II, the analysis for the isopropanol precipitated samples, Ara1b and Ara2b (refer to paper II), also reveals the peak signals at *m/z* 309, 441, 573, 705; corresponding to a series of "dehydrated" arabinose (Ara*f*) moieties with a single feruloyl ester substitution (Figure 2.5), however further analysis using MS/MS has not been performed on these samples.

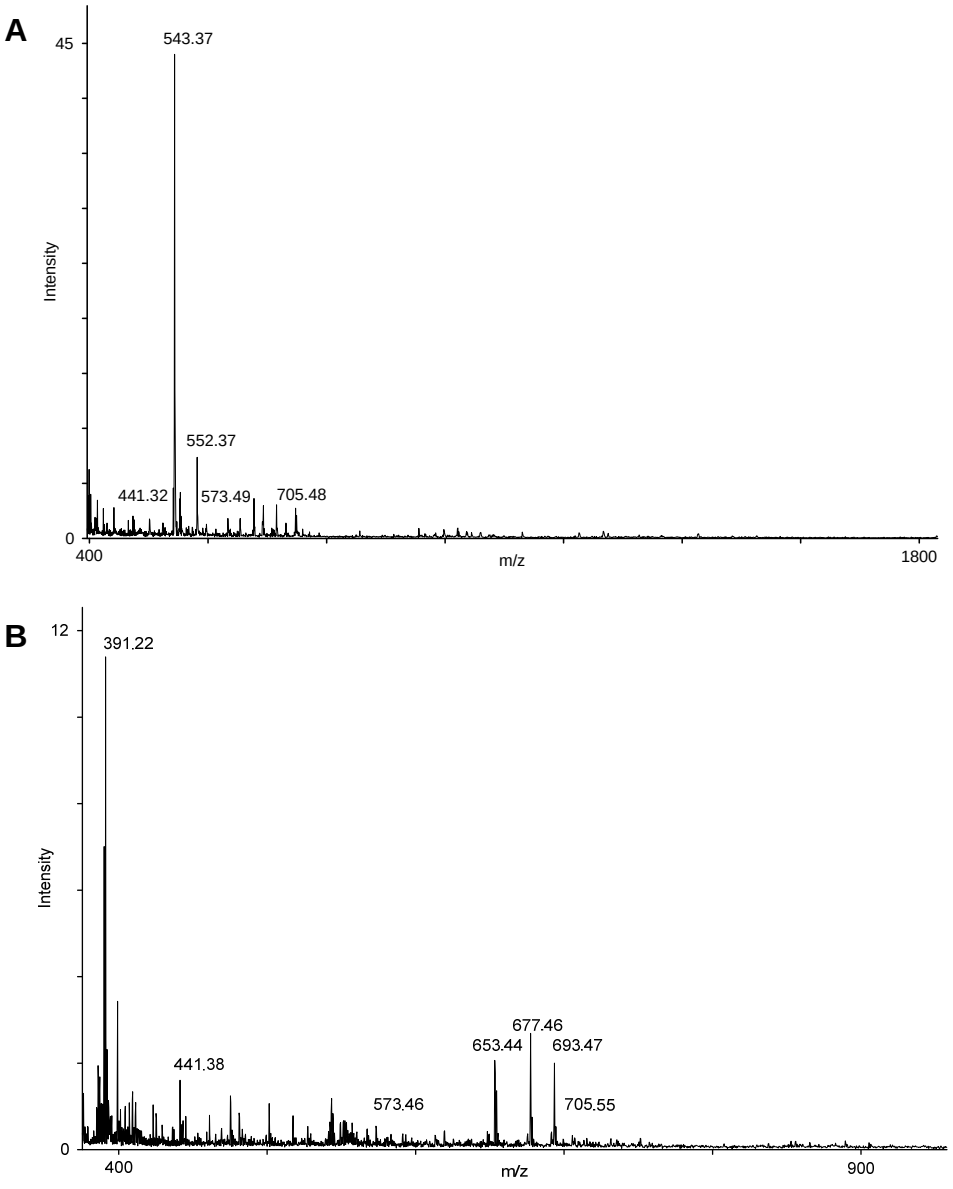

Figure 2.5: Mass spectra from MALDI-TOF analysis of (A) Ara1b and (B) Ara2b.

CHAPTER 3

Biocatalytic cross-linking of pectic polysaccharide

A number of studies have recently been reported in the literature examining the enzyme catalyzed modification of pectic polysaccharides in relation to obtaining novel functionalities, notably gelation. In order to provide a proper knowledge base, an insight into the mechanisms of pectic polysaccharide gelation, and an overview of the options for enzymatic gelation of sugar beet pectic polysaccharides, a detailed review on the structure of pectic polysaccharide, the different mechanisms and reactions of cross-linking are presented. In order to understand the functional effects, particular focus is directed towards the enzymatic modification and the methodologies used for the functionality assessment. This chapter is based on the review as presented in paper I "Biocatalytic cross-linking of pectic polysaccharide for designed food functionality: structures, mechanisms and reactions".

3.1 Cross-links in pectic polysaccharides

In nature, the matrices of plant cell wall consist of various covalent and non-covalent cross-linked polysaccharides, within the cell wall and intercellular linkage, which include ionic bridges, borate-diol esters, hydrophobic interactions and ferulic acid linkages (Sila *et al*., 2009). The complexity of cross-linked polysaccharide network structure contribute strength, flexibility and functionality to the plant cell wall which has led to several researches in modified *in situ* polysaccharide cross-linking for application in food (Norsker *et al*., 2000; Waldron *et al*., 2003; Willats *et al*., 2006). Among the many covalent cross-links, the main cross-linking involve in pectic polysaccharides include divalent-cation (Ca^{2+}) promoted ionic cross-linking, ferulic acid oxidative cross-linking, RGII cross-linking *via* borate ester, and cross-linking of uronyl ester of pectin with a hydroxyl group of another polysaccharide chain (Figure 3.1). These cross-links have been actively investigated *in vitro* and *in situ* for its use in food application which is reported to influence the properties and functionality of food, as well as the physiological effect on human.

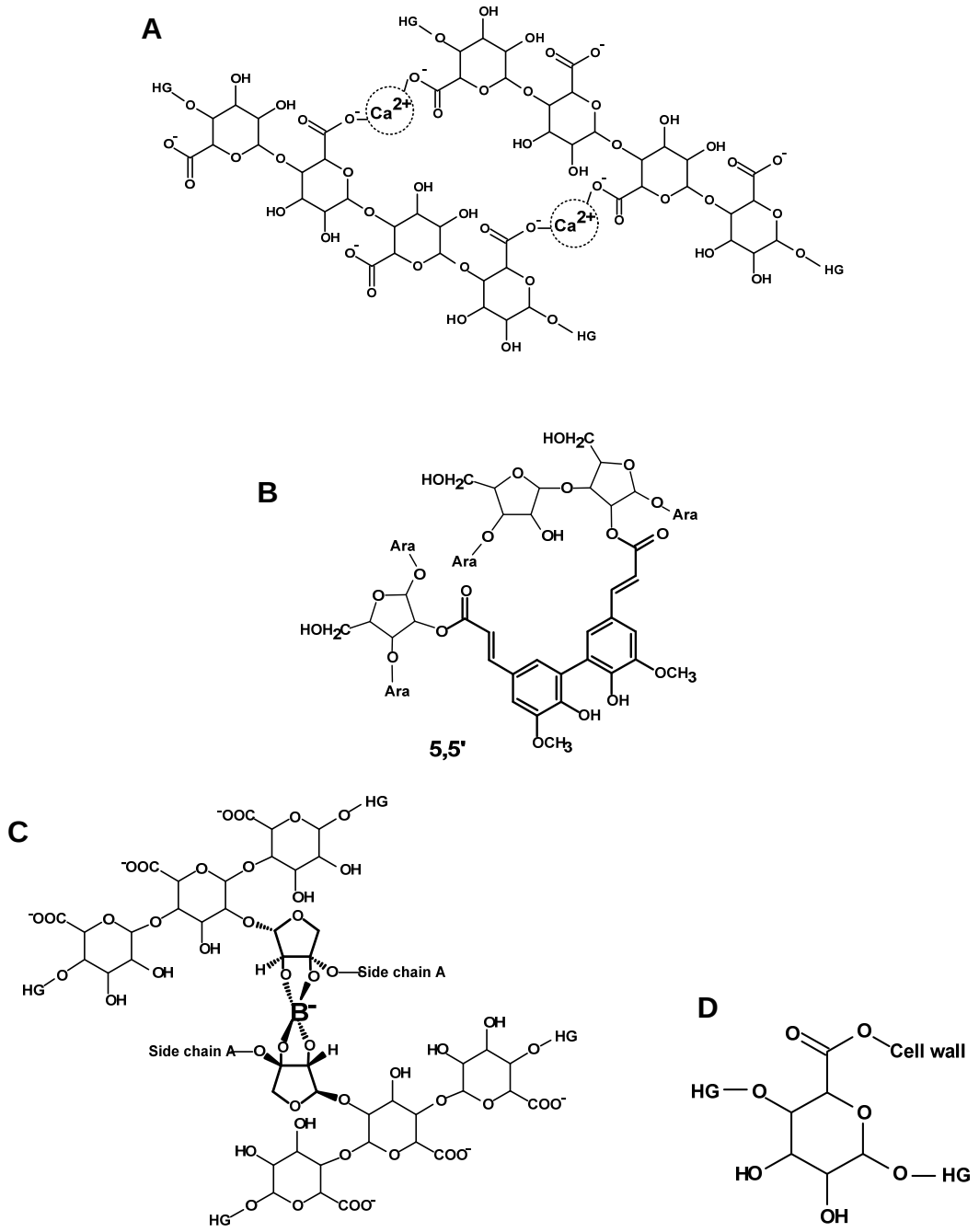

Figure 3.1: Covalent cross-linking in pectic polysaccharides as described in literature: (A) calcium bridges; (B) ferulic acid oxidation; (C) RGII-borate ester; (D) uronyl ester-hydroxyl group (adapted from Hilz, 2007).

3.1.1 Ionic cross-links

Ionic cross-links mainly refer to the interaction *via* divalent cations between two non-esterified Gal*p*A moieties in (low methylated) HG. The divalent cations are very often Ca^{2+} ions that can interact with the negatively charged carboxylate ions on C-6 of non-esterified Gal*p*A. These interactions, widely referred to as ionic junction zones (Kastner *et al.*, 2012), form a pectate

network, actually connecting two anti-parallel HG chains at a time (Figure 3.1A). At least 7 to 14 consecutive unmethylated Gal*p*A residues are required to form stable cross-links and produce stable gel (Fraeye *et al*., 2010). Degree of methoxylation (DE) has significant influence on the physical properties of the gel produced (Willats *et al*., 2006). Pectins with a low DE (< 50%), for example from citrus peel or apple pomace, produce a strong gel in the presence of Ca^{2+}. Enzymatic reaction of pectin methyl esterases (PMEs) in high DE pectin improves the interaction of unmethylated HG with the divalent cations to form calcium-pectate network. Due to this type of cross-linking, pectin mainly from citrus or apple, is commonly used in food industry as gelling agent in jams and jellies, stabilizer or fat agent replacer.

3.1.2 Ferulic acid oxidative cross-links

The reaction mechanism of ferulic acid oxidative cross-linking involves formation of ferulates radicals by the action of oxidoreductive enzymes and/or oxidizing agent and thus coupling of two ferulates to form FA dehydrodimers: 5,5', 8-O-4', 8,8', 8,5' (Figure 3.2). The proposed mechanism of the oxidative cross-linking by oxidoreductase enzymes, such as peroxidase and laccase, is the dehydrogenation of the hydroxyl group at C-4 of FA into reactive phenoxy radicals that can further dimerize or react with another radical to form C-C or C-O linkages (Ralph *et al*., 1994). This type of cross-linking occurred mainly in pectic polysaccharides originating from sugar beet and spinach, in which the feruloyl group is esterified to the *O*-2 position of the Ara*f* residues in the α-(1,5)-linked-arabinan backbone, or to the *O*-5 on the terminal arabinose (Levigne *et al*., 2004), or, to a much lesser extent, at the *O*-6 position of the galactopyranosyl (Gal*p*) residues in the β-(1,4)-galactan chains (Colquhoun *et al*., 1994). Recently, higher oligomers than dehydrodimers of ferulic acid, *i.e*. dehydrotrimers (triFA) and dehydrotetramers (tetraFA), were isolated and structurally identified in the cell wall (Figure 3.3) (Bunzel *et al*., 2005; 2006), indicating that cross-linking of higher oligomers are possible *via* enzyme catalyzed oxidative reaction. It was proposed that triFA most likely cross-link two polysaccharides chain, rather than three chains (Bunzel, 2010).

3.1.3 RGII-borate ester cross-links

Two RGII monomers are covalently cross-linked by borate esters at the 3-linked-Api*f* residues of the 2-O-Me-Xyl-containing side-chains (side-chain A) of the two RGII subunits to form RGII dimer (dRGII-B) (Figure 3.1C). This dimer formation could lead to the formation of a three-dimensional pectic network *in muro* (Ishii *et al*., 2001), which contributes to the mechanical properties of primary cell wall, and plant growth and development. This cross-link improves gel formation and hinders the processability of fruits, for example in black currant, which could be an

advantage in fruit juice processing (Hilz *et al*., 2006). The ability of dRGII-B to complex with metal ions, such as lead, barium and strontium, has been shown to improve physiological effect of dRGII-B in the intestinal and thus makes it a potential nutritional product (Tahiri *et al*., 2000).

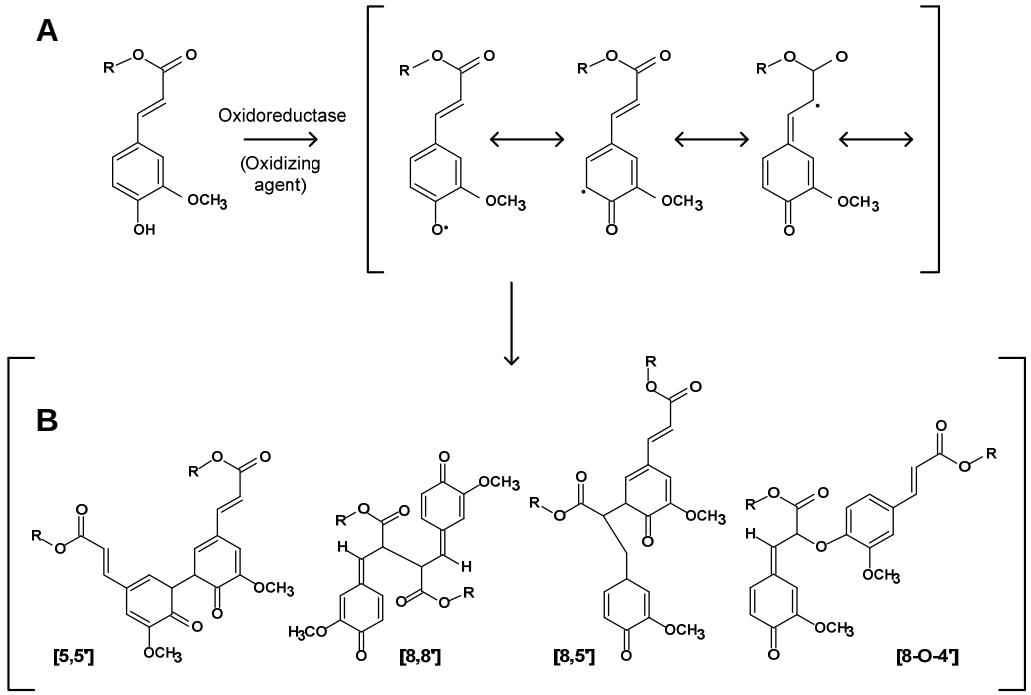

Figure 3.2: (A) Formation of ferulic acid radicals by oxidoreductase enzyme and/or oxidizing agent; (B) Intermediate products from cross-link of two ferulic acid radicals which leads to formation of dehydroferulates esters (as shown in Figure 1.7) (adapted from Ralph *et al*., 1994).

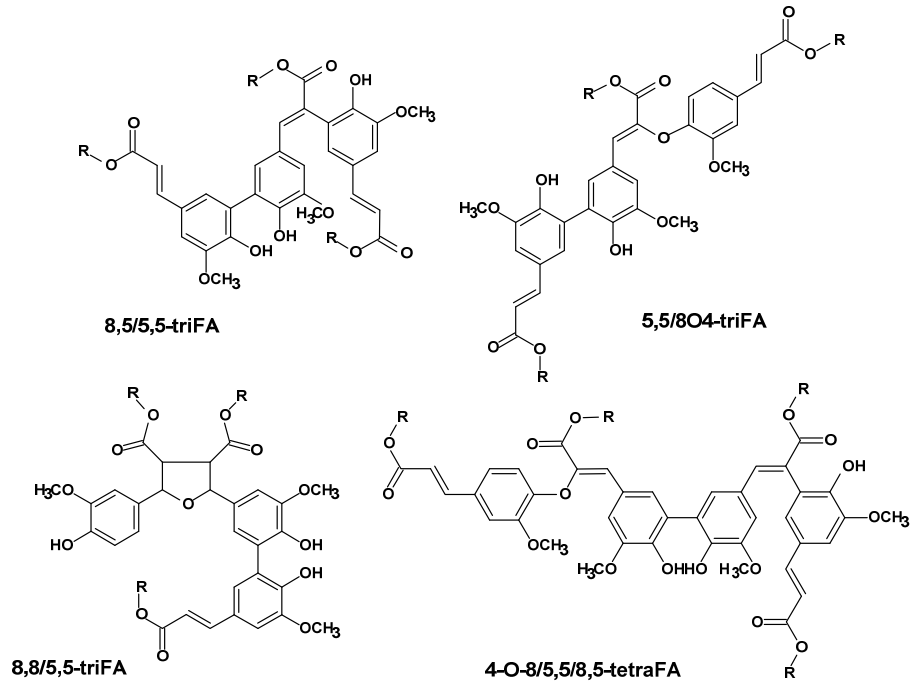

Figure 3.3: Possible formation of ferulic acid dehydrotrimers and dehydrotetramers from oxidative cross-linking (adapted from Bunzel *et al*., 2005; 2006).

3.1.4 Uronyl ester cross-links

It was proposed that HG could be covalently cross-linked to a hydroxyl group of other components in polysaccharides by uronyl esters (Brown & Fry, 1993) (Figure 3.1D). Up to 2% of the Gal*p*A residues could be linked *via* this type of cross-linking. Pectin methyl esterase (PME) was reported to catalyze the transesterification reaction between the HG linkages, which contains methyl esters that have great potential for cross-linking (Hou & Chang, 1996). During transesterification, methanol was released and the carboxyl group of the Gal*p*A moiety is attached to an -OH group of a Gal*p*A moiety of another HG chain.

3.2 Oxidoreductase enzymes

Oxidoreductase is an enzyme which catalyzes the transfer of electrons from one molecule (an oxidant, a hydrogen donor or electron donor) to another (a reductant, a hydrogen acceptor or electron acceptor) (Burton, 2003). In general, oxidoreductase enzymes, such as tyrosinase and laccase, use molecular oxygen (O_2) as the electron acceptor, whereas peroxidases require hydrogen peroxide (H_2O_2). Oxidoreductase enzymes are widely used in food processing and other emerging biotechnology industries which involves oxidation/reduction reactions, making these enzymes a prime target for exploitation. Two of the oxidoreductase enzymes, *i.e.* horseradish peroxidase and laccase, have been proposed to be the *in vivo* generators of phenoxyl radicals (Henriksen *et al*., 1999). Description of the mechanisms of these two oxidoreductase enzymes, which are used in this study and have been reported to be involved in the oxidative cross-linking of feruloylated polysaccharides, are presented briefly.

3.2.1 Peroxidase

Peroxidases (EC 1.11.1.7) are a diverse group of oxidative enzymes occurring in plants, animals and microbes, and the substrate specificity varies with the origin. For this study, one of the most commonly available peroxidase isolated from horseradish roots (*Amoracia rusticana*) was used. Horseradish peroxidase (HRP), a heme-containing enzyme, catalyzes the oxidative coupling of a variety of organic and inorganic compounds using H_2O_2 as the oxidizing agent. The reaction is a three-step cyclic reaction (equation (1) to (3)) by which the native enzyme (HRP_N) is first oxidized by H_2O_2 to HRP_I and then reduced to HRP_{II} in two sequential one-electron transfer steps from reducing substrates (R-H), typically a small molecule phenol derivative, producing one molecule of water (H_2O) and returning the enzyme to its native state. In this process, the substrate is oxidized to free radicals ($R^{\bullet}$) (Henriksen *et al*., 1999).

$$HRP_N + H_2O_2 \xrightarrow{k_1} HRP_I + H_2O \quad (1)$$

$$HRP_I + R\text{-}H \xrightarrow{k_2} HRP_{II} + R^{\bullet} \quad (2)$$

$$HRP_{II} + R\text{-}H \xrightarrow{k_3} HRP_N + R^{\bullet} + H_2O \quad (3)$$

HRP is widely used in biochemistry applications for example as a component of clinical diagnostic kits and for immunoassays (Veitch, 2004), treatment of wastewaters (Wagner & Nicell, 2002), and as oxidative agent in food application due to its ability to cross-link polysaccharides or proteins in food (Norsker *et al*., 2000).

3.2.2 Laccase

Laccases (EC 1.10.3.2) are blue multicopper oxidases enzymes commonly found in nature and occur widely in fungi, but limited in higher plants. For this study, laccase from *Trametes versicolor*, which belongs to the white-rot fungi, was used. Laccases use molecular oxygen (O_2) to oxidize various aromatic and non-aromatic compounds by a radical-catalyzed reaction mechanism (Thurston, 1994), with the assistance of a cluster of four copper atoms forming the catalytic core of the enzyme. The substrates are oxidized by reduction of one molecule of O_2 to two molecules of water (H_2O) and simultaneously producing four oxygen-centered free radicals (equation (4)):

$$4 \text{ substrate } (\text{reduced}) + O_2 + 4H^+ \xrightarrow{laccase} 4 \text{ substrate } (\text{oxidized}) + 2H_2O \quad (4)$$

Variety applications of laccase have been useful in industrial oxidative processes such as delignification, dye or stain bleaching (Pazarlıoğlu *et al*., 2005), plant fiber modification (Stoilova *et al*., 2010), biosensors application (Yaropolov *et al*., 1994) and in food application as it could cross-link polysaccharide *via* ferulic acid side-chains (Norsker *et al*., 2000).

3.3 Review – Biocatalytic cross-linking of pectic polysaccharide for designed food functionality: structures, mechanisms and reactions

Paper I: Biocatalytic cross-linking of pectic polysaccharide for designed food functionality: structures, mechanisms and reactions

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Paper I

Biocatalytic cross-linking of pectic polysaccharide
for designed food functionality:
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Manuscript

Title

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 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.

Keywords

Cross-link, polysaccharide, enzymatic, food functionality

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* [1-3]. Plant cell walls consist of complex matrices of polysaccharides, which (particularly in the primary cell walls of monocots) include cellulose, hemicelluloses, and pectin, as well as various levels of lignin. Each of these components have different structural complexities and play different physiological roles in plants [3]. 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 [4]. These different types of polysaccharide 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 [5-7]. 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 [8], and pectic polysaccharide structures have also recently been proposed to be potentially suitable for use in non-food applications such as for biomedical and biopharmaceutical purposes [9-11] or as new components in (bio)plastic manufacture [12].

Significant progress has recently been achieved within exploitation of enzymatic reactions for improving pectic polysaccharide functionality for these applications [13-17], 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 pectins [15,16,18,19]. 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 reological 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 [8,20,21] (Figure 1A). HG mainly consists of linear α-(1,4)-linked-galacturonic acid residues, and the galacturonic acid (Gal*p*A) 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)-Xyl*p* substitutions) to form xylogalacturonan [22]. The RGI backbone is made up of consecutive repeating units of [→2)-α-L-Rha*p*–(1,4)-α-D-Gal*p*A-(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 [23] and/or arabinogalactan I (AGI) or arabinogalactan II (AGII) [24] (Figure 1A). AGI is composed of a β-(1,4)-linked-galactan backbone with Ara*f* residues attached to *O*-3 of the galactosyl residues whereas AGII is substituted by short chains of α-(1,6)-linked-Ara*f*-[β-(1,6)-linked-Gal*p*]$_n$ (n = 1, 2 or 3) [2], and the galactosyl residues of the side chains can be substituted with α-(1,3)-linked-Ara*f*. 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*f* residues in the α-(1,5)-linked-arabinan backbone, but may also be bound to the *O*-5 on the terminal arabinose [25], or, to a much lesser extent, at the *O*-6 position of the galactopyranosyl (Gal*p*) residues in the β-(1,4)-galactan chains [26]. 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 [15,27] whereas in e.g. potato RGI side chains there is less than 0.05 wt% FA and hardly any diFAs [28]. 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-Gal*p*A residues, hence despite the name RGII there is no rhamnogalacturonan backbone made up of repeating units of [→2)-α-L-Rha*p*–(1,4)-α-D-Gal*p*A-(1→] and the side chains protrude directly from the C-2 and C-3 of the Gal*p*A moieties [2,29]. In spite of its complexity and low amount in the cell wall (typically ~1 to 4 wt%) [30], RGII is thought to have a highly conserved structure and to provide an important role in the plant cell wall functionality [8].

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 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 Gal*p*A moieties in (low methylated) HG. The divalent cations are very often Ca^{2+} ions that can interact with the negatively charged carboxylate ions on C-6 of non-esterified Gal*p*A. These interactions are widely referred to as ionic junction zones once a number of consecutive Gal*p*A moieties are aligned in the crosslinking [63]. The junctions connect two HG chains in an anti-parallel fashion at a time, and may form larger pectate networks (Figure 1B). This type of ionic cross-linking and the properties of the gel 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 [31], *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*p*A residues are coordinated in this way [64] (Figure 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 Gal*p*As by Ca^{2+} with higher DP [37].

Demethoxylation of methylated Gal*p*A 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 Gal*p*A 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 *pI*, form relatively random patterns of carboxylic acids in HG, whereas PMEs from plants, which often have neutral or alkaline *pI*, catalyze the demethoxylation of longer consecutive stretches of the methoxylated Gal*p*As in HG before attacking the next chain, resulting in blockwise demethoxylation patterns in the HG [43]. 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 [8]. 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 carboxyls group on the HG backbone [65]. 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 demethoxylation patterns [43]. Gelation of HG "pectin" may also take place directly *via* hydrophobic interactions between methylated Gal*p*As or *via* hydrogen bonding of undissociated carboxyl groups on neighbouring HG chains [63]. 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 [34]. Depending on the DE, the level of Ca^{2+} added into the low methylated HG pectin also influence the properties of the pectin gel; a higher concentration of Ca^{2+} generally promotes a stronger gel [66], but excess Ca^{2+} has been shown to induce aggregation of the ionic binding *via* weak electrostatic interactions [67].

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 [8]. 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 [14,31,43,45]. 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 Gal*p*As on HG has shown a synergistic effect on the Ca^{2+}-pectin gel; producing a more stable and stronger gel [14,35]. A tailored demethoxylation of Gal*p*As on HG by chemical (NaOH) reactions produced weaker Ca^{2+}-pectin gels compared to the enzymatic demethoxylation induced by plant PMEs [38]. 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 [33], 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 neighbouring molecules [63]. 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 [36].

Functionality 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 [32,40,41]. Modified

pectins catalyzed by PMEs were also reported to have a better interaction with acidified caseins than non-PME treated controls [44]. In a recent study, debranching of pectin, catalyzed by endo-arabinanase and α-L-arabinofuranosidase treatment, produced weaker gels, indicating a potential influence of the arabinose containing side-chains in the properties of Ca^{2+}-pectin gel [13]. In addition to modification of methylated pectins e.g. from apple or citrus, enzymatic modification of pectin from sugar beet (SBP) as catalyzed by PMEs, has been shown to lead to an increased calcium binding ability of SBP [39,42]. However, the calcium binding depends on very low degree of methoxylation (DE) and moreover requires low acetylation, since the acetylation has been shown to hinder the Ca^{2+} binding to the Gal*p*A of HG in SBP.

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 [33], NMR analysis to determine the mode of action of PME catalysis [33,65], 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 [33]. 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 [31,43] or oscillatory rheometry measurements [13,14,42,45] (Table 1).

In compression testing, the elasticity of the gel in the low strain region is calculated by measuring the force acting on the cross sectional area of the gel [31]. 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 [13,14,42,45]. 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 [32].

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 [68] or oxidoreductase enzymes e.g. peroxidase or laccase catalysis to promote gelation [16,47,48]. The cross-linking reaction of two feruloylated polysaccharide takes place *via* radical coupling of the FA resulting in the formation of intermediate products (Figure 2B) which then leads to formation of different diFAs: 5-5′, 8-O-4′, 8-5′ and 8-8′ (Figure 1C; Figure 2C) [69,70]. Recently, FA trimer and tetramer compounds were identified, which mainly involve the coupling of 8-O-4- and/or 5-5-diFAs [71,72].

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 [68]. 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 [68]. 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 [46]. 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 to 6.5 and temperatures between 55 to 65^{o}C depending on the enzyme source, whereas horseradish peroxidase (HRP) works optimally at pH 6 and room temperature [47]. 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 O_2 present in the reaction mixture). Laccases use molecular oxygen (O_2) to oxidize the substrate by a radical-catalyzed reaction mechanism [73], 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 O_2 to two molecules of water (H_2O). HRP catalyzes the oxidation reactions using H_2O_2 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 H_2O_2 or the available FA are consumed [74]. 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 [16]. For HRP catalysis to proceed, a sufficient amount of H_2O_2 must be added to oxidize the cross-linking reaction. However, reaction with excess amount of H_2O_2 has been shown to cause a significantly decreased level of FA but no increase in diFAs [15], possibly because the H_2O_2 led to direct oxidation of the FA without cross-linking and/or partial inactivation of the enzyme [75].

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, (Ca^{2+}) induced gelation mechanism. Modification of SBP by different extraction methods have been shown to change the gelling ability of SBP [23,46,47]. 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 [46,47]. 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 [16,48]. Laccase catalyzed gels have generally been found to be firmer or stronger, i.e. having higher *G'* 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 [16]. 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 *G'*). The rates of oxidative gelation of laccase catalysis has also been shown to be influenced by addition of Ca^{2+} into the laccase catalysis [49], hence, Ca^{2+} addition retarded the laccase catalyzed rate of gelation of SBP but improved the texture of the gel at high enzyme activities [49]. Several reaction parameters that affect the enzyme catalyzed oxidative gelation of SBP e.g. pectin level, temperature, enzyme dosage, and, for HRP, the H_2O_2 concentration, can be tuned to adjust the rate of gelation and thus the properties of the gels produced [16].

Oxidative gelation of SBP catalyzed by laccase [17,50-52] or HRP [53] 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 H_2O_2) 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* [48]. 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 [48].

For the sake of completeness it should be mentioned that principally the same type of enzyme catalyzed cross-linking has been reported for feruloylated arabinoxylan (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* [74,76] and *Trametes versicolor* [80,81], 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 [82], whereas the FA moieties are esterified to the O-5 position of the arabinose residues [83] - 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 have been reported to improve the protein stability *via* catalysis of bond formation between tyrosine and feruloylated AX [84,85]. 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 [23,47,86]. Both oxidoreductase enzymes and oxidative agents, respectively, have been shown to induce 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 was 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 were unaccessible for cross-linking or the reactions were slowed down by the increased viscosity, preventing the consumption of FA to form diFAs [15].

The levels of FA and diFAs can be analyzed using a GLC-MS [69] or HPLC after saponification [86]. Decrement of FA and increment of diFAs after the addition of oxidoreductase enzymes demonstrate the formation of diFAs *via* oxidative cross-linking of FA [15]. Gel formation through the oxidative cross-linking of feruloylated polysaccharide 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 [16, 23,49], 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 socalled 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 [47].

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 boric acid- or 1:1 borate-mRGII-intermediates. These intermediates react rapidly with RGII monomers (mRGII) to form the RGII-borate dimer (dRGII-B) (Figure 3) [54]. In general, the cross-linking is based on that two RGII monomers (mRGII) are covalently cross-linked by borate esters at the 3-linked-Api*f* residues of the 2-O-Me-Xyl-containing side chains (side chain A) of the RGII to form a RGII-borate dimer (dRGII-B) (Figure 1D; Figure 3). This formation of dRGII-B may lead to the development of a three-dimensional pectic network [87]. The

optimal pH for formation of dRGII-B has been reported to be between pH 2.2 to 4.8 with the maximum formation of dimers at pH 3.0 and 3.4 at room temperature [54,55]. In red wine, RGII has been found predominantly as dRGII-B since this form contains relatively high amounts of boric 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 [54,55]. Other divalent cations such as Ca^{2+}, Ni^{2+}, Cd^{2+} and Zn^{2+} were found to be less effective in increasing the rate of cross-linking, while Mg^{2+} and Cu^{2+} caused 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) [54] hypothesized that the hydrolysis and formation of borate ester(s) are enzymatically catalysed *in vivo* but it has not yet been unequivocally proven that enzymes are involved in the cross-linking of RGII by boric 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 [30]. The mechanism and reaction of the 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 [30]. 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 [54]. 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 [57] (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 [56] (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 [87]. 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 thus establishment of the cross-linking. The molecular masses and tentative structures of the mRGII and dRGII-B have been determined by GLC-MS and MALDI-TOF-MS [54].

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 uronyl esters [88]. In addition to catalyzing the hydrolysis of the methyl ester linkages on methylated Gal*p*A in the HG backbone, some PMEs may also catalyze transacylation, i.e. catalyze the transfer of the C-6 carboxyl group of a Gal*p*A 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 (Figure 4) [58]. These ester linkages are more stable and resistant to the action of PME compared to the methyl ester linkages on the methylated Gal*p*A 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 [58]. 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 [59,60] and an induced turbidity of the pectin solution [61,62] (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 [59]. 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 [62]. Thus, cross-linking of polysaccharide through transacylation *in situ* could be used in the food industry to prepare reduced sugar-pectin 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-D-xylose, β-D-galactose and α-L-fucose residues, and is usually considered to be a part of the hemicellulose fraction [89]. 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 RGI side chains (Figure 5). Popper & Fry (2008) [89] proposed two

models for this covalent cross-linking between XG and RGI. The first reaction involves an enzymatic transglycosylation reaction between XG-RGI by an endo-transglycosylase using XG as the donor substrate and 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 [91,92], 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 properties 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.

Abbreviations

AX	arabinoxylan
DE	degree of methoxylation
DP	degree of polymerization
diFA	dehydromer of ferulic acid
FA	ferulic acid
Gal*p*A	galacturonic acid
HG	homogalacturonan
PME	pectin methyl esterase
RG	rhamnogalacturonan
SBP	sugar beet pectin

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Figure captions

Figure 1: (A) Structure of pectic polysaccharides backbone and side chains, and three types of covalent cross-links by (B) calcium bridges (C) ferulic acid oxidation (D) RGII-borate ester

Figure 2: (A) Formation of ferulic acid radicals by oxidoreductase enzyme and/or oxidizing agent; (B) Intermediate products from cross-linking of two ferulic acid radicals; (C) Formation of dehydroferulate esters (adapted from Ralph et al., 1994 [69])

Figure 3: Proposed mechanism of two 3-linked-Api*f* residues (I) with boric acid (II). The 1:1 boric acid ester intermediate (III) and the 1:1 borate ester intermediate (IV) of an Api*f* residue can each react with a second Api*f* residue to form RGII-B dimer (V) (adapted from O'Neill et al., 1996 [54]).

Figure 4: 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 & Chang, 1996 [58])

Figure 5: Proposed RGI-xyloglucan cross-links (Hilz, 2007 [90])

References

[1] Cosgrove DJ. Expansive growth of plant cell walls. Plant Physiol Biochem 2000;38:109–24.

[2] Ridley BL, O'Neill MA, Mohnen D. Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. Phytochemistry 2001;57:929–67.

[3] Caffall KH, Mohnen D. The structure, function, and biosynthesis of plant cell wall pectic polysaccharides. Carbohydr Res 2009;344:1879–1900.

[4] Sila DN, Buggenhout SV, Duvetter T, Fraeye I, de Roeck A, Loey AV, et al. Pectins in processed fruits and vegetables: part II - structure-function relationships. Compr Rev Food Sci Food 2009;8:86–104.

[5] Parr AJ, Waldron KW, Ng A, Parker ML. The wall-bound phenolics of Chinese water chestnut (*Eleocharis dulcis*). J Sci Food Agric 1996;71:501–7.

[6] Waldron KW, Parker ML, Smith AC. Plant cell walls and food quality. Compr Rev Food Sci Food Saf 2003;2:101–19.

[7] Singh DP, Liu LH, Øiseth SK, Beloy J, Lundin L, Gidley MJ, et al. Influence of boron on carrot cell wall structure and its resistance to fracture. J Agric Food Chem 2010;58:9181–9.

[8] Willats WGT, Knox JP, Mikkelsen JD. Pectin: new insights into an old polymer are starting to gel. Trends Food Sci Technol 2006;17:97–104.

[9] Itoh K, Hatakeyama T, Shimoyama T, Miyazaki S, D'Emanuele A, Attwood D. *In situ* gelling formulation based on methylcellulose/pectin system for oral-sustained drug delivery to dysphagic patients. Drug Dev Ind Pharm 2011;37:790–7.

[10] Munarin F, Guerreiro SG, Grellier MA, Tanzi MC, Barbosa MA, Petrini P, et al. Pectin-based injectable biomaterials for bone tissue engineering. Biomacromolecules 2011;12:568–77.

[11] Takei T, Sugihara K, Ijima H, Kawakami K. *In situ* gellable sugar beet pectin *via* enzyme-catalyzed coupling reaction of feruloyl groups for biomedical applications. J Biosci Bioengineering 2011;112:491–4.

[12] Liu B, Bhaladhare S, Zhan P, Jiang L, Zhang J, Liu L, et al. Morphology and properties of thermoplastic sugar beet pulp and poly(butylenes adipate-co-terepthalate) blends. Ind Eng Chem Res 2011;50:13859–65.

[13] Ngouémazong DE, Kabuye G, Fraeye I, Cardinaels R, Loey AV, Moldenaers P, et al. Effect of debranching on the rheological properties of Ca^{2+}-pectin gels. Food Hydrocolloids 2012;26:44–53.

[14] Ngouémazong DE, Nkemamin NF, Cardinaels R, Jolie RP, Fraeye I, Loey AV, et al. Rheological properties of Ca^{2+}-gels of partially methylesterified polygalacturonic acid: Effect of "mixed" patterns of methylesterification. Carbohydr Polym 2012;88:37–45.

[15] Zaidel DNA, Arnous A, Holck J, Meyer AS. Kinetics of enzyme-catalyzed cross-linking of feruloylated arabinan from sugar beet. J Agric Food Chem 2011;59:11598–607.

[16] Zaidel DNA, Chronakis IS, Meyer AS. Enzyme catalyzed oxidative gelation of sugar beet pectin: Kinetics and rheology. Food Hydrocolloids 2012;28:130–40.

[17] Zeeb B, Gibis M, Fischer L, Weiss J. Crosslinking of interfacial layers in multilayered oil-in-water emulsions using laccase: Characterization and pH-stability. Food Hydrocolloids 2012;27:126–36.

[18] Min B, Lim J, Ko S, Lee KG, Lee SH, Lee S. Environmentally friendly preparation of pectins from agricultural byproducts and their structural/rheological characterization. Bioresource Technol 2011;102:3855–60.
[19] Fissore EN, Rojas AM, Gerschenson LN. Rheological performance of pectin-enriched products isolated from red beet (*Beta vulgaris L. var. conditiva*) through alkaline and enzymatic treatments. Food Hydrocolloids 2012;26:246–60.
[20] Coenen GJ, Bakx EJ, Verhoef RP, Schols HA, Voragen AGJ. Identification of the connecting linkage between homo- or xylogalacturonan and rhamnogalacturonan type I. Carbohydr Polym 2007;70:224–35.
[21] Holck J, Hjernø K, Lorentzen A, Vigsnæs LK, Hemmingsen L, Licht TR, et al. Tailored enzymatic production of oligosaccharides from sugar beet pectin and evidence of differential effects of a single DP chain length difference on human faecal microbiota composition after *in vitro* fermentation. Process Biochem 2011;46:1039–49.
[22] Voragen AGJ, Coenen GJ, Verhoef RP, Schols HA. Pectin, a versatile polysaccharide present in plant cell walls. Struct Chem 2009;20:263–75.
[23] Oosterveld A, Beldman G, Schols HA, Voragen AGJ. Characterization of arabinose and ferulic acid rich pectic polysaccharides and hemicelluloses from sugar beet pulp. Carbohydr Res 2000;328:185–97.
[24] Lerouge P, O'Neill MA, Darvill AG, Albersheim P. Structural characterization of endo-glycanase-generated oligoglycosyl side chains of rhamnogalacturonan I. Carbohydr Res 1993;243:359–71.
[25] Levigne SV, Ralet MCJ, Quéméner BC, Pollet BNL, Lapierre C, Thibault JFJ. Isolation from sugar beet cell walls of arabinan oligosaccharides esterified by two ferulic acid monomers. Plant Physiol 2004;134:1173–80.
[26] Colquhoun IJ, Ralet MC, Thibault JF, Faulds CB, Williamson G. Structure identification of feruloylated oligosaccharides from sugar beet pulp by NMR spectroscopy. Carbohydr Res 1994;263:243–56.
[27] Micard V, Grabber JH, Ralph J, Renard MGC, Thibault JF. Dehydrodiferulic acids from sugar beet pulp. Phytochemistry 1997;44:1365–8.
[28] Singh A, Sabally K, Kubow S, Donnelly DJ, Gariepy Y, Orsat V, Raghavan GSV. Microwave-assisted extraction of phenolic antioxidants from potato peels. Molecules 2011;16:2218–32.
[29] Whitcombe AJ, O'Neill MA, Steffan W, Albersheim P, Darvill AG. Structural characterization of the pectic polysaccharide, rhamnogalacturonan-II. Carbohydr Res 1995;271:15–29.
[30] O'Neill MA, Ishii T, Albersheim P, Darvill AG. Rhamnogalacturonan II: Structure and function of a borate cross-linked cell wall pectic polysaccharide. Annu Rev Plant Biol 2004;55:109–39.
[31] Willats WGT, Orfila C, Marcus SE, Knox JP, Limberg G, Christensen TMIE, et al. Modulation of the degree and pattern of methyl-esterification of pectic homogalacturonan in plant cell walls: Implications for pectin methyl esterase action, matrix properties, and cell adhesion. J Biol Chem 2001;276:19404–13.
[32] Corredig M, Kerr W, Wicker L. Particle size distribution of orange juice cloud after addition of sensitized pectin. J Agric Food Chem 2001;49:2523–6.
[33] Hotchkiss ATJ, Savary BJ, Cameron RG, Chau HK, Brouillette J, Luzio GA, et al. Enzymatic modification of pectin to increase its calcium sensitivity while preserving its molecular weight. J Agric Food Chem 2002;50:2931–7.

[34] Yoo SH, Fishman ML, Savary BJ, Hotchkiss ATJ. Monovalent salt-induced gelation of enzymatically deesterified pectin. J Agric Food Chem 2003;51:7410–7.
[35] Yoo SH, Lee BH, Savary BJ, Lee S, Lee HG, Hotchkiss AT. Characteristics of enzymatically-deesterified pectin gels produced in the presence of monovalent ionic salts.Food Hydrocolloids 2009;23:1926–9.
[36] Yoo YH, Lee S, Kim Y, Kim KO, Kim YS, Yoo SH. Functional characterization of the gels prepared with pectin methylesterase (PME)-treated pectins. Int J Biol Macromol 2009;45:226–30.
[37] Luzio GA, Cameron RG. Demethylation of a model homogalacturonan with the salt-independent pectin methylesterase from citrus: Part II. Structure-function analysis. Carbohydr Polym 2008;71:300–9.
[38] Tanhatan-Nasseri A, Crépeau MJ, Thibault JF, Ralet MC. Isolation and characterization of model homogalacturonans of tailored methylesterification patterns. Carbohydr Polym 2011;86:1236–43.
[39] Ralet MC, Crépeau MJ, Buchholt HC, Thibault JF. Polyelectrolyte behavior and calcium binding properties of sugar beet pectins differing in their degrees of methylation and acetylation. Biochem Eng J 2003;16:191–201.
[40] Wicker L, Ackerley JL, Hunter JL. Modification of pectin by pectinmethylesterase and the role in stability of juice beverages. Food Hydrocolloids 2003;17:809–14.
[41] Croak S, Corredig M. The role of pectin in orange juice stabilization: Effect of pectin methylesterase and pectinase activity on the size of cloud particles. Food Hydrocolloids 2006;20:961–5.
[42] Lee H, Rivner J, Urbauer JL, Garti N, Wicker L. De-esterification pattern of Valencia orange pectinmethylesterases and characterization of modified pectins. J Sci Food Agric 2008;88:2102–10.
[43] Fraeye I, Colle I, Vandevenne E, Duvetter T, Buggenhout SV, Moldenaers P, et al. Influence of pectin structure on texture of pectin-calcium gels. Innovative Food Sci Emerging Technol 2010;11:401–9.
[44] Kim Y, Wicker L. Charge domain of modified pectins influence interaction with acidified caseins. Food Hydrocolloids 2011;25:419–25.
[45] Ngouémazong DE, Tengweh FF, Fraeye I, Duvetter T, Cardinaels R, Loey AV, et al. Effect of de-methylesterification on network development and nature of Ca^{2+}-pectin gels: Towards understanding structure-function relations of pectin. Food Hydrocolloids 2012;26:89–98.
[46] Guillon F, Thibault JF. Oxidative cross-linking of chemically and enzymatically modified sugar-beet pectin. Carbohydr Polym 1990;12:353–74.
[47] Micard V, Thibault JF. Oxidative gelation of sugar-beet pectins: use of laccases and hydration properties of the cross-linked pectins. Carbohydr Polym 1999;39:265–73.
[48] Norsker M, Jensen M, Adler-Nissen J. Enzymatic gelation of sugar beet pectin in food products. Food Hydrocolloids 2000;14:237–43.
[49] Kuuva T, Lantto R, Reinikainen T, Buchert J, Autio K. Rheological properties of laccase-induced sugar beet pectin gels. Food Hydrocolloids 2003;17:679–84.
[50] Littoz FF, McClements DJ. Bio-mimetic approach to improving emulsion stability: Cross-linking adsorbed beet pectin layers using laccase. Food Hydrocolloids 2008;22:1203-11.

[51] Chen B, McClements DJ, Gray DA, Decker EA. Stabilization of soybean oil bodies by enzyme (laccase) cross-linking of adsorbed beet pectin coatings. J Agric Food Chem 2010;58:9259–65.
[52] Jung J, Wicker L. Laccase mediated conjugation of sugar beet pectin and the effect on emulsion stability. Food Hydrocolloids 2012;28:168–73.
[53] Li JL, Cheng YQ, Wang P, Zhao WT, Yin LJ, Saito M. A novel improvement in whey protein isolate emulsion stability: Generation of an enzymatically cross-linked beet pectin layer using horseradish peroxidase. Food Hydrocolloids 2012;26:448–55.
[54] O'Neill MA, Warrenfeltz D, Kates K, Pellerin P, Doco T, Darvill AG, et al. Rhamnogalacturonan-II, a pectic polysaccharide in the walls of growing plant cell, forms a dimer that is covalently cross-linked by a borate ester: In vitro conditions for the formation and hydrolysis of the dimer. J Biol Chem 1996;271:22923–30.
[55] Ishii T, Matsunaga T, Pellerin P, O'Neill MA, Darvill A, Albersheim P. The plant cell wall polysaccharide rhamnogalacturonan II self-assembles into a covalently cross-linked dimer. J Biol Chem 1999;274:13098–104.
[56] Tahiri M, Pellerin P, Tressol JC, Doco T, Pepin D, Rayssiguier Y, et al. The rhamnogalacturonan-II dimer decreases intestinal absorption and tissue accumulation of lead in rats. J Nutr 2000;130:249–53.
[57] Hilz H, Williams P, Doco T, Schols HA, Voragen AGJ. The pectic polysaccharide rhamnogalacturonan II is present as a dimer in pectic populations of bilberries and black currants in muro and in juice. Carbohydr Polym 2006;65:521–8.
[58] Hou WC, Chang WH. Pectinesterase-catalyzed firming effects during precooking of vegetables. J Food Biochem 1996;20:397–416.
[59] Jiang CM, Lai YJ, Lee BH, Chang WH, Chang HM. De-esterification and transacylation reactions of pectinesterase from Jelly fig (*Ficus awkeotsang Makino*) achenes. J Food Sci 2001; 66: 810–5.
[60] Jiang CM, Wu MC, Chang WH, Chang HM. Change in particle size of pectin reacted with pectinesterase isozymes from pea (*Pisum sativum L.*) sprouts. J Agric Food Chem 2001;49:4383–7.
[61] Hwang JY, Lee CW, Wu MC, Chang HM. Transacylation and de-esterification reactions of pectin as catalyzed by pectinesterases from tomato and citrus. J Agric Food Chem 2003;51:6287–92.
[62] Wu MC, Chen YW, Hwang JY, Lee BH, Chang HM. Transacylation of citrus pectin as catalyzed by pectinesterase from tendril shoots of chayote [*Sechium edule* (Jacq.) Swartz]. Food Res Int 2004;37:759–65.
[63] Kastner H, Einhorn-Stoll U, Senge B. Structure formation in sugar containing pectin gels – influence of Ca^{2+} on the gelation of low-methoxylated pectin at acidic pH. Food Hydrocolloids 2012;27:42–9.
[64] Fraeye I, Duvetter T, Doungla E, Loey AV, Hendrickx M. Fine-tuning the properties of pectin-calcium gels by control of pectin fine structure, gel composition and environmental conditions. Trends Food Sci Technol 2010;21:219–28.
[65] Denès JM, Baron A, Renard CMGC, Péan C, Drilleau JF. Different action patterns for apple pectin methylesterase at pH 7.0 and 4.5. Carbohydr Res 2000;327:385–93.
[66] Ngouémazong DE, Jolie RP, Cardinaels R, Fraeye I, Loey AV, Moldenaers P, et al. Stiffness of Ca^{2+}-pectin gels: combined effects of degree and pattern of methylesterification for various Ca^{2+} concentractions. Carbohyr Res 2012;348:69–76.

[67] Braccini I, Pérez S. Molecular basis of Ca^{2+}-induced gelation in alginates and pectins: the egg-box model revisited. Biomacromolecules 2001;2:1089–96.
[68] Thibault JF, Rombouts FM. Effect of some oxidizing agents, especially ammonium peroxysulfate, on sugar-beet pectins. Carbohydr Res 1986;154:205–15.
[69] Ralph J, Quideau S, Grabber JH, Hatfield RD. Identification and synthesis of new ferulic acid dehydrodimers present in grass cell walls. J Chem Soc Perkin Trans 1 1994; 3485–98.
[70] Saulnier L, Thibault JF. Ferulic acid and diferulic acids as components of sugar-beet pectins and maize bran heteroxylans. J Sci Food Agric 1999;79:396–402.
[71] Bunzel M, Ralph J, Funk C, Steinhart H. Structural elucidation of new ferulic acid-containing phenolic dimers and trimers isolated from maize bran. Tetrahedron Lett 2005;46:5845–50.
[72] Bunzel M, Ralph J, Bruning P, Steinhart, H. Structural identification of dehydrotriferulic and dehydrotetraferulic acids isolated from insoluble maize bran fiber. J Agric Food Chem 2006;54:6409–18.
[73] Thurston CF. The structure and function of fungal laccases. Microbiology 1994;140:19–26.
[74] Figueroa-Espinoza MC, Rouau X. Oxidative cross-linking of pentosans by a fungal laccase and horseradish peroxidase: mechanism of linkage between feruloylated arabinoxylans. Cereal Chem 1998;75:259–65.
[75] Bunzel M, Heuermann B, Kim H, Ralph J. Peroxidase-catalyzed oligomerization of ferulic acid esters. J Agric Food Chem 2008;56:10368–375.
[76] Vansteenkiste E, Babot C, Rouau X, Micard V. Oxidative gelation of feruloylated arabinoxylan as affected by protein. Influence on protein enzymatic hydrolysis. Food Hydrocolloids 2004;18:557–64.
[77] Carvajal-Millan E, Guigliarelli B, Belle V, Rouau X, Micard V. Storage stability of laccase induced arabinoxylan gels. Carbohydr Polym 2005;59:181–8.
[78] Carvajal-Millan E, Guilbert S, Morel MH, Micard V. Impact of the structure of arabinoxylan gels on their rheological and protein transport properties. Carbohydr Polym 2005;60:431–38.
[79] Robertson JA, Faulds CB, Smith AC, Waldron KW. Peroxidase-mediated oxidative cross-linking and its potential to modify mechanical properties in water-soluble polysaccharide extracts and cereal grain residues. J Agric Food Chem 2008;56:1720–6.
[80] Berlanga-Reyes CM, Carvajal-Millan E, Juvera GC, Rascon-Chu A, Marquez-Escalante JA, Martinez-Lopez AL. Laccase induced maize bran arabinoxylan gels: structural and rheological properties. Food Sci Biotechnol 2009;18:1027–29.
[81] Berlanga-Reyes CM, Carvajal-Millan E, Lizardi-Mendoza J, Islas-Rubio AR, Rascón-Chu A. Enzymatic cross-linking of alkali extracted arabinoxylans: gel rheological and structural characteristics. Int J Mol Sci 2011;12:5853–61.
[82] Izydorczyk MS, Biliaderis CG. Cereal arabinoxylans: Advances in structure and physicochemical properties. Carbohydr Polyms 1995;28:33–48.
[83] Smith MM, Hartley RD. Occurance and nature of ferulic acid substitution of cell-wall polysaccharides in graminaceous plants. Carbohydr Res 1983;118:65–80.
[84] Boeriu CG, Oudgenoeg G, Spekking WTJ, Berendsen LBJM, Vancon L, Boumans H, et al. Horseradish peroxidase-catalyzed cross-linking of feruloylated arabinoxylans with β-casein. J Agric Food Chem 2004;52:6633–39.

[85] Selinheimo E, Lampila P, Mattinen ML, Buchert J. Formation of protein-oligosaccharide conjugates by laccase and tyrosinase. J Agric Food Chem 2008;56:3118–28.
[86] Waldron KW, Parr AJ, Ng A, Ralph J. Cell wall esterified phenolic dimers: Identification and quantification by reverse phase high performance liquid chromatography and diode array detection. Phytochem Anal 1996;7:305–12.
[87] Ishii T, Matsunaga, T. Pectic polysaccharide rhamnogalacturonan II is covalently linked to homogalacturonan. Phytochemistry 2001;57:969–74.
[88] Brown JA, Fry SC. Novel O-D-galacturonoyl esters in the pectic polysaccharides of suspension-cultured plant cells. Plant Physiol 1993;103:993–9.
[89] Popper ZA, Fry SC. Xyloglucan-pectin linkages are formed intra-protoplasmically, contribute to wall-assembly, and remain stable in the cell wall. Planta 2008;227:781–94.
[90] Hilz H. Characterisation of cell wall polysaccharides in bilberries and black currants. PhD Thesis. Wageningen University, The Netherlands; 2007.
[91] Thompson JE, Fry SC. Evidence for covalent linkage between xyloglucan and acidic pectins in suspension-cultured rose cells. Planta. 2000;211:275–86.
[92] Popper ZA, Fry SC. Widespread occurrence of a covalent linkage between xyloglucan and acidic polysaccharides in suspension-cultured angiosperm cells. Ann Bot 2005;96:91–9.

Table

Table 1: Different types and mechanisms of cross linking in polysaccharides *in situ* by direct or indirect enzymatic reaction/modification for designed functionality

Type of cross linking	Reaction mechanism	Substrate	Enzyme / Catalyst	Functionality / Modification / Application	Assessment	References
Ionic	Decreasing the degree of methyl esterification of GalA on HG and promoting the cross-linking of HG in the presence of Ca^{2+}	Lime pectin	PME from orange peel and *Aspergillus niger*	Degree and patterns of methoxylation of HG	Compression test, water holding capacity, porosity	Willats *et al*., 2001 [31]
		Citrus pectin	PME from grapefruit pulp	Demethoxylation of pectin for orange juice clarification	Cloud stability test	Corredig *et al*., 2001 [32]
			PME from orange peel	Calcium sensitivity; degree of methoxylation of HG	Viscosity, calcium sensitivity assay, molecular size distribution, NMR	Hotchkiss *et al*., 2002 [33]
			PME from orange peel and/or *Aspergillus aculeatus*	Addition of monovalent salt for pectin gelation; synergistic effect of combined plant and fungal derived PMEs on gel strength, flavor release	Compression; syneresis test; flavor release determination	Yoo *et al*., 2003 [34]; Yoo *et al*., 2009 [35,36]
			PME from plant	Demethoxylation of HG	Calcium activity, viscosity, degree of blockiness	Luzio & Cameron, 2008 [37]; Tanhatan-Nasseri et al., 2011 [38]
		Sugar beet pectin	PMEs from plant and fungi	Deacetylation and/or demethoxylation of SBP for improved calcium binding properties	Calcium sensitivity test	Ralet et al., 2003 [39]
		Orange (juice)	PME from orange peel	Stabilization of orange juice	Cloud stability test	Wicker et al., 2003 [40]; Croak & Corredig, 2006 [41]
		Citrus and sugar beet pectins	PME from Valencia orange peel	Degree and patterns of methoxylation of HG	Controlled dynamic oscillatory test	Lee et al., 2008 [42]
		Apple pectin	PMEs from tomatoes and from *Aspergillus aculeatus*	Degree and patterns of methoxylation of HG	Compression test; titration	Fraeye *et al*., 2010 [43]
		High ester methyl pectin	PME from Valencia orange peel	Demethoxylation of HG and interaction with acidified caseins	Particle size, surface charge, viscosity, light microscopy	Kim & Wicker, 2011 [44]
		Carrot pectin	PME from carrots and/or *Aspergillus aculeatus*	Debranching or (mixed patterns of) demethoxylation of HG	Viscosity; small amplitude oscillatory shear tests	Ngouémazong et al., 2012 [13,14, 45]

Phenolic ester	Oxidative cross linking of FA esterified to arabinose residues and formation of FA dimers	Sugar beet pectin	Ammonium persulfate	Oxidative cross-linking for differently extracted SBP (Structural change of SBP)	Gelation visual observation, spectrophotometry	Guillon and Thibault, 1990 [46]
			Laccase from *Polyporus insitius* and *Myceliophtora thermophili*	Oxidative cross-linking for differently extracted SBP; Water-absorption capacities of pectin gels	Gelation visual observation, swelling capacity, spectrophotometry	Micard and Thibault, 1999 [47]
			Horseradish peroxidase and hydrogen peroxide; Ammonium persulfate	Oxidative cross-linking for differently extracted SBP	Small amplitude shear strain oscillatory measurement, spectrophotometry	Oosterveld *et al*., 2000 [23]
			Horseradish peroxidase and hydrogen peroxide; laccase	Oxidative cross-linking of SBP in different food products	Gelation test on texture analyzer (hardness, adhesiveness, springiness, chewiness)	Norsker *et al*., 2000 [48]
			Laccase from *Trametes hirsuta*	Addition of Ca^{2+} into laccase induced gelation and laccase activity on the gelation kinetics	Small deformation viscoelastic measurement, compression test	Kuuva et al., 2003 [49]
			Laccase from *Trametes versicolor*	Oxidative cross-linking for improved emulsion stability	Emulsion: particle size, zeta-potential, creaming stability, optical, spectrophotometry	Littoz and McClements, 2008 [50]; Chen et al., 2010 [51]; Jung & Wicker 2012 [52]; Zeeb et al., 2012 [17]
			Horseradish peroxidase and hydrogen peroxide	Oxidative cross-linking for improved emulsion stability	Emulsion: particle size, zeta-potential, creaming stability	Li et al., 2012 [53]
			Horseradish peroxidase hydrogen peroxide; laccase from *Trametes versicolor*	Different enzyme catalysis mechanisms, various reaction parameters	Small angle oscillatory measurement	Zaidel et al., 2012 [16]

Borate ester	Cross linking of two RGII monomers at the 3-linked Api*f* residues of the 2-O-MeXyl containing side chains by borate diol ester	Red wine, pea	Boric acid	Formation of dRGII-b by addition of divalent cations	Molecular size distribution	O'Neill *et al*., 1996 [54]
		Sugar beet, potato, bamboo shoots and red wine		Formation of dRGII-b as influenced by addition of mono-, di-, tri-, tetravalent cations	Molecular size distribution	Ishii *et al*., 1999 [55]
		Apple		Formation of dRGII-b for improved intestinal absorption and tissue retention of heavy metal	Molecular size distribution; heavy metal absorption	Tahiri *et al*., 2000 [56]
		Black currant and bilberries		Formation of dRGII-b for improved juice processability	Molecular size distribution	Hilz *et al*., 2006 [57]
Other	Uronyl ester cross-linking to another polysaccharide through transacylation reaction catalyzed by pectinesterase (PE)	Citrus pectin	PE from pea sprouts	Transacylation reaction in a model pectin system catalyzed by PE and the correlation between the ester linkages to the texture of cooked vegetables	Molecular size distribution	Hou & Chang 1996 [58]
			PE from Jelly fig (*Ficus awleotsang* Makino) achenes or pea (*Pisum sativum L.*) sprout	Transacylation reaction in a model pectin system catalyzed by PE	Molecular size distribution	Jiang et al., 2001 [59,60]
		Citrus pectin (and apple pectin)	PE from pea pod, tomato and citrus; PE from tendril shoots of chayote (*Sechium edule* (Jacq.) Swartz)	Transacylation reaction in a model pectin system catalyzed by PE	Turbidity evaluation monitored by spectrophotometer; compression test; viscosity of pectin solution, compression test	Hwang et al., 2003 [61]; Wu et al., 2004 [62]

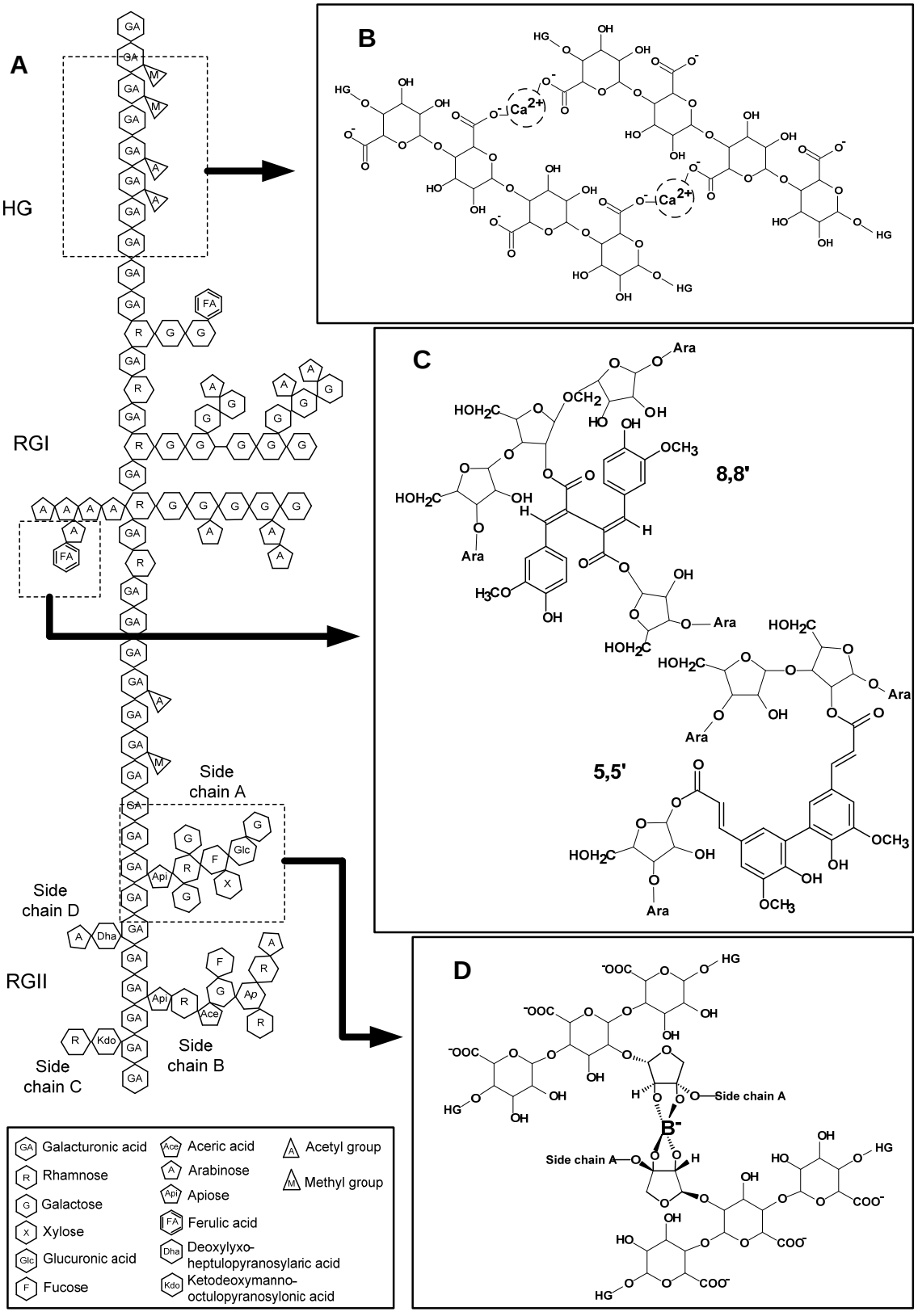
HG homogalacturonan; PME pectin methyl esterase; PE pectinesterase; SBP sugar beet pectin

Table 2: Phenolic ester cross-linking in arabinoxylan *in situ* by enzymatic reaction/modification for designed functionality

Reaction mechanism	**Substrate**	**Enzyme/ Catalyst**	**Functionality/ Modification/ Application**	**Assessment**	**References**
Oxidative cross linking of FA esterified to AX and formation of FA dimers	Wheat AX	Horseradish peroxidase and hydrogen peroxide; laccase from *Pycnoporus cinnabarinus*	Different enzyme catalysis	Capillary viscometric measurement	Figuera-Espinoza *et al*., 1998 [74]
		Laccase from *Pycnoporus cinnabarinus*	Addition of bovine serum albumin to the oxidative cross-linking reaction	Small amplitude shear oscillatory measurement	Vansteenkiste *et al*., 2004 [76]
		Laccase from *Pycnoporus cinnabarinus*	Stability of the gel; Structural of AX impact on gel properties	Small amplitude shear oscillatory measurement, swelling capacity	Carvajal-Millan *et al*., 2005 [77,78]
	AX from wheat and rye, sugar beet pectin	Horseradish peroxidase and hydrogen peroxide	Mechanical properties of gel from different substrates	Gelation visual observation, swelling capacity	Robertson *et al*., 2008 [79]
	AX from maize; wheat bran	Laccase from *Trametes versicolor*	Mechanical properties of AX gels at different AX concentrations; influenced by extraction methods	FA and diFA: RPHPLC; Gelation: small amplitude oscillatory shear by rheometer, swelling capacity	Berlanga-Reyes *et al*., 2009; 2011 [80,81]

Figures

Figure 1:



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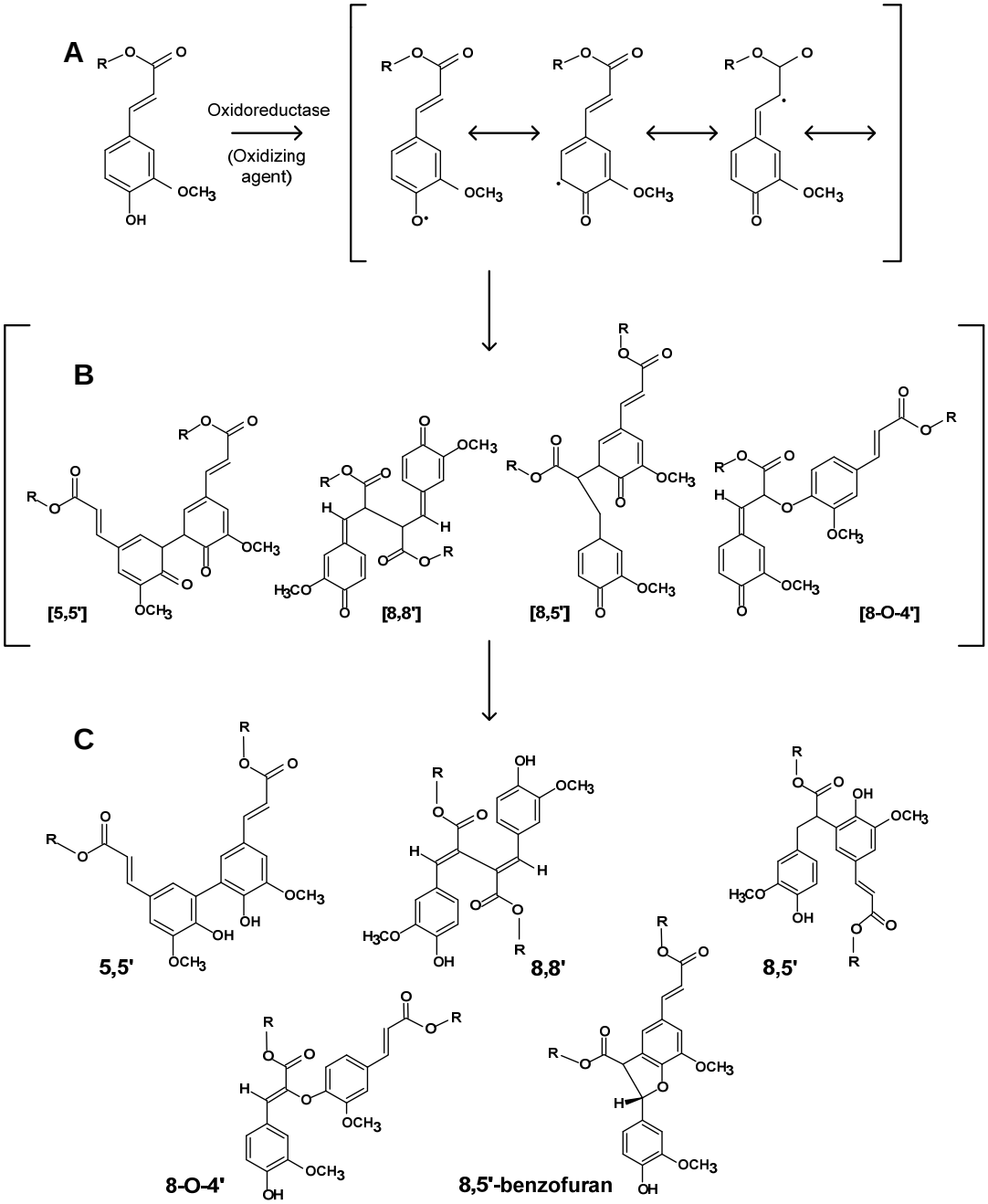

A
Oxidoreductase
(Oxidizing agent)
B
[5,5']
[8,8']
[8,5']
[8-O-4']
C
5,5'
8,8'
8,5'
8-O-4'
8,5'-benzofuran

Figure 3:

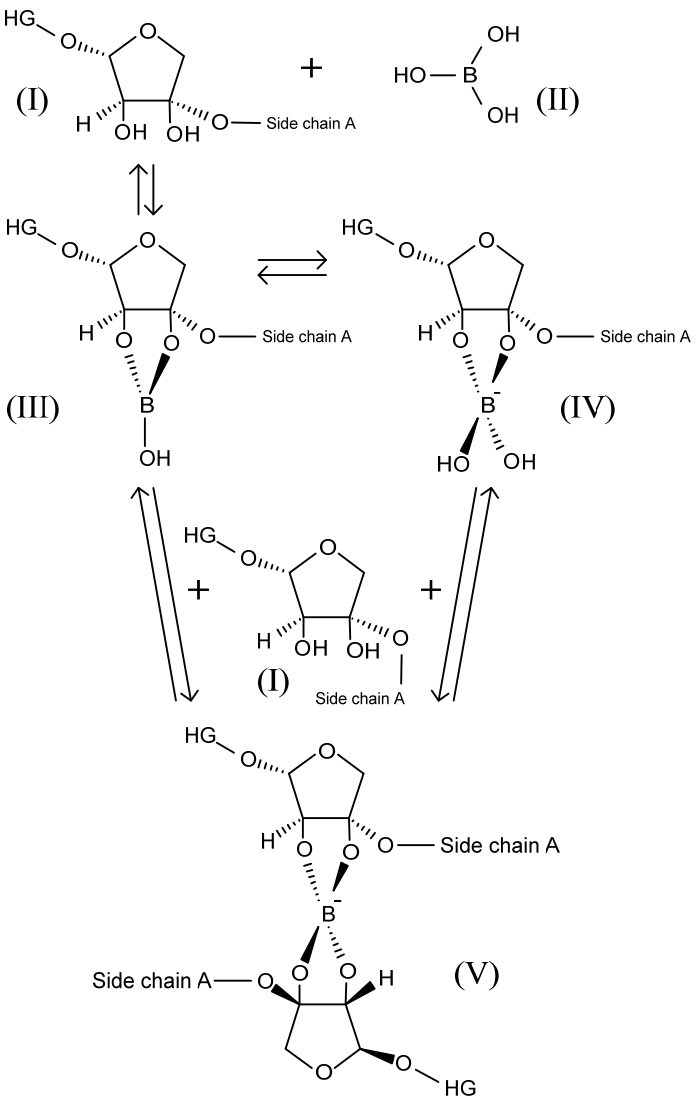

HG
O
O
H
OH
OH
O
Side chain A
(I)
+
OH
HO
B
OH
(II)
(III)
(IV)
B
OH
HO
OH
(I)
Side chain A
(V)
Side chain A
HG

Figure 4:

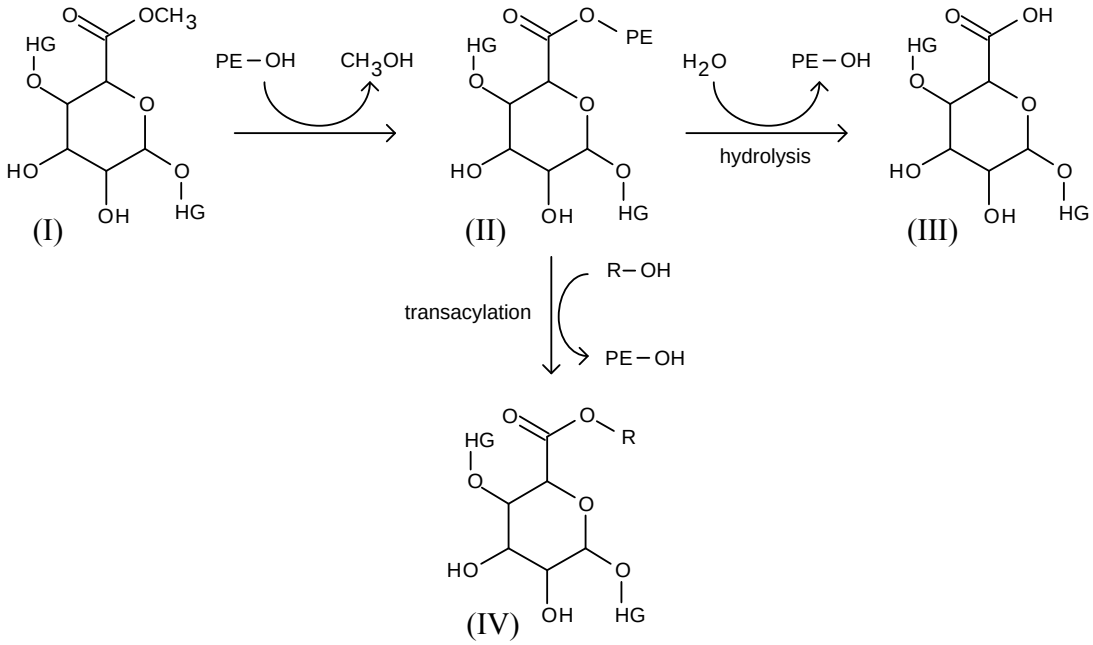
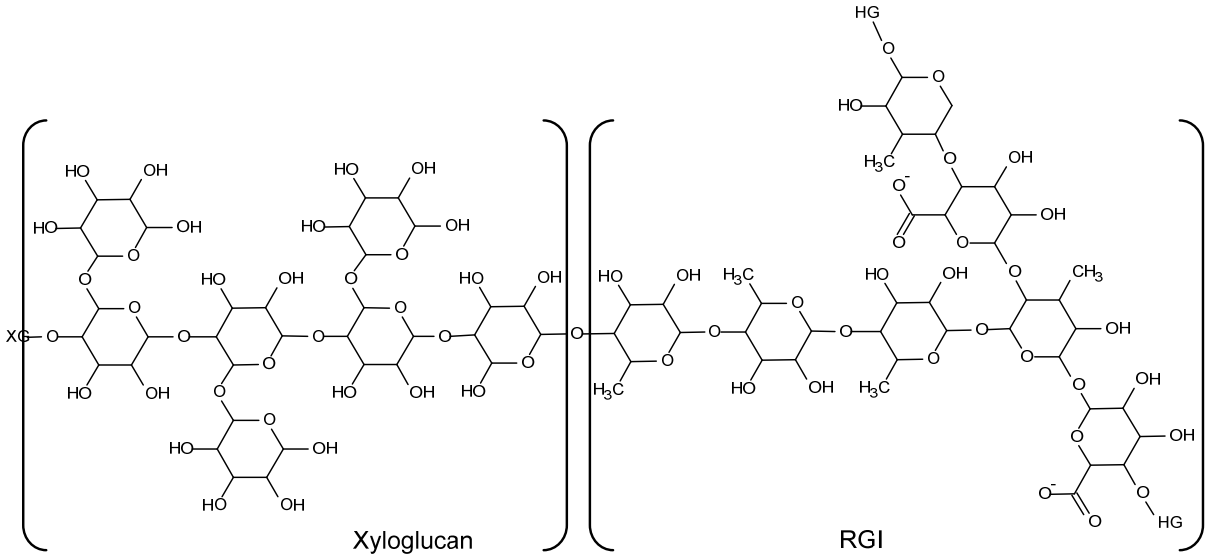

PE – OH
CH3OH
H2O
PE – OH
hydrolysis
transacylation
R – OH
PE – OH
(I)
(II)
(III)
(IV)

Figure 5:



CHAPTER 4

Kinetics of enzyme catalyzed cross-linking of feruloylated oligosaccharide

In order to understand the kinetics of arabinan-oligosaccharide from sugar beet, it is important to characterize the substrate in accordance to the structure and molecular sizes, and thus investigate how the differences in the oligosaccharide structure or molecular sizes can affect the kinetics of the cross-linking reaction. Other reaction factors such as enzyme dosage, substrate concentration, and hydrogen peroxide concentration are investigated in this study to elucidate the effect of the differences in the structural make up on the kinetics of enzyme catalyzed oxidative cross-linking reaction. This chapter is based on the results presented in paper II "Kinetics of enzyme-catalyzed cross-linking of feruloylated arabinan from sugar beet".

4.1 Kinetics of enzyme-catalyzed cross-linking of feruloylated arabinan from sugar beet

Paper II: Kinetics of enzyme-catalyzed cross-linking of feruloylated arabinan from sugar beet.
Dayang Norulfairuz Abang Zaidel, Anis Arnous, Jesper Holck, & Anne S. Meyer.
Journal of Agricultural and Food Chemistry, 2011, 59, 11598 – 11607.
See page 31.

4.1.1 Significance of study

In the past, ample studies on oxidative cross-linking of feruloyl substitutions in pectic polysaccharides have been reported (Micard & Thibault, 1999; Oosterveld *et al*., 1997; 2000b). However, there are no reports on the effect of the length of the backbone of the arabinans on the rate of the cross-linking of feruloylated arabinan. Currently the arabinan fraction from sugar beet pulp is a byproduct, but the utilization of the arabinan as a food additive could help maintain the competitiveness of the sugar beet based industry. Thus the provision of knowledge regarding the cross-linking of feruloylated arabinan would for example be important for the rational design and control of enzymatic processes for upgrading of sugar beet pulp arabinans for use as viscosity-regulating or gelling agents in foods and beverages.

In this study, we hypothesized that the rate of cross-linking of feruloylated arabinans obtained from sugar beet pulp might be affected by the backbone length of the arabinan to which the FA moieties are attached. Hypothetically, the rate of cross-linking between FA on longer arabinan chains is expected to be slower than the rate of cross-linking between FA on shorter arabinan chains mainly because of the relatively slower movement (diffusion) of larger molecules. However, once some initial diFAs formation has taken place with the longer chains, hence cross-linking two arabinan chains, the rates of diFAs formation of long and short arabinan chains may approach each other since the diFAs on the longer chains will be in closer proximity, resulting in a sort of "zipping" of the FA esterified to the arabinan chains (Paper II- Zaidel *et al.*, 2011). The purpose of this study was to test the hypothesis that the kinetics of the cross-linking of feruloylated arabinans is affected by the chain length of the arabinan.

4.1.2 Experimental considerations

Only the liquid arabinan fractions were used for this cross-linking experiment to start a basis for the kinetics of feruloylated polysaccharides. The two batches of arabinan (Ara1 and Ara2) used as the starting material in this study were almost similar in molecular size (Figure 2.2). Thus, precipitation using isopropanol was performed to obtain longer oligosaccharide fractions (Ara1b and Ara2b). Prior to the cross-linking experiment analyses on the monosaccharide composition, molecular size and structure were performed on these samples to verify the identity of the polysaccharide recovered from the precipitation.

Since the two arabinan were of different starting FA concentration, for the cross-linking experiment, equimolar FA levels (0.0025–0.05 mM) were compared, but with slightly different levels (0.004–1.02 g L^{-1}) in the different samples designed to achieve significant differences in the reaction rates. The FA levels were designed based on a standard curve obtained from an authentic external FA standard in spectrophometry. The disappearance of FA absorbance at 316 nm was monitored at 25^{o}C for 20 min after addition of HRP and H_2O_2. At 316 nm, the FA dehydrodimers (diFAs) absorbed five times lower than FA as verified by HPLC results in a separate phenolic analysis.

After preliminary runs, an amount of H_2O_2 (14 μM) was determined to be moderate and sufficient for all the measurements within the FA concentrations range (0.0025–0.05 mM) of this cross-linking experiment as higher concentration of H_2O_2 did not increase the rate. Addition of 0.2 mg L^{-1} HRP (equivalent to 0.2 U mL^{-1}) into the reaction mixture reduced the absorbance at a moderate rate allowing for precise absorbance measurement after the addition of the HRP. Due to

the fast reaction rates for short length arabinan, the rates were calculated from the slope of the decreasing absorbance for the first 10 seconds, while for the longer length arabinan, the rates were calculated for the first minute of the cross-linking reaction. All cross-linking experiments were run in duplicate.

Higher sample concentration (50 g L^{-1}) was used in phenolic analysis using HPLC due to differences in sensitivity between spectrophotometry and HPLC. To determine the phenolic content after cross-linking, the reaction (1 mL) was performed with addition of 2.0 U mL^{-1} HRP and 85 μM H_2O_2 to the sample for 1 min and the reaction was stopped by addition of 2 M NaOH (1 mL) prior to triple ethyl acetate extraction. A fix amount of H_2O_2 was used for all samples regardless of the starting FA concentration.

4.1.3 Highlights

Characterization of the arabinan oligosaccharides using HPAEC-PAD, HPSEC, HPLC, HILIC and MALDI-TOF confirmed that arabinose was the main component in the fractions (88-95 mol%), with abundant existence of feruloyl substitutions (1.1-7.0 mg·g^{-1} DM) esterified to the arabinose moieties, and larger molecular weight arabinans were obtained from the precipitation of the original arabinan samples with isopropanol. At similar FA concentrations, the rates of cross-linking for short chain arabinans were significantly higher ($P < 0.05$), that is, approximately five times faster, than the rates of cross-linking for the longer chain arabinans (Figure 4.1). Thus, it was shown that the rate of cross-linking of feruloylated arabinan from sugar beet was influenced by the arabinan oligosaccharides backbone chain length. Longer arabinans exhibit a slower cross-linking rate than shorter, all other things being equal.

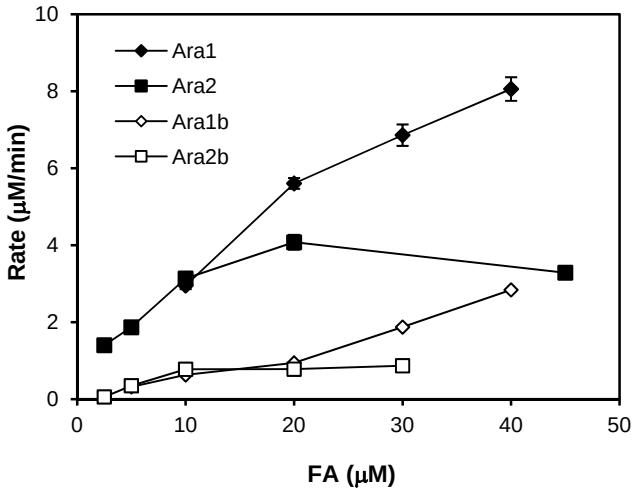


Figure 4.1: Initial rate of enzyme catalyzed oxidative cross-linking of FA in feruloylated arabinans (figure from paper II- Zaidel *et al.*, 2011).

Feruloyl dehydrodimers (5,5', 8-O-4' and 8,5' benzofuran-diFA) were produced from the oxidative enzymatic cross-linking of feruloylated arabinan from sugar beet. Disappearance of FA and formation of diFAs from the cross-linking reaction was confirmed by HPLC analysis, with 8,5' benzofuran-diFA predominated, followed by 5,5' and 8-O-4'-diFA (Figure 4.2). A small peak of 8,8'-diFA was identified but could not be quantified. It was also assumed that higher feruloyl oligomers such as trimers and tetramers might be produced in this reaction. The pattern for diFAs profile was similar for all arabinan samples except for Ara2b, where the levels of all three types of diFAs in fact decreased after cross-linking. We interpret that the decrease in diFAs in Ara2b was due to the oxidation by excess H_2O_2 because the H_2O_2:FA level was relatively higher for Ara2b due to the low FA levels in this sample.

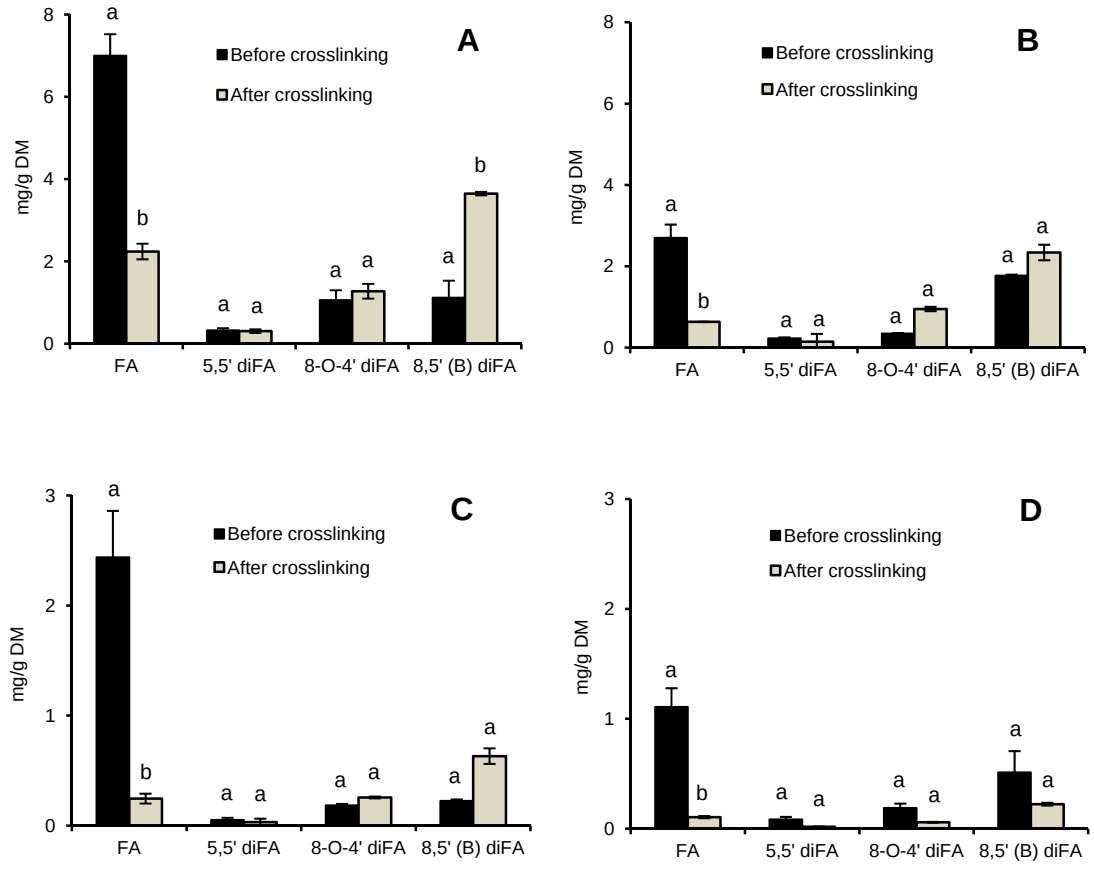


Figure 4.2: The profile of FA and diFAs after the addition of HRP and H_2O_2 in (A) Ara1; (B) Ara2; (C) Ara1b; (D) Ara2b. Error bars are SD from duplicate measurements. Different letters (a, b) within each set of comparison of the content (before and after cross-linking) indicate statistically significant difference at $P<0.05$ using one way ANOVA. Pooled SDs were: (A) 0.4231; (B) 0.1909; (C) 0.2155; (D) 0.4162. (figure from paper II- Zaidel *et al.*, 2011).

Paper II

Kinetics of enzyme-catalyzed cross-linking of feruloylated arabinan from sugar beet

Dayang Norulfairuz Abang Zaidel, Anis Arnous, Jesper Holck, Anne S. Meyer

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ARTICLE

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Kinetics of Enzyme-Catalyzed Cross-Linking of Feruloylated Arabinan from Sugar Beet

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ABSTRACT: Ferulic acid (FA) groups esterified to the arabinan side chains of pectic polysaccharides can be oxidatively cross-linked in vitro by horseradish peroxidase (HRP) catalysis in the presence of hydrogen peroxide (H_2O_2) to form ferulic acid dehydrodimers (diFAs). The present work investigated whether the kinetics of HRP catalyzed cross-linking of FA esterified to α-(1,5)-linked arabinans are affected by the length of the arabinan chains carrying the feruloyl substitutions. The kinetics of the HRP-catalyzed cross-linking of four sets of arabinan samples from sugar beet pulp, having different molecular weights and hence different degrees of polymerization, were monitored by the disappearance of FA absorbance at 316 nm. MALDI-TOF/TOF-MS analysis confirmed that the sugar beet arabinans were feruloyl-substituted, and HPLC analysis verified that the amounts of diFAs increased when FA levels decreased as a result of the enzymatic oxidation treatment with HRP and H_2O_2. At equimolar levels of FA (0.0025−0.05 mM) in the arabinan samples, the initial rates of the HRP-catalyzed cross-linking of the longer chain arabinans were slower than those of the shorter chain arabinans. The lower initial rates may be the result of the slower movement of larger molecules coupled with steric phenomena, making the required initial reaction of two FAs on longer chain arabinans slower than on shorter arabinans.

KEYWORDS: arabinan, dehydrodimers, ferulic acid, horseradish peroxidase kinetics

INTRODUCTION

Pectic polysaccharides extracted from sugar beet pulp mainly consist of homogalacturonan and rhamnogalacturon I (RGI), the latter notably being arabinan- and arabinogalactan-substituted RGI.[1] As in other dicot plant cell walls the backbone of sugar beet RGI is made up of repeating units of [→2)-α-L-Rha*p*−(1→4)-α-D-Gal*p*A-(1→].[2] The rhamnose moieties of the RGI in sugar beet pectin may be substituted at the *O*-4 position with α-(1,5)-linked arabinans having a high degree of α-(1,2)- and α-(1,3)-arabinofuranosyl (Ara*f*) substitutions and with minor amounts of β-(1,4)-linked galactan.[1,3] Some of the Ara*f* moieties in the α-(1,5)-linked arabinans are substituted with feruloyl groups, as single ferulic acid (FA) moieties or in the form of ferulic acid dehydrodimers (diFAs) that may even cross-link two different arabinan chains.[4,5] The FA moieties are mainly esterified to the *O*-2 position of the Ara*f* residues in the α-(1,5)-linked arabinan backbone, but may also be bound to the *O*-5 on the terminal arabinose,[4] or, to a much lesser extent, at the *O*-6 position of the galactopyranosyl (Gal*p*) residues in the β-(1,4)-galactan chains.[6]

The FA bound to the side chains of pectic polysaccharides have long been known to have the ability to be oxidatively cross-linked via enzyme catalysis by oxidoreductases, that is, peroxidase or laccase activities.[7,8] Previous work has shown that addition of, for example, peroxidase, together with H_2O_2 or laccase, results in a reduction of the amount of FA esterified to the arabinan chains while the levels of diFAs increase simultaneously.[8,9] The enzyme-catalyzed cross-linking of two feruloylated arabinan oligosaccharides takes place via radical coupling of the FA resulting in the formation of different diFAs: 5,5′, 8-*O*-4′, 8,5′, and 8,8′.[8−10] The formation of diFA cross-linking between feruloylated arabinans or feruloylated arabino-oligosaccharides may result in gel formation.[1,8,11] Although studies on the oxidative cross-linking of feruloyl substitutions in polysaccharides have been amply reported,[7−9] there are no reports on the effect of the length of the backbone of the arabinans on the rate of the cross-linking of feruloylated arabinan. Currently, the arabinan fraction from sugar beet pulp is a byproduct, but the utilization of the arabinan as a food additive could help maintain the competitiveness of the sugar beet based industry. Thus, the provision of knowledge regarding the cross-linking of feruloylated arabinan would, for example, be important for the rational design and control of enzymatic processes for upgrading sugar beet pulp arabinans for use as viscosity-regulating or gelling agents in foods and beverages.

We hypothesized that the rate of cross-linking of feruloylated arabinans obtained from sugar beet pulp might be affected by the backbone length of the arabinan to which the FA moieties are attached. Hypothetically, the rate of cross-linking between FA on longer arabinan chains is expected to be slower than the rate of cross-linking between FA on shorter arabinan chains mainly because of the relatively slower movement (diffusion) of larger molecules. However, once some initial diFAs formation has taken place with the longer chains, hence cross-linking two arabinan chains, the rates of diFA formation of long and short arabinan chains may approach each other because the diFAs on the longer chains will be in closer proximity, resulting in a sort of

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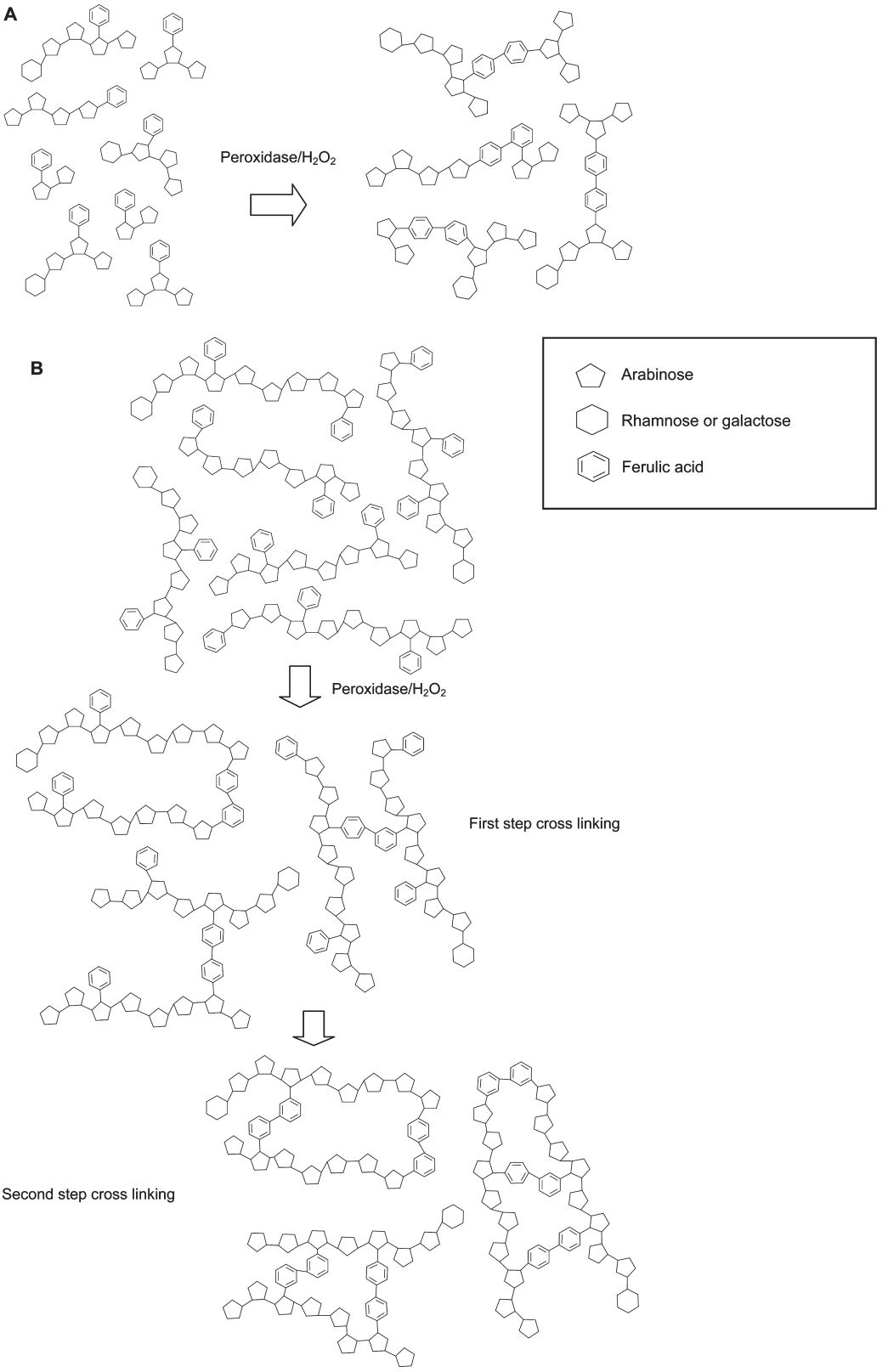

Figure 1. (A) Schematic representative structures of feruloylated arabinans from sugar beet pectin illustrating the one-step enzyme-catalyzed oxidative cross-linking model of short-chain arabinans. (B) Two-step enzyme-catalyzed oxidative cross-linking or "zipping" model for long-chain feruloylated arabinans.

"zipping" of the FA esterified to the arabinan chains (Figure 1). The purpose of this study was to test the hypothesis that the kinetics of the cross-linking of feruloylated arabinans is affected by the chain length of the arabinan.

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MATERIALS AND METHODS

Enzyme and Chemicals. Horseradish peroxidase (HRP) (EC 1.11.1.7) type VI-A, 1000 U mg^{-1}, ferulic acid (4-hydroxy-3-methoxycinnamic acid) 99%, isopropanol, sodium hydroxide (NaOH) 50% (w/w), ethyl acetate, acetonitrile, hydrogen peroxide (H_2O_2) 50% (w/w), sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$), trifluoroacetic acid (TFA) 98% (w/w), pullulan standard with molecular weight of 1.3 kDa, and monosaccharide standards including D-galactose, L-arabinose, D-fucose, L-rhamnose monohydrate, and D-galacturonic acid monohydrate were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). D-Glucose, D-xylose, anhydrous sodium sulfate (Na_2SO_4), and disodium hydrogen phosphate (Na_2HPO_4) were from Merck (Darmstadt, Germany). *trans*-Cinnamic acid 99% was from Alfa Aesar GmbH & Co. (Karlsruhe, Germany). Dextran standards with average molecular weights of 10 and 40 kDa, respectively, were from Pharmacia (Uppsala, Sweden). Arabino-oligosaccharide standards from DP2 to DP5 were purchased from Megazyme (Bray, Wicklow, Ireland).

Arabinan Raw Material. Arabinan samples were supplied by Danisco A/S (Nakskov, Denmark). The two batches of samples, Ara1 and Ara2, respectively, were two different permeate streams obtained from the preparation of pectin from sugar beet pulp via nitric acid hydrolysis involving removal of insoluble cellulose, ultrafiltration, and diafiltration with a 50 kDa cutoff.[2]

Monosaccharide Composition of the Raw Material. The monosaccharide compositions of these starting materials were determined by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on a Dionex BioLC system (Dionex Corp., Sunnyvale, CA), after acid hydrolysis (4 g L^{-1} substrate, 2 M TFA, 2 h, 121 °C) as described previously.[12]

Oligosaccharide Profiles of the Raw Materials . Separation and quantification of arabino-oligosaccharides were done by HPAEC-PAD using a Dionex BioLC system equipped with a CarboPac PA1 analytical column (4 × 250 mm) controlled via Chromeleon 6.80 Sp8 Build 2623 software (Dionex Corp.) as described in ref 13. In addition to arabinose, the arabino-oligosaccharide standards used were from DP2 to DP5.

Precipitation with Isopropanol. To obtain longer chain arabinan fractions, the Ara1 and Ara2 samples were treated with isopropanol in a 30:70 (v/v) water/isopropanol ratio and centrifuged at 9000*g* for 15 min. In each case, the supernatant was discarded, and the pellet of the alcohol-insoluble longer chain arabinans was dried by evaporation under N_2, then frozen, and lyophilized. The longer chain (feruloylated) arabinans from Ara1 were referred to as Ara1b and the longer chain arabinans from Ara2 as Ara2b. Ara1b and Ara2b constituted approximately 3.5 and 4.5% of the weight of Ara1 and Ara2 after precipitation, respectively.

Phenolics Analysis. The contents of FA and diFAs were determined by reverse phase high-performance liquid chromatography (HPLC) after alkaline saponification (under nitrogen, N_2) with 2 M NaOH at 25 °C, acidification, and triple ethyl acetate extraction as described previously.[14,15] For each extracted sample, the ethyl acetate phases were pooled and water was removed by adding anhydrous Na_2SO_4. The pooled samples were dried under N_2 at 30 °C. Fifty percent (v/v) aqueous methanol was added to the samples that were then filtered using a 0.20 μm nylon membrane filter (VWR International, USA). The sample (40 μL) was injected into an HPLC Chemstation 1100 series equipped with an ODS-L Optimal (250 × 4.6 mm, 5 μm) column from Capital HPLC and a diode array detector (Hewlett-Packard, Palo Alto, CA). The gradient elution was performed using solvents A (5% (v/v) acetonitrile, 1 mM TFA) and B (acetonitrile) starting with 20% (v/v) acetonitrile at 0.5 mL min^{-1}. The gradient was run up to 40% (v/v) acetonitrile for 35 min and further up to 100% for another 3 min, with a final regeneration to 20% (v/v) acetonitrile for 2 min.[16] The column temperature was maintained at 40 °C. FA was detected and quantified at 316 nm using an authentic external standard for retention time and spectral recognition; quantification was by area linear regression. diFAs were also detected at 316 nm but quantified at 280 nm according to response factors as described in ref [17]. Due to differences in sensitivity between HPLC and spectrophotometry, higher sample concentrations (50 g L^{-1}) were used for the HPLC analysis.

Size Exclusion Chromatography (HPSEC). HPSEC was performed using a system consisting of a P680 HPLC pump, an ASI-100 automated sample injector, and an RI-101 refractive index detector (Dionex Corp.). Lyophilized arabinan samples were redissolved and diluted in 0.1 M sodium acetate buffer (pH 6) and filtered with a syringe through a 0.20 μm nylon membrane filter (VWR International, USA). The sample (25 μL) was injected and separated using a Shodex SB-806 HQ OHpak column (300 × 8 mm) with a Shodex SB-G guard column (50 × 6 mm) (Showa Denko K.K., Tokyo, Japan) and eluted with 0.1 M sodium acetate buffer (pH 6) at 30 °C and a flow rate of 0.5 mL min^{-1}.[18] Standards were a monomer (arabinose), pullulan standard with a molecular weight of 1.3 kDa, and dextran standards with molecular weights of 10 and 40 kDa, respectively.

Analysis of Feruloylated Arabino-oligosaccharides by Hydrophilic Interaction Chromatography (HILIC). Lyophilized (feruloylated) arabinan samples were suspended in deionized water followed by cleanup using 70% (v/v) final concentration of acetonitrile. The acetonitrile cleanup step was included to eliminate the noise from the presence of peptides and polar compounds prior to chromatographic separation of the arabinan samples. Acetonitrile (70% (v/v)) treated samples were immediately centrifuged for 3 min at 16000*g*, and each supernatant was then injected (10 μL) into the HILIC system. The HILIC separation of the feruloylated arabinans according to hydrophilic interaction was performed using an ICS-3000 system coupled to a photodiode array detector (PDA-100) (Dionex Corp.) and equipped with a TSKgel Amide-80 column (250 × 4.6 mm i.d., 5 μm, Tosoh Bioscience) at 55 °C. The eluent system consisted of three components (A, acetonitrile; B, deionized water containing 1% (v/v) TFA; C, deionized water) that were delivered in a gradient at a flow rate of 1 mL min^{-1}. The gradient was run from 75% (v/v) of A and 25% (v/v) of B to 25% (v/v) of A and 75% (v/v) of B for 35 min and then run at 100% of C for 2 min. The gradient was then returned to 75% (v/v) of A and 25% (v/v) of B for the final 2 min for reconditioning. Feruloylated compounds were detected and quantified by UV absorption at 316 nm using ferulic acid as external standard.

MALDI-TOF/TOF Analysis. Mass spectrometric analyses of the arabinan samples were performed on a 4800 *Plus* MALDI TOF/TOF (AB SCIEX) mass spectrometer. The sample (0.5 μL) was deposited onto a stainless steel MALDI target with 0.5 μL of the matrix (α-cyano-4-hydroxycinnamic acid from Sigma-Aldrich (St. Louis, MO)) and dried. The matrix was prepared at a concentration of 10 mg mL^{-1} in a 70% ACN/0.1% TFA/water (v/v) solution. The instrument was operated in positive ion reflector mode, and the m/z range from 100 to 3500 was monitored. A total of 1000 laser shots were applied to each sample in MS mode. In MS/MS mode 2000 shots were obtained. The laser intensity was manually varied to obtain the best possible spectra (MS, from 4500 to 4800; MS/MS, from 4800 to 5100). MALDI-TOF-MS and MS/MS data were exported as text files using DataExplorer (version 4.0). Each spectrum were smoothed, labeled, and analyzed manually in M/Z (Genomic Solutions).

Cross-Linking Kinetics. Stock solutions of 10 mg L^{-1} HRP and 85 μM H_2O_2, respectively, were prepared in 0.1 M sodium phosphate buffer, pH 6 (both were made fresh every day). Reaction mixtures for the cross-linking were prepared by adding H_2O_2 (14 μM) and HRP (0.2 mg L^{-1} = 0.2 U mL^{-1}) to different defined FA concentrations (0.0025−0.05 mM) of the feruloylated arabinan samples. After preliminary

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Table 1. Total Ferulic Acid and Diferulates Content in Arabinan Samples before and after Cross-Linking Using HRP and H_2O_2 Analyzed Using RP-HPLC[a]

	FA (mg g^{-1} dry matter)		total diFAs[b] (mg g^{-1} dry matter)	
sample	before cross-linking	after cross-linking	before cross-linking	after cross-linking
Ara1	7.0 ± 0.5	2.4 ± 0.2	2.4 ± 0.6	5.1 ± 1.3
Ara2	2.5 ± 0.6	1.4 ± 0.3	2.32 ± 0.03	2.82 ± 0.04
Ara1b	2.4 ± 0.5	0.29 ± 0.06	0.44 ± 0.04	1.01 ± 0.08
Ara2b	1.1 ± 0.2	0.1 ± 0.01	0.6 ± 0.2	0.30 ± 0.02

[a] Results are shown as average data ± SD of duplicates. [b] Total diFAs = 5,5′-diFA + 8,5-diFA benzofuran + 8-*O*-4′-diFA.

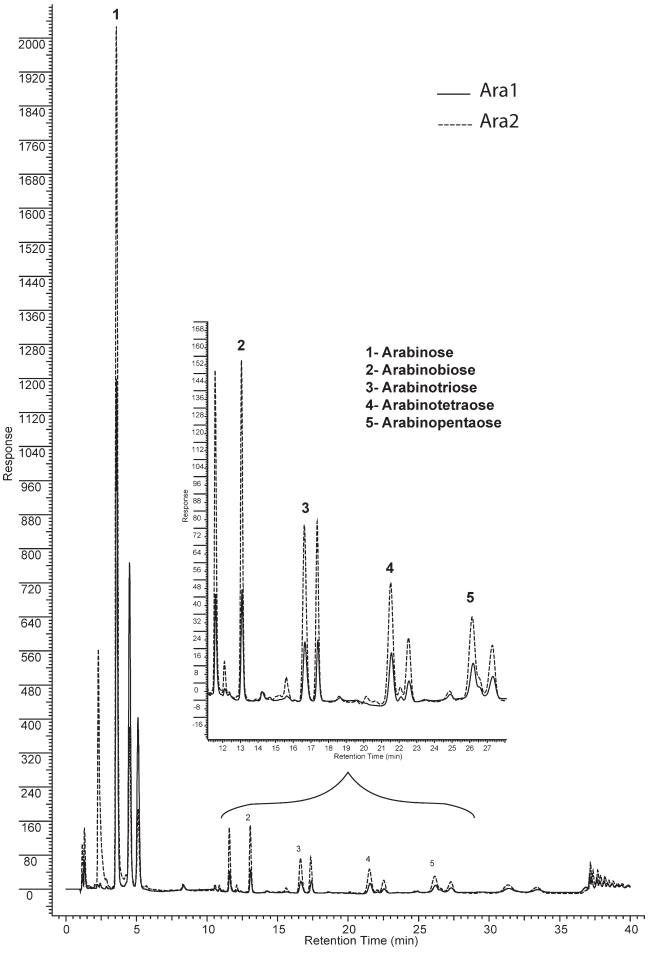

Figure 2. Arabino-oligosaccharide profiles of the feruloylated sugar beet pulp arabinan samples Ara1 and Ara2 as analyzed by HPAEC. The numbers over the peaks represent α-(1,5)-arabinose to α-(1,5)-arabinopentaose.

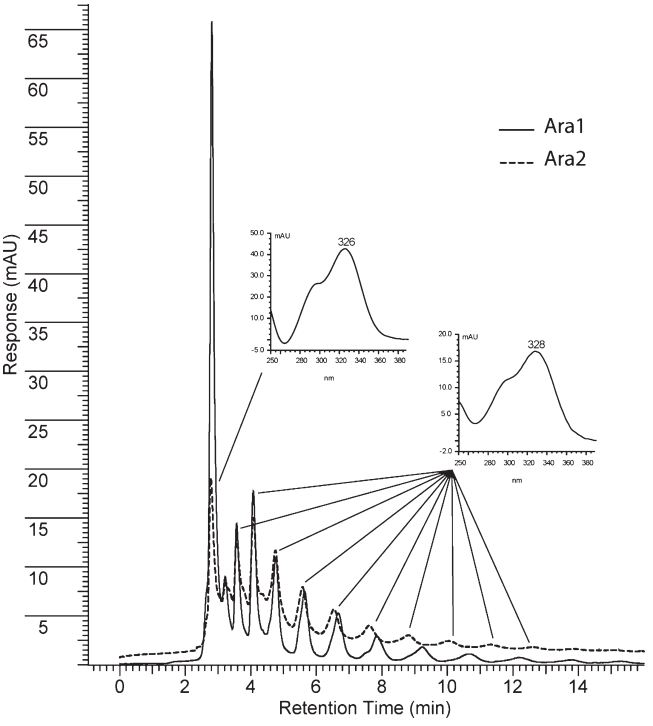

Figure 3. HILIC gradient chromatogram illustrating the presence of *trans*-feruloyl groups attached to sugar beet pulp derived arabinan oligosaccharides of different lengths eluting in the order of chain length, shorter first. The FA was detected at 316 nm, but the peak of the spectra reached the maximum at 326−328 nm.

runs, an amount of H_2O_2 (14 μM) was determined to be moderate and sufficient for all of the measurements within the FA concentrations range (0.0025−0.05 mM) of this cross-linking experiment. In blanks, the enzyme solution was substituted with 0.1 M sodium phosphate buffer, pH 6. The cross-linking reaction was monitored by the disappearance of FA at an absorbance of 316 nm at 25 °C for 20 min in a Lambda20 UV−vis spectrophotometer (PerkinElmer Inc., USA). For analysis of FA and diFAs content by HPLC after the reaction, the reaction (1 mL) was stopped by the addition of 2 M NaOH (1 mL) and extracted as described above. For each cross-linking experiment the reactions were run in duplicate, and hence the recordings of the absorbance were given as the average value ± standard deviation of two replicate runs.

Statistics. One-way analyses of variances (one-way ANOVA; 95% confidence intervals) were compared as Tukey−Kramer intervals calculated from pooled standard deviations (Minitab Statistical Software, Addison-Wesley, Reading, MA).

RESULTS AND DISCUSSION

Composition of Sugar Beet Arabinan Substrates. The monosaccharide profiles of the starting materials, Ara1 and Ara2, confirmed that the substrates could be classified as arabinans, and Ara1 has also been characterized previously.[19] Their compositions (in mmol g^{-1} dry matter) were as follows: for Ara1, arabinose, 3.4 (equivalent to 88 mol %); glucose, 0.27 (equivalent to 7 mol %), with traces of <1 mol % of rhamnose, galactose, and galacturonic acid; for Ara2, arabinose, 2.9

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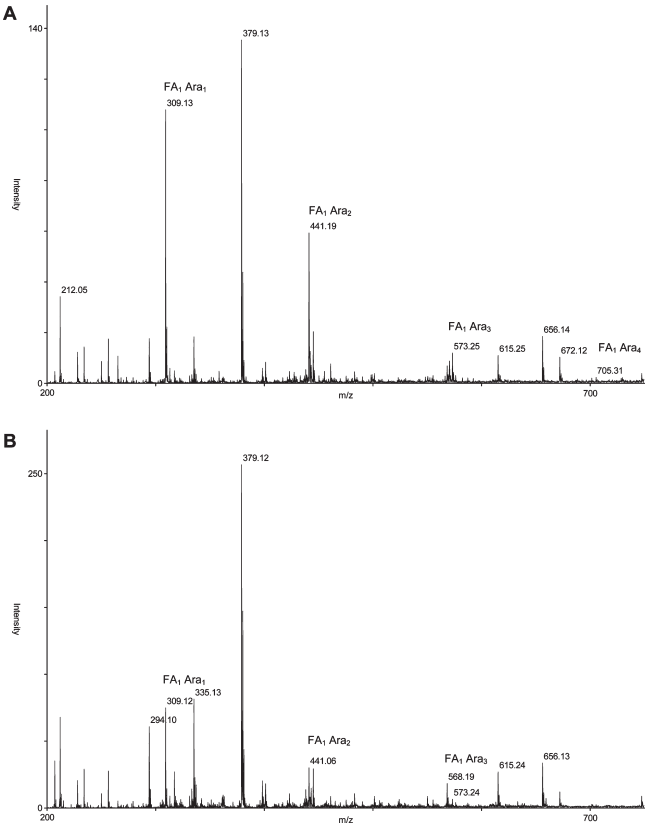

Figure 4. Mass spectra from MALDI-TOF analysis of (A) Ara1 and (B) Ara2.

(equivalent to 95 mol %) and traces of <1 mol % of rhamnose, glucose, galactose, and galacturonic acid. The total FA content in the four different arabinan samples ranged from 1.1 to 7.0 mg g^{-1} dry matter arabinan (Table 1). Ara1 contained the highest amount of FA esterified to the arabinan (7.0 ± 0.5 mg g^{-1}) followed by Ara2 (2.5 ± 0.6 mg g^{-1}), Ara1b (2.4 ± 0.5 mg g^{-1}), and Ara2b (1.1 ± 0.2 mg g^{-1}) (Table 1). Hence, the extent of feruloyl substitution was apparently 50−60% less in the longer chain arabinan samples than in the starting material. The molar ratio of FA to arabinose (FA:Ara) in the samples was calculated to be 1:90 (Ara1), 1:200 (Ara2), 1:40 (Ara1b), and 1:20 (Ara2b). Naturally occurring diFAs made up 2.4 and 2.3 mg g^{-1} of the Ara1 and Ara2 samples and 0.44 and 0.60 mg g^{-1} of Ara1b and Ara2b, respectively (Table 1). Other naturally occurring diFAs such as 8,8′ and 4-*O*-5′-diFAs were not detected in the samples, most probably due to very low concentrations in different species of sugar beet or loss due to the extraction conditions.

Arabino-oligomer Profile of the Arabinan Substrates. Analysis by HPAEC indicated that Ara1 and Ara2 mainly consisted of short DP arabino-oligomers, including mainly monomers (notably arabinose) and α-(1,5)-arabino-oligosaccharides up to DP5 (arabinopentaose), with the Ara2 being relatively richer in all of the detected short oligomers, including the monosaccharide peak, when evaluated at the same dry matter concentration (Figure 2). The peaks of Ara1b and Ara2b eluted similarly to Ara1 and Ara2 but at much lower levels (approximately 7−15% of Ara1 and Ara2) when analyzed at 1 g L^{-1} for DP2−DP4, but the large MW molecules escaped this analysis (data not shown). The HPAEC spectra also revealed triple peaks, of which the latest eluted peak in each set was

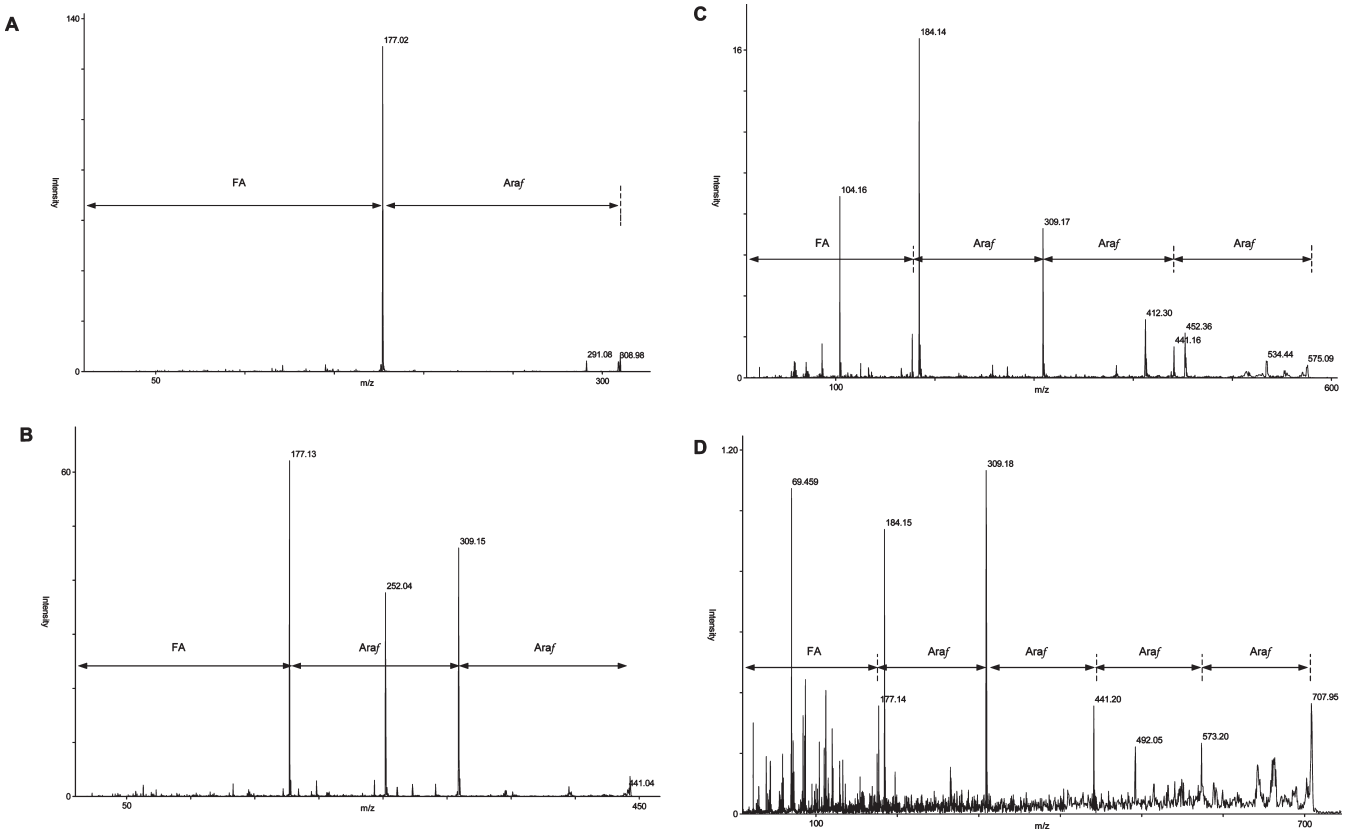

Figure 5. MS/MS spectra for arabinan oligosaccharides for ions at *m*/*z* (A) 309, (B) 441, (C) 573, and (D) 705 of Ara1.

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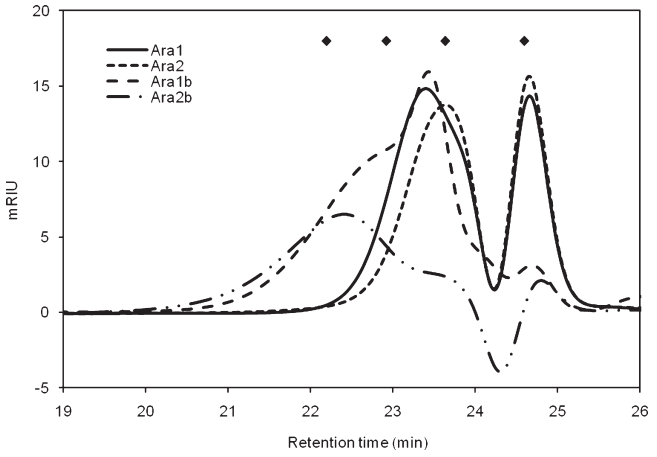

Figure 6. HPSEC chromatogram for Ara1, Ara2, Ara1b, and Ara2b. Standards (◆), from left to right: 40 kDa, 10 kDa, 1.3 kDa, and 150 Da (arabinose).

unidentifiable, except for the latest peak designated peak 2 in the first triplet oligomer peak set, which was identified as α-(1,5)-arabinobiose from the elution of an authentic standard (Figure 2). With the extent of feruloyl substitution of ∼1:90 and ∼1:200 in Ara1 and Ara2 (Table 1), respectively, it seems unlikely that the "additional" peaks were due to the feruloyl substitutions. Rather, the triplet sets of peaks may be a result of branched arabino-oligosaccharides because sugar beet pectin is rich in those and branched arabino-oligosaccharides are known to elute slightly differently from the corresponding (same number of monomers) linear arabino-oligosaccharides.[3] For disaccharides, the different retention times may be due to different glycosyl-linkage positions of the arabino-oligosaccharides. Therefore, because the peak labeled 2 was the α-(1,5)-arabinobiose, it is suggested that there was an approximately equal amount of α-(1,3)-arabinobiose, whereas the third smaller peak between these two peaks might indicate a small amount of α-(1,2)-arabinobiose. However, a further analysis with different standards is needed to confirm this result.

Feruloyl Group Distribution. The UV−vis spectra from HILIC separation confirmed the identity of the FA attached to different arabino-oligosaccharide chains (Figure 3) as also reported previously.[19] The amide stationary phase provided a unique selectivity for the arabino-oligosaccharides under HILIC as the hydrogen-bonding retention mechanism between hydroxyl groups of the oligosaccharides and the carbamoyl group in the stationary phase made it possible to elute different chain lengths based on the oligosaccharide chains' polarity and degree of solvation.

MALDI-TOF-MS analysis of the Ara1 and Ara2 samples confirmed the structural makeup of the substrates with the series of peak signals at m/z 309, 441, 573, and 705, corresponding to a series of "dehydrated" arabinose (Araf) moieties (of 132 Da) with a single feruloyl ester substitution (m/z 177) (Figure 4). The fragmentation pattern thus revealed the formation of C type ions corresponding to loss of arabinose moieties. Because the MS/MS analysis was performed in positive mode, protonated $[M + H]^+$ adducts were formed, measuring ferulic acid as 176 + 1: m/z = 177. The structure was confirmed by MS/MS fragmentation as exemplified with m/z 441 (Figure 5). The MS/MS data could not, however, determine the degree of branching or the exact points of feruloyl substitution on each type of molecule, because molecules with different substitution patterns have the same fragmentation pattern.[19] In a separate study further purification of the Ara1 arabinan sample has revealed that this sample also contained arabino-oligosaccharide series (DP7−14) with double feruloyl substitutions.[19] No feruloyl substitutions on galactose residues were detected. Neither were any diFAs detected by MS/MS, even though the HPLC analysis indicated their presence (Table 1).

Determination of Molecular Weight. HPSEC analysis showed that Ara1 and Ara2 gave dual peaks with one peak at ∼24.5 min corresponding to monomers and an earlier peak at ∼23.5 min (Figure 6). These peaks were shaped differently for Ara1 and Ara2, corresponding to molecular weights somewhat higher than 1.3 kDa for Ara1 and ∼1.3 kDa for Ara2 (Figure 6). The SEC profile of Ara1b showed that this sample contained

Table 2. Ferulic Acid (FA) Content per Amount of Dry Matter (DM) Arabinan in the Samples Used for Cross-Linking of (Feruloylated) Arabinan Samples with HRP and H_2O_2 Treatment[a]

sample	FA (mM)[b]	dry matter (g L^{-1})	FA[c] (μM)	FA/DM (mg g^{-1})
Ara1	0.010	0.19	9.87 ± 0.01	9.87 ± 0.01
	0.020	0.40	20.72 ± 0.01	10.04 ± 0.01
	0.030	0.61	32.10 ± 0.01	10.26 ± 0.01
	0.040	0.81	43.37 ± 0.03	10.35 ± 0.01
	0.050	1.02	57.43 ± 0.04	10.93 ± 0.01
Ara2	0.0025	0.08	5.81 ± 0.02	15.1 ± 0.1
	0.005	0.10	8.80 ± 0.10	14.6 ± 0.1
	0.010	0.20	15.8 ± 0.2	15.2 ± 0.1
	0.020	0.40	30.5 ± 0.2	15.9 ± 0.1
	0.045	0.80	63.4 ± 0.2	15.4 ± 0.1
Ara1b	0.010	0.20	14.0 ± 0.3	13.6 ± 0.3
	0.020	0.47	37.3 ± 0.4	15.5 ± 0.2
	0.030	0.73	54.7 ± 0.3	14.5 ± 0.1
	0.040	1.00	75.8 ± 0.1	14.7 ± 0.02
Ara2b	0.0025	0.004	0.27 ± 0.03	12.7 ± 1.6
	0.005	0.06	2.11 ± 0.03	7.20 ± 0.10
	0.010	0.16	11.2 ± 0.7	13.4 ± 0.9

[a] Results are shown as mean ± SD for samples analyzed as duplicates. [b] Initial FA value. [c] FA measured by absorbance measurement at 316 nm.

Table 3. Initial Rate for Oxidative Cross-Linking Ara1 and Ara2 at Various Enzyme Dosages of HRP at 30 μM FA Equivalents in Arabinan Samples

	initial rate (μM min^{-1})	
HRP (U mL^{-1})	Ara1	Ara2
0.1	5.7 ± 0.3	3.1 ± 0.5
0.2	9.7 ± 0.2	5.1 ± 0.3
0.4	11.4 ± 0.8	7.3 ± 0.1
0.6	11.1 ± 0.7	7.5 ± 0.1

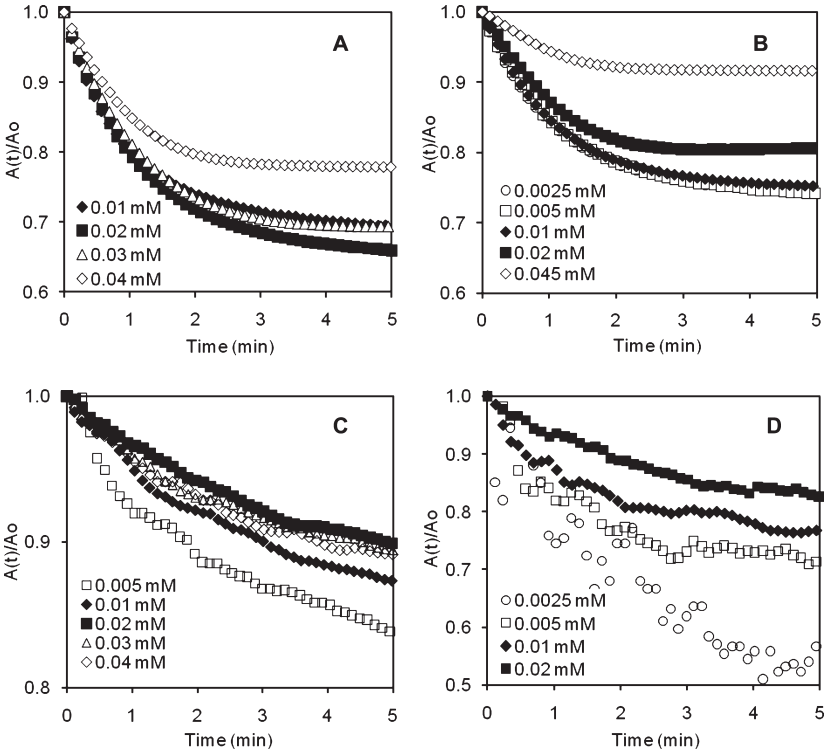

Figure 7. Cross-linking for the first 5 min of the reaction of (A) Ara1, (B) Ara2, (C) Ara1b, and (D) Ara2b using 0.2 mg L^{-1} HRP and 14 μM H_2O_2 at various arabinan concentrations. $A(t)/A_0$ is the relative absorbance as a function of time: $Abs_{316\ nm}$ at the particular time (t) divided by the initial the $Abs_{316\ nm}$ at time 0.

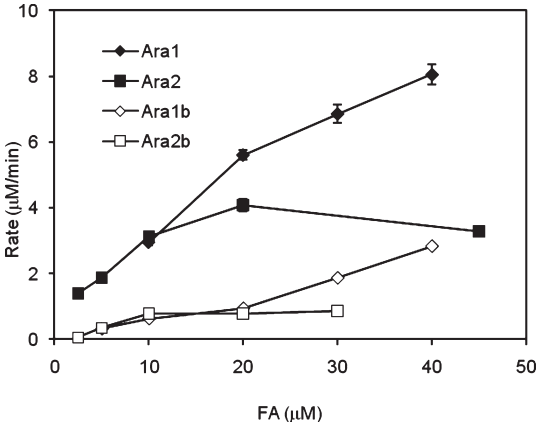

Figure 8. Initial rate of enzyme-catalyzed oxidative cross-linking of FA in feruloylated arabinans. Rate was calculated during the first 10 s of the reaction for the original arabinan samples, Ara1 and Ara2, and during the first minute of enzyme-catalyzed reaction for the longer chain arabinans, Ara1b and Ara2b.

larger molecular weight polysaccharides eluting broadly from 22 to 24 min with a shoulder on the peak with a maximum at ~23.5 min indicating molecular weights around and above 1.3 kDa. The Ara2b contained relatively more of the larger molecular weight polysaccharides than Ara1b with a broad peak eluting mainly around 22 min (Figure 6). Hence, the SEC profile confirmed that larger molecules, that is, longer chain arabinans, were extracted from the samples by the isopropanol precipitation and also revealed that Ara1 and Ara2 contained slightly differently sized arabinan and arabino-oligosaccharides around 1.3 kDa and above.

Cross-Linking Kinetics of Feruloylated Arabinan. To ensure that the rate of cross-linking was not affected by the different levels of FA in the different arabinan samples, the cross-linking experiments were compared at equimolar FA levels (0.0025–0.050 mM), but with slightly different levels in the different samples designed to achieve significant differences in the reaction rates (Table 2). The enzyme-catalyzed oxidative cross-linking experiments were done at 25 °C and pH 6 with 14 μM H_2O_2 and 0.2 mg L^{-1} HRP (equivalent to 0.2 U mL^{-1}) as defined from preliminary experiments as being the optimal conditions of reaction for monitoring the rate of the HRP-catalyzed reaction (Table 3). At this concentration the changes in absorbance took place at moderate rates, allowing for precise absorbance measurement after the addition of the HRP into the reaction mixture. The level of H_2O_2 was sufficient for the cross-linking as a higher concentration of H_2O_2 did not increase the rate (data not shown). Rather, a higher concentration of H_2O_2 may lead to partial inactivation of the enzyme.[20] In general, for the Ara1 and Ara2, the relative absorbance at 316 nm decreased steeply at the beginning of the reaction (from 0 to 1 min) and then decreased more gradually until it reached a pseudosteady state (Figure 7A,B). Presumably, at this point there were still some FA groups left that were unable to react to form the diFAs. The corresponding slopes of the reactions of the longer chain arabinan samples, Ara1b and Ara2b, were much lower, indicating relatively slower consumption of the FA as compared to the consumption in the shorter arabinans at the same FA concentrations (Figure 7C,D).

Rates of Cross-Linking of Different Arabinan Sizes. Due to the different initial absorbances of the different substrate concentrations,

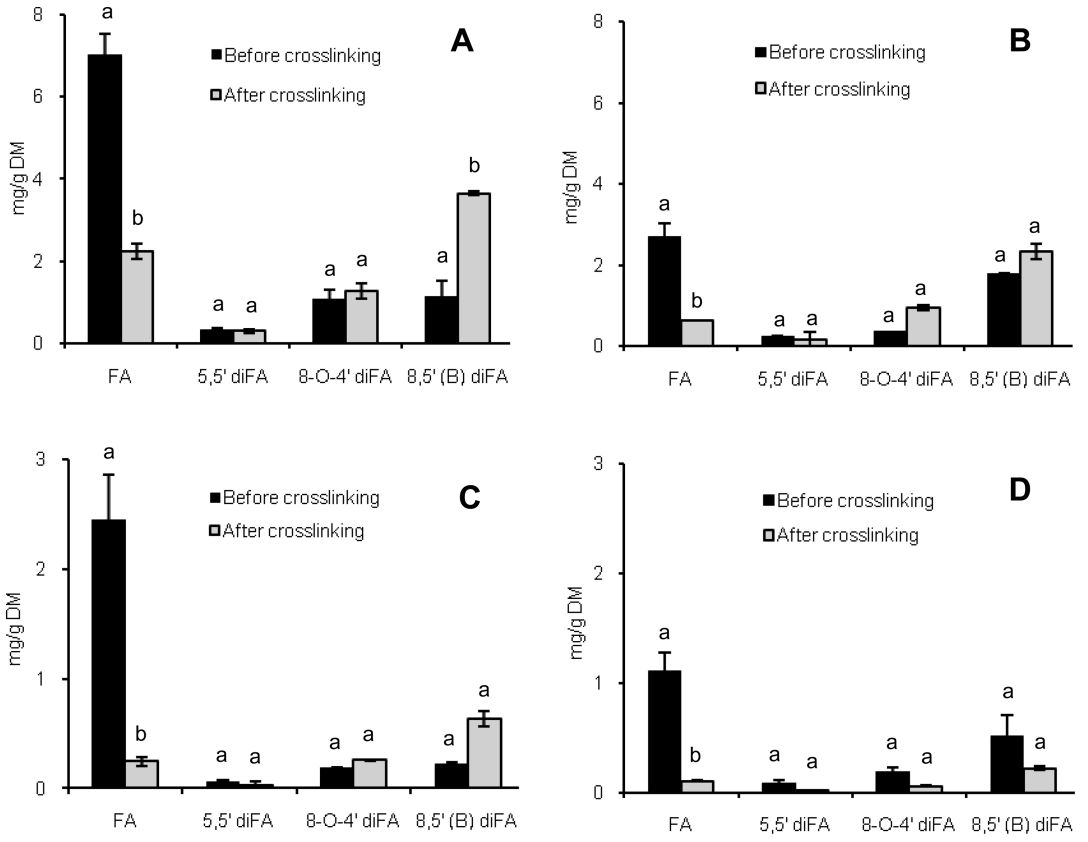

Figure 9. Profile of FA and diFAs after the addition of HRP and H_2O_2 in (A) Ara1, (B) Ara2, (C) Ara1b, and (D) Ara2b. Error bars are SD from duplicate measurements. Different letters (a, b) within each set of comparison of the content (before and after cross-linking) indicate statistically significant difference at $P < 0.05$ using one-way ANOVA. Pooled SDs were (A) 0.4231, (B) 0.1909, (C) 0.2155, and (D) 0.4162.

the data for the cross-linking reactions at different substrate concentrations were plotted as a graph of normalized absorbance ($A(t)/A_0$) (Figure 7). When the reaction rates were assessed as the amount of FA consumed per minute (μM min^{-1}), it became evident that the initial reaction rates increased with increased FA substrate concentration and, moreover, that the reaction rates of Ara1 and Ara2 in general were several-fold higher than the reaction rates of samples Ara1b and Ara2b (Figure 8). Hence, at similar FA concentrations of, for example, 20 μM, the rates of cross-linking for Ara1 and Ara2 were 5.6 and 4.1 μM min^{-1}, respectively, and thus statistically significantly higher ($P < 0.05$) than the rates of cross-linking for the longer chain arabinans, which were approximately 5 times slower at 0.94 and 0.78 μM min^{-1} for Ara1b and Ara2b, respectively (Figure 8). Due to the fast reaction rates for Ara1 and Ara2, rates were calculated from the slope of the decreasing absorbance for the first 10 s, whereas for Ara1b and Ara2b the rates were calculated for the first minute of the cross-linking reaction due to absorbance fluctuation in the first few minutes of the longer chain cross-linking reactions (Figure 7C,D). At the relatively low FA levels, from 2.5 to 10 μM, the reaction rates of Ara1 and Ara2 were similar and ranged from ~1.5 to 3.0 μM min^{-1}. The reaction rates of the Ara1b and Ara2b substrates were also similar, even from 2.5 to 20 μM, and ranged from 0 to 0.4 μM min^{-1} (Figure 8). At higher substrate concentration, the reaction rates of the pairs of Ara1 and Ara1b were significantly higher ($P < 0.05$) than those of Ara2 and Ara2b. Apart from Ara1b having a higher content of the relatively shorter arabinans, of molecular weight 1.3 kDa, than the Ara2b sample (Figure 6), it was not possible to relate the differences in the rates directly to the subtle differences in the molecular weight profiles among the pairs of shorter arabinans Ara1 and Ara2 and among the longer chain arabinan samples Ara1b and Ara2b, respectively. The data did indicate, however, that the "zipping" between FAs in the arabinan to form diFAs was very slow and increased less with increased FA concentration in the longer chain arabinans than in the shorter, probably due to the limited access of FA for the cross-linking to occur. It appeared to be more difficult to initiate the reaction between the FA on the longer chains than on the shorter arabinan chains, corroborating the interpretation that longer chains react more slowly as illustrated in Figure 1. Hence, the overall result that the cross-linking rates of the longer chain (feruloylated) arabinans were much slower than the corresponding rates of the shorter (feruloylated) arabinans when reacted at the same FA concentration supported the hypothesis that the rate of cross-linking is affected by the extent of the backbone length of the arabinan.

Formation of Ferulic Acid Dehydrodimers. Enzymatic cross-linking of FA was an oxidative reaction that produced feruloyl dehydrodimers (5,5′, 8-*O*-4′, and 8,5′ benzofuran-diFA). Disappearance of FA and formation of diFAs from the cross-linking reaction was further confirmed by HPLC analysis (Table 1 and Figure 9), which is in complete agreement with previous studies.[8,9] Of the diFAs identified, the 8,5′-diFA and 8-*O*-4′-diFA forms predominated, with traces of 5,5′-diFA detected (Figure 9). A minimal peak was observed at the retention time equivalent to 8,8′-diFA, but the level was too low to be quantified. It has been reported that 8,8′-diFA is one of the most favorable forms resulting from the oxidative cross-linking

of FA with different peroxidases and laccases in arabinoxylan,[21] whereas in pectic polysaccharides, 8-*O*-4′ and 8,5′-diFA appear to dominate.[9] As suggested in the previous study,[21] one of the possibilities that the 8,8′-diFA form was not detected in the analysis of the cross-linked arabinan might be due to its transient properties and involvement in further polymerization to form more complex dehydropolymers. However, it has previously been reported that 8,8′-diFA can be found in pectic polysaccharides with a small decrease after the addition of HRP and H_2O_2.[9]

Among the different types of substrate samples, the amount of 8,5-diFA increased significantly in Ara1 and with a lower relative increase in the 5,5′-diFA form by cross-linking (35%) and no change in the 8-*O*-4′-diFA (Figure 9A). A similar pattern was evident for Ara2 and Ara1b, whereas Ara2b deviated somewhat from this trend as the levels of all three types of diFAs in fact decreased after cross-linking (Figure 9D). We interpret the decrease in diFAs in Ara2b as being due to the oxidation by excess H_2O_2 because the H_2O_2:FA level was relatively higher for Ara2b due to the low FA levels in this sample (Figure 9D). Reaction with a further excess amount of H_2O_2 caused a significantly decreased level of FA but no increase in diFAs (data not shown). This is because a higher ratio of H_2O_2/FA in the reaction caused a higher extent of oxidation of FA and diFAs and, hence, reduced the amount of diFAs as well. A previous study also reported a decrease in oligomerization with higher ratios of H_2O_2/FA, possibly because higher amounts of H_2O_2 led to partial inactivation of the enzyme.[20] There is also a possibility that a portion of the FA and natural occurring diFAs was converted to other products not detectable by the HPLC method employed, for example, trimers or tetramers.[20,22] The latter types of reactions may in any case explain the imbalanced total amount of FA and diFAs before and after the cross-linking as such reactions have been reported previously to cause deviations in the stoichiometry of cross-linking reactions of FA to diFAs.[23] In this study, a few unidentified peaks did occur on the chromatograms after cross-linking. The spectra (UV−vis) were similar to the spectra of trimers reported,[24] but our analysis did not allow a firm identification or quantification of these compounds.

It may be speculated that the hypothesis that the length of the arabinan backbone influenced the kinetics of the cross-linking was an oversimplification of the events, but the results nevertheless confirmed this initial hypothesis. Hence, it was proven that the rate of FA cross-linking depends on the arabinan oligosaccharide backbone chain length; that is, longer arabinans exhibit a slower cross-linking rate than shorter arabinans, all other things being equal. It is not only the substrate concentration but also the molecular size, that is, the arabinan backbone chain length, that affects the rate of cross-linking. For further investigation, this work will be used as a basis to investigate how the rates of enzyme-catalyzed cross-linking affect the rates of gelation and the resulting gel strength.

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ABBREVIATIONS USED

Ara, arabinan; diFAs, ferulic acid dehydrodimers; DP, degree of polymerization; FA, ferulic acid groups; HRP, horseradish peroxidase; H_2O_2, hydrogen peroxide; NaOH, sodium hydroxide; $NaH_2PO_4 \cdot H_2O$, sodium dihydrogen phosphate monohydrate; Na_2HPO_4, disodium hydrogen phosphate; Na_2SO_4, anhydrous sodium sulfate.

REFERENCES

(1) Oosterveld, A.; Beldman, G.; Schols, H. A.; Voragen, A. G. J. Characterization of arabinose and ferulic acid rich pectic polysaccharides and hemicelluloses from sugar beet pulp. *Carbohydr. Res.* **2000**, *328*, 185–197.

(2) Holck, J.; Hjernø, K.; Lorentzen, A.; Vigsnæs, L. K.; Hemmingsen, L.; Licht, T. R.; Mikkelsen, J. D.; Meyer, A. S. Tailored enzymatic production of oligosaccharides from sugar beet pectin and evidence of differential effects of a single DP chain length difference on human faecal microbiota composition after *in vitro* fermentation. *Process Biochem.* **2011**, *46*, 1039–1049.

(3) Westphal, Y.; Khnel, S.; de Waard, P.; Hinz, S. W. A.; Schols, H. A.; Voragen, A. G. J.; Gruppen, H. Branched arabino-oligosaccharides isolated from sugar beet arabinan. *Carbohydr. Res.* **2010**, *245*, 1180–1189.

(4) Levigne, S. V.; Ralet, M.-C. J.; Quéméner, B. C.; Pollet, B. N.-L.; Lapierre, C.; Thibault, J.-F. J. Isolation from sugar beet cell walls of arabinan oligosaccharides esterified by two ferulic acid monomers. *Plant Physiol.* **2004**, *134*, 1173–1180.

(5) Saulnier, L.; Thibault, J. Ferulic acid and diferulic acids as components of sugar-beet pectins and maize bran heteroxylans. *J. Sci. Food Agric.* **1999**, *402*, 396–402.

(6) Colquhoun, I. J.; Ralet, M. C.; Thibault, J. F.; Faulds, C. B.; Williamson, G. Structure identification of feruloylated oligosaccharides from sugar beet pulp by NMR spectroscopy. *Carbohydr. Res.* **1994**, *263*, 243–256.

(7) Micard, V.; Thibault, J. F. Oxidative gelation of sugar-beet pectins: use of laccases and hydration properties of the cross-linked pectins. *Carbohydr. Polym.* **1999**, *39*, 265–273.

(8) Oosterveld, A.; Beldman, G.; Voragen, A. G. J. Oxidative cross-linking of pectic polysaccharides from sugar beet pulp. *Carbohydr. Res.* **2000**, *328*, 199–207.

(9) Oosterveld, A.; Grabber, J. H.; Beldman, G.; Ralph, J.; Voragen, A. G. J. Formation of ferulic acid dehydrodimers through oxidative cross-linking of sugar beet pectin. *Carbohydr. Res.* **1997**, *300*, 179–181.

(10) Waldron, K. W.; Ng, A.; Parker, M. L.; Parr, A. J. Ferulic acid dehydrodimers in the cell walls of *Beta vulgaris* and their possible role in texture. *J. Sci. Food Agric.* **1997**, *74*, 221–228.

(11) Kuuva, T.; Lantto, R.; Reinikainen, T.; Buchert, J.; Autio, K. Rheological properties of laccase-induced sugar beet pectin gels. *Food Hydrocolloids* **2003**, *17*, 679–684.

(12) Arnous, A.; Meyer, A. S. Comparison of methods for compositional characterization of grape (*Vitis vinifera* L.) and apple (*Malus domestica*) skins. *Food Bioprod. Process.* **2008**, *86*, 79–86.

(13) Rasmussen, L. E.; Sørensen, J. F.; Meyer, A. S. Kinetics and substrate selectivity of a *Triticum aestivum* xylanase inhibitor (TAXI) resistant D11F variant of *Bacillus subtilis* XynA xylanase. *J. Biotechnol.* **2010**, *146*, 207–214.

(14) Andreasen, M. F.; Christensen, L. P.; Meyer, A. S.; Hansen, Å. Release of hydroxycinnamic and hydroxybenzoic acids in rye by commercial plant cell wall degrading enzyme preparations. *J. Sci. Food Agric.* **1999**, *7*, 411–413.

(15) Andreasen, M. F.; Christensen, L. P.; Meyer, A. S.; Hansen, Å. Content of phenolic acids and ferulic acid dehydrodimers in 17 rye (*Secale cereale* L.) varieties. *J. Agric. Food Chem.* **2000**, *48*, 2837–2842.

(16) Agger, J.; Viksø-Nielsen, A.; Meyer, A. S. Enzymatic xylose release from pretreated corn bran arabinoxylan: differential effects of deacetylation and deferuloylation on insoluble and soluble substrate fractions. *J. Agric. Food Chem.* **2010**, *58*, 6141–6148.

(17) Waldron, K. W.; Parr, A. J.; Ng, A.; Ralph, J. Cell wall esterified phenolic dimers: Identification and quantification by reverse phase high performance liquid chromatography and diode array detection. *Phytochem. Anal.* **1996**, *7*, 305–312.

(18) Rasmussen, L. E.; Meyer, A. S. Size exclusion chromatography for the quantitative profiling of the enzyme-catalyzed hydrolysis of xylo-oligosaccharides. *J. Agric. Food Chem.* **2010**, *58*, 762–769.

(19) Holck, J.; Lorentzen, A.; Vigsnæs, L. K.; Licht, T. R.; Mikkelsen, J. D.; Meyer, A. S. Feruloylated and nonferuloylated arabino-oligosaccharides from sugar beet pectin selectively stimulate the growth of *Bifidobacterium* spp. in human fecal in vitro fermentations. *J. Agric. Food Chem.* **2011**, *59*, 6511–6519.

(20) Bunzel, M.; Heuermann, B.; Kim, H.; Ralph, J. Peroxidase-catalyzed oligomerization of ferulic acid esters. *J. Agric. Food Chem.* **2008**, *56*, 10368–10375.

(21) Robertson, J. A.; Faulds, C. B.; Smith, A. C.; Waldron, K. W. Peroxidase-mediated oxidative cross-linking and its potential to modify mechanical properties in water-soluble polysaccharide extracts and cereal grain residues. *J. Agric. Food Chem.* **2008**, *56*, 1720–1726.

(22) Bunzel, M.; Ralph, J.; Steinhart, H. Phenolic compounds as cross-links of plant derived polysaccharides. *Czech J. Food Sci.* **2004**, *22*, 64–67.

(23) Bunzel, M.; Ralph, J.; Funk, C.; Steinhart, H. Isolation and identification of a ferulic acid dehydrotrimer from saponified maize bran insoluble fiber. *Eur. Food Res. Technol.* **2003**, *217*, 128–133.

(24) Bunzel, M.; Ralph, J.; Funk, C.; Steinhart, H. Structural elucidation of new ferulic acid-containing phenolic dimers and trimers isolated from maize bran. *Tetrahedron Lett.* **2005**, *46*, 5845–5850.

CHAPTER 5

Oxidative gelation of sugar beet pectin

This chapter discusses the rheological properties of sugar beet pectin gels produced *via* enzyme catalyzed oxidative cross-linking. Main results are discussed based on the work presented in paper III "Enzyme catalyzed oxidative gelation of sugar beet pectin: Kinetics and rheology".

5.1 Rheological measurement

To define food gels in a rheological term, they are often categorized as "soft solids" that exhibit a yield stress, that have a viscoelastic properties and have a moderate modulus ($<10^6$ Pa) (Walstra, 1996). Viscoelastic properties of a gel, exhibiting both solid and liquid characteristic, could be well described or characterized using a dynamic oscillatory rheological test. Oscillatory tests provide measurement of two dynamic mechanical properties: an elastic modulus (G') which exhibits a pronounced plateau after extended of time (or frequency) and a viscous (G'') modulus, which is relatively smaller than the elastic modulus in the plateau region. The physical characteristic of the material can be further expressed by complex modulus (G^*), complex viscosity (η^*), and phase shift angle (δ) (Table 5.1) (Mezger, 2006).

Table 5.1: Definition of rheological parameters demonstrating the physical properties of a material (built from several references: Morris, 1995; Mezger, 2006).

Parameter (Unit)	**Definition**
Elastic modulus, G' (Pa)	A measure of the deformation energy stored by the sample during the shear process; represents the elastic behavior of a material
Viscous modulus, G'' (Pa)	A measure of deformation energy used up by the sample during the shear process; represents the viscous behavior of a material
Complex modulus, G^* (Pa)	Overall resistance to deformation of a material; $\lvert G^* \rvert = (G'^2 + G''^2)^{1/2}$
Complex viscosity, η^* (Pa)	A measure of difference between the dynamic viscosity and elasticity; $\lvert \eta^* \rvert = (\eta'^2 - \eta''^2)^{1/2}$; or a ratio between complex modulus and angular frequency, $\lvert \eta^* \rvert = G^*/\omega$
Phase shift angle, δ (rad)	Reflecting the phase shift between the strain and stress amplitude due to viscoelasticity; A measure of the presence and extent of elastic behavior in a fluid
Loss factor, $\tan\delta$	A measure of the relative magnitude of the viscous to elastic component; $\tan\delta = G''/G'$; elastic material: $\tan\delta < 1$; viscous material: $\tan\delta > 1$

For a typical polysaccharide gel system, dynamic viscosity decreases steeply with increasing frequency, and the slope of log η^* vs. log ω approaches -1 when *G'* and *G"* are constant (Figure 5.1A) (Morris, 1995). Oscillatory test performed on SBP gel produced *via* enzyme catalyzed oxidative gelation shows an increment of *G'* with time as the reaction of enzyme catalysis progresses, as exemplified in Figure 5.1B. The gel formation starts at the point when the *G' > G"* (Figure 5.1B), hence this point is known as the point of gelling (Morris, 1995). As the time of reaction increases *G'* reached a plateau demonstrating formation of a stable gel.

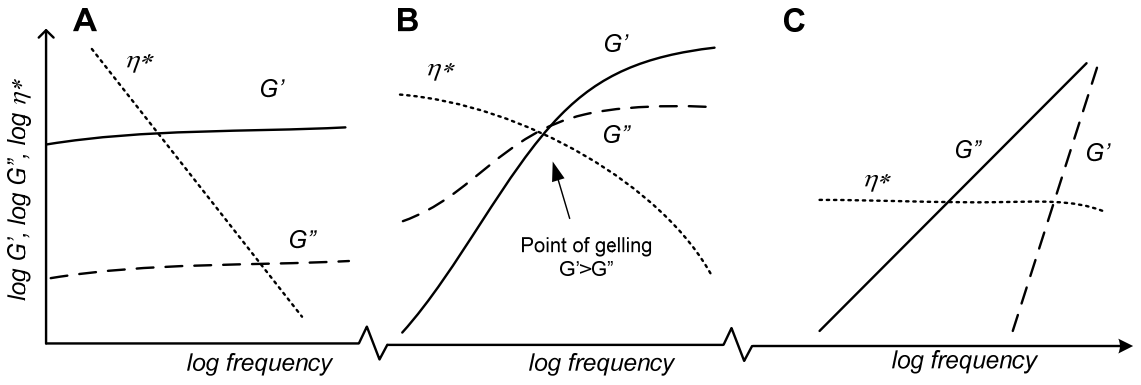

Figure 5.1: Typical dynamic mechanical spectrum for a polysaccharide system showing (A) a typically strong polysaccharide gel; (B) concentrated entangled polysaccharide solution; (C) dilute polysaccharide solution (adapted from Morris, 1995).

5.2 Properties of pectin gels from sugar beet and other sources

Gels produced from enzyme catalyzed oxidative covalent cross-linking of feruloylated polysaccharide are thermo-irreversible (Carvajal-Millan *et al.*, 2005), which is a feature of particular significance for food applications. Other pectin gels produced using high DE pectins (>50% DE) are thermo-reversible, but only at low pH, and in the presence of sucrose, whereas the gels produced using low DE pectins (<50% DE), by addition of Ca^{2+}, the melting temperature are influenced by the DE and concentration of pectins (Clark & Farrer, 1996). Our investigation on the effect of temperature on the SBP gels produced *via* oxidative gelation catalyzed by HRP or laccase confirmed the thermo-irreversible properties of the gels (Figure 5.2). The cross-linked sugar beet pectin gels can be dried easily and the resulting powders have remarkable water-absorption capacities (or known as swelling properties) in contrast to the gels obtained from apple and citrus pectins (Thibault, 1986). In relation to this characteristic, a notable textural deficiency known as syneresis, which is due to the release of water from the gel network, was associated mostly with

low DE pectin gels (El-Nawawi & Heikal, 1994) but this feature has not been reported in the literature for oxidative cross-linked SBP gel.

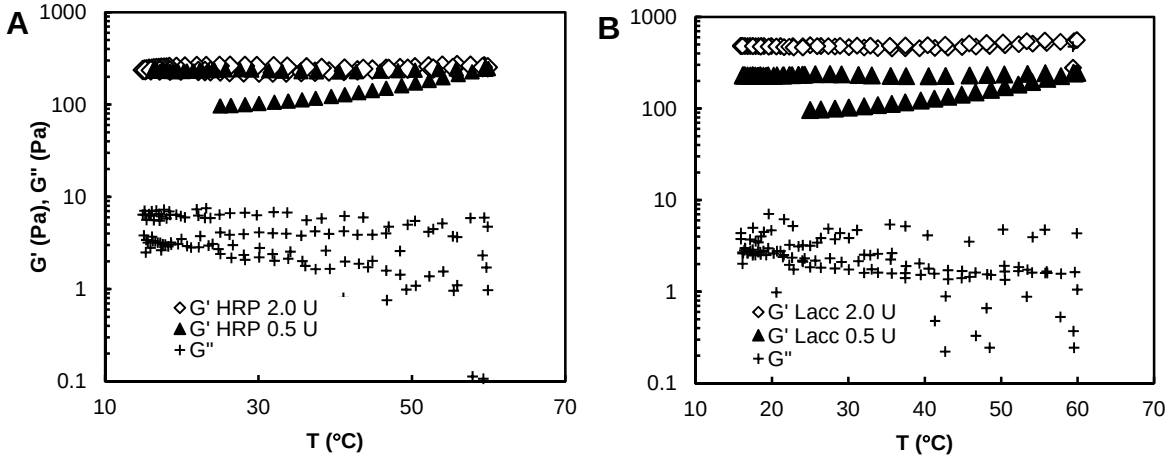

Figure 5.2: Thermal-irreversible stability measurement of oxidative gelation of 2.5% (w/v) SBP catalyzed by (A) HRP; (B) laccase

Factors affecting the rates of oxidative gelation of SBP

The kinetic behavior during gelation of pectin is an important factor in the manufacture of many food products, for example a slow rate of gelation is preferred to obtain a clear gel product without air bubbles (May, 1992). It has been reported that the rate of oxidative gelation of SBP catalyzed by laccase affected the properties of the gels produced (Kuuva *et al*., 2003); high final *G'* was achieved for the gels produced at slow gelation rate compared to the gels produced at high gelation rate. Addition of Ca^{2+} to the SBP solution catalyzed by laccase has been shown to retard the rate of gelation of SBP but improved the texture of the gel formed at high enzyme activities (Kuuva *et al*., 2003). Hence, in our investigation, addition of Ca^{2+} into the oxidative gelation of SBP by HRP or laccase has been shown to reduce the rate of gelation which supported the previous work by Kuuva *et al*. (2003), however lower *G'* was achieved at the end of 20 min reaction (Figure 5.3). This result indicates the influence of cations on the enzyme activity which slowed down the cross-linking of FA in SBP. However, it has been reported that enzyme activity and thermal stability of peroxidase from various sources was increased in the presence of Ca^{2+} (Sutherland & Aust, 1996; Hiner *et al*., 2004). Furthermore, it has been shown that halide ions (F^-, Cl^-, Br^-) inhibited the activity of laccase (Xu, 1996). Thus, it might be that the oxidative gelation of SBP catalyzed by HRP or laccase was hindered by the halide ion (which was Cl^-) instead of Ca^{2+}. Addition of a chelating agent, ethylenediaminetetraacetic acid (EDTA), on the oxidative gelation of SBP catalyzed by HRP or laccase (to chelate any Ca^{2+} or other divalent cations) did not produce

gelation (data not shown), which underlined that the gelation was not a result of polygalacturonic acid-Ca^{2+} interactions in the SBP substrate.

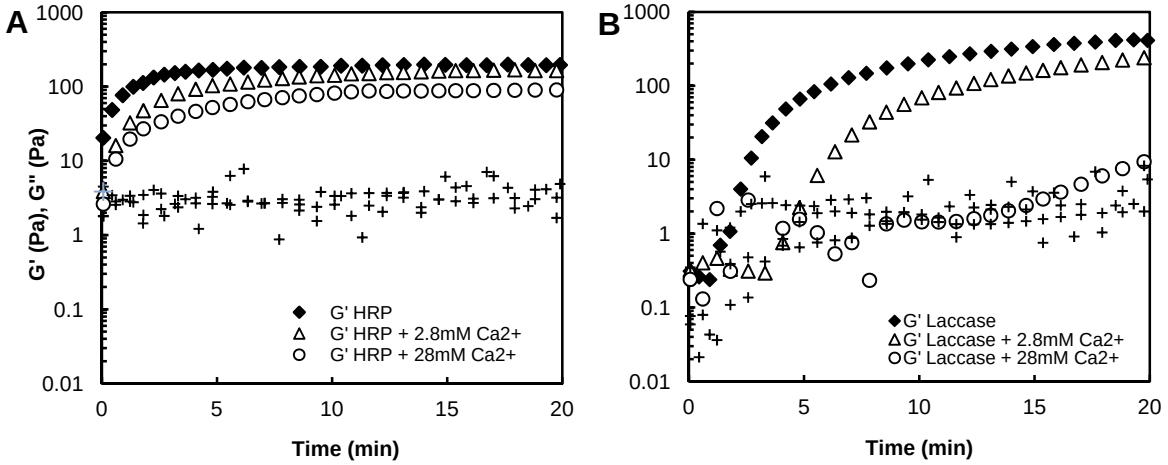

Figure 5.3: Influence of addition of $CaCl_2$ on the oxidative gelation of 2.5% (w/v) SBP catalyzed by (A) 2.0 U·mL^{-1} HRP; (B) 2.0 U·mL^{-1} laccase.

As shown in our work on the kinetics of enzyme catalyzed oxidative cross-linking of feruloylated arabinan-oligosaccharide, the backbone chain length of the oligosaccharide (Paper II- Zaidel *et al.*, 2011) influenced the rate of cross-linking of ferulic acid. Therefore, it was our initial intention to relate this finding to the influence of the backbone length on the rate of gelation of arabinan-oligosaccharide. From our preliminary result on the oxidative gelation of arabinan samples performed using oscillatory measurement, no significant increment has been detected in *G'* after addition of the enzyme (HRP or laccase), even after an extended reaction time (>12 hrs). Increasing the concentration of the arabinan sample to 50% has not improved the result and thus it can be deduced that the relatively short chain or low molecular weight of the arabinan sample (approximately 1.3 kDa) prevented the measurement of the macromolecular properties of arabinan. This is in fact one of the critical parameters for gelation, given that the FA content of arabinan were 7.0 mg·g^{-1} DM (Ara1) and 2.5 mg·g^{-1} DM (Ara2), which are rather sufficient for oxidative cross-linking of FA to occur. By taking the HM fraction (SBP) from the extraction of sugar beet pulp, which has molecular weight of >110 kDa, gelation has been performed successfully. This demonstrates that the length or molecular size of polysaccharide influence the oxidative gelation of pectic polysaccharide although our result shows the occurrence of oxidative cross-linking of feruloylated arabinan oligosaccharide (Zaidel *et al.*, 2011). Furthermore, extraction process is one of the important factors that influence the characteristic of sugar beet pectin gels (Micard & Thibault, 1999; Oosterveld *et al.*, 2000b); non-gellable pectins can be obtained despite high contents in feruloyl groups. The accessibility of the feruloyl groups is apparently one critical

parameter for the gelation, however the quantification of accessible feruloyl groups is not simple (Thibault, 1988; Guillon & Thibault, 1987; 1990).

As the properties of pectin gels formed from Ca^{2+} complexation depends on the DE of HG and concentration of Ca^{2+}, the properties of sugar beet pectin gels formed *via* enzyme catalyzed oxidative cross-linking are influenced by various factors such as the enzyme catalysis, degree of formation of cross-linked diFAs and pectin concentration. Oxidoreductase enzymes catalyze the oxidative coupling of a variety of organic and inorganic compounds, by utilizing oxygen (laccase) or H_2O_2 (HRP) as the oxidizing agent. The different mechanisms of oxidation by these two enzymes might lead to different properties of the gels produced from their catalysis. It was reported that SBP gels catalyzed by HRP was softer than the SBP gels catalyzed by laccase (Norsker *et al*., 2000). However, in their study they did not investigate the amount of diFAs after the gelation which might explain the reason for the different properties of the gels. Furthermore, the availability of oxygen or oxidizing agent for the oxidative cross-linking of FA to occur is one of the important factors for an optimum rate of gelation and thus producing a strong gel. However, an excess amount of H_2O_2 was predicted to lead to partial inactivation of the enzyme which decreases the oligomerization of FA in SBP (Bunzel *et al*., 2008), which has also been reported in Paper II (Zaidel *et al.*, 2011). Thibault (1986) reported that gelation of SBP catalyzed by HRP or ammonium persulphate was observed when pectin concentration was higher than 0.8% (w/v), showing only an increase in viscosity at low pectin concentration and firmer gels were obtained when pectin concentrations were increased.

5.3 Gelation kinetics and rheological properties of sugar beet pectin gels

Paper III: Enzyme catalyzed oxidative gelation of sugar beet pectin: Kinetics and rheology
Dayang Norulfairuz Abang Zaidel, Ioannis S. Chronakis, & Anne S. Meyer
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5.3.1 Significance of study

Due to its acetylation and relatively short HG stretches, the classic, divalent-cation (Ca^{2+}) promoted pectin gelation is less significant for SBP than for *e.g.* citrus pectin. However, covalent cross-linking of feruloylated SBP catalyzed by oxidoreductase enzyme produces a relatively stable and thermo-irreversible gel which is a feature of particular significance for food applications. In this study we

hypothesized that the different mechanisms of the two oxidoreductase enzymes (HRP and laccase) might influence the kinetics of the cross-linking and consequently the properties of the gels formed. Another hypothesis was that a slower rate of gelation would give better gel strength (presented by *G'*). The objective of this study was to examine the correlations between rates of gelation, catalyzed by either HRP or laccase, with the properties of the gels produced. The conditions at which the rate of gelation and the gel strength were optimized by use of response surface methodology. An understanding of any differences in the reaction kinetics and/or any differences in the resulting gel properties of gels produced by enzyme catalysis is an important prerequisite for rational design of enzymatic gelation reactions *e.g.* for food applications.

5.3.2 Experimental considerations

During the preliminary experiment, the total reaction time was determined in order to produce a good oxidative cross-linking reaction of the SBP and at the end of the reaction a stable gel can be obtained. Formation of gels *via* oxidative gelation of feruloylated polysaccharide can take place at a relatively short time, given the right amount of substrate concentration and reaction conditions. Various reaction times were used previously to determine the oxidative gelation of SBP catalyzed by different oxidoreductase enzyme, *e.g.* gelation of SBP catalyzed by HRP was performed at only 8 min (Oosterveld *et al.*, 2000b) and longer reaction time *i.e.* 5.5 hr was applied to investigate the oxidative gelation of SBP catalyzed by laccase (Kuuva *et al.*, 2003). Preliminary experiment was conducted to determine the reaction time for the oxidative gelation of SBP catalyzed by HRP or laccase, and 20 min was determined to be sufficient in producing a relatively good gel. After an extended period of time >1hr the gel has been shown to produce an unstable system (unstable *G'* and *G"*) (data not shown).

Preliminary data showed that gelation was observed when SBP concentration was >2.0% for HRP catalysis, and >1.0% for laccase catalysis (data not shown). For this study, the concentration of 2.5% SBP was chosen to depict the gelation reaction, and the enzymes were compared at a unit activity level. When compared in the same assay using ABTS as substrate (25^{o}C, pH 5.0), HRP had a 50 times higher specific activity (U mg^{-1}) than laccase. In preliminary experiment, already at 1.0 U·mL^{-1} the oxidative gelation of SBP catalyzed by HRP showed an instantaneous formation of gel, whereas for laccase at similar enzyme activity a slower gel formation was observed. Higher enzyme unit (> 2.0 U mL^{-1}) of HRP catalyzes a very fast gelation, *i.e.* rapid increase in *G'*, which makes it a bit difficult to measure the gelation on the rheometer. The amount of H_2O_2 used for HRP catalysis was the critical factors for the oxidative gelation to occur. We have determined that 0.5 mM H_2O_2 was sufficient for this study as further increase of H_2O_2 concentration did not show

any increase in the rate of gelation or the strength of the gel (data not shown). Gelation of SBP was further investigated *via* randomized, quadratic, Box-Behnken statistically designed experiments. For this experiment, several reaction factors were considered, *i.e*. pectin level, temperature, pH, enzyme dosage, and, for HRP, the H_2O_2 concentration. The effect of temperature has not been reported previously, but it has shown a significant effect in the initial rate of gelation.

5.3.3 Highlights

Analysis on monosaccharide and phenolic compounds showed that SBP contains feruloylated arabinan side-chains (arabinose: 22.9 mol%; FA: 7.3 $mg \cdot g^{-1}$ DM) to allow enzyme catalyzed gelation *via* oxidative cross-linking of ferulic acid to ferulic acid dehydrodimers. The amount of FA decreased after addition of HRP and laccase, and this decrease was accompanied by an increase in diFAs (Figure 5.4). At equal enzyme addition levels (equal units of activity), the rates of laccase catalyzed oxidative gelation of SBP were slower than the rates of HRP catalyzed oxidative gelation. However, laccase catalysis produced stronger gels. This could be explained by the significantly (P<0.05) higher amount of diFAs produced at the end of the oxidative cross-linking reaction catalyzed by laccase.

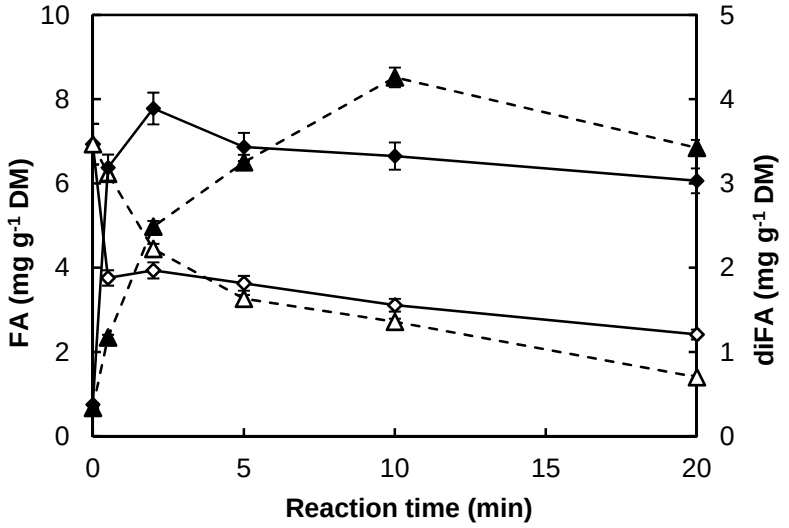

Figure 5.4: FA decrement and diFAs formation during oxidative cross-linking of SBP catalyzed by HRP and laccase at 2.0 $U \cdot mL^{-1}$ for both enzymes (0.14 mM H_2O_2 was used for HRP since SBP was 1% (w/v) which contains FA equivalent to approximately 0.5 mM). Coefficients of variation based on calibration curves were in the range of 2.7-4.8%. (◇): FA, HRP; (◆): diFA, HRP; (△): FA, laccase; (▲): diFA, laccase. (figure from paper III- Zaidel *et al.*, 2012)

Gel strengths correlate positively to the rates of gelation for laccase catalyzed gels, whereas no such correlation exists for HRP catalyzed gels (Figure 5.5). The straight lines represent the correlation between G'_{10} and rate of gelation; slope > 1 for laccase whereas slope < 0 for HRP. Our finding showed that it was possible to tune the enzyme catalyzed gelation rate *via* adjustment of the reaction factors, *i.e.* pectin level, temperature, enzyme dosage, and, for HRP, the H_2O_2 concentration, to produce desirable properties of the gel for a particular food application.

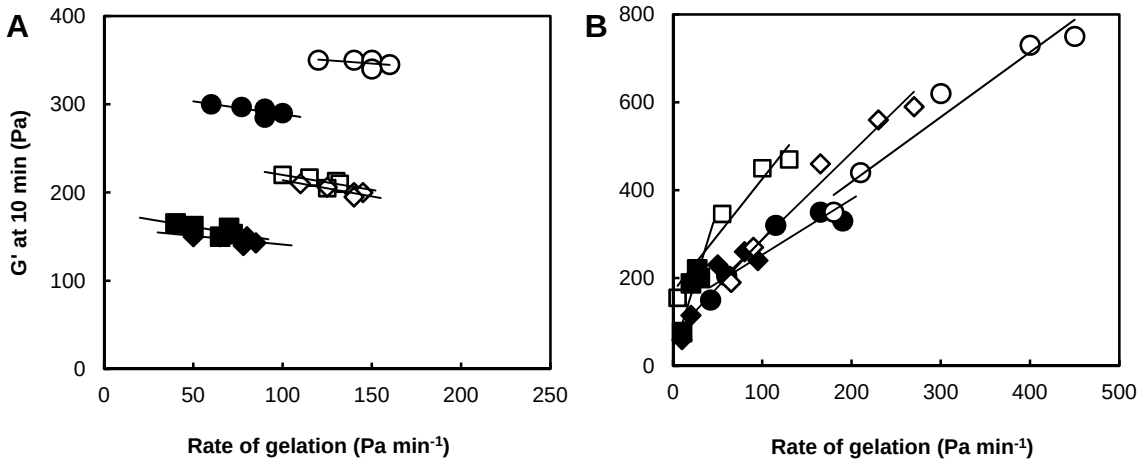

Figure 5.5: Correlation between the rate of gelation and G'_{10} at 25, 40 and 55 °C for 2.5% (w/v) SBP or 4.0% (w/v) SBP catalyzed using either (**A**) HRP (at 1 mM H_2O_2) or (**B**) laccase. (■): 2.5%, 25 °C; (◆): 2.5%, 40 °C; (●): 2.5%; 55 °C; (□): 4.0%; 25 °C; (◇): 4.0%, 40 °C; (○): 4.0%, 55 °C. Data were extracted from the statistically design experiments (figure from paper III- Zaidel *et al.*, 2012). The straight lines represent the correlation between the rate of gelation and G'_{10}.

Paper III

Enzyme catalyzed oxidative gelation of sugar beet pectin: Kinetics and rheology

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Enzyme catalyzed oxidative gelation of sugar beet pectin: Kinetics and rheology

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Sugar beet pectin

ABSTRACT

Sugar beet pectin (SBP) is a marginally utilized co-processing product from sugar production from sugar beets. In this study, the kinetics of oxidative gelation of SBP, taking place via enzyme catalyzed cross-linking of ferulic acid moieties (FA), was studied using small angle oscillatory measurements. The rates of gelation, catalyzed by horseradish peroxidase (HRP) (EC 1.11.1.7) and laccase (EC 1.10.3.2), respectively, were determined by measuring the slope of the increase of the elastic modulus (G') with time at various enzyme dosages (0.125–2.0 U mL^{-1}). When evaluated at equal enzyme activity dosage levels, the two enzymes produced different gelation kinetics and the resulting gels had different rheological properties: HRP (with addition of H_2O_2) catalyzed a fast rate of gelation compared to laccase (no H_2O_2 addition), but laccase catalysis produced stronger gels (higher G'). The main effects and interactions between different factors on the gelation rates and gel properties were examined in response surface designs in which enzyme dosage (0.125–2.0 U mL^{-1} for HRP; 0.125–10 U mL^{-1} for laccase), substrate concentration (1.0–4.0%), temperature (25–55 °C), pH (3.5–5.5), and H_2O_2 (0.1–1.0 mM) (for HRP only) were varied. Gelation rates increased with temperature, substrate concentration, and enzyme dosage; for laccase catalyzed SBP gelation the gel strengths correlated positively with increased gelation rate, whereas no such correlation could be established for HRP catalyzed gelation and at the elevated gelation rates (>100 Pa min^{-1}) gels produced using laccase were stronger (higher G') than HRP catalyzed gels at similar rates of gelation. Chemical analysis confirmed the formation of ferulic acid dehydrodimers (diFAs) by both enzymes supporting that the gelation was a result of oxidative cross-linking of FAs.

1. Introduction

Sugar beet pectin is a byproduct from sugar (sucrose) production based on sugar beets. Pectic polysaccharides in sugar beet mainly consist of homogalacturonan and rhamnogalacturonan I (RGI), the latter notably being arabinan and arabinogalactan substituted RGI (Oosterveld, Beldman, & Voragen, 2000). The backbone of sugar beet RGI is made up of repeating units of [→2)-α-L-Rha*p*-(1 → 4)-α-D-Gal*p*A-(1→] (Holck et al., 2011). The rhamnose moieties of the RGI in sugar beet pectin may be substituted at the *O*-4 position with α-(1,5)-linked-arabinans having a high degree of α-(1,2)- and α-(1,3)-arabinofuranosyl (Ara*f*) substitutions, and minor amounts of β-(1,4)-linked-galactan (Oosterveld et al., 2000; Westphal et al., 2010). Some of the Ara*f* moieties in the α-(1,5)-linked-arabinans are substituted with feruloyl groups. These feruloyl groups occur either as single ferulic acid (FA) moieties or are present as ferulic acid dehydrodimers (diFAs) that may cross-link two different arabinan chains (Levigne et al., 2004).

Due to its acetylation and relatively short homogalacturonan stretches, the classic, divalent-cation (Ca^{2+}) promoted pectin gelation is less significant for SBP than for e.g. citrus pectin. However, the existence of the feruloyl groups on the arabinan side-chains of RGI in SBP provides a way for enzyme catalyzed oxidative cross-linking of SBP to promote gelation (Micard & Thibault, 1999). The FA moieties are mainly esterified to the *O*-2 position of the Ara*f* residues in the α-(1,5)-linked-arabinan backbone, but may also be bound to the *O*-5 on the terminal arabinose (Levigne et al., 2004), or at the *O*-6 position of the galactopyranosyl (Gal*p*) residues in the β-(1,4)-galactan chains (Colquhoun, Ralet, Thibault, Faulds, & Williamson, 1994).

The ability of horseradish peroxidase (HRP) (EC 1.11.1.7) or laccase (EC 1.10.3.2) to catalyze cross-linking of FAs in SBP to produce a gel has been shown in previous studies (Kuuva, Lantto, Reinikainen, Buchert, & Autio, 2003; Norsker, Jensen, & Adler-Nissen, 2000). During the reaction, the FAs are enzymatically

Abbreviations: DM, dry matter; FA, ferulic acid; diFAs, ferulic acid dehydrodimers; HRP, horseradish peroxidase; H_2O_2, hydrogen peroxide; SBP, sugar beet pectin.

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oxidized into free radicals and 5,5′, 8-O-4′, 8,5′ and 8,8′ ferulic acid dehydrodimers (diFAs) are formed (Oosterveld et al., 2000; Oosterveld, Grabber, Beldman, Ralph, & Voragen, 1997). HRP and laccase catalyze the cross-linking reaction via different oxidizing mechanisms. The peroxidase requires hydrogen peroxide as the oxidizing agent while laccase oxidizes the substrate via direct interaction of oxygen with the copper cluster in the enzyme (Ercili Cura et al., 2009).

Gels produced from enzyme catalyzed covalent cross-linking of feruloyl groups are thermo-irreversible (Carvajal-Millan, Guilbert, Morel, & Micard, 2005), which is a feature of particular significance for food applications. The rheological properties of SBP gels produced by HRP and laccase catalysis have been compared previously (Norsker et al., 2000), and it is known that the rate of gelation catalyzed by laccase affects the gel properties (Kuuva et al., 2003), but the gelation kinetics of the HRP and laccase catalyzed SBP gelation have not been explored previously. We hypothesized that the different mechanisms of these two enzymes might influence the kinetics of the cross-linking and consequently the properties of the gels formed. Another hypothesis was that a slower rate of gelation would give better gel strength (presented by G'). The objective of this study was to test these hypotheses. This was done by examining correlations between rates of gelation, catalyzed by either HRP or laccase, with the properties of the gels produced. The conditions at which the rate of gelation and the gel strength were optimal, *i.e*. had maximum gel strength, were optimized by use of response surface methodology. An understanding of any differences in the reaction kinetics and/or any differences in the resulting gel properties of gels produced by enzyme catalysis is an important prerequisite for rational design of enzymatic gelation reactions e.g. for food applications. A recent study revealed the potential suitability of *in situ* SBP gels prepared through HRP-catalyzed oxidative reaction for biomedical and biopharmaceutical applications (Takei, Sugihara, Ijima, & Kawakami, 2011).

2. Materials and methods

2.1. Substrate

Sugar beet pectin (SBP) was obtained from Danisco A/S (Nakskov, Denmark). The pectin had been prepared from sugar beet pulp by sequential extraction with nitric acid, involving removal of insoluble cellulose, ultrafiltration, and diafiltration with a 50 kDa cut off essentially as described by Buchholt, Christensen, Fallesen, Ralet, and Thibault (2004), except that precipitation in isopropanol was replaced by spray drying. The degrees of methoxylation and acetylation of the pectin were ~59% and 20%, respectively (Holck et al., 2011).

2.2. Chemicals

Trans-cinnamic acid 99% was purchased from Alfa Aesar GmbH & Co. (Karlsruhe, Germany). Ferulic acid 99%, sodium hydroxide (NaOH) 50% (w/w), ethyl acetate, hydrogen peroxide (H_2O_2) 50% (w/w), sodium acetate, trifluoroacetic acid (TFA) 98% (w/w), and monosaccharide standards including: D-galactose, L-arabinose, D-fucose, L-rhamnose monohydrate, and D-galacturonic acid monohydrate were purchased from Sigma–Aldrich (Steinheim, Germany). D-glucose, D-xylose, disodium hydrogen phosphate (Na_2HPO_4) and anhydrous sodium sulfate (Na_2SO_4) were purchased from Merck (Darmstadt, Germany).

2.3. Enzymes

Horseradish peroxidase (HRP) (EC 1.11.1.7) type VI-A 1000 U mg^{-1} and laccase (EC 1.10.3.2) from *Trametes versicolor* 20 U mg^{-1} were purchased from Sigma–Aldrich (Stein-heim, Germany). One HRP unit is defined as oxidation of one μmole of 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) per minute at 25 °C and pH 5.0. One laccase unit is defined as conversion of one μmole of catechol per minute at 25 °C and pH 4.5. In this study, the enzymes were compared at a unit activity level. When compared in the same assay using ABTS as substrate (25 °C, pH 5.0), HRP had a 50 times higher specific activity (U mg^{-1}) than laccase. Viscozyme L 9 (VL9) produced from a selected strain of *Aspergillus aculeatus* was a commercial liquid preparation obtained from Novozymes A/S (Bagsvaerd, Denmark). VL9 was purified as described in Garna, Mabon, Wathelet, and Paquot (2004) prior to use.

2.4. Monosaccharides composition of SBP

Sugar beet pectin was hydrolyzed for monosaccharide composition analysis by means of the combined chemical and enzymatic hydrolysis method by Garna et al. (Garna, Mabon, Nott, Wathelet, & Paquot, 2006; Garna et al., 2004), briefly as follows: The SBP solution (0.25% w/v) was hydrolyzed with TFA (0.2 M) at 80 °C for 72 h. The hydrolyzate was adjusted to pH 5, diluted, and treated with purified VL9 at 50 °C for 24 h. The hydrolyzate was then diluted and filtered through a 0.22 μm nylon membrane filter (VWR International, USA) before injection in HPAEC-PAD using Dionex BioLC system (Dionex Corp., Sunnyvale, CA, USA) equipped with a PA20 column for monosaccharide analysis, and the monosaccharide analysis was done as described previously (Arnous & Meyer, 2008).

2.5. Phenolics analysis

The content of FAs and diFAs were determined by reverse phase high performance liquid chromatography (HPLC) after alkaline saponification (under nitrogen, N_2) with NaOH (1 M) at 25 °C, acidification and triple ethyl acetate extraction as described previously (Andreasen, Christensen, Meyer, & Hansen, 1999, 2000). For each extracted sample, the ethyl acetate phases were pooled and water was removed by adding anhydrous Na_2SO_4. The pooled samples were dried by evaporation under N_2 at 30 °C. Methanol (50% v/v) was added to the samples which were then filtered using a 0.22 μm nylon membrane filter (VWR International, USA). The sample (40 μL) was injected into an HPLC Chemstation 1100 series equipped with an ODS-L Optimal (250 × 4.6 mm, 5 μm) column from Capital HPLC, and a diode array detector (Hewlett Packard, Palo Alto, CA). The gradient elution was performed using solvents A (5% acetonitrile, 1 mM TFA) and B (acetonitrile) starting with 20% B at 0.5 mL min^{-1}. The gradient was running up to 40% B for 35 min and further up to 100% for another 3 min, with a final regeneration to 20% B for 2 min (Agger, Viksø-Nielsen, & Meyer, 2010). The column temperature was maintained at 40 °C. FA was detected and quantified at 316 nm using an authentic external standard for retention time and spectral recognition; quantification by linear area regression. diFAs were also detected and recognized at 316 nm but quantified at 280 nm according to response factors reported previously (Waldron, Parr, Ng, & Ralph, 1996).

2.6. Gelation

For each gelation experiment, the SBP, diluted in 0.05 M sodium acetate buffer, pH 5, to give a concentration of 2.5% w/v in the final reaction mixture, was mixed with enzyme at various dosages (0.125, 0.25, 0.5, 1.0 and 2.0 U mL^{-1}) and 0.5 mM H_2O_2 (for HRP only) at 25 °C. A H_2O_2 stock solution was prepared fresh every day. The enzymes were dosed according to their specific activity (see 2.3) and compared at similar activity levels (U mL^{-1}). The rheological analyses of the oxidative gelation of SBP, were based on

small angle oscillatory rheological measurements on serrated parallel plates using a HAAKE MARS rotational rheometer (Thermo Scientific Inc., Germany). Each gelation was initiated by adding the required amount of enzyme to the SBP solution and the storage (G') and loss (G'') modulus were recorded for 20 min at a frequency of 1 Hz and 0.1 Pa stress. Frequency sweeps (0.1–100 Hz) at 0.1 Pa stress were carried out after 20 min of the gel formation for two enzyme dosages (0.5 and 2.0 U mL^{-1}). Stress sweeps were performed at stress of 0.1–2000 Pa and a frequency of 1 Hz. All measurements were done within the linear viscoelastic region and at 25 °C. For the frequency and stress sweep evaluations silicon oil was used to cover the edges of the samples to avoid evaporation during measurements.

2.7. Statistically designed optimization of the gelation

Gelation of SBP was further investigated via randomized, quadratic, Box–Behnken statistically designed experiments. The experimental designs for HRP and laccase contained 41 and 25 different combinations, respectively, with three replications of the center point (the difference in number of experimental combinations were due to the inclusion of H_2O_2 concentration in the experimental design for HRP). Since the gelation rate catalyzed by laccase did not reach a maximum (plateau) in the first experimental design a further design was conducted for laccase using higher enzyme dosage and substrate concentrations. The factor limits for the statistically designed experiments are listed in Table 1.

2.8. Statistics

The program MODDE version 7.0.0.1 (Umetrics AB, Umeå, Sweden) was used as an aid to design the experimental templates and to evaluate main factor effects and interactions by multiple linear regression analysis. One-way analyses of variances (one-way ANOVA): 95% confidence intervals were compared as Tukey–Kramer intervals calculated from pooled standard deviations (Minitab Statistical Software, Addison-Wesley, Reading, MA).

2.9. Correlation between rate of gelation and G'_{10}

The data for rate of gelation and G' at 10 min (G'_{10}) for 2.5% and 4.0% w/v SBP catalyzed at various enzyme dosages (HRP or laccase) were extracted from the results obtained from the statistically design experiments.

3. Results and discussion

3.1. Sugar beet pectin composition

As expected, the monosaccharide profile of sugar beet pectin showed a significant amount of galacturonic acid (52.5 mol%) as well as relatively high levels of arabinose (22.9 mol%) and galactose (17.8 mol%), with lower levels of rhamnose (6.0 mol%) and only a trace of glucose (<1 mol%) (Table 2). These data were in accordance with data obtained previously for a similarly prepared SBP sample (different batch) (Holck et al., 2011). The total levels of FA and diFAs extracted from SBP were approximately 7.3 ± 0.3 mg g^{-1} DM (36 ± 2 μmol g^{-1}) and 0.36 ± 0.01 mg g^{-1} DM (1.0 ± 0.1 μmol g^{-1}), respectively (Table 2).

Table 1
Factor limits for the response surface experimental designs.

Enzyme	Enzyme dosage (U mL^{-1})	Substrate (% w/v)	Temperature (°C)	pH	H_2O_2 (mM)
HRP	0.125–2.0	1.0–4.0	25–55	3.5–5.5	0.1–1.0
Laccase	0.125–10.0	1.0–4.0	25–55	3.5–5.5	–
Laccase[a]	5.0–15.0	2.5–5.5	25, 55	4.5	–

[a] Further experiments were done with higher enzyme dosage and substrate concentration.

Table 2
Monosaccharide profiles and amounts of FA and diFAs in sugar beet pectin.

Component	mmol g^{-1} DM	mol%
Rhamnose	0.36	6.0
Arabinose	1.35	22.9
Galactose	1.06	17.8
Glucose	0.04	<1.0
Galacturonic acid	3.11	52.5
FA	0.036	–
diFAs	0.0010	–

3.2. Gelation kinetics

After a few preliminary experiments, a relatively fast rate of gelation and short reaction time (20 min) was obtained using a concentration of 2.5% (w/v) SBP while lower SBP concentrations (1.0–2.0% w/v) gave slower rates of gelation (data not shown). The development of storage (G') and loss (G'') moduli of the 2.5% (w/v) SBP solutions undergoing oxidative gelation catalyzed by HRP or laccase was investigated at various enzyme dosages (0.125–2.0 U mL^{-1}) (Fig. 1). The G' increased with time according to a regular hyperbola curve, and there were clear dose-response effects for both enzymes (Fig. 1). The increase of G' indicated that both enzymes catalyzed the formation of a gel with time. The results were in accordance with data reported previously for SBP gel formation catalyzed by HRP (Oosterveld et al., 2000) and laccase (Kuuva et al., 2003), but the data also showed that HRP and laccase exhibited somewhat different gelation kinetics. Hence, when compared at similar enzyme dosage, G' increased faster when SBP gelation was catalyzed by HRP than by laccase (Fig. 1). This result corresponded to a faster initial rate of gelation catalyzed by HRP than by laccase at equal enzyme dosage levels, notably at enzyme dosages above 0.5 U mL^{-1} (Fig. 2). The initial rate of gelation was determined from the initial slope of the G' as a function of time when $G' > G''$. At lower enzyme dosages, for example at 0.125 and 0.25 U mL^{-1}, HRP and laccase produced similar initial rates of gelation, approximately 2.4–3.3 and 10.0–11.3 Pa min^{-1}, respectively (Fig. 2). At higher enzyme dosages (0.5–2.0 U mL^{-1}), the rate of gelation catalyzed by HRP increased linearly with enzyme dosage and the rates were significantly ($P < 0.05$) higher than the corresponding rates catalyzed by laccase. For example, at 1.0 and 2.0 U mL^{-1} the rates of gelation catalyzed by HRP were 48 ± 9 and 94 ± 8 Pa min^{-1} whereas laccase catalysis produced gelation rates of 24 ± 3 and 26 ± 4 Pa min^{-1}, respectively (Fig. 2). The gelation catalyzed by laccase also had a longer 'lag phase' than the equivalent HRP catalyzed reaction, i.e. where $G' < G''$. Hence, a 'lag phase' of at least 3 min with addition of 2.0 U mL^{-1} and one of approximately 12 min with 0.125 U mL^{-1} of laccase added were recorded (Fig. 1B). Taken together with the initial rate data these results corresponded to a more rapid oxidation of FAs by H_2O_2 catalyzed by HRP relative to the oxidation by direct interaction of oxygen catalyzed via the copper cluster in the laccase enzyme. Addition of H_2O_2 had no effect on the laccase catalyzed reaction of the SBP gelation (data not shown). At similar enzyme dosage, the gels produced using HRP also reached a plateau faster than those produced by laccase catalysis, but the laccase catalyzed gels produced with enzyme addition levels of 1.0 and 2.0 U mL^{-1} reached a higher final G' value than the corresponding HRP catalyzed gels at the end of the 20 min reaction (Fig. 1). For example, at 2.0 U mL^{-1}, the G'

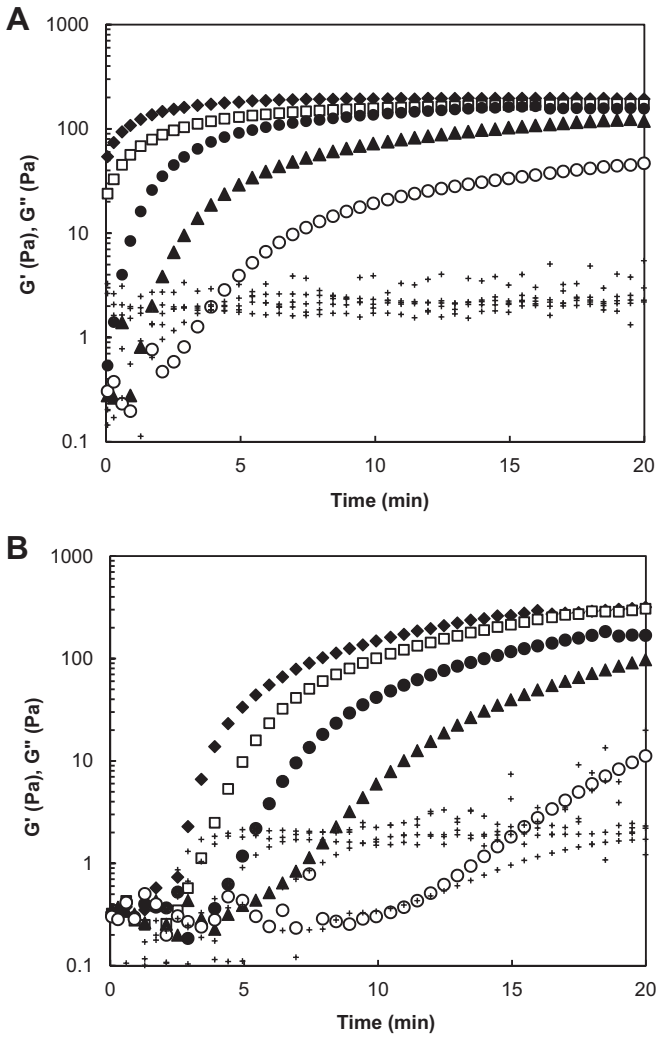

Fig. 1. Oxidative gelation kinetics of 2.5% (w/v) SBP catalyzed by (A) HRP; (B) laccase at various enzyme dosages at 25 °C. (◆): G' 2.0 U mL^{-1}; (□): G' 1.0 U mL^{-1}; (●): G' 0.5 U mL^{-1}; (▲): G' 0.25 U mL^{-1}; (○): G' 0.125 U mL^{-1}; (+): G''.

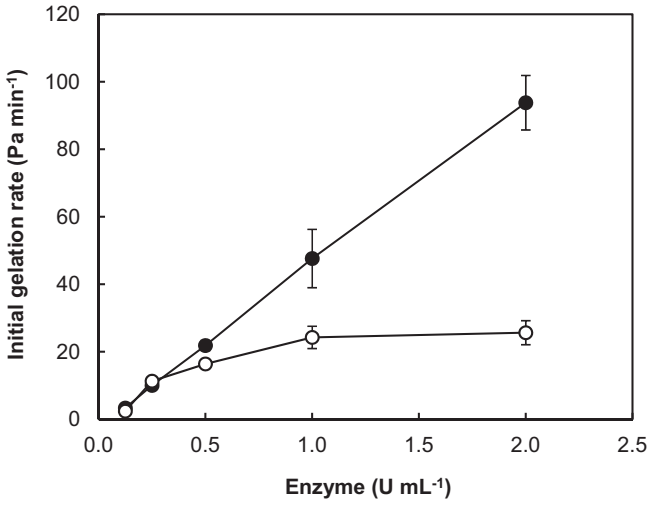

Fig. 2. Initial rate of gelation (Pa min^{-1}) of 2.5% (w/v) SBP catalyzed by HRP and laccase at various enzyme dosages (0.125–2.0 U mL^{-1}). (●): HRP; (○): laccase. Data are averages of two replicate gelation experiments and error bars are standard deviation for the two replicates.

achieved with HRP catalysis was approximately 200 Pa at 5 min and remained so during the 20 min of reaction, whereas for the gel catalyzed by laccase the G' value was approximately 50 Pa at 5 min but reached 300 Pa at 15 min and remained at this level during the 20 min reaction (Fig. 1). Kuuva et al. (2003) also reported that gels produced using laccase had higher G' (after 5.5 h of reaction) when the gels were produced at a slower rate of gelation than gels produced at a higher gelation rate. Control reactions of SBP without enzyme and with and without EDTA addition (to chelate any Ca^{2+} or other divalent cations) did not produce gelation (data not shown), which underlined that the gelation was not a result of polygalacturonic acid-Ca^{2+} interactions in the SBP substrate.

3.3. Rheological properties of diFA cross-linked sugar beet pectin gels

Frequency sweep and stress sweep tests were performed on the enzymatically oxidized SBP gels after 20 min of reaction with 0.5 and 2.0 U mL^{-1} dosages of HRP and laccase, respectively. The frequency sweep data showed that the gels produced by HRP and laccase, respectively generally exhibited typical gel behavior with $G' > G''$, with the G' values being independent of the frequency from 0.1 to 100 Hz whereas G'' was highly dependent on frequency (Fig. 3A, B). The elastic modulus values (G') tended to be higher for gels produced with the higher enzyme dosage (2.0 U mL^{-1} vs. 0.5 U mL^{-1}), and were generally higher for the laccase catalyzed gels than for the HRP catalyzed gels at similar enzyme addition levels. The trendlines for the slope indicating the complex dynamic viscosity ($|\eta^*|$) for the gels were also similar: Hence, at 2.0 U mL^{-1} the $|\eta^*|$ values were −0.869 (HRP) and −0.934 (laccase), and these gels thus had dynamic viscosities close to the limiting value of −1 (Fig. 3A and B). The tan δ values ($=G''/G'$) were in the range of 0.01–0.13 for both gels produced with 2.0 U mL^{-1} of enzyme addition (data not shown) supporting that the SBP system was elastic, since low tan δ values (<0.1) indicate an elastic network system whereas higher tan δ (>1) values imply a more liquid-like character of the network (Oosterveld et al., 2000). The stress sweep data showed that the strength of each gel (measured as G') was constant for stress applied from 0.1 Pa to above 500 Pa (Fig. 3C). At higher stress the solid-like character dropped sharply for each gel, indicating an abrupt breakdown of the gel network (Fig. 3C), but the gels produced using HRP and laccase, respectively, exhibited different responses to the breaking stresses. The gel produced with laccase at 2.0 U mL^{-1} needed a higher stress (1100 Pa) to break the network than the gel produced with 0.5 U mL^{-1} laccase (994 Pa) (Fig. 3C). In contrast, for the HRP oxidized gels, the gel produced using 0.5 U mL^{-1} needed a higher stress (900 Pa) to break the network than the gel produced using 2.0 U mL^{-1} (667 Pa). Presumably, this difference in stress response with enzyme dosage between the gels formed via laccase and HRP catalysis, respectively, may be related to differences in gelation rates, and in turn related to the relationship between cross-linking of FA and the formation of the gel network. Hence, we propose that the unexpected lower stress resistance of the gel produced with the higher HRP dosage was due to a too fast gelation rate, i.e. in this reaction system a rate significantly higher than 20–25 Pa min^{-1} (Fig. 2). Hence, if the enzyme catalyzed cross-linking of the FAs was much faster than this rate, the mass transfer of the pectin molecules was too slow to allow them to be arranged and packed properly, in turn resulting in a relatively weak gel network (in terms of elastic modulus, G'). This presumption would also account for the relatively higher gel stress resistance of the laccase catalyzed gels versus the HRP catalyzed gels when compared at the same enzyme dosage U mL^{-1} (Fig. 3A and B) and are in accord with the higher gel strengths obtained for the laccase catalyzed gels than the HRP catalyzed gels (Fig. 1). The data obtained for this 2.5% (w/v) SBP gel system thus indicated that

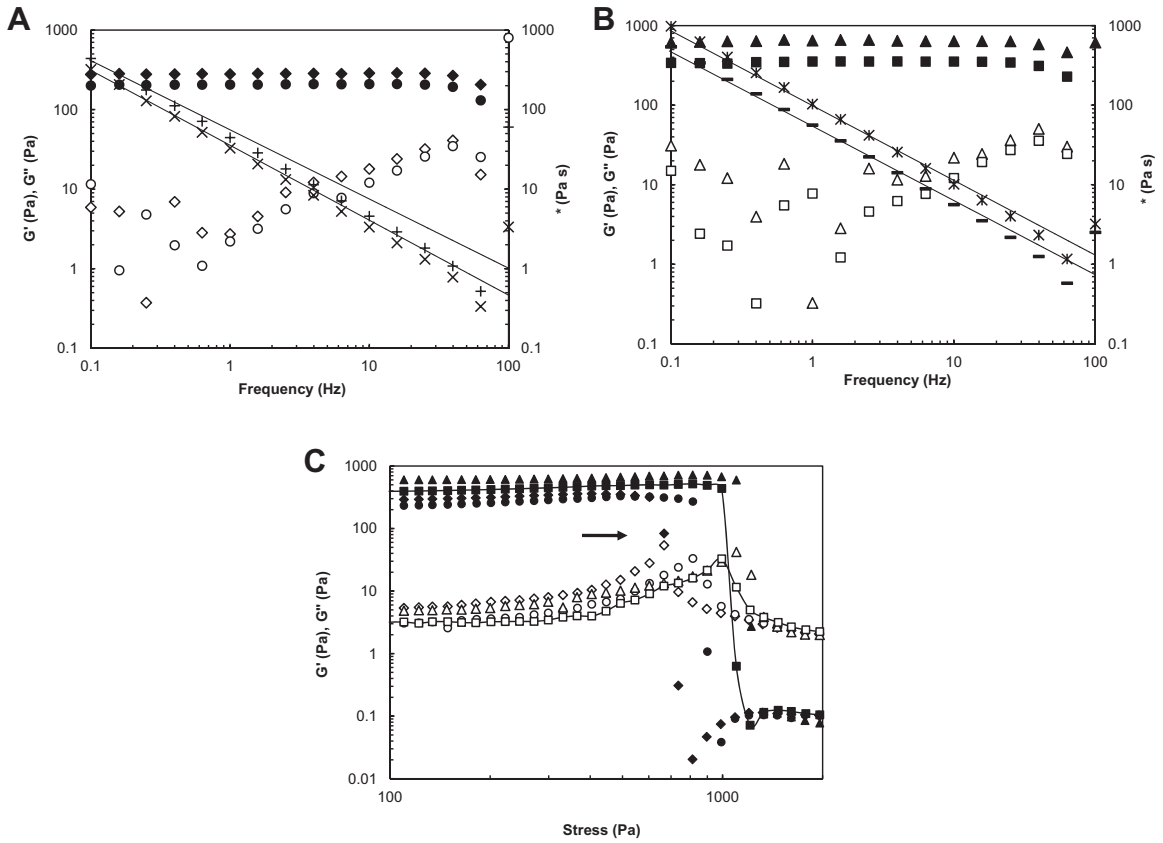

Fig. 3. Frequency sweep (0.1–100 Hz) performed at 0.1 Pa stress for 2.5% (w/v) SBP gels catalyzed by (A) HRP; (B) laccase. Data recorded after 20 min reaction; straight lines represent trendlines of the complex dynamic viscosity, $|\eta^*|$ (Pa s); (+): $|\eta^*|_{\text{HRP 2.0U}} = -0.869$; (×): $|\eta^*|_{\text{HRP 0.5U}} = -0.942$; (✳): $|\eta^*|_{\text{laccase 2.0U}} = -0.934$; (−): $|\eta^*|_{\text{laccase 0.5U}} = -0.94$. (C) Stress sweep (0.1–2000 Pa) performed at a frequency of 1 Hz on 2.5% (w/v) SBP gels produced using 0.5 and 2.0 U mL^{-1} enzymes at 25 °C showing the breaking point of the gels. (◆): G' HRP 2 U mL^{-1}; (●): G' HRP 0.5 U mL^{-1}; (▲): G' Laccase 2.0 U mL^{-1}; (■): G' Laccase 0.5 U mL^{-1}; (◇): G'' HRP 2 U mL^{-1}; (○): G'' HRP 0.5 U mL^{-1}; (△): G'' Laccase 2.0 U mL^{-1}; (□): G'' Laccase 0.5 U mL^{-1}. Arrow indicates the breaking point.

at the slower rates of gelation a stronger gel network was developed via laccase catalysis than via HRP catalysis, which took place at higher rates of gelation. This conclusion is in agreement with the previously reported observation that sugar beet pectin gels produced using HRP/H_2O_2 were softer than gels produced using laccase (Norsker et al., 2000).

When assessing the viscous resistance, measured as G'', the evolution with increased stress showed that G'' values increased gradually, notably when the stress came above ~500 Pa, and then decreased after reaching a peak resistance 'breaking point' (Fig. 3C). The gradual decrease in G'' with applied stress beyond the 'breaking point', was most likely due to fragments of the broken network conferring a contribution to viscosity. This contribution will decrease as the fragments are broken down further with increased stress (Fig. 3C).

3.4. Factors affecting gelation kinetics and rheological properties of sugar beet pectin gel

3.4.1. Gelation kinetics

Multiple linear regression analysis results for rate of gelation of SBP showed a significant increase in the rate of gelation of SBP catalyzed by HRP and laccase, respectively, with an increase in enzyme dosage ($P < 0.01$), temperature ($P < 0.05$) and substrate ($P < 0.0001$), whereas pH did not have significant effect on the rate of gelation for the range tested in this study (Table 3). For the gelation catalyzed by HRP, an increase in H_2O_2 also increased the SBP gelation rate significantly ($P < 0.0001$) (Table 3). Furthermore, interactions between temperature and pH, and substrate and H_2O_2 concentration, each had a significant effect ($P < 0.05$) on the rate of gelation catalyzed by HRP. For laccase, significantly positive interactions ($P < 0.05$) between enzyme dosage and temperature, enzyme dosage and substrate, and temperature and substrate, on the rate of gelation were found (Table 3). The validity of the multiple linear regression models were confirmed by the mean value of the center points (75.5 ± 1.9 Pa min^{-1} and 52.8 ± 2.4 Pa min^{-1}, for HRP and laccase, respectively) being close to the coefficient of the constant (68.4 ± 8.3 Pa min^{-1} and 52.8 ± 21.2 Pa min^{-1}, respectively). The regression models were as given in Eqs. (1) and (2) for HRP and laccase, respectively. The summary of fit of the models was satisfactory with $R^2 = 0.816$ and 0.909; $Q^2 = 0.499$ and 0.631, respectively.

$$\text{Rate}_{\text{HRP}} = 68.4 + 9.93x_1 + 7.62x_2 + 1.77x_3 + 21.4x_4 + 20.4x_5 - 9.33x_2x_3 + 17.2x_4x_5 \quad (1)$$

$$\text{Rate}_{\text{Lacc}} = 52.8 + 48.5x_1 + 46.9x_2 + 4.59x_3 + 80.4x_4 + 36.6x_1x_2 + 68.1x_1x_4 + 81.7x_2x_4 \quad (2)$$

where x_1 is enzyme dosage, x_2 is temperature, x_3 is pH, x_4 is substrate, and x_5 is hydrogen peroxide.

3.4.2. Kinetics of gelation catalyzed by horseradish peroxidase

3D surface plots obtained for the different reaction factor combinations for SBP gelation catalyzed using HRP expanded the

Table 3
Multiple linear regression results showing the coefficients of the parameters and interactions for the initial rates of gelation and G' at 10 min (G'_{10}) from gelation of (2.5% w/v) SBP by HRP and laccase, respectively. Hydrogen peroxide only used with HRP catalysis.

Response	Parameters and interactions (x)	Coefficient	
		HRP	Laccase
Rate of gelation (Pa min^{-1})	Constant	68.4[d]	52.8[a]
	Enzyme dosage (x_1)	9.93[b]	48.47[c]
	Temperature (x_2)	7.62[a]	46.93[c]
	pH (x_3)	1.77[ns]	4.59[ns]
	Substrate (x_4)	21.37[d]	80.36[d]
	Hydrogen peroxide (x_5)	20.41[d]	–
	Enzyme dosage × temperature	–	36.55[ns]
	Enzyme dosage × substrate	–	68.06[b]
	Temperature × pH	−9.33[a]	–
	Temperature × substrate	–	81.71[c]
	Substrate × hydrogen peroxide	17.16[c]	–
	R^2	0.816	0.909
	Q^2	0.499	0.631
G'_{10} (Pa)	Constant	133.8[c]	230.1[c]
	Enzyme dosage (x_1)	−4.75[ns]	80.76[c]
	Temperature (x_2)	42.11[b]	44.71[a]
	pH (x_3)	1.48[ns]	−2.92[ns]
	Substrate (x_4)	34.42[a]	137.76[d]
	Hydrogen peroxide (x_5)	40.83[b]	–
	Enzyme dosage × substrate	–	55.99[b]
	Temperature × pH	−35.82[a]	−30.65[ns]
	Temperature × substrate	–	34.06[ns]
	Substrate × hydrogen peroxide	26.25[ns]	–
	R^2	0.609	0.902
	Q^2	0.298	0.581

[ns]Not significant.
[a] Significant at $P < 0.05$.
[b] Significant at $P < 0.01$.
[c] Significant at $P < 0.001$.
[d] Significant at $P < 0.0001$.

understanding of how the gelation rate responded to different factor combinations (Fig. 4). Although the enzyme dosage × substrate interaction was not statistically significant, the significantly positive effects of substrate concentration and enzyme dosage (Table 3) manifested that the rate of gelation of SBP catalyzed using HRP/H_2O_2 reached an optimum point at a combination of high substrate concentration (3.5–4.0%) and enzyme at approximately 1.6–1.8 U mL^{-1} at the center point (40 °C, H_2O_2 0.55 mM, and pH 4.5) (Fig. 4A). The model for the data at 55 °C showed a similar trend, but the maximal gelation rate was higher, namely above 95 Pa min^{-1} at the higher temperature (Fig. 4B). A further increase in maximal rate was achieved when also the hydrogen peroxide concentration was increased, i.e. from H_2O_2 0.55–1.0 mM, in the high temperature reaction (55 °C, pH 4.5) and the response of the rate of gelation with increased substrate concentration was higher, whereas the effect of enzyme dosage tended to be less (Fig. 4C). Hence, at the higher temperature, 55 °C and the higher H_2O_2 concentration, the model produced a maximal rate of gelation of ~150 Pa min^{-1} at the highest substrate concentration (4.0% w/v) (Fig. 4C). The elevated gelation rate at higher temperature could be due to a decrease in viscosity of the substrate coupled with an increased reaction rate of the enzyme during the reaction to cross-link the FA to form a gel. For the gelation catalyzed by HRP, the H_2O_2 level is one of the limiting factors for the FA cross-linking as also indicated by the highly significant, positive effect of H_2O_2 on the gelation rate (Table 3). However, the positive interaction between H_2O_2 and the SBP substrate concentration (Table 3), and notably the response surface plot (Fig. 4D), also revealed a complex influence of the H_2O_2 on the gelation rate. Hence, at low H_2O_2 levels, the HRP catalyzed gelation did not occur at either the low or high substrate levels, but at high H_2O_2 levels neither did a gelation occur at the low substrate levels (Fig. 4D). This essentially zero gelation at high H_2O_2 and low SBP substrate level could be related to oxidative degradation or peroxidation of the feruloyl groups and/or the diFAs caused by the relatively high H_2O_2 to substrate ratio, preventing gelation. Such an explanation would be in accord with our recent observation on HRP catalyzed oxidation of feruloylated sugar beet-derived arabinans in which diFA levels tended to decrease at high H_2O_2:FAs ratios (Zaidel, Arnous, Holck, & Meyer, 2011).

The surface response plot for gelation rate in response to pH and temperature revealed that also the temperature-pH interaction, which produced a negative multiple regression coefficient (Table 3) was complex; hence, the positive effect of pH as a main factor, and the positive effect of increased temperature on the rate of gelation (Table 3), produced a saddle-like surface response plot exhibiting that a maximum rate of gelation of ~85 Pa min^{-1} would be achieved at ~pH 4.0–4.5, 55 °C (with the other factors at center point). At lower pH, the rate of gelation increased steeply as the temperature increased but as the pH increased the effect of temperature became less pronounced and essentially insignificant (Fig. 4E).

3.4.3. Kinetics of gelation catalyzed by laccase

As compared to gelation using HRP, the reaction factors for laccase catalysis produced a slightly different response on the rate of gelation. Due to the 'lag phase' in the gelation using laccase (Fig. 1B), higher dosages of laccase were used (0.1–10 U mL^{-1}) to investigate the interactions between different factors on the gelation kinetics and gel properties. 3D surface plots obtained from the gelation using laccase at 40 °C and pH 4.5 exhibited the significantly positive interaction between substrate concentration and enzyme dosage, i.e. that the enzyme dosage increased the rate of gelation significantly at higher substrate concentration (Fig. 5A). At 55 °C, the rate of gelation exhibited a similar response to the enzyme dosage × substrate interaction, but the gelation rate increased up to approximately 450 Pa min^{-1} (Fig. 5B). The pH had no effect within the range, which is why reactions were compared at pH 4.5.

3.4.4. Gelation kinetics of laccase at higher enzyme dosage and substrate levels

The 3D surface plots of the data from the experiments performed for the gelation catalyzed by laccase using higher enzyme dosages (5–15 U) and substrate levels (2.5–5.5% w/v) showed that for reactions at 25 °C the rate reached a maximum when the substrate was increased to approximately 4.0% w/v and decreased as the substrate increased further. At 55 °C the pattern was similar, but the peak substrate concentration was at ~4.75–5.0% w/v, and the maximum gelation rate achieved was higher than 700 Pa min^{-1} (Fig. 5C, D). Besides the increased enzyme activity at higher temperature, these data corroborated the significant interactions of enzyme dosage × temperature, and temperature × substrate, respectively (Table 3).

3.4.5. Effect of temperature

At higher temperature the initial viscosity of the substrate was lowered, and these interactions indicated that higher reaction temperature increased gelation via both increasing the mass transfer for the enzyme catalysis, i.e. the enzyme–substrate interactions, and allowed a faster gelation due to the higher mass transfer of the pectin molecules at elevated temperature and lowered viscosity. Since O_2 solubility decreases with temperature, being e.g. 0.26 mM in water at 25 °C vs. ~0.18 mM at 45 °C, the data (Fig. 5C, D) also indicated that oxygen solubility was not limiting for laccase catalysis at elevated temperature. The effect of temperature on the properties of gels produced via HRP catalysis was investigated further by comparing two sets of experiments at 25 and 55 °C, respectively, for gelation of 2.5% SBP, at an enzyme dosage of

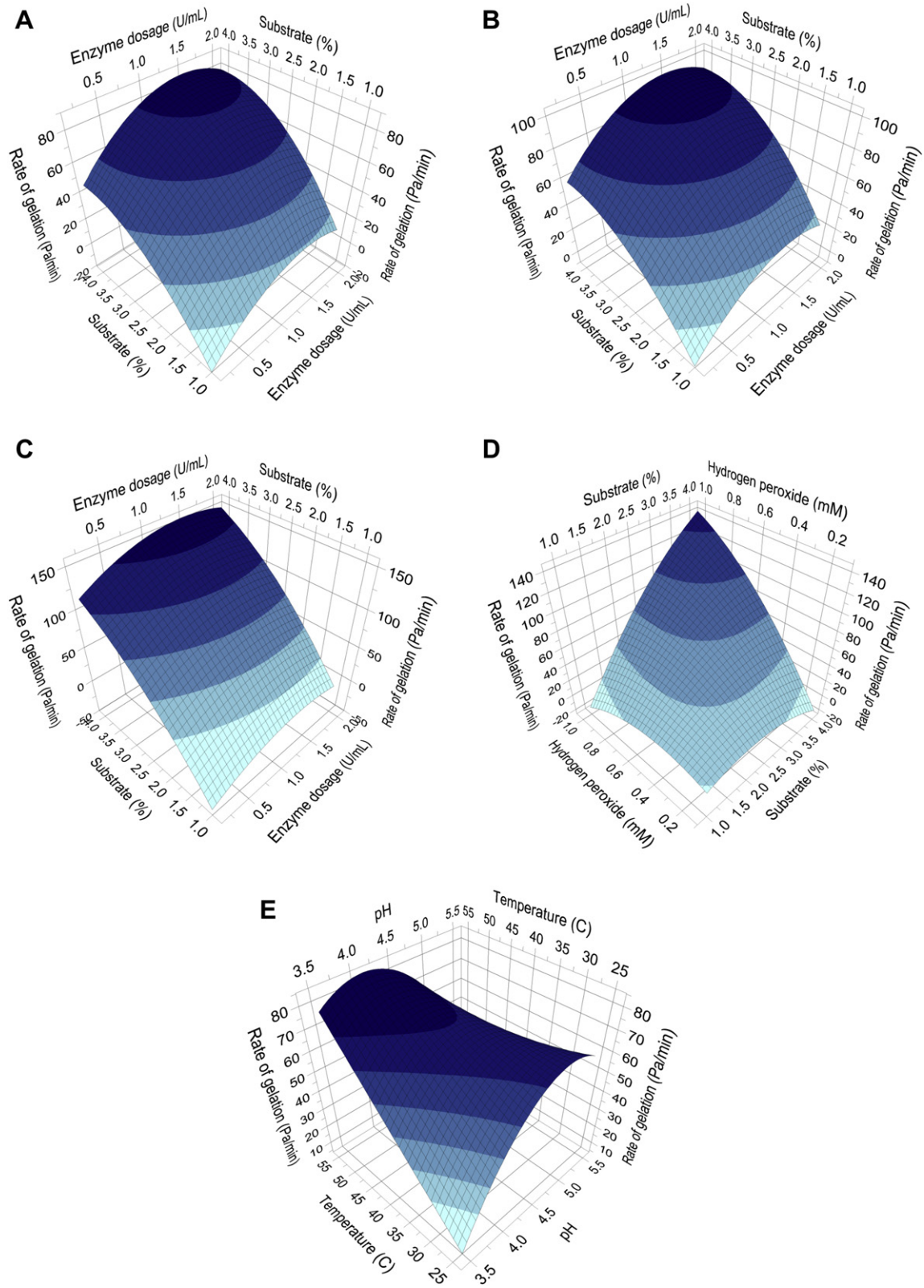

Fig. 4. 3D response surface plot showing the rate of gelation catalyzed using HRP at different combinations of factors (A) SBP substrate (1.0–4.0% w/v) and enzyme dosage (0.125–2.0 U mL^{-1}) at 40 °C, pH 4.5, 0.55 mM H_2O_2; (B) substrate (1.0–4.0% w/v) and enzyme dosage (0.125–2.0 U mL^{-1}) at 55 °C, pH 4.5, 0.55 mM H_2O_2; (C) substrate (1.0–4.0% w/v) and enzyme dosage (0.125–2.0 U mL^{-1}) at 55 °C, pH 4.5, 1.0 mM H_2O_2; (D) H_2O_2 (0.1–1.0 mM) and substrate (1.0–4.0% w/v) at 40 °C, pH 4.5, 1 U mL^{-1} HRP; (E) temperature (25–55 °C) and pH (3.5–5.5) at 2.5% w/v SBP, 1 U mL^{-1} HRP, 0.55 mM H_2O_2. Light to dark tones represent low to high values.

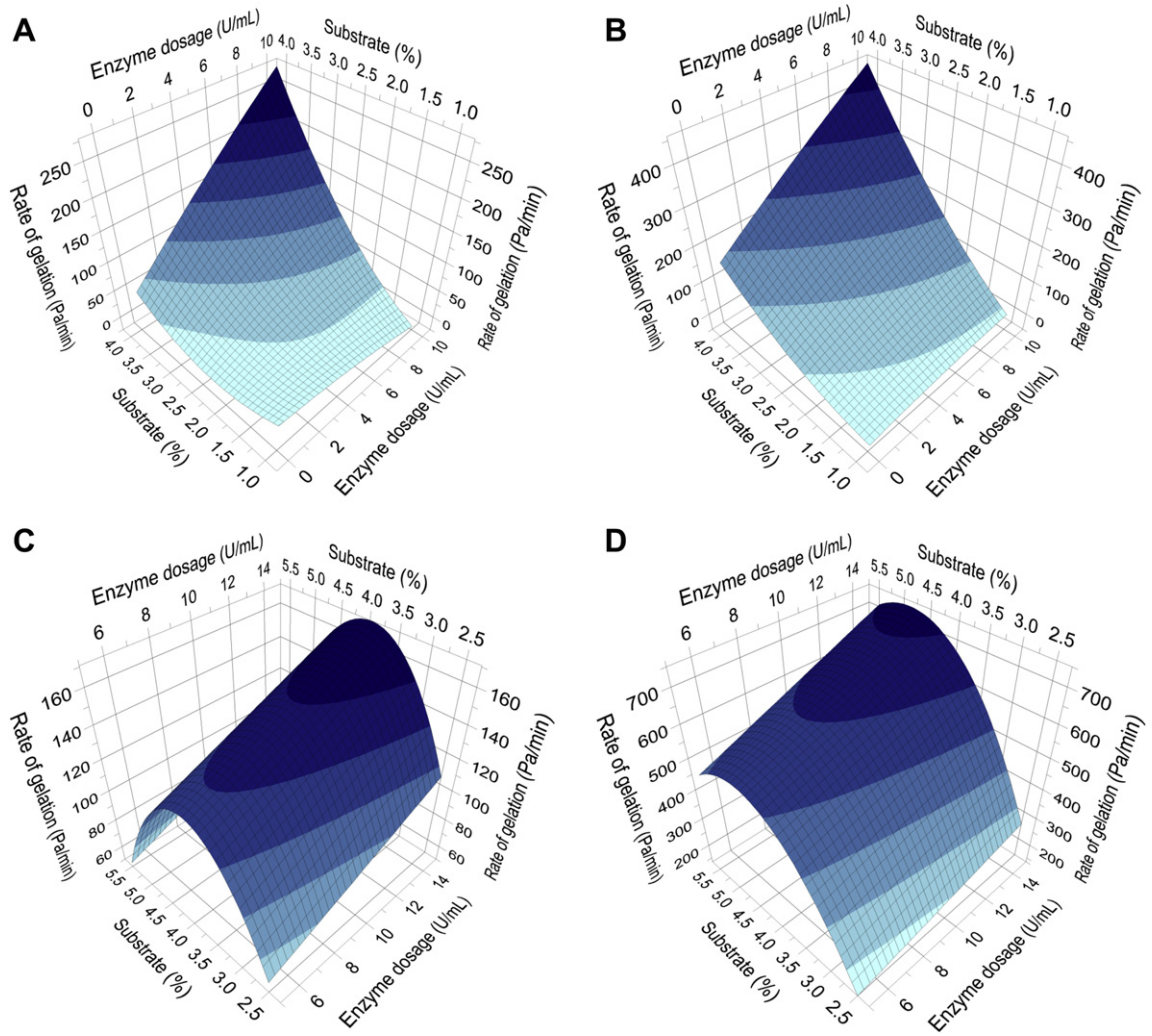

Fig. 5. 3D response surface plot showing the rate of gelation catalyzed using laccase at different combinations of factors (A) substrate (1.0–4.0%) and enzyme dosage (0.125–10 U mL^{-1}) at 40 °C, pH 4.5; (B) substrate (1.0–4.0%) and enzyme dosage (0.125–10 U mL^{-1}) at 55 °C, pH 4.5; (C) substrate (2.5–5.5%) and enzyme dosage (5–15 U mL^{-1}) at 25 °C, pH 4.5; (D) substrate (2.5–5.5%) and enzyme dosage (5–15 U mL^{-1}) at 55 °C, pH 4.5. Light to dark tones represent low to high values.

2.0 U mL^{-1} HRP, 0.55 mM H_2O_2, and pH 4.5. Increasing the temperature from 25 to 55 °C doubled the initial rate of gelation from 50.3 to 107 Pa min^{-1} (Fig. 6A). The G' reached a plateau at 100 Pa after approximately 2.5 min at both temperatures (Fig. 6A); the G' for the gel produced at 25 °C remained at 100 Pa for 20 min, whereas at 55 °C the G' value remained at 100 Pa for about 10 min and then it increased to extremely high values (>500 Pa) (Fig. 6B). This increase of G' at 55 °C might be due to crystallization, hardening, and/or drying of the gel at the higher temperature. A similar behavior was observed for the gels produced by laccase (data not shown). Due to this, G' at 10 min (G'_{10}) was used to investigate the strength of the gel network developed by the oxidative gelation of SBP catalyzed by HRP and laccase.

3.5. Properties of enzymatically oxidized sugar beet pectin gels

Multiple linear regression analysis of G'_{10} responses for gels produced using HRP and laccase, respectively, showed that both the temperature and the substrate concentration had positive, main effects and increased the G'_{10} significantly ($P < 0.05$) (Table 3). The enzyme dosage only affected the G'_{10} for gels produced using laccase and the reaction pH had no significant effect on G'_{10} for either gel type (Table 3). For the SBP gels produced using HRP, an increase in the concentration of H_2O_2 increased the G'_{10} significantly ($P < 0.01$) (Table 3). The only factor interaction for the HRP catalyzed G'_{10} was between temperature and pH that produced a significantly negative interaction effect ($P < 0.05$) (Table 3). For laccase catalyzed gels, the enzyme dosage and substrate produced a significantly positive interaction ($P < 0.01$) on the G'_{10}, whereas there was no significant interaction between these factors on the G'_{10} for HRP catalyzed gels (Table 3). The validity of the models were confirmed by the mean value of the center points (220.7 ± 32.2 Pa and 290.3 ± 53.2 Pa, for HRP and laccase, respectively) being close to the coefficient of the constant (133.8 ± 36.7 Pa and 230.1 ± 47.3 Pa, respectively). The regression models are given in Eqs. (3) and (4) for HRP and laccase, respectively. The summary of fit of the models was satisfactory with $R^2 = 0.609$ and 0.902; $Q^2 = 0.298$ and 0.581, respectively.

$$G'_{10,\,\mathrm{HRP}} = 133.8 - 4.75x_1 + 42.11x_2 + 1.48x_3 + 34.42x_4 + 40.83x_5 - 35.82x_2x_3 + 26.25x_4x_5 \quad (3)$$

$$G'_{10,\,\mathrm{Lacc}} = 230.1 + 80.76x_1 + 44.71x_2 - 2.92x_3 + 137.76x_4 + 55.99x_1x_4 - 30.65x_2x_3 + 34.06x_2x_4 \quad (4)$$

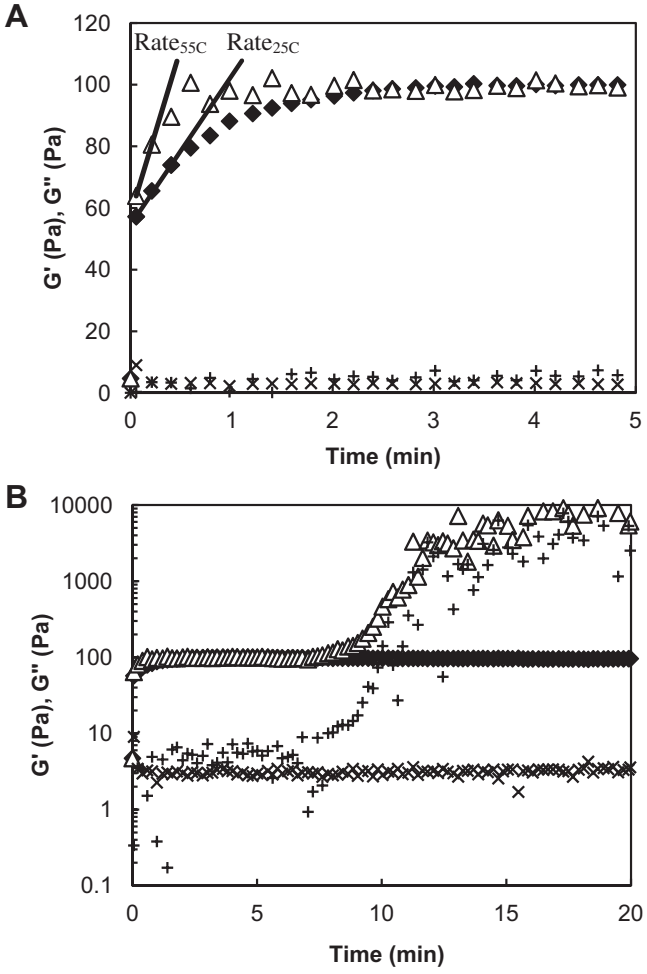

Fig. 6. Increase of G' for 2.5% (w/v) SBP as influenced by temperature at pH 4.5, 2.0 U mL^{-1} HRP, 0.55 mM H_2O_2 (A) for the first 5 min reaction time; the rate of gelation were 50.29 and 107.42 Pa min^{-1} at 25 and 55 °C respectively; (B) for 20 min reaction where G' and G'' increased extremely high ($G' > 500$ Pa) at approximately 10 min reaction time. (◆): G' at 25 °C; (△) G' at 55 °C; (×): G'' at 25 °C; (+): G'' at 55 °C.

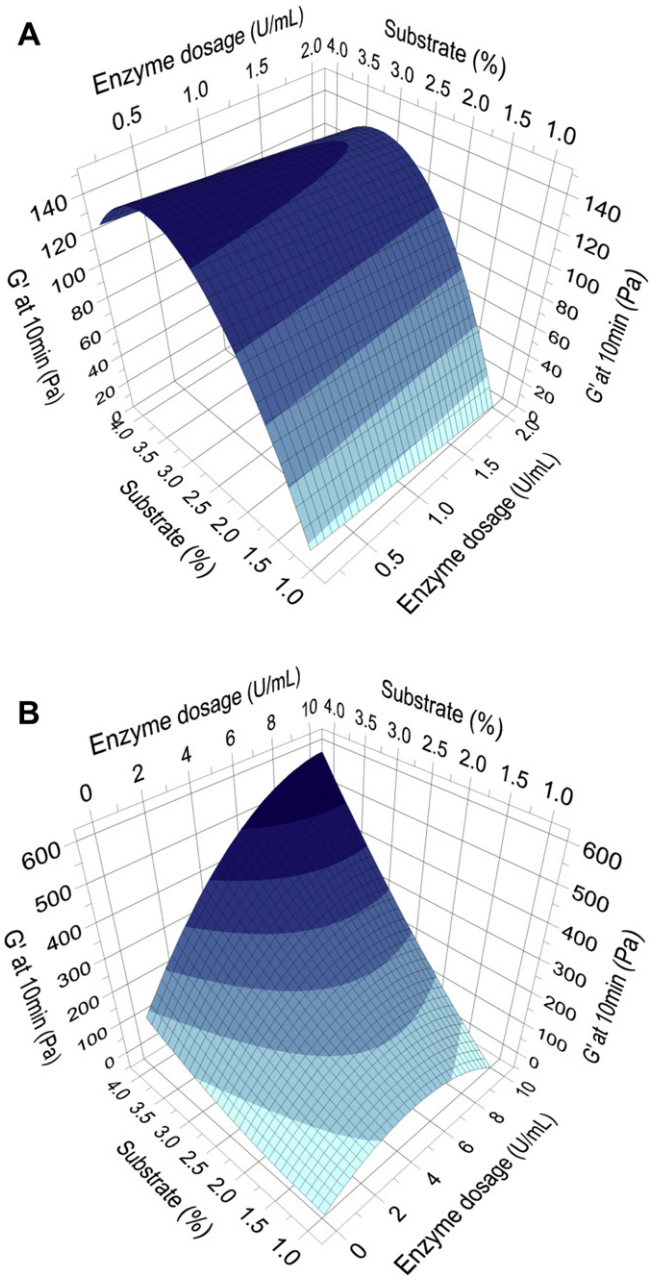

Fig. 7. 3D response surface plot showing the G' at 10 min (G'_{10}) of the SBP gels at different combinations of factors (A) SBP substrate (1.0–4.0%) and enzyme dosage (0.125–2.0 U mL^{-1} of HRP) at 40 °C, pH 4.5, 0.55 mM H_2O_2; (B) substrate (1.0–4.0%) and enzyme dosage (0.125–10 U mL^{-1} of laccase) at 40 °C, pH 4.5. Light to dark tones represent low to high values.

where x_1 is enzyme dosage, x_2 is temperature, x_3 is pH, x_4 is substrate and x_5 is hydrogen peroxide.

The 3D surface response plots corroborated that G'_{10} for the HRP catalyzed gels increased significantly as the SBP substrate level increased and reached a maximum at approximately 3.2–3.5% w/v independent of the enzyme dosage (no enzyme dosage × substrate interaction) (Fig. 7A), so the 3D model indicated that at substrate concentrations >3.5% w/v the G'_{10} decreased. For the gels produced by laccase catalysis, the surface response plot highlighted the highly positive interaction between enzyme dosage × substrate concentration as the G'_{10} increased steeply when both enzyme dosage and substrate concentration were high, and began to reach plateau at 570 Pa at 10 U mL^{-1} enzyme dosage and 4.0% w/v substrate (Fig. 7B). At low substrate concentration the enzyme dosage had no significant effect on G'_{10}, which is why the interaction effect was positive.

No general correlation could be deduced between the rate of gelation and G'_{10} for the gels produced using either HRP or laccase. G'_{10} for the gels produced using HRP were not affected by the rate of gelation whereas for gels produced using laccase the G'_{10} increased with increasing rate of gelation (Fig. 8). When comparing the G'_{10} for the gels produced by each of the two enzymes at 25 °C, laccase catalysis gave higher G'_{10} at a slower rate of gelation than HRP catalyzed gels, while for reactions at 40 °C, the gels produced using laccase still had higher G'_{10}, even though the rate of gelation was similar to the equivalent HRP catalyzed gelation (Fig. 8). At the very high rates of gelation, i.e. >200 Pa min^{-1}, which were only achieved with laccase catalyzed gels at high SBP levels (4.0% w/v) and elevated temperature, either 40 °C or 55 °C, the G'_{10} values were higher than those obtained for the equivalent HRP catalyzed gels (Fig. 8). Thus it can be concluded that gels produced using laccase were stronger compared to the equivalent HRP catalyzed gels at all rates of gelation.

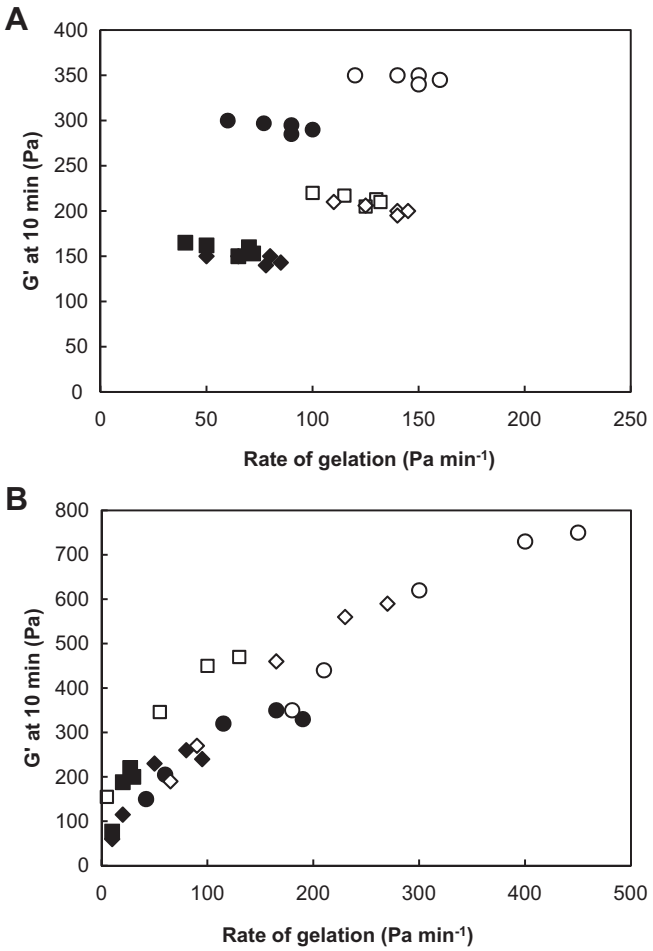

Fig. 8. Correlation between rate of gelation and G'_{10} at 25, 40 and 55 °C for 2.5% (w/v) SBP or 4.0% (w/v) SBP catalyzed using either (A) HRP (at 1 mM H_2O_2) or (B) laccase. (■): 2.5%, 25 °C; (◆): 2.5%, 40 °C; (●): 2.5%; 55 °C; (□): 4.0%; 25 °C; (◇): 4.0%, 40 °C; (○): 4.0%, 55 °C. Data were extracted from the statistically design experiments.

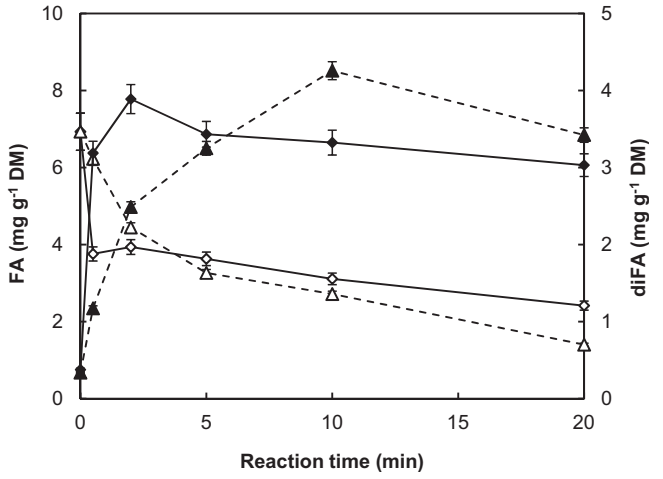

Fig. 9. FA decrement and diFAs formation during oxidative cross-linking of SBP catalyzed by HRP and laccase at 2.0 U mL^{-1} for both enzymes (0.14 mM H_2O_2 was used for HRP since SBP was 1% (w/v) which contains FA equivalent to approximately 0.5 mM). Coefficients of variation based on calibration curves were in the range of 2.7–4.8%. (◇): FA, HRP; (◆): diFA, HRP; (△): FA, laccase; (▲): diFA, laccase.

3.6. Formation of diFAs

The amount of FA decreased after addition of HRP and laccase, respectively, and this decrease was accompanied by an increase in diFAs (Fig. 9, Table 4). At similar enzyme dosage of 2.0 U mL^{-1} a slower decrease in FAs occurred with laccase catalysis as compared to HRP catalysis during the first few minutes of the reaction and the formation of diFAs was in turn slower with addition of laccase than with HRP (Fig. 9). This result correlated well with the differences in rates of gelation and G' values obtained for gels catalyzed by the two enzymes where laccase catalyzed a slower rate of gelation of SBP than HRP (Fig. 1). The formation of diFAs was also higher for laccase catalyzed gelation as the reaction progressed, although no pronounced differences in diFA levels were found after 20 min of reaction (Table 4). The rapid formation of diFAs and the accompanying rapid drop in FA level for the HRP catalyzed gels, can be explained by the rapid HRP catalyzed oxidation of FA by H_2O_2. Grabber, Hatfield, Ralph, Zon, and Amrhein (1995) reported that several active sites (C-5, C-8 and O-4) were present on FA radicals during H_2O_2/peroxidase reaction which resulted in the formation of several diFAs. The data recored in the present work showed that the profile of diFAs formed, were relatively similar for HRP and laccase catalysis (Table 4). The proposed mechanism for FA oxidation by laccase is dehydrogenation of the hydroxyl group at C-4 of FA into a reactive phenoxy radical that can further dimerize or react with another radical from C–C or C–O linkages (Ercili Cura et al., 2009). This mechanism may produce a slower reaction rate for laccase catalyzed oxidation of the FAs by direct interaction between O_2 and the copper cluster in the enzyme while H_2O_2 can rapidly oxidize the FA and HRP therefore catalyze rapid radical dimerization. An excess amount of H_2O_2 in the reaction might also affect the reaction by further oxidation to trimers and tetramers which explained the decrease in diFAs after 6 min reaction (Fig. 9). Despite of their different mechanisms, three main diFAs were detected for both enzymes; 5,5, 8-O-4 and 8,5-Benzofuran-diFAs with 8,5-Benzofuran diFA being most dominant contributing approximately 45–47% of the total diFAs, with 38–40% and 15–17% contributed by 8-O-4-diFA and 5,5-diFA respectively (Table 4).

Table 4
Profile of diFAs formation within 20 min reaction after addition of 2 U• mL^{-1} HRP (0.14 mM H_2O_2) and 2 U mL^{-1} laccase: 5-5′, 8-O-4′ and 8-5′-Benzofuran diFA (mg g^{-1} DM).

Reaction time (min)	HRP/H_2O_2			Laccase		
	5,5′ (mg g^{-1})	8-0-4′ (mg g^{-1})	8,5′-Benzofuran (mg g^{-1})	5,5′ (mg g^{-1})	8-0-4′ (mg g^{-1})	8,5′-Benzofuran (mg g^{-1})
0	0.075 ± 0.004	0.159 ± 0.008	0.142 ± 0.007	0.068 ± 0.002	0.138 ± 0.004	0.131 ± 0.004
0.5	0.55 ± 0.03	1.27 ± 0.06	1.37 ± 0.07	0.204 ± 0.006	0.44 ± 0.01	0.53 ± 0.01
2	0.64 ± 0.03	1.57 ± 0.08	1.68 ± 0.08	0.41 ± 0.01	0.93 ± 0.03	1.15 ± 0.03
5	0.57 ± 0.03	1.36 ± 0.07	1.51 ± 0.07	0.51 ± 0.01	1.20 ± 0.03	1.55 ± 0.04
10	0.45 ± 0.02	1.28 ± 0.06	1.60 ± 0.08	0.59 ± 0.02	1.66 ± 0.05	2.01 ± 0.06
20	0.40 ± 0.02	1.16 ± 0.06	1.47 ± 0.07	0.40 ± 0.01	1.27 ± 0.04	1.75 ± 0.05

4. Conclusions

At similar enzyme dosage, the rate of SBP gelation catalyzed by laccase was slower than the rate catalyzed by HRP, but the gels produced by laccase catalysis were consistently stronger than the corresponding HRP catalyzed gels, as shown by higher G' and by the higher stress needed to break the gels. Multiple linear regression analysis results showed that the reaction parameters enzyme dosage, temperature and substrate concentration each had a significant effect ($P < 0.05$) on the rate of gelation for HRP and laccase, respectively, while the pH, (pH 3.5–5.5), did not have any significant effect on the gelation rates. H_2O_2 significantly affected the rate of gelation catalyzed by HRP and was one of the limiting factors which influenced the gelation reaction for HRP catalyzed gels. From the correlation obtained between rate of gelation and gel strength, there was no significant effect of rate of gelation on the gel properties catalyzed by HRP, whereas the gel strength for gels catalyzed by laccase were positively correlated to rate of gelation. This finding rejected our hypothesis that slower rate of gelation would produce a stronger gel. Reaction at high temperature affected the properties of the gel, as very high G' values were produced after 10 min. A good gel could be produced at a moderate temperature (25–40 °C) with the correct combination of substrate (3.5–4.0%) and enzyme dosage (1.6–1.8 U mL^{-1} for HRP; 15 U mL^{-1} for laccase), but the strongest gels were produced at 55 °C at higher initial rates of gelation. The data showed that SBP obtained from an industrial byproduct stream is a useful substrate for gelation via enzyme catalyzed oxidative cross-linking of FA. From this investigation, the use of laccase to catalyze such gelation seem to be a better choice than HRP – also because HRP requires H_2O_2 for the reaction. At lower enzyme dosage and slower rate of gelation, laccase could produce stronger gels than HRP and the use of H_2O_2 would be avoided, which is considered advantageous for food applications of this technology.

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References

Agger, J., Viksø-Nielsen, A., & Meyer, A. S. (2010). Enzymatic xylose release from pretreated corn bran arabinoxylan: differential effects of deacetylation and deferuloylation on insoluble and soluble substrate fractions. *Journal of Agricultural & Food Chemistry, 58*, 6141–6148.

Andreasen, M. F., Christensen, L. P., Meyer, A. S., & Hansen, Á (1999). Release of hydroxycinnamic and hydroxybenzoic acids in rye by commercial plant cell wall degrading enzyme preparations. *Journal of the Science of Food & Agriculture, 79*, 411–413.

Andreasen, M. F., Christensen, L. P., Meyer, A. S., & Hansen, Á (2000). Content of phenolic acids and ferulic acid dehydrodimers in 17 rye (*Secale cereale* L.) varieties. *Journal of Agricultural & Food Chemistry, 48*, 2837–2842.

Arnous, A., & Meyer, A. S. (2008). Comparison of methods for compositional characterization of grape (*Vitis vinifera* L.) and apple (*Malus domestica*) skins. *Food & Bioproducts Processing, 86*, 79–86.

Buchholt, H. C., Christensen, T. M. I. E., Fallesen, B., Ralet, M. C., & Thibault, J. F. (2004). Preparation and properties of enzymatically and chemically modified sugar beet pectins. *Carbohydrate Polymers, 58*, 149–161.

Carvajal-Millan, E., Guilbert, S., Morel, M.-H., & Micard, V. (2005). Impact of the structure of arabinoxylan gels on their rheological and protein transport properties. *Carbohydrate Polymers, 60*, 431–438.

Colquhoun, I. J., Ralet, M. C., Thibault, J. F., Faulds, C. B., & Williamson, G. (1994). Structure identification of feruloylated oligosaccharides from sugar beet pulp by NMR spectroscopy. *Carbohydrate Research, 263*, 243–256.

Ercili Cura, D., Lantto, R., Lille, M., Andberg, M., Kruus, K., & Buchert, J. (2009). Laccase-aided protein modification: effects on the structural properties of acidified sodium caseinate gels. *International Dairy Journal, 19*, 737–745.

Garna, H., Mabon, N., Nott, K., Wathelet, B., & Paquot, M. (2006). Kinetic of the hydrolysis of pectin galacturonic acid chains and quantification by ionic chromatography. *Food Chemistry, 96*, 477–484.

Garna, H., Mabon, N., Wathelet, B., & Paquot, M. (2004). New method for a two-step hydrolysis and chromatographic analysis of pectin neutral sugar chains. *Journal of Agricultural & Food Chemistry, 52*, 4652–4659.

Grabber, J. H., Hatfield, R. D., Ralph, J., Zon, J., & Amrhein, N. (1995). Ferulate cross-linking in cell walls isolated from maize cell suspensions. *Phytochemistry, 40*, 1077–1082.

Holck, J., Hjernø, K., Lorentzen, A., Vigsnæs, L. K., Hemmingsen, L., Licht, T. R., et al. (2011). Tailored enzymatic production of oligosaccharides from sugar beet pectin and evidence of differential effects of a single DP chain length difference on human faecal microbiota composition after *in vitro* fermentation. *Process Biochemistry, 46*, 1039–1049.

Kuuva, T., Lantto, R., Reinikainen, T., Buchert, J., & Autio, K. (2003). Rheological properties of laccase-induced sugar beet pectin gels. *Food Hydrocolloids, 17*, 679–684.

Levigne, S. V., Ralet, M.-C. J., Quéméner, B. C., Pollet, B. N.-L., Lapierre, C., & Thibault, J.-F. J. (2004). Isolation from sugar beet cell walls of arabinan oligosaccharides esterified by two ferulic acid monomers. *Plant Physiology, 134*, 1173–1180.

Micard, V., & Thibault, J. F. (1999). Oxidative gelation of sugar-beet pectins: use of laccases and hydration properties of the cross-linked pectins. *Carbohydrate Polymers, 39*, 265–273.

Norsker, M., Jensen, M., & Adler-Nissen, J. (2000). Enzymatic gelation of sugar beet pectin in food products. *Food Hydrocolloids, 14*, 237–243.

Oosterveld, A., Beldman, G., & Voragen, A. G. J. (2000). Oxidative cross-linking of pectic polysaccharides from sugar beet pulp. *Carbohydrate Research, 328*, 199–207.

Oosterveld, A., Grabber, J. H., Beldman, G., Ralph, J., & Voragen, A. G. J. (1997). Formation of ferulic acid dehydrodimers through oxidative cross-linking of sugar beet pectin. *Carbohydrate Research, 300*, 179–181.

Takei, T., Sugihara, K., Ijima, H., & Kawakami, K. (2011). *In situ* gellable sugar beet pectin *via* enzyme-catalyzed coupling reaction of feruloyl groups for biomedical applications. *Journal of Bioscience & Bioengineering, 112*, 491–494.

Waldron, K. W., Parr, A. J., Ng, A., & Ralph, J. (1996). Cell wall esterified phenolic dimers: identification and quantification by reverse phase high performance liquid chromatography and diode array detection. *Phytochemical Analysis, 7*, 305–312.

Westphal, Y., Kühnel, S., de Waard, P., Hinz, S. W. A., Schols, H. A., Voragen, A. G. J., et al. (2010). Branched arabino-oligosaccharides isolated from sugar beet arabinan. *Carbohydrate Research, 345*, 1180–1189.

Zaidel, D. N. A., Arnous, A., Holck, J., & Meyer, A. S. (2011). Kinetics of enzyme-catalyzed cross-linking of feruloylated arabinan from sugar beet. *Journal of Agricultural & Food Chemistry, 59*, 11598–11607.

CHAPTER 6

Emulsion stabilization and oxidative gelation of emulsion-sugar beet pectin mixture

Emulsion stabilization is an important issue in food industry and we exemplify this attribute by means of a mixture of emulsion and polysaccharide, particularly SBP. We examine the stabilization of emulsion in the presence of SBP in the emulsion systems *via* enzyme catalyzed oxidative cross-linking of SBP. The work presented in this chapter is based on the paper IV "Stabilization of oil-in-water emulsions by enzyme catalyzed oxidative gelation of sugar beet pectin".

6.1 Emulsion

In food system, an emulsion consists of two immiscible liquids, particularly oil and water, mixed together through an intense mechanical agitation known as homogenization. Oil-in-water emulsion, containing oil droplets dispersed throughout the water phase, is most commonly found in food products for example mayonnaise, salad dressings, gravies, and cream soups (Brown, 2008). The other type of emulsion, water-in-oil which consists of water droplets dispersed in oil phase, is found in foods such as butter and margarine.

6.1.1 Emulsion stability

When discussing properties of emulsion, stability is one of the most important determining factors in producing good quality food colloids. Stability of emulsion can be related to its ability to resist changes in its physicochemical properties over time (McClements, 2007). Food emulsions may become unstable due to different physicochemical mechanisms such as gravitational separation (creaming/sedimentation), flocculation and coalescence (McClements, 2007). A number of previous studies examined the factors influencing stability of emulsion for example types of stabilizers or emulsifiers, environmental stresses (*e.g*. pH, temperature, ionic strength) and homogenization conditions (*e.g*. pressure, heating) (Dickinson, 1997; Demetriades & McClements, 1998; Chanamai & McClements, 2002; Ciron *et al*., 2010). Various methods and techniques for characterizing emulsion stability are available; although the emulsion stability evaluation can be easily visualized by creaming layer, *i.e*. phase separation of cream and serum regions. Commonly,

particle size distribution has been employed to characterize emulsion; emulsion with smaller particle size (< 1 μm) tends to be more stable than the one with larger particle size.

6.1.2 Whey proteins as emulsifier

In an oil-in-water emulsion preparation, it is important to include a surface-active substance *i.e.* emulsifier, such as protein, to adsorb at the surface of the oil droplets which can facilitate the formation of a fine emulsion, improve the stability and produce desirable physicochemical properties of the emulsion. The type of emulsifier present at the droplet surface determines the colloidal forces responsible for emulsion stability (Demetriades & McClements, 1998). Whey proteins are amphiphilic molecules that are commonly used as emulsifiers, which stabilize the emulsion against aggregation or flocculation by a combination of electrostatic and steric repulsion (Demetriades & McClements, 1998; Kulmyrzaev *et al*., 2000). Their properties are mainly influenced by pH and ionic strength. Investigation on the influence of pH and ionic strength on the emulsion stability revealed that emulsion tends to aggregate when the pH is close to isoelectric point of the protein (Kulmyrzaev *et al*., 2000).

6.1.3 Emulsion stabilization by polysaccharide

Polysaccharides are commonly used as food additives to improve texture and stability of food products. The use of polysaccharides as stabilizing agent in food has been known, due to their surface-active properties, hydrophilic properties *i.e.* interaction with water and other polymers, and ability to modify the rheological properties of the colloid systems *e.g* viscosity regulating (Garti, 1999). Once it is strongly adsorbed to the oil-water interface, polysaccharide can stabilize emulsion in a longer period compared to protein (Dickinson, 2003), possibly by a mechanism involving adsorption, but not necessarily so (Garti, 1999). This stabilization induced by the polysaccharides presumably takes place through formation of polysaccharide-protein complexes that coat the emulsified droplets and increase the repulsion among the particles (Schmitt *et al*., 1998).

Pectic polysaccharide from sugar beet has been reported to have more surface-active properties than other high-methoxyl or low-methoxyl pectic polysaccharide, most probably due to high acetyl group on the HG backbone of sugar beet pectin (Dea & Madden, 1986). A recent study has reported a lower emulsifying performance in the SBP which has been through enzymatic treatments with protease, arabinanase/galactanase or polygalacturonase, compared to the untreated SBP (Funami *et al*., 2011), indicating the importance of the lateral chains of SBP in accounting for emulsifying properties of SBP besides the proteinaceous moiety. In another study, oxidative cross-

linked SBP catalyzed by laccase (Littoz & McClements, 2008; Zeeb *et al*., 2012) or HRP (Li *et al*., 2012) has been shown to improve emulsion stabilization in multilayered emulsion systems, and laccase oxidized cross-linking of SBP in dilute (0.3% w/v) solutions has recently been shown to improve stabilization of a model emulsion system (10% tetradecane, pH 6.5) during 30 days of incubation (Jung & Wicker, 2012). These studies have shown that emulsion containing cross-linked SBP was more stable to pH changes, ions addition and thermal processing, and better long term stability compared to one-layer and/or non-cross-linked multilayered emulsions. This attribute indicates stable adsorption of cross-linked SBP on the (coated) droplets surface resisting attractive interactions between the particles.

6.2 Stabilization of emulsions by enzyme catalyzed oxidative gelation of sugar beet pectin

Paper IV: Stabilization of oil-in-water emulsions by enzyme catalyzed oxidative gelation of sugar beet pectin

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6.2.1 Significance of study

In this present work, we hypothesized that the presence (or not) of SBP during the homogenization for emulsion preparation could influence both the rates of enzyme catalyzed SBP cross-linking and the emulsion stabilization by the enzyme catalyzed reaction. The purpose of this work was to test these hypotheses by investigating the emulsion stability of oil-in-water emulsion in the presence of SBP (or not) during homogenization for emulsion preparation *i.e*. two sets of emulsions were prepared by different methods of preparation as described in paper IV. Another objective was to investigate the rates of gelation of the SBP containing emulsions, as catalyzed by HRP or laccase. The rationale of preparing the emulsion differently was to evaluate the flexibility of the emulsion preparation on the stability of the emulsion and the rheological properties of the SBP gel. From a practical point of view, the provision of knowledge of enzyme catalyzed oxidative gelation of SBP in emulsion systems is important in designing SBP as a gelling agent in food application.

6.2.2 Experimental considerations

At the beginning of this work, the initial objective was to determine the effect of cross-linked arabinan oligosaccharide on the stability of multilayered emulsions. However, the properties of the arabinan oligosaccharide as stabilizer were not as significant as the cross-linked sugar beet pectin (data not shown). Further investigation on the stabilization of emulsion using arabinan oligosaccharide fraction was not performed due to the fact that short backbone length of arabinan might be the reason for the less significant effect on the emulsion stabilization compared to sugar beet pectin. This can be related to the previous study (Funami *et al*., 2011) indicating the importance of lateral chains of SBP and acetyl group (Dea & Madden, 1986) in emulsifying properties of SBP.

Investigation of emulsion stabilization was conducted as a continuation based on the data obtained from the oxidative gelation in paper III (Zaidel *et al.*, 2012). The primary oil-in-water emulsion was prepared at pH 4.5 since our result revealed that pH range from 3.5 to 5.5 did not significantly affect the gelation of SBP catalyzed by HRP or laccase. From our initial evaluation on the emulsion stability, the emulsion was unstable without the presence of SBP *i.e.* top cream layer took place after 30 min of incubation at room temperature. This result was due to the lower electrostatic repulsion than attractive interactions in the emulsion, at pH range 4 to 6, which leads to aggregation.

Mixing the SBP into the prepared emulsion was able to stabilize the emulsion and the top cream layer was not observed until after 24 hr. Hence, using this as a benchmark, further experiment was conducted to compare the step at which the SBP should be added into the emulsion system during the preparation. Thus, two different modes of preparation of emulsions were performed *i.e.* emulsion prepared separately and subsequently mixed with SBP (referred as Mix A) or emulsion prepared by homogenizing oil, protein and SBP all together (referred as Mix B). In a practical point of view, it is preferable to produce a good quality food product with a simpler and more flexible way in order to fully utilize the ingredient and other resources. Thus, two different steps of emulsion preparation were compared to differentiate the effect of the presence of SBP on the emulsion stabilization and the rates of gelation of SBP in emulsion systems.

6.2.3 Highlights

From this study, we reveal that the enzyme catalyzed oxidative cross-linking of SBP was able to stabilize emulsions. Our data showed that the emulsion preparation methodology in the presence of SBP affected the average particle size, $d_{3,2}$ of the emulsions and thus the emulsions stability.

The presence of SBP during homogenization (Mix B) was able to stabilize the emulsion, as opposed to the weak emulsion stabilization shown when the emulsions were prepared separately and subsequently mixed with SBP (Mix A).

Our data showed that the rates of gelation of SBP in emulsion systems and the gels properties were affected by the mode of emulsions preparation. For both enzymes, at higher enzyme dosages of 2.0 $U \cdot mL^{-1}$, the rates of gelation of Mix B were significantly slower ($P < 0.05$) than the rates of gelation of Mix A. In laccase catalyzed gels, Mix B tended to have lower *G'* values than the corresponding gels in Mix A, but no such behaviour was obtained for HRP catalyzed gels. The gels produced in Mix A had higher strengths than the gels prepared in Mix B, regardless of the types of enzyme and enzyme dosage. Moreover, the strengths of the gels produced in SBP containing emulsion systems (Mix A and Mix B) were slightly higher than the strength of the gels produced by the SBP control without oil or WPI added. This result indicated that the interactions between proteins and polysaccharides (the pectin) coupled with the covalent cross-links created from the enzyme catalyzed oxidative cross-linking of SBP enhanced the properties of the gels produced.

Furthermore, this finding confirmed our previous work in paper III that the rates of laccase catalyzed oxidative gelation was slower than HRP catalyzed gelation, but laccase catalysis produced stronger gels compared to HRP catalysis, even in the presence of emulsion systems.

Paper IV

Stabilization of oil-in-water emulsions by enzyme catalyzed oxidative gelation of sugar beet pectin

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Manuscript

Title

Stabilization of oil-in-water emulsions by enzyme catalyzed oxidative gelation of sugar beet pectin

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Abstract

Enzyme catalyzed oxidative cross-linking of feruloyl groups can promote gelation of sugar beet pectin (SBP). It is uncertain how the enzyme kinetics of this cross-linking reaction are affected in emulsion systems and whether the gelation affects emulsion stability. In this study, SBP (2.5% w/v) was mixed into an oil-in-water emulsion system (4.4% w/w oil, 0.22% w/w whey protein, pH 4.5). Two separate, identically composed, emulsion systems were prepared by different methods of preparation. The emulsions prepared separately and subsequently mixed with SBP (referred as Mix A) produced significantly larger average particle sizes than the emulsions in which the SBP was homogenized into the emulsion system during emulsion preparation (referred as Mix B). Mix B type emulsions were stable. Enzyme catalyzed oxidative gelation of SBP helped stabilize the emulsions in Mix A. The kinetics of the enzyme catalyzed oxidative gelation of SBP was evaluated by small angle oscillatory measurements for horseradish peroxidase (HRP) (EC 1.11.1.7) and laccase (EC 1.10.3.2) catalysis, respectively. HRP catalyzed gelation rates, determined from the slopes of the increase of elastic modulus (G') with time, were higher ($P < 0.05$) than the corresponding laccase catalyzed rates, but the final G' values were higher for laccase catalyzed gels, regardless of the presence of emulsions or type of emulsion preparation (Mix A or Mix B). For both enzymes, rates of gelation in Mix A were higher ($P < 0.05$) than in Mix B, and higher stress was needed to break the gels in Mix A than in Mix B at similar enzyme dosage levels. These differences may be related to a lower availability of the feruloyl-groups for cross-linking when the SBP was homogenized into the emulsion system during preparation.

Keywords

Emulsion; gelation; horseradish peroxidase; laccase; sugar beet pectin

1. Introduction

Whey proteins are amphiphilic molecules that are commonly used as emulsifiers because of their ability to facilitate the formation and stabilization of oil-in-water emulsions (Kulmyrzaev, Sivestre, & McClements, 2000). When preparing the emulsion at pH close to the isoelectric point of the whey proteins (4<pH<6), the emulsion droplets tend to aggregate because the electrostatic repulsion is not large enough to overcome attractive interactions. The emulsion stability can be improved by addition of polysaccharides to the emulsified oil-water-protein mixture. This stabilization induced by the polysaccharides presumably takes place through formation of polysaccharide-protein complexes that coat the emulsified droplets and increase the repulsion among the particles (Schmitt, Sanchez, Desobry-Banon, & Hardy, 1998). Pectic polysaccharides are some of the natural polysaccharides which have been used as such stabilizers in food emulsion systems. Sugar beet pectin (SBP) is a marginally used byproduct from sugar beet based sugar production. Due to a high degree of acetylation and relatively short homogalacturonan stretches of SBP, the divalent-cation (Ca^{2+}) gelation of SBP is less significant than for example that of citrus pectin. The existence of ferulic acid (FA) moieties esterified to the arabinose side-chain at the backbone of rhamnogalacturonan I (RGI) in SBP prepared from sugar beet pulp provides a way for enzyme catalyzed oxidative cross-linking of SBP by oxidoreductase enzymes to promote gelation (Micard & Thibault, 1999; Norsker, Jensen, & Adler-Nissen, 2000; Zaidel, Chronakis, & Meyer, 2012). The FA moieties in SBP are mainly esterified to the *O*-2 position of the arabinofuranosyl (Ara*f*) residues in the α-(1,5)-linked-arabinan backbone, but may also be bound to the *O*-5 on terminal arabinose (Levigne, Ralet, Quéméner, Pollet, Lapierre, & Thibault, 2004), or at the *O*-6 position of the galactopyranosyl (Gal*p*) residues in the β-(1,4)-galactan side-chains of the RGI (Colquhoun, Ralet, Thibault, Faulds, & Williamson, 1994). During the enzyme

catalyzed oxidative cross-linking of SBP, the esterified FA moieties on the RGI side-chains are enzymatically oxidized into ferulic acid dehydrodimers (diFAs) in the forms of 5,5', 8-O-4', 8,5' and 8,8'-diFAs (Oosterveld, Grabber, Beldman, Ralph, & Voragen, 1997; Oosterveld, Beldman, & Voragen, 2000; Zaidel et al., 2012). Such covalently cross-linked gels are generally strong and thermo-irreversible and provide for the gel to be stable upon heat processing (Carvajal-Millan, Guilbert, Morel, & Micard, 2005) - a feature of particular significance for food applications. Improved stabilization of multilayered emulsions containing SBP has been explored previously via cross-linking of SBP by addition of laccase (Littoz & McClements, 2008; Zeeb, Gibis, Fischer, & Weiss, 2012) or horseradish peroxidase (Li, Cheng, Wang, Zhao, Yin, & Saito, 2010), and laccase oxidized cross-linking of SBP in dilute (0.3% w/v) solutions has recently been shown to improve stabilization of a model emulsion system (10% tetradecane, pH 6.5) during 30 days of incubation (Jung, & Wicker, 2012). However, the significance of the enzyme catalyzed oxidative gelation rates and the influence of the presence of the SBP during the emulsion preparation (as opposed to subsequently adding SBP after emulsion preparation) on the emulsion stabilization have not been investigated. An understanding of the enzyme catalyzed oxidative cross-linking rates in emulsions systems and/or any influences of the enzymatic gelation on the emulsion stabilization and the gel properties are prerequisites for rational employment of such SBP stabilized emulsion systems in food applications. In this present work, we hypothesized that the presence (or not) of SBP during the homogenization for emulsion preparation could influence both the rates of enzyme catalyzed SBP cross-linking and the emulsion stabilization by the enzyme catalyzed reaction. The purpose of this work was to test these hypotheses by investigating the rates of gelation of the SBP containing emulsions, catalyzed by either HRP or laccase, using two sets of emulsions prepared by different methods of preparation. The rationale

of preparing the emulsion differently was to evaluate the flexibility of the emulsion preparation on the stability of the emulsion and the rheological properties of the SBP gel.

2. Materials and Methods

2.1 Materials

SBP was obtained from Danisco A/S (Nakskov, Denmark). The SBP had been prepared from sugar beet pulp as previously described (Buchholt, Christensen, Fallesen, Ralet, & Thibault, 2004; Holck et al., 2011). The SBP used in this study was the same as used in our previous study of enzyme catalyzed oxidative gelation of sugar beet pectin (Zaidel et al., 2012) and its composition in $mmol \cdot g^{-1}$ DM was: rhamnose 0.36; arabinose 1.35; galactose 1.06; glucose 0.04; galacturonic acid 3.11; FA 0.036; diFAs 0.001, as analyzed by HPAEC (Arnous, & Meyer, 2008) and HPLC (Agger , Viksø-Nielsen, & Meyer, 2010), respectively. Hydrogen peroxide (H_2O_2) 50% w/w and sodium acetate were purchased from Sigma-Aldrich (Steinheim, Germany). Rapeseed oil was obtained from Inco Danmark (Copenhagen, Denmark) and whey protein isolate (WPI) was purchased from Arla Foods Ingredients (Videbæk, Denmark).

2.2 Enzymes

Horseradish peroxidase (HRP) (EC 1.11.1.7) type VI-A 1000 U mg^{-1} and laccase (EC 1.10.3.2) from *Trametes versicolor* 20 U mg^{-1} were purchased from Sigma-Aldrich (Steinheim, Germany). One HRP unit is defined as oxidation of one µmole of 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) per minute at 25°C and pH 5.0. One laccase unit is defined as conversion of one µmole of catechol per minute at 25°C and pH 4.5. In this study, the enzymes were

compared at their unit activity level. When compared in the same assay using ABTS as substrate (25°C, pH 5.0), HRP had a 50 times higher specific activity (U mg^{-1}) than laccase, however.

2.3 Preparation of oil-in-water emulsions

2.3.1 Preparation of Mix A

Rapeseed oil (10% w/w) was mixed into an aqueous emulsifier solution of 0.5% w/w WPI in 0.1 M acetate buffer, pH 4.5, in a high speed blender (Ystral, Ballrechten-Dottingen, Germany) for 7 min, followed by five passes at 40 psi through a microfluidizer (Microfluidics, Massachusetts, USA). Then the resulting emulsion was mixed with the SBP solution by magnetic stirring prior to the gelation experiments to result in an emulsion system (Mix A) containing 4.4% w/w oil, 0.22% w/w WPI, and 2.5% w/v SBP.

2.3.2 Preparation of Mix B

Rapeseed oil (4.4% w/w) was mixed directly into an aqueous emulsifier solution of 0.22% w/w WPI and 2.5% w/v SBP, pH 4.5, in a high speed blender (Ystral, Ballrechten-Dottingen, Germany) for 7 min, followed by five passes at 40 psi through a microfluidizer (Microfluidics, Massachusetts, USA).

2.3.3 Emulsion control without SBP

Rapeseed oil (10% w/w) was mixed into an aqueous emulsifier solution of 0.5% w/w WPI in 0.1 M acetate buffer, pH 4.5, in a high speed blender (Ystral, Ballrechten-Dottingen, Germany) for 7 min, followed by five passes at 40 psi through a microfluidizer (Microfluidics, Massachusetts, USA). The resulting emulsion contained 4.4% w/w oil and 0.22% w/w WPI, pH 4.5.

2.4 Particle size distribution and emulsion stability evaluation

Particle size distributions of the emulsion control, Mix A and Mix B were measured using a light scattering analyzer (Mastersizer, Zetamaster, Malvern Instruments Ltd.). The average particle size was characterized by surface weighted mean diameter, $d_{3,2}$. Measurements of particle size were done at least in duplicate. For emulsion stability evaluation, 5 mL sample (emulsion control, Mix A and Mix B, respectively) was placed in a sealed transparent glass tube (2 cm diameter x 6 cm height) and the evaluation of the emulsion stability was done by measurement of the creaming layer relative to the total height of the emulsion at 1, 24 and 72 hours at room temperature.

2.5 Gelation

For each gelation measurement, the sample (Mix A or Mix B; each containing 2.5% w/v SBP, 4.4% w/w oil, 0.22% w/w WPI) was mixed with enzyme at various dosages (0.125, 0.25, 0.5, 1.0 and 2.0 U mL^{-1}) and 0.5 mM H_2O_2 (for HRP only) at 25°C. The H_2O_2 stock solution was prepared fresh every day. The enzymes were dosed according to their specific activity (see 2.2) and compared at similar dosage levels (U mL^{-1}). Enzyme catalyzed cross-linking of SBP (2.5% w/v) in 0.05 M sodium acetate buffer, pH 5 (without any emulsion) was used as control. The rheological analyses of the oxidative gelation of SBP containing emulsion were based on small angle oscillatory rheological measurements on serrated parallel plates using a HAAKE MARS rotational rheometer (Thermo Scientific Inc., Germany). Each gelation reaction was initiated by adding the required amount of enzyme to the sample and the storage (*G'*) and loss (*G"*) moduli were recorded for 20 min at a frequency of 1 Hz and 0.1 Pa stress. Frequency sweeps (0.1 to 100 Hz) at 0.1 Pa stress were carried out after 20 min of the oxidative gelation reaction for enzyme

dosage of 0.5 and 2.0 U mL^{-1}. Stress sweeps were performed at stress of 0.1 – 2000 Pa and a frequency of 1 Hz. All measurements were done within the linear viscoelastic region and at 25°C. For the frequency and stress sweep evaluations silicon oil was used to cover the edges of the samples to avoid evaporation during measurements.

2.6 Modeling of the rheological data

According to the Bohlin's cooperative theory of flow (Bohlin, 1980), the properties of the gels produced from the oxidative gelation, using the SBP control, Mix A and Mix B, catalyzed by HRP or laccase were further investigated using the following power law model (equation (1)):

$$G^* = A\omega^{1/z} \qquad (1)$$

where G^* (Pa) = $(G'^2 + G''^2)^{1/2}$ is the complex modulus, ω (Hz) is the frequency, A (Pa) is a constant that can be interpreted as the magnitude of the interactions between the molecules responsible for the gelation of the sample, and z (dimensionless) is the coordination number of the cooperative flow units in the structure which measures the extent (or level) of the three dimensional network in the gel (Manoi & Rizvi, 2009). The rheological data were fitted into the power law model (equation 1) to obtain the Bohlin's parameters A and z.

2.7 Statistics

One-way analyses of variances (one-way ANOVA): 95% confidence intervals were compared as Tukey-Kramer intervals calculated from pooled standard deviations (Minitab Statistical Software, Addison-Wesley, Reading, MA).

3. Results and Discussion

3.1 Particle size distributions and emulsion stability evaluation

Emulsions with a significantly ($P < 0.05$) smaller average particle size, $d_{3,2}$ (0.12 μm), could be produced by homogenizing the oil, WPI and SBP all together (Mix B), whereas, the emulsion prepared separately and subsequently mixed with SBP (Mix A) produced a larger average particle size, $d_{3,2}$ (8.3 μm), (Table 1). The average particle size distribution data thus showed that the emulsion preparation methodology affected the average particle size, $d_{3,2}$ of the emulsions. In addition to this, the emulsion stability evaluation showed that Mix B, having the smaller particle size, had better stability than Mix A and the emulsion control *i.e.* no creaming took place in the emulsions prepared as Mix B after 72 hours, even without enzymatic cross-linking (Table 1). Hence the results indicated that the presence of SBP during homogenization was able to stabilize the emulsion. This effect was presumably a result of increased interaction of protein-polysaccharides for smaller particle sized and thus large interfacial area at the isoelectric point of the WPI, since the same stabilization was not achieved when the SBP was added only after the homogenization forming the emulsion or for the emulsion control (Table 1). The difference between the emulsion stability of the emulsion control (without any SBP added) and Mix A also signified that the addition of SBP *per se* had a weak stabilizing effect.

3.3 Gelation kinetics of SBP in the emulsion systems

The development of storage (*G'*) and loss (*G"*) moduli of the SBP during oxidative gelation in the two differently prepared emulsion mixtures, *i.e.* Mix A and Mix B (each containing 2.5% w/v SBP, 4.4% w/w oil, 0.22% w/w WPI), as catalyzed by HRP or laccase was investigated at various enzyme dosages (0.125 – 2.0 U mL^{-1}) (Fig. 1). In all cases the *G'* increased with time according

to a regular hyperbola curve. For both enzymes there were clear dose-response effects with respect to the gel formation regardless of how the emulsion/gelation mixtures had been prepared (Fig. 1). After 20 min of reaction, the gels catalyzed in the presence of emulsions had higher *G'* values than the SBP control particularly at high enzyme dosages, *i.e.* ≥0.5 U mL^{-1} for the gels catalyzed by HRP (Fig. 1A; 1B) and ≥1.0 U mL^{-1} for the gels catalyzed by laccase (Fig. 1C). In general the HRP catalyzed gelation was faster than the laccase catalyzed gelation (at the same enzyme dosage), and the rate of gelation, to be discussed below, was faster in Mix A than in Mix B, all other things being equal (Fig. 1). Compared to the SBP control (*i.e* 2.5% w/v SBP without emulsion) the increases in *G'* (*i.e.* when *G'* > *G"*) for the HRP catalyzed gelation of Mix A and Mix B, respectively, were approximately similar to the gelation of the SBP control (2.5% w/v SBP) at high dosage of HRP (Fig. 1A; 1B). However, at lower enzyme dosages of HRP the increases in *G'* (*i.e.* when *G'* > *G"*) were slightly slower compared to the increase in *G'* in the SBP control (Fig. 1A; 1B). This gelation 'lag phase' (*i.e.* where *G'* < *G"*) in the presence of the emulsion was even more apparent when the SBP gelation was catalyzed by laccase, especially for Mix B (Fig. 1C; 1D). In spite of the slower increase of *G'* at lower enzyme dosages, a direct comparison of the initial rates of gelation of Mix A and Mix B showed no significant difference ($P < 0.05$) to the rates of gelation of the SBP control (2.5% w/v SBP) at low enzyme dosages (≤1.0 U·mL^{-1}) for either HRP or laccase catalyzed gelation (Fig. 2). However, for both HRP and laccase at higher enzyme dosages of 2.0 U mL^{-1} the rate of gelation of Mix B was significantly slower ($P < 0.05$) than the rates of gelation of Mix A and the SBP control (Fig. 2). Taken together with the evaluation of the particle size of the emulsions (Table 1) the data imply that in Mix B the interaction between the SBP and the WPI coating the particles in fact means that despite the presumed larger surface area of the (presumed protein-polysaccharide coated) droplets, the rate of

the cross-linking was slower in Mix A having larger particle size $d_{3,2}$ (Table 1). The present study does not allow a firm mechanistic explanation for this paradox, unless the larger surface area in Mix B in fact produces a substrate deficiency of feruloyl groups for the cross-linking.

3.4 Rheological properties of SBP gels in the emulsion systems

3.4.1 Frequency sweep

Frequency sweep and stress sweep tests were performed on the enzymatically oxidized SBP gels in the differently prepared emulsion systems; *i.e*. in Mix A and Mix B, that had reacted for 20 min with 0.5 and 2.0 U mL^{-1} dosages of HRP and laccase, respectively. The frequency sweep data showed that the gels produced in both Mix A and Mix B catalyzed by HRP or laccase, respectively, exhibited typical gel behavior with *G'* > *G"* (Fig 3). This gel behavior also implied that the *G'* values were independent of the applied frequency from 0.1 to 100 Hz whereas *G"* was highly dependent on frequency (Fig. 3). In general the *G'* obtained with HRP had the same *G'* in Mix A and Mix B, but the lower enzyme dosage apparently produced higher *G'* values (Fig. 3A) whereas no immediate differences in the *G"* were obvious. In contrast, for the laccase catalyzed gels in the emulsion systems there was an impact of the way the emulsion was prepared with SBP and the gels resulting from laccase catalyzed reactions in Mix B tended to have lower *G'* than the corresponding gels in Mix A (Fig. 3B). Another difference between the HRP and laccase catalyzed gels was that the effect of enzyme dosage was reversed; in the HRP catalyzed gels the higher *G'* were obtained at the lower enzyme dosage of 0.5 U mL^{-1} as compared to those prepared with 2.0 U mL^{-1} (Fig. 3A). For the laccase catalyzed gels higher enzyme dosage produced higher *G'* regardless of the emulsion system (Mix A or Mix B) (Fig. 3B). Apparently, the lower rates of gelation produced higher *G'* confirming the result from the gels formation (Fig. 1).

The slopes of the complex dynamic viscosity $|\eta^*|$ of the gels, obtained from the (Pa·s) data in response to the frequency sweep (Zaidel et al., 2012), were in the range of -0.40 to -0.96 (Table 2). The $|\eta^*|$ of the gels catalyzed by HRP were all in the range from -0.9 to -0.96 and thus close to the limiting value of -1, signifying the solid-like gel behavior, for all enzyme dosages, whereas for the gels catalyzed by laccase the $|\eta^*|$ was only close to the limiting value of -1 at enzyme dosages above 0.5 U mL^{-1}. For the lower laccase dosages the lower value of the $|\eta^*|$ indicated a more liquid structure of the gels network. For neither the HRP or the laccase catalyzed gels no significant differences or effects from the type of emulsion system (Mix A or Mix B) was obtained (Table 2).

In general, similar conclusions could be drawn from the tan δ ($= G''/G'$) data: the tan δ data were in the range of 0.05 to 2.2 across all the gels (Table 2). Gels produced from catalysis of lower enzyme dosage, especially for laccase, had a high tan δ (>1) which indicated a more liquid-like character of the gel produced. Low tan δ values (<0.1) produced from gels catalyzed by high enzyme dosage indicated presence of the elastic network in the system. Even though low laccase dosages produced differences in the tan δ values none of the other values of tan δ produced from gels using Mix A and Mix B were statistically significantly different (Table 2). Neither were the tan δ values different from the tan δ values of the SBP controls ($0.01 <$ tan $\delta < 0.13$) (data not shown).

3.4.2 Stress sweep

The stress sweep data showed that the strength of each gel (measured as G') was constant for the stress applied from 0.1 Pa to above 500 Pa (Fig. 4). At higher stress the solid-like character dropped sharply for each gel, indicating an abrupt breakdown of the gel network (Fig. 4). When

assessing the viscous resistance, measured as *G"*, the evolution with increased stress showed that *G"* values increased gradually, notably when the stress came above ~500 Pa, and then decreased after reaching a peak resistance 'breaking point'(Fig. 4). The gradual decrease in *G"* with applied stress beyond the 'breaking point', was most likely due to fragments of the broken network conferring a contribution to viscosity. This contribution will decrease as the fragments are broken down further with increased stress. For the HRP catalyzed gels, at 0.5 U mL^{-1}, the gels needed higher stress to break the network (SBP control: 814 Pa; Mix A: 995 Pa; Mix B: 899 Pa) compared to the stress needed to break the gel network produced using 2.0 U·mL^{-1} HRP (SBP control: 667 Pa; Mix A: 736 Pa; Mix B: 603 Pa) (Fig. 4A). On the contrary, the gels catalyzed by laccase at 2.0 U mL^{-1} needed high stress to break the network (SBP control: 1100 Pa; Mix A: 1210 Pa; Mix B: 1100 Pa) than the gels catalyzed using 0.5 U mL^{-1} laccase (SBP control: 995 Pa; Mix A: 1100 Pa; Mix B: 814 Pa) (Fig. 4B). The gels produced in Mix A had higher strengths than the gels prepared in Mix B regardless of the enzyme type and enzyme dosage (Fig. 4). The strengths of the gels produced in Mix A or Mix B were slightly higher than the strength of the gels produced by the SBP control without oil or WPI added (Fig. 4). This latter result indicates that interactions between proteins and polysaccharides (the pectin) coupled with the covalent cross-links created from the enzyme catalyzed oxidative cross-linking of SBP enhanced the properties of the gels produced.

3.4.3 Bohlin's parameters

According to the Bohlin's theory, emulsions are modeled as a network of rheological units, which interact for establishing system structure (Peressini, Sensidoni, & de Cindio, 1998). Using the power law model (equation (1)), Bohlin's parameters *A* and *z* were obtained for all the gels

produced from the oxidative gelation experiment. The constant *A* in this model is interpreted as the magnitude of the interactions and *z* is the coordination number of the cooperative flow units in the structure which measures the extent (or level) of the three dimensional network (Manoi & Rizvi, 2009). For the gels catalyzed by HRP, the type of emulsion preparation had no consistent or significant effects on the Bohlin's parameters *A* and *z* (Table 3). In contrast, for the laccase catalyzed gels the *A* values tended to be higher than the corresponding values for the HRP catalyzed systems at high enzyme dosages. More importantly, the *A* values for gels produced in Mix A were consistently higher than the corresponding values from Mix B irrespective of the laccase dosage, except for the lowest enzyme dosage, but no such consistent differences could be discerned from the *z* data (Table 3). The result for the Bohlin's parameter *A* indicated that stronger interactions between the molecules (*i.e*. the SBP network and/or the protein/SBP network) prevailed in the laccase catalyzed gels as compared to the HRP catalyzed gels at higher enzyme dosages and especially so when the laccase catalyzed gels were prepared in Mix A as opposed to in Mix B. This result confirms that laccase apparently catalyzed the formation of stronger gels (higher *G'*) than HRP at the same, higher enzyme dosages of 1.0-2.0 U mL^{-1}, confirming the results shown in Fig. 1. These data also agree with enzyme catalyzed oxidative gelation data obtained in SBP gels without emulsions (Zaidel et al., 2012). Hence, higher *A* values indicated that the gel emulsion has better ability to keep the original microstructure, which prevents the creaming of the emulsion droplets. Another significant finding was that higher *G'* values were obtained in Mix A than in Mix B for laccase catalyzed gels which correspond to the result of Bohlin's parameter *A* (Table 3).

4. Conclusions

The presence of SBP regardless of the preparation mode of the emulsion systems improved the emulsion stability. Especially when SBP was homogenized into the emulsion during its preparation, the creaming was prevented. The particle size of such prepared emulsion was also much smaller than emulsion prepared separately and subsequently mixed with SBP.

Enzyme catalyzed oxidative gelation of SBP was also affected by the mode of preparation of the SBP containing emulsions (these emulsions contained WPI). Both HRP (with H_2O_2) and laccase (without H_2O_2) readily catalyzed the gelation of SBP even in the presence of emulsified oil in emulsion systems. The rate of the enzyme catalyzed oxidative gelation was however slower for the systems in which the emulsions had been homogenized together with the SBP (Mix B) as opposed to when prepared separately (Mix A). This effect was most likely attributable to differences in the structural make-up of the emulsion systems, that might have provided different availabilities of the SBP-feruloyl groups for cross-linking in Mix A and Mix B, respectively.

Another observation was that laccase catalyzed gelation was slower than HRP catalyzed gelation, but the laccase catalysis produced stronger gels (having higher *G'* values) regardless of the presence of emulsions and the preparation mode of the emulsions. That the enzyme catalyzed cross-linking of SBP can stabilize emulsions paves the way for employing SBP as techno-functional stabilizers in emulsified food systems. The finding that this enzyme catalyzed gelation stabilization of the emulsion overrides the effect of the preparation mode of the emulsion systems provides for a flexible application of SBP and enables diversity in the food formulation.

Abbreviations

FA	ferulic acid
diFAs	ferulic acid dehydrodimers
HRP	horseradish peroxidase
H_2O_2	hydrogen peroxide
RGI	rhamnogalacturonan I
SBP	sugar beet pectin
WPI	whey protein isolate

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Figure captions

Fig. 1: Gelation of (A) Mix A catalyzed by HRP and 0.5 mM H_2O_2; (B) Mix B catalyzed by HRP and 0.5 mM H_2O_2; (C) Mix A catalyzed by laccase; (D) Mix B catalyzed by laccase, at 25°C. *G'* for SBP control: (——): 2.0 U·mL^{-1}; (– –): 1.0 U·mL^{-1}; (·····): 0.5 U·mL^{-1}; (— — -): 0.25 U·mL^{-1}; (— · —): 0.125 U·mL^{-1}; *G'* for emulsions: (diamond): 2.0 U·mL^{-1}; (square): 1.0 U·mL^{-1}; (circle): 0.5 U·mL^{-1}; (triangle): 0.25 U·mL^{-1}; (∗) or (×): 0.125 U·mL^{-1}; (+): *G"*.

Fig. 2: Rate of gelation of Mix A (filled symbols) or Mix B (open symbols) catalyzed by HRP or laccase at various enzyme dosages. Gelation of 2.5% w/v SBP was used as control. The data shown are averages of two replicate gelation experiments and error bars are standard deviation from the two replicate. (×): SBP control, HRP; (∗): SBP control, laccase; (diamond): HRP; (triangle): laccase.

Fig. 3: Frequency sweep (0.1 to 100 Hz) performed at 0.1 Pa stress for SBP gels containing emulsion (2.5% w/v SBP, 4.4% w/w oil, 0.22% w/w WPI) catalyzed by (**A**) HRP; (**B**) laccase. Data recorded after 20 min reaction. (filled symbols): Mix A; (open symbols): Mix B; *G'* for emulsions: (diamond): 2.0 U·mL^{-1} HRP or laccase; (circle): 0.5 U·mL^{-1} HRP or laccase; *G"* for emulsions: (+): 2.0 U·mL^{-1} HRP or laccase; (×): 0.5 U·mL^{-1} HRP or laccase.

Fig. 4: Stress sweep (0.1 to 2000 Pa) performed at a frequency of 1 Hz on 2.5% w/v SBP gels produced using 0.5 and 2.0 U·mL^{-1} enzymes at 25 °C showing the breaking point of the gels (**A**) HRP; (**B**) laccase. Filled symbols: Mix A; Open symbols: Mix B; (——): SBP control (2.5% w/v SBP), 2.0 U·mL^{-1}; (– –): SBP control (2.5% w/v SBP), 0.5 U·mL^{-1}; (diamond): *G'* 2.0 U·mL^{-1} HRP or laccase; (circle): *G'* 0.5 U·mL^{-1} HRP or laccase; (+): *G"* 2 U·mL^{-1}; (×): *G"* 0.5 U·mL^{-1}; (·····): *G"* SBP control.

References

Agger, J., Viksø-Nielsen, A., & Meyer, A. S. (2010). Enzymatic xylose release from pretreated corn bran arabinoxylan: Differential effects of deacetylation and deferuloylation on insoluble and soluble substrate fractions. *Journal of Agricultural and Food Chemistry,* 58, 6141-6148.

Arnous, A., & Meyer, A. S. (2008). Comparison of methods for compositional characterization of grape *(Vitis vinifera L.)* and apple *(Malus domestica)* skins. *Food and Bioproducts Processing,* 86, 79-86.

Bohlin, L. (1980). A theory of flow as a cooperative phenomenon. *Journal of Colloid and Interfacial Science*, 74, 423-434.

Buchholt, H. C., Christensen, T. M. I. E., Fallesen, B., Ralet, M. C., & Thibault, J. F. (2004). Preparation and properties of enzymatically and chemically modified sugar beet pectins. *Carbohydrate Polymers*, 58, 149-161.

Carvajal-Millan, E., Guilbert, S., Morel, M.-H., & Micard, V. (2005). Impact of the structure of arabinoxylan gels on their rheological and protein transport properties. *Carbohydrate Polymers,* 60, 431-438.

Colquhoun, I. J., Ralet, M. C., Thibault, J. F., Faulds, C. B., & Williamson, G. (1994). Structure identification of feruloylated oligosaccharides from sugar beet pulp by NMR spectroscopy. *Carbohydrate Research,* 263, 243-256.

Holck, J., Hjernø, K., Lorentzen, A., Vigsnæs, L. K., Hemmingsen, L., Licht, T. R., Mikkelsen, J. D., & Meyer, A. S. (2011). Tailored enzymatic production of oligosaccharides from sugar beet pectin and evidence of differential effects of a single DP chain length difference on

human faecal microbiota composition after *in vitro* fermentation. *Process Biochemistry,* 46, 1039-1049.

Kulmyrzaev, A., Sivestre, M. P. C., & McClements, D. J. (2000). Rheology and stability of whey protein stabilized emulsions with high $CaCl_2$ concentrations. *Food Research International*, 33, 21-25.

Jung, J., & Wicker, L. (2012). Laccase mediated conjugation of sugar beet pectin and the effect on emulsion stability. *Food Hydrocolloids*, 28, 168-173.

Levigne, S. V., Ralet, M. -C. J., Quéméner, B. C., Pollet, B. N. -L., Lapierre, C., & Thibault, J.-F. J. (2004). Isolation from sugar beet cell walls of arabinan oligosaccharides esterified by two ferulic acid monomers. *Plant Physiology,* 134, 1173-1180.

Li, J. L., Cheng, Y. Q., Wang, P., Zhao, W. T., Yin, L. J., & Saito, M. (2012). A novel improvement in whey protein isolate emulsion stability: Generation of an enzymatically cross-linked beet pectin layer using horseradish peroxidase. *Food Hydrocolloids*, 26, 448-455.

Littoz, F., & McClements, D. J. (2008). Bio-mimetic approach to improving emulsion stability: Cross-linking adsorbed beet pectin layers using laccase. *Food Hydrocolloids*, 22, 1203-1211.

Manoi, K. & Rizvi, S. S. H. (2009). Emulsification mechanisms and characterizations of cold, gel-like emulsions produced from texturized whey protein concentrate. *Food Hydrocolloids*, 23, 1837-1847.

Micard, V., & Thibault, J. F. (1999). Oxidative gelation of sugar-beet pectins: use of laccases and hydration properties of the cross-linked pectins. *Carbohydrate Polymers,* 39, 265-273.

Norsker, M., Jensen, M., & Adler-Nissen, J. (2000). Enzymatic gelation of sugar beet pectin in food products. *Food Hydrocolloids*, 14, 237-243.

Oosterveld, A., Beldman, G., & Voragen, A. G. J. (2000). Oxidative cross-linking of pectic polysaccharides from sugar beet pulp. *Carbohydrate Research,* 328, 199-207.

Oosterveld, A., Grabber, J. H., Beldman, G., Ralph, J., & Voragen, A. G. J. (1997). Formation of ferulic acid dehydrodimers through oxidative cross-linking of sugar beet pectin. *Carbohydrate Research,* 300, 179-181.

Peressini, D., Sensidoni, A., & de Cindio, B. (1998). Rheological characterization of traditional and light mayonnaises. *Journal of Food Engineering*, 35, 409-417.

Schmitt, C., Sanchez, C., Desobry-Banon, S., & Hardy, J. (1998). Structure and technofunctional properties of protein-polysaccharide complexes: A review. *Critical Reviews in Food Science and Nutrition,* 38, 689-753.

Zaidel, D. N. A., Chronakis, I. S., & Meyer, A. S. (2012). Enzyme catalyzed oxidative gelation of sugar beet pectin: Kinetics and rheology. *Food Hydrocolloids*, 28, 130-140.

Zeeb, B., Gibis, M., Fischer, L., & Weiss, J. (2012). Crosslinking of interfacial layers in multilayered oil-in-water emulsions using laccase: Characterization and pH-stability. *Food Hydrocolloids*, 27, 126-136.

Table 1: Average particle size $d_{3,2}$ and the ratio between the cream layer to the total height of the emulsions after 1, 24 and 72 hr stored at room temperature. Different superscript letters (a, b, c) in the same columns signify significantly different values ($P < 0.05$).

Emulsion	Average particle size $d_{3,2}$ (μm)	With or without enzyme (HRP or laccase)	Cream layer:total height		
			1 hr	24 hr	72 hr
Emulsion control	8.4 ± 0.2	–	0.38 ± 0.04[a]	0.40 ± 0.00[a]	0.43 ± 0.04[a]
Mix A	8.3 ± 0.7	–	0.18 ± 0.04[b]	0.20 ± 0.00[b]	0.22 ± 0.03[b]
		+	0[c]	0[c]	0[c]
Mix B	0.12 ± 0.00	–	0[c]	0[c]	0[c]
		+	0[c]	0[c]	0[c]

Table 2: Data for tan δ and slope of the complex dynamic viscosity, $|\eta^*|$ for gels using Mix A and Mix B after enzymatic oxidative cross-linking catalyzed by HRP or laccase. The values are averages of two replicate gelation experiments and pooled standard deviations were calculated.

Enzyme ($U \cdot mL^{-1}$)	**$\|\eta^*\|$ (Pa·s)**		**tan δ**	
	Mix A[1]	**Mix B[1]**	**Mix A[2]**	**Mix B[2]**
HRP				
2.0	-0.93	-0.92	0.039	0.033
1.0	-0.90	-0.93	0.030	0.019
0.5	-0.93	-0.96	0.020	0.018
0.25	-0.93	-0.90	0.033	0.035
0.125	-0.92	-0.91	0.045	0.120
Laccase				
2.0	-0.93	-0.94	0.030	0.025
1.0	-0.89	-0.93	0.006	0.050
0.5	-0.90	-0.84	0.036	0.135
0.25	-0.85	-0.40	0.170	1.67
0.125	-0.49	-0.42	0.580	2.20

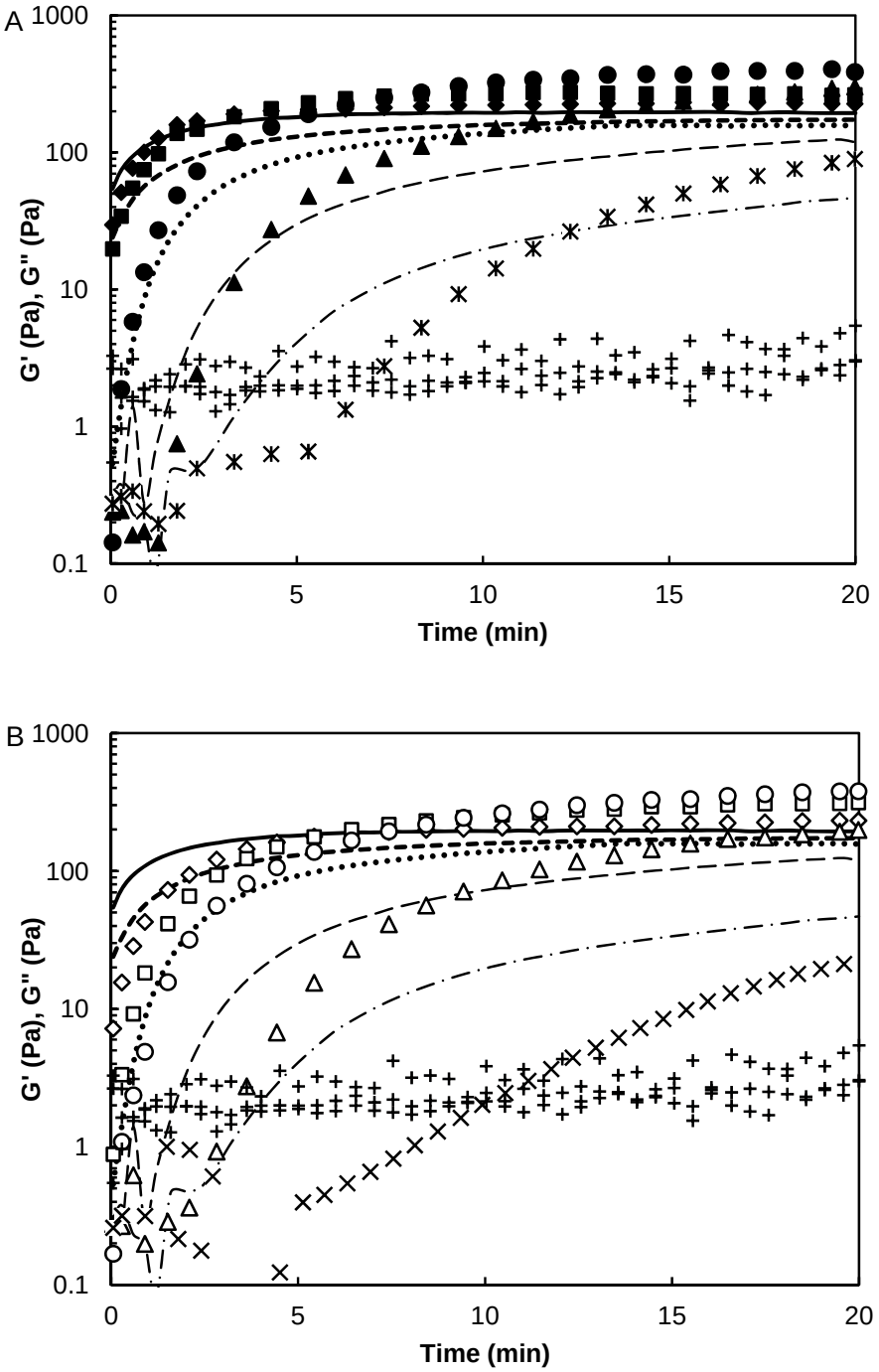
Pooled SD: [1]0.02; [2]0.019

Table 3: Bohlin's parameters, *A* and *z* of gels produced from 2.5% w/v SBP solution (control), Mix A and Mix B after 20 min oxidative gelation catalyzed by HRP or laccase at various enzyme dosages. Values are averages of two replicate gelation experiments. For Bohlin's parameter A, different superscript letters (a, b, c, d, e) in the same column signify significantly different values ($P < 0.05$), *i.e*. across different enzymes and enzyme dosages. Different superscript letters (v, w, x) in the same rows signify significant different values ($P < 0.05$) *i.e*. across different emulsion preparations and the SBP control. For Bohlin's parameter z there were no significant differences across the columns nor the rows except an effect in the SBP control catalyzed by HRP that gave a higher z at the highest HRP dosage.

Enzyme (U·mL^{-1})	Control		Mix A		Mix B	
	A [1]	***z*** [2]	***A*** [1]	***z*** [2]	***A*** [1]	***z*** [2]
HRP						
2.0	181 $^{b,c\ [w]}$	26.9	306 $^{a\ [v,w]}$	14.1	237 $^{a,b\ [w]}$	12.9
1.0	293 $^{b\ [v,w]}$	15.2	327 $^{c\ [v,w]}$	12.6	312 $^{a\ [v,w]}$	14.7
0.5	326 $^{b\ [v,w]}$	18.0	472 $^{b,c\ [v]}$	14.9	388 $^{a\ [v]}$	20.2
0.25	221 $^{b,c\ [v,w]}$	11.5	436 $^{b,c\ [v]}$	14.0	303 $^{a\ [v,w]}$	10.2
0.125	221 $^{b,c\ [v,w]}$	13.9	212 $^{c,d\ [w]}$	12.1	84 $^{b,c\ [w]}$	11.7
Laccase						
2.0	460 $^{a\ [v,w]}$	17.9	694 $^{a\ [v]}$	21.9	391 $^{a\ [w]}$	16.7
1.0	281 $^{b\ [w,x]}$	10.3	542 $^{a,b\ [v]}$	10.5	330 $^{a\ [w]}$	14.3
0.5	113 $^{c\ [x]}$	6.7	342 $^{c\ [w]}$	7.2	146 $^{b\ [x]}$	6.8
0.25	180 $^{b,c\ [x]}$	8.0	130 $^{d,e\ [x]}$	6.7	11 $^{b,c\ [x]}$	1.7
0.125	188 $^{b,c\ [x]}$	8.7	10 $^{e\ [x]}$	1.9	8 $^{b,c\ [x]}$	1.7

Pooled SD: [1]43; [2]4.2

Fig. 1:



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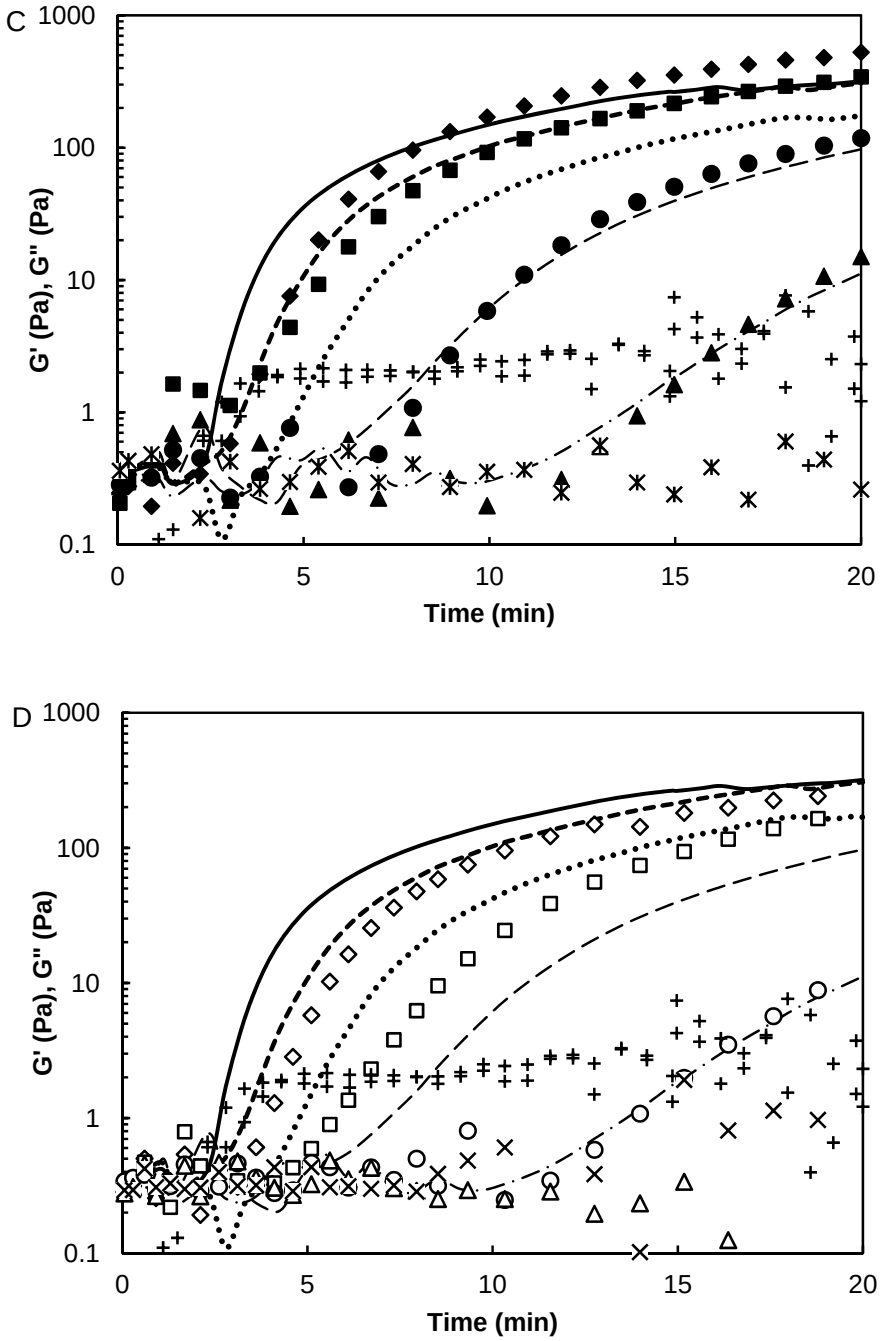
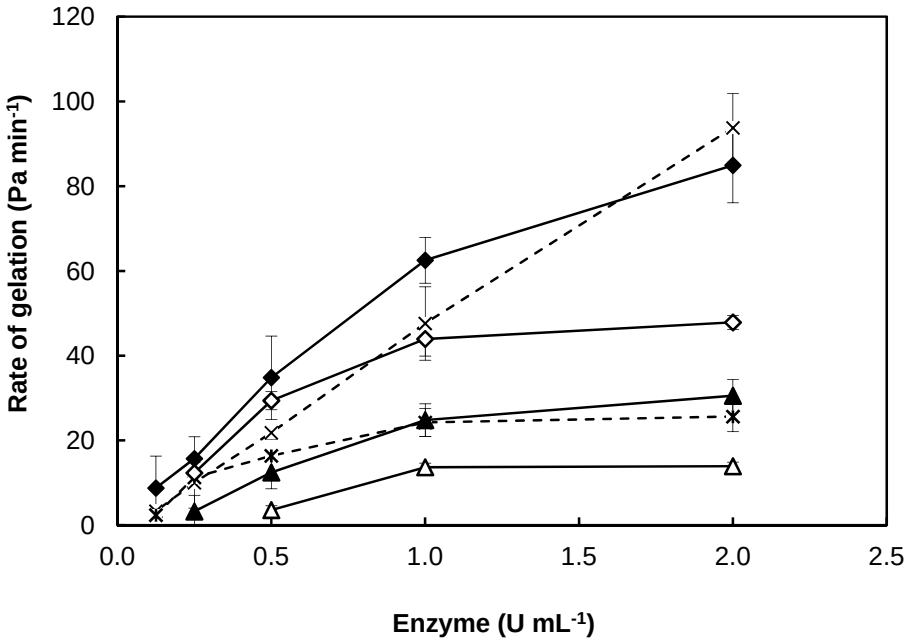


Fig. 2:


120
100
80
60
40
20
0
0.0
0.5
1.0
1.5
2.0
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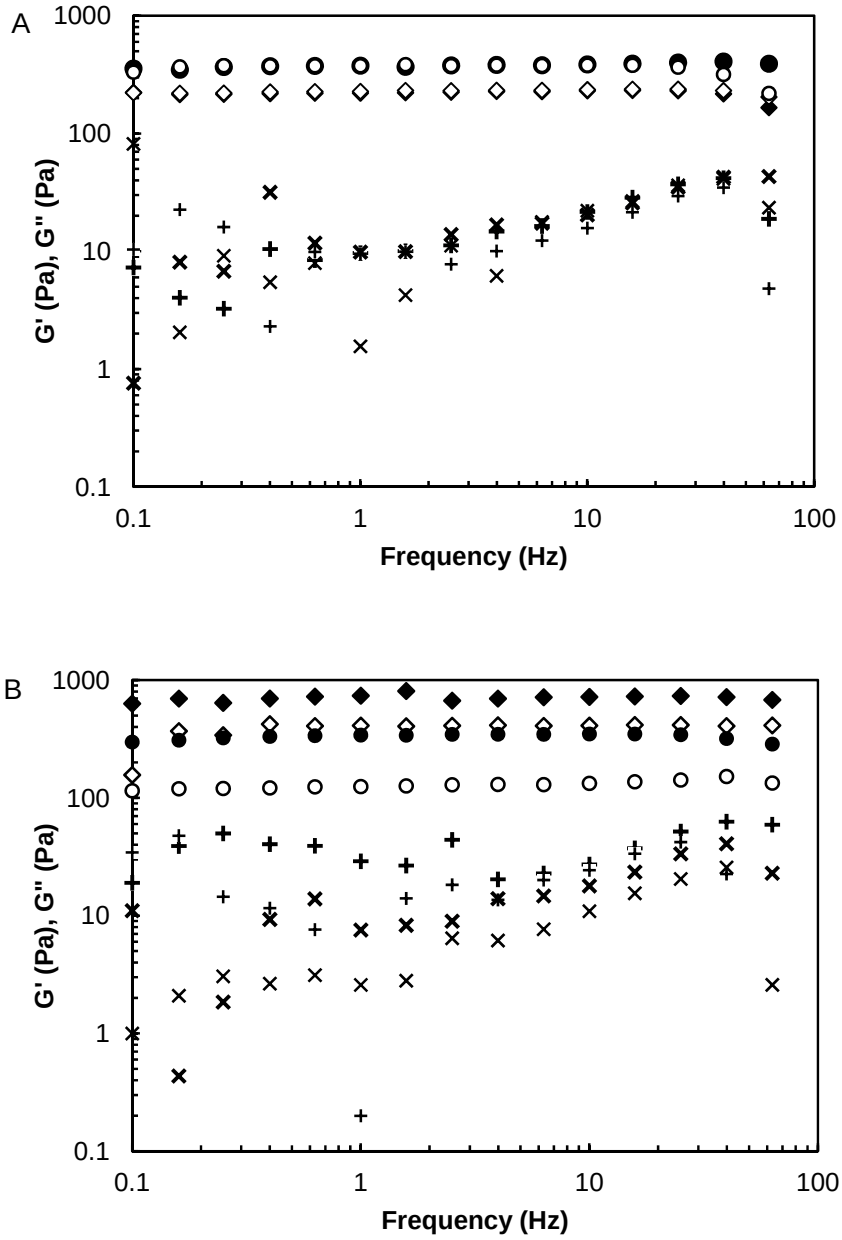
Rate of gelation (Pa min-1)
Enzyme (U mL-1)

Fig. 3:


A
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G' (Pa), G" (Pa)
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G' (Pa), G" (Pa)
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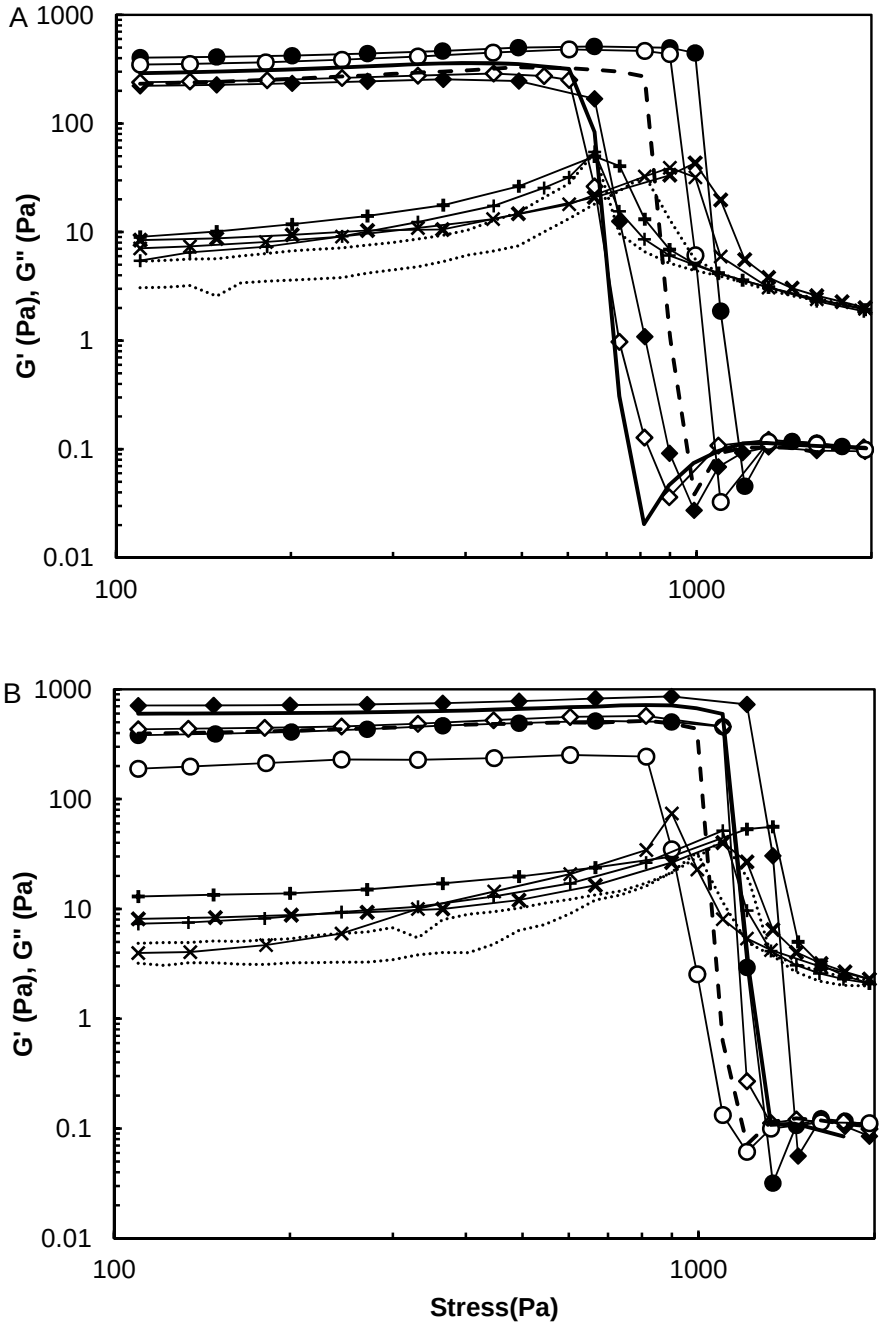
Frequency (Hz)

Fig. 4:



CHAPTER 7
Conclusions

The overall aim of this PhD study has been to understand and describe the kinetics of enzyme catalyzed oxidative cross-linking of feruloylated pectic polysaccharide substrates from sugar beet and relate the kinetics to the properties of the cross-linked products. A prerequisite for the PhD study was to have an overview of the different types of cross-links and gelation mechanisms of pectic polysaccharides, and notably to examine how the cross-linking can be promoted enzymatically to design particular macromolecular properties. In order to provide this overview a review of the available literature was presented (Paper I). The review confirmed that several different types of enzyme catalyzed modification of pectic polysaccharides can be promoted to design specific cross-links and functionalities.

Regarding the kinetics of enzyme catalyzed oxidative cross-linking, the overall objective was met by correlating the oxidative cross-linking reaction kinetics to the backbone length of the substrate, investigating the reaction factors that influence the kinetics of cross-linking reaction, and characterizing the macromolecular properties of the cross-linked product in terms of rheological properties. Our initial hypothesis was based on that the extent of ferulic acid substrate and the size of the substrate affect the kinetic of the enzymatic reaction, that is, longer the length, slower the reaction. The first part of this study utilized the arabinan-oligosaccharide fraction as the substrate, which has shown substantial amount of FA for oxidative cross-linking reaction catalyzed by HRP. Our analysis from HILIC separation and MALDI-TOF confirmed the identity and distribution of the FA attached to different arabino-oligosaccharides chains. Furthermore, we were able to separate the arabinan-oligosaccharides according to their molecular weight. The results presented in paper II illustrate the influence of the backbone length which supports our hypothesis, that is, longer arabinans exhibit a slower cross-linking rate than shorter, all other things being equal. It is not only substrate concentration, but also the molecular size *i.e.* the arabinan backbone chain length, that affects the rate of cross-linking.

The work from paper II was then used as a basis to investigate how the rates of enzyme catalyzed cross-linking affect the rates of gelation and the resulting gel strength. We have foreseen the utilization of cross-linked arabinan-oligosaccharide in beverages taking its low viscosity into consideration. However, our investigation on the macromolecular properties of the cross-linked arabinan-oligosaccharide has not shown any significant increment in viscosity (and *G'*). This

might be due to the relatively short chain of arabinan or low molecular weight of the sample (~1.3 kDa) which prevented the measurement of the rheological properties of arabinan. This suggests that the length (or molecular size), or structural make up of the substrate is one of the critical parameters for gelation, given the fact that our result in paper II has shown the ability of the arabinan-oligosaccharide to be cross-linked by HRP. Therefore, this leads to the work on the SBP solid fraction which has a higher molecular weight (>100 kDa) and contains sufficient feruloylated arabinan side-chains for oxidative enzyme catalyzed cross-linking.

For the second hypothesis that the different mechanisms of two oxidoreductase enzymes (HRP and laccase) might influence the kinetics of the cross-linking and consequently the properties of the gels formed, the result has shown a significant difference between the SBP gels produced from the catalysis of HRP and laccase. At equal enzyme activity level, laccase catalysis produced stronger SBP gels albeit a slower rate of gelation than the HRP catalysis. A higher formation of diFAs at the end of the reaction from laccase catalysis than HRP catalysis supports the stronger properties of the gels produced from the laccase catalysis. This result reveals that not only the rate of gelation affects the properties of the gels produced, but rather the availability of the oxidizing agent (O_2 or H_2O_2) and the reaction of the enzyme on the substrate itself that enable formation of diFAs at the end of the reaction. Our study also demonstrates that the reaction factors, particularly the pectin level, temperature, enzyme dosage, and, for horseradish peroxidase, the H_2O_2 concentration, significantly affecting the kinetics of the oxidative gelation for HRP or laccase catalysis. These reaction factors could be tuned in order to adjust the enzyme catalyzed gelation and thus the properties of the gels produced. In addition, we have obtained a positive correlation between the rates of gelation and gel strengths for laccase catalyzed gels, but no such correlation exists for HRP catalyzed gels. From this investigation, laccase seems to be a better choice over HRP for the gelation of SBP; at lower enzyme dosage and slower rate of gelation, laccase could produce stronger gels than HRP and the use of H_2O_2 would be avoided, which is considered advantageous for food applications of this technology.

Our investigation on emulsion stabilization demonstrates the stabilizing properties of SBP in emulsion systems. The mixture of emulsion and SBP could undergo enzyme catalyzed oxidative gelation using HRP or laccase. Our result reveals that SBP could stabilize an emulsion in a gel. Furthermore, the rheological measurement shows that the enzyme catalyzed oxidative gelation using the mixture of emulsion and SBP produced stronger gels compared to the SBP only. We have shown that a flexible way of mixing the emulsion and SBP was able to stabilize the gels and produce good gel *via* enzyme catalyzed oxidative gelation.

The idea of utilizing agro-industrial byproduct streams which are rich sources of non-digestible plant fibers has already been subject to many scientific researches and therefore in this study we employ the byproduct from sugar beet pulp. Our study reveals that arabinan-oligosaccharide and SBP fractions extracted from sugar beet pulp byproduct could undergo oxidative cross-linking of the feruloyl group which abundantly esterified to the arabinan side-chains through enzyme catalyzed reaction. Furthermore our result exemplify that the properties of the cross-linked products could be tuned *via* adjustment of the reaction factors of the enzyme catalyzed reaction. Hence, the prospect of utilizing this byproduct as starting materials for obtaining bioactive carbohydrates has an enormous potential in food industry.

7.1 Future perspectives

As we have shown in this study that the utilization of pectic polysaccharide extracted from sugar beet pulp has a great potential as a food ingredient, there are a lot more possibilities to be explored for this substrate. Further investigation on the influence of chain length or molecular size on the kinetics of cross-linking of feruloylated polysaccharide can be done if the SBP is subject to enzymatic hydrolysis. Hydrolytic action of monocomponent enzyme on the SBP, for example pectin lyase and polygalacturonase, has shown to degrade SBP into smaller molecular sizes. Pectin lyase catalyzes the hydrolysis of the α-(1,4)-linked-Gal*p*A in high methoxylated HG backbone while polygalacturonase hydrolyzes mainly in un-esterified HG. From this catalysis, HG of SBP is degraded and thus cut-off from RGI backbone. Separation of the different molecular sizes fractions of SBP after the enzymatic degradation by membrane technology is considered to provide a better size distribution profile of the different fraction, with a specific MWCO membrane size. The investigation of the cross-linking of the RGI fraction which contains the arabinan side-chains to which ferulic acid is esterified and smaller molecular size than the native SBP might describe the influence of backbone chain length on the oxidative cross-linking and the gelation of feruloylated pectic polysaccharide.

Investigation of the cross-linked SBP gel by visualization or microscopic techniques like confocal laser scanning microscopy (CLSM) may provide an insight of the gel network built from the diFA linkages. Arltoft *et al*. (2006) has investigated the use of direct immunostaining technique to localize the pectin in a gelled dairy dessert and assessed using CLSM. In their study, they have shown that the antibody bound specifically to pectin network in gelled dairy product. This technique can possibly be applied to the enzyme catalyzed oxidative cross-linked SBP-emulsion gels, to illustrate the gel network formed. In a recent study, an antibody that could bind feruloyl

group has been discovered (Clausen *et al*., 2004), which might be interesting to further investigate the structure of the gels network through the dehydrodimers of ferulic acid links.

The prospective of arabinan and pectin fractions from sugar beet as health promoting biopolymer has been explored previously (Al-Tamimi *et al*., 2006; Holck *et al*., 2011a;b). The bioavailability of dihydrodimers of ferulic acid as antioxidant was reported (Andreasen *et al*., 2001) by using cereal brans as the substrate. By taking these into consideration, future study involving cross-linked products from sugar beet pectin as prebiotics or antioxidant could be an interesting area to be explored.

REFERENCES

Al-Tamimi, M. A. H. M., Palframan, R. J., Cooper, J. M., Gibson, G. R., & Rastall, R. A. (2006). In vitro fermentation of sugar beet arabinan and arabino-oligosaccharides by the human gut microflora. *Journal of Applied Microbiology*, 100, 407-414.

Andreasen, M. F., Kroon, P. A., Williamson, G., & Garcia-Conesa, M. T. (2001). Intestinal release and uptake of phenolic antioxidant diferulic acids. *Free Radical Biology & Medicine*, 31, 304-314.

Arltoft, D., Ipsen, R., Christensen, N., & Madsen, F. (2006). Localising pectin in dairy products using direct immunostaining, In P. A. Williams & G. O. Phillips (Eds.), *Gums and stabilisers for the food industry 13* (pp. 41-51). Cambridge, UK: Royal Society of Chemistry.

BeMiller, J. N., & Whistler, R. L. (1996). Carbohydrates. In O. R. Fennema (Eds.), *Food chemistry.* (pp. 158-223). New York: Marcel Dekker, Inc.

Berlanga-Reyes, C. M., Carvajal-Millán, E., Juvera, G. C., Raseón-Chu, A., Marquez-Escalante, J. A., & Martinez-Lopez, A. L. (2009). Laccase induced maize bran arabinoxylan gels: structural and rheological properties. *Food Science and Biotechnology*, 18, 1027-1029.

Brown, A. C. (2008). Fats and oils. In A. C. Brown (Eds.) *Understanding food: Principles & preparation*. Third Edition. (pp. 419-443). Belmont: Thomson Learning, Inc.

Brown, J. A., & Fry, S. C. (1993). Novel O-D-galacturonoyl esters in the pectic polysaccharides of suspension-cultured plant cells. *Plant Physiology*, 103, 993-999.

Buchholt, H. C., Christensen, T. M. I. E., Fallesen, B., Ralet, M. C., & Thibault, J. -F. (2004). Preparation and properties of enzymatically and chemically modified sugar beet pectins. *Carbohydrate Polymers*, 58, 149-161.

Bunzel, M. (2010). Chemistry and occurance of hydroxycinnamate oligomers. *Phytochemistry Reviews*, 9, 47-64.

Bunzel, M., Heuermann, B., Kim, H., & Ralph, J. (2008). Peroxidase-catalyzed oligomerization of ferulic acid esters. *Journal of Agricultural and Food Chemistry*, 56, 10368-10375.

Bunzel, M., Ralph, J., Bruning, P., & Steinhart, H. (2006). Structural identification of dehydrotriferulic and dehydrotetraferulic acids isolated from insoluble maize bran fiber. *Journal of Agricultural and Food Chemistry*, 54, 6409-6418.

Bunzel, M., Ralph, J., Funk, C., & Steinhart, H. (2005). Structural elucidation of new ferulic acid-containing phenolic dimers and trimers isolated from maize bran. *Tetrahedron Letters*, 46, 5845-5850.

Burton, S. G. (2003) Oxidizing enzymes as biocatalysts. *Trends in Biotechnology*, 21, 543–549.

Carvajal-Millan, E., Guilbert, S., Morel, M. -H., & Micard, V. (2005). Impact of the structure of arabinoxylan gels on their rheological and protein transport properties. *Carbohydrate Polymers,* 60, 431-438.

Chanamai, R., & McClements, D. J. (2002). Comparison of gum arabic, modified starch, and whey protein isolate as emulsifiers: Influence of pH, $CaCl_2$ and temperature. *Journal of Food Science*, 67, 120-125.

Ciron, C. I. E., Gee, V. L., Kelly, A. L., & Auty, M. A. E. (2010). Comparison of the effects of high-pressure microfluidization and conventional homogenization of milk on particle size, water retention and texture of non-fat and low-fat yoghurts. *International Dairy Journal*, 20, 314-320.

Clark, A. H., & Farrer, D. B. (1996). Shear modulus-concentration relationships for low DE pectin-calcium gels in the temperature range 20-85°C. *Food Hydrocolloids*, 10, 31-39.

Clausen, M. H., Ralet, M. C., Willats, W. G. T., McCartney, L., Marcus, S. E., Thibault, J. F., & Knox, J. P. (2004). A monoclonal antibody to feruloylated-(1→4)-β-D-galactan. *Planta*, 219, 1036-1041.

Coenen, G. J., Bakx, E. J., Verhoef, R. P., Schols, H. A., Voragen, A. G. J. (2007). Identification of the connecting linkage between homo- or xylogalacturonan and rhamnogalacturonan type I. *Carbohydrate Polymers*, 70, 224-235.

Colquhoun, I. J., Ralet, M. C., Thibault, J. F., Faulds, C. B., & Williamson, G. (1994). Structure identification of feruloylated oligosaccharides from sugar-beet pulp by NMR spectroscopy. *Carbohydrate Research*, 263, 243-256.

Dea, I. C. M., & Madden, J. K. (1986). Acetylated pectic polysaccharides of sugar beet. *Food Hydrocolloids*, 1, 71-88.

Demetriades, K., & McClements, D. J. (1998). Influence of pH and heating on physicochemical properties of whey protein-stabilized emulsions containing a nonionic surfactant. *Journal of Agricultural and Food Chemistry*, 46, 3936-3942.

Dickinson, E. (1997). Properties of emulsions stabilized with milk proteins: Overview of some recent developments. *Journal of Dairy Science*, 80, 2607-2619.

Dickinson, E. (2003). Hydrocolloids at interfaces and the influence on the properties of dispersed systems. *Food Hydrocolloids*, 17, 25-39.

El-Nawawi, S. A., & Heikal, Y. A. (1995). Factors affecting the production of low-ester pectin gels. *Carbohydrate Polymers*, 26, 189-193.

FAO. (2011). Crops production in 2009. Food and Agriculture Organization of the United Nations (FAO), http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#ancor Accessed on October 6, 2011.

Fraeye, I., Duvetter, T., Doungla, E., Loey, A. V., Hendrickx, M. (2010). Fine-tuning the properties of pectin-calcium gels by control of pectin fine structure, gel composition and environmental conditions. *Trends in Food Science & Technology*, 21, 219-228.

Fry, S. C. (1983). Feruloylated pectins from the primary cell wall: their structure and possible functions. *Planta*, 157, 111-123.

Funami, T., Nakauma, M., Ishihara, S., Tanaka, R., Inoue, T., & Philips, G. O. (2011). Structural modifications of sugar beet pectin and the relationship of structure to functionality. *Food Hydrocolloids*, 25, 221-229.

Garti, N. (1999). Hydrocolloids as emulsifying agents for oil-in-water emulsions. *Journal of Dispersion Science and Technology*, 20, 327-355.

Guillon, F., & Thibault, J. F. (1987). Characterization and oxidative cross-linking of sugar beet pectins after mild acid hydrolysis and arabanases and galactanases degradation. *Food Hydrocolloids*, 1, 547-549.

Guillon, F., & Thibault, J. F. (1989). Methylation analysis and mild acid hydrolysis of the "hairy" fragments of sugar-beet pectins. *Carbohydrate Research*, 190, 85-96.

Guillon, F., & Thibault, J. F. (1990). Oxidative cross-linking of chemically and enzymatically modified sugar-beet pectin. *Carbohydrate Polymers*, 12, 353-374.

Henriksen, A., Smith, A. T., & Gajhede, M. (1999). The structures of the horseradish peroxidase C-Ferulic acid complex and the ternary complex with cyanide suggest how peroxidases oxidize small phenolic substrates. *The Journal of Biological Chemistry*, 274, 35005-35011.

Hilz, H. (2007). Characterization of cell wall polysaccharides in bilberries and black currants. PhD thesis. Wageningen University, The Netherlands.

Hilz, H., Williams, P., Doco, T., Schols, H. A., Voragen, A. G. J. (2006). The pectic polysaccharide rhamnogalacturonan II is present as a dimer in pectic populations of bilberries and black currants in muro and in juice. *Carbohydrate Polymers*, 65, 521-528.

Hiner, A. N. P., Sidrach, L., Chazarra, S., Varón, R., Tudela, J., García-Cánovas, F., & Rodríguez-López, J. N. (2004). Kinetic study of the effects of calcium ions on cationic artichoke (*Cynara scolymus* L.) peroxidase: calcium binding, steady-state kinetics and reactions with hydrogen peroxide. *Biochimie*, 86, 667-676.

Holck, J., Hjernø, K., Lorentzen, A., Vigsnæs, L. K., Hemmingsen, L., Licht, T. R., Mikkelsen, J. D., & Meyer, A. S. (2011a). Tailored enzymatic production of oligosaccharides from sugar beet pectin and evidence of differential effects of a single DP chain length difference on human faecal microbiota composition after *in vitro* fermentation. *Process Biochemistry,* 46, 1039-1049.

Holck, J., Lorentzen, A., Vigsnæs, L. K., Licht, T. R., Mikkelsen, J. D., & Meyer, A. S. (2011b). Feruloylated and nonferuloylated arabino-oligosaccharides from sugar beet pectin selectively stimulate the growth of *Bifidobacterium* spp. in human fecal *in vitro* fermentations. *Journal of Agricultural and Food Chemistry,* 59, 6511-6519.

Hou, W. C., Chang, W. H. (1996). Pectinesterase-catalyzed firming effects during precooking of vegetables. *Journal of Food Biochemistry*, 20, 397-416.

Ishii, T. (1997). Structure and functions of feruloylated polysaccharides. *Plant Science*, 127, 111-127.

Ishii, T., & Matsunaga, T. (2001). Pectic polysaccharide rhamnogalacturonan II is covalently linked to homogalacturonan. *Phytochemistry*, 57, 969-974.

Ishii, T., Matsunaga, T., & Hayashi, N. (2001). Formation of rhamnogalacturonan II-borate dimer in pectin determines cell wall thickness of pumpkin tissue. *Plant Physiology*, 126, 1698-1705.

Itoh, K., Hatakeyama, T., Shimoyama, T., Miyazaki, S., D'Emanuele, A., & Attwood, D. (2011). *In situ* gelling formulation based on methylcellulose/pectin system for oral-sustained drug delivery to dysphagic patients. *Drug Development and Industrial Pharmacy*, 37, 790-797.

Jung, J., & Wicker, L. (2012). Laccase mediated conjugation of sugar beet pectin and the effect on emulsion stability. *Food Hydrocolloids*, 28, 168-173.

Kastner, H., Einhorn-Stoll, U., & Senge, B. (2012). Structure formation in sugar containing pectin gels – influence of Ca^{2+} on the gelation of low-methoxylated pectin at acidic pH. *Food Hydrocolloids*, 27, 42-49.

Kulmyrzaev, A., Sivestre, M. P. C., & McClements, D. J. (2000). Rheology and stability of whey protein stabilized emulsions with high $CaCl_2$ concentrations. *Food Research International*, 33, 21-25.

Kuuva, T., Lantto, R., Reinikainen, T., Buchert, J., & Autio, K. (2003). Rheological properties of laccase-induced sugar beet pectin gels. *Food Hydrocolloids,* 17, 679-684.

Laurent, M. A., & Boulenguer, P. (2003). Stabilization mechanism of acid dairy drinks (ADD) induced by pectin. *Food Hydrocolloids*, 17, 445-454.

Lerouge, P., O'Neill, M. A., Darvill, A. G., & Albersheim, P. (1993). Structural characterization of endo-glycanase-generated oligoglycosyl side chains of rhamnogalacturonan I. *Carbohydrate Research*, 243, 359-371.

Levigne, S. V., Ralet, M. -C. J., Quéméner, B. C., Pollet, B. N. -L., Lapierre, C., & Thibault, J. -F. J. (2004). Isolation from sugar beet cell walls of arabinan oligosaccharides esterified by two ferulic acid monomers. *Plant Physiology*, 134, 1173-1180.

Levigne, S., Thomas, M., Ralet, M. -C., Quemener, B., & Thibault, J. -F. (2002). Determination of the degrees of methylation and acetylation of pectins using a C18 column and internal standards. *Food Hydrocolloids*, 16, 547-550.

Li, J. L., Cheng, Y. Q., Wang, P., Zhao, W. T., Yin, L. J., & Saito, M. (2012). A novel improvement in whey protein isolate emulsion stability: Generation of an enzymatically cross-linked beet pectin layer using horseradish peroxidase. *Food Hydrocolloids*, 26, 448-455.

Littoz, F., & McClements, D. J. (2008). Bio-mimetic approach to improving emulsion stability: Cross-linking adsorbed beet pectin layers using laccase. *Food Hydrocolloids*, 22, 1203-1211.

Liu, B., Bhaladhare, S., Zhan, P., Jiang, L., Zhang, J., Liu, L., & Hotchkiss, A. L. (2011). Morphology and properties of thermoplastic sugar beet pulp and poly(butylenes adipate-co-terepthalate) blends. *Industrial & Engineering Chemistry Research*, 50, 13859-13865.

May, C. D. (1992). Pectins. In A. Imeson (Eds.), *Thickening and gelling agents for food*, (pp. 124-152). Glasgow: Blackie Academic & Professional.

McClements, D. J. (2007). Critical review of techniques and methodologies for characterization of emulsion stability. *Critical Reviews in Food Science and Nutrition*, 47, 611-649.

Mezger, T. G. (2006). Oscillatory tests. In T. G. Mezger (Eds.), *The rheology handbook: for users of rotational and oscillatory rheometers*, 2nd revised edition (pp. 114-170). Hannover: Vincentz Network, GmbH.

Micard, V., Grabber, J. H., Ralph, J., Renard, M. G. C., & Thibault, J. –F. (1997). Dehydrodiferulic acids from sugar-beet pulp. *Phytochemistry*, 44, 1365-1368.

Micard, V., & Thibault, J. F. (1999). Oxidative gelation of sugar-beet pectins: use of laccases and hydration properties of the cross-linked pectins. *Carbohydrate Polymers,* 39, 265-273.

Mohnen, D. (2008). Pectin structure and biosynthesis. *Current Opinion in Plant Biology*, 11, 266-277.

Morris, E. R. (1995). Polysaccharide rheology and in-mouth perception. In A. M. Stephen (Eds.), *Food polysaccharides and their applications*, (pp. 517-546). New York: Marcel Dekker, Inc.

Munarin, F., Guerreiro, S. G., Grellier, M. A., Tanzi, M. C., Barbosa, M. A., Petrini, P., & Granja P. L. (2011). Pectin-based injectable biomaterials for bone tissue engineering. *Biomacromolecules*, 12, 568-577.

Nakamura, A., Furuta, H., Maeda, H., Takao, T., & Nagamatsu, Y. (2002). Structural studies by stepwise enzymatic degradation of the main backbone of soybean soluble polysaccharides consisting of galacturonan and rhamnogalacturonan. *Bioscience, Biotechnology and Biochemistry*, 66, 1301-1313.

Norsker, M., Jensen, M., & Adler-Nissen, J. (2000). Enzymatic gelation of sugar beet pectin in food products. *Food Hydrocolloids*, 14, 237-243.

Oosterveld, A., Beldman, G., Schols, H. A., & Voragen, A. G. J. (1996). Arabinose and ferulic acid rich pectic polysaccharides extracted from sugar beet pulp. *Carbohydrate Research*, 288, 143-153.

Oosterveld, A., Beldman, G., Schols, H. A., & Voragen, A. G. J. (2000a). Characterization of arabinose and ferulic acid rich pectic polysaccharides and hemicelluloses from sugar beet pulp. *Carbohydrate Research*, 328, 185-197.

Oosterveld, A., Beldman, G., & Voragen, A. G. J. (2000b). Oxidative cross-linking of pectic polysaccharides from sugar beet pulp. *Carbohydrate Research*, 328, 199-207.

Oosterveld, A., Grabber, J. H., Beldman, G., Ralph, J., & Voragen, A. G. J. (1997). Formation of ferulic acid dehydrodimers through oxidative cross-linking of sugar beet pectin. *Carbohydrate Research*, 300, 179-181.

Pazarlıoğlu, N. K., Sariişik, M., & Telefoncu, A. (2005). Laccase: production by *Trametes versicolor* and application to denim washing. *Process Biochemistry*, 40, 1673-1678

Ralph, J., Quideau, S., Grabber, J. H., & Hatfield, R. D. (1994). Identification and synthesis of new ferulic acid dehydrodimers present in grass cell walls. *Journal of the Chemical Society, Perkin Transactions 1*, 3485-3497.

Ridley, B. L., O'Neill, M. A., & Mohnen, D. (2001). Pectins: structure, biosynthesis, and oligogalacturonide-related signaling. *Phytochemistry*, 57, 929-967.

Rombouts, F. M., & Thibault, J. -F. (1986). Feruloylated pectic substances from sugar-beet pulp. *Carbohydrate Research*, 154, 177-187.

Sakamoto, T., & Sakai, T. (1995). Analysis of structure of sugar-beet pectin by enzymatic methods. *Phytochemistry*, 39, 821-823.

Saulnier, L., & Thibault, J. F (1999). Ferulic acid and diferulic acids as components of sugar-beet pectins and maize bran heteroxylans. *Journal of the Science of Food and Agriculture*, 79, 396-402.

Schmitt, C., Sanchez, C., Desobry-Banon, S., & Hardy, J. (1998). Structure and technofunctional properties of protein-polysaccharide complexes: A review. *Critical Reviews in Food Science and Nutrition,* 38, 689-753.

Sila, D. N., van Buggenhout, S., Duvetter, T., Fraeye, I., de Roeck, A., van Loey, A., & Hendrickx, M. (2009). Pectins in processed fruits and vegetables: part II - structure-function relationships. *Comprehensive Reviews in Food Science and Food Safety*, 8, 86-104.

Stoilova, I., Krastanov, A., & Stanchev, V. (2010). Properties of crude laccase from *Trametes versicolor* produced by solid-substrate fermentation. *Advances in Bioscience and Biotechnology*, 1, 208-215.

Sutherland, G. R. J., & Aust, S. D. (1996). The effects of calcium on the thermal stability and activity of manganese peroxidase. *Archives of Biochemistry and Biophysics*, 332, 128-134.

Tahiri, M., Pellerin, P., Tressol, J. C., Doco, T., Pepin, D., Rayssiguier, Y., & Coudray C. (2000). The rhamnogalacturonan-II dimer decreases intestinal absorption and tissue accumulation of lead in rats. *Journal of Nutrition*,130, 249-253.

Takei, T., Sugihara, K., Ijima, H., & Kawakami, K. (2011). *In situ* gellable sugar beet pectin *via* enzyme-catalyzed coupling reaction of feruloyl groups for biomedical applications. *Journal of Bioscience & Bioengineering*, 112, 491-494.

Thibault, J. -F. (1986). Some physicochemical properties of sugar-beet pectins modified by oxidative cross-linking. *Carbohydrate Research*, 155, 183-192.

Thibault, J. -F. (1988). Characterisation and oxidative crosslinking of sugar-beet pectins extracted from cossettes and pulps under different conditions. *Carbohydrate Polymers,* 8, 209-223.

Thibault, J. -F. & Rombouts, F. M. (1986). Effects of some oxidising agents, especially ammonium peroxysulfate, on sugar-beet pectins. *Carbohydrate Research*, 154, 205-215.

Thurston, C. F. (1994). The structure and function of fungal laccases. *Microbiology*, 140, 19-26.

Veitch, N. C. (2004). Horseradish peroxidase: a modern view of a classic enzyme. *Phytochemistry*, 65, 249-259.

Voragen, A. G. J., Coenen, G. -J., Verhoef, R. P., & Schols, H. A. (2009). Pectin, a versatile polysaccharide present in plant cell walls. *Structural Chemistry*, 20, 263–275.

Wagner, M. & Nicell, J. A. (2002). Impact of dissolved wastewater constituents on peroxidase-catalyzed treatment of phenol. *Journal of Chemical Technology and Biotechnology*, 77, 419-428.

Waldron, K. W., Ng, A., Parker, M. L., & Parr, A. J. (1997). Ferulic acid dehydrodimers in the cell walls of *Beta vulgaris* and their possible role in texture. *Journal of the Science of Food and Agriculture,* 74, 221-228.

Waldron, K. W., Parr, A. J., Ng, A., & Ralph, J. (1996). Cell wall esterified phenolic dimers: Identification and quantification by reverse phase high performance liquid chromatography and diode array detection. *Phytochemical Analysis*, 7, 305-312.

Waldron, K. W., Parker, M. L., & Smith, A. C. (2003). Plant cell walls and food quality. *Comprehensive Reviews in Food Science and Food Safety*, 2, 101-119.

Walstra, P. (1996). Dispersed systems: Basic considerations. In O. R. Fennema (Eds.), *Food chemistry* (pp. 95-156). New York: Marcel Dekker, Inc.

Westphal, Y., Kühnel, S., de Waard, P., Hinz, S. W. A., Schols, H. A., Voragen, A. G. J., & Gruppen, H. (2010). Branched arabino-oligosaccharides isolated from sugar beet arabinan. *Carbohydrate Research*, 345, 1180-1189.

Whitcombe, A. J., O'Neill, M. A., Steffan, W., Albersheim, P., & Darvill, A. G. (1995). Structural characterization of the pectic polysaccharide, rhamnogalacturonan-II. *Carbohydrate Research*, 271, 15-29.

Willats, W. G. T., Knox, J. P., & Mikkelsen, J. D. (2006). Pectin: new insights into an old polymer are starting to gel. *Trends in Food Science & Technology*, 17, 97-104.

Xu, F. (1996). Oxidation of phenols, anilines, and benzenethiols by fungal laccases: Correlation between activity and redox potentials as well as halide inhibition. *Biochemistry*, 35, 7608-7614.

Yaropolov, A. I., Skorobogaťko, O. V., Vartanov, S. S., & Varfolomeyev, S. D. (1994). Laccase. Properties, catalytic mechanism, and applicability. *Applied Biochemistry and Biotechnology*, 49, 257-280.

Zaidel, D. N. A., Arnous, A., Holck, J., & Meyer A. S. (2011). Kinetics of enzyme-catalyzed cross-linking of feruloylated arabinan from sugar beet. *Journal of Agricultural and Food Chemistry*, 59, 11598-11607.

Zaidel, D. N. A., Chronakis, I. S., & Meyer, A. S. (2012). Enzyme catalyzed oxidative gelation of sugar beet pectin: Kinetics and rheology. *Food Hydrocolloids*, 28, 130-140.

Zeeb, B., Gibis, M., Fischer, L., & Weiss, J. (2012). Crosslinking of interfacial layers in multilayered oil-in-water emulsions using laccase: Characterization and pH-stability. *Food Hydrocolloids*, 27, 126-136.

Zhan, D., Janssen, P., & Mort, A. J. (1998). Scarcity or complete lack of single rhamnose residues interspersed within the homogalacturonan regions of citrus pectin. *Carbohydrate Research*, 308, 373-380.