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

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A B S T R A C T
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 H₂O₂) catalyzed a fast rate of gelation compared to laccase (no H₂O₂ 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 H₂O₂ (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.

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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-Rhap–(1 → 4)α-1-D-GalpA–(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 (Araf) substitutions, and minor amounts of β-(1,4)-linked-galactan (Oosterveld et al., 2000; Westphal et al., 2010). Some of the Araf 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 arabian chains (Levigne et al., 2004).

Due to its acetylation and relatively short homogalacturonan stretches, the classic, divalent-cation (Ca²⁺) promoted pectin gelation is less significant for SBP than for e.g. citrus pectin. However, the existence of the feruloyl groups on the arabian 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 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 (Galp) 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, Reiniikainen, Buchert, & Autio, 2003; Norsker, Jensen, & Adler-Nissen, 2000). During the reaction, the FAs are enzymatically
oxidized into free radicals and 5,5', 8-O-4', 8,5' and 8,8' furulic acid dehydrodimers (difAs) are formed (Oosterveld et al., 2000; Oosterveld, Grabber, Baldman, Ralph, & Voragen, 1997). HRP and laccase catalyze the cross-linking reaction via different oxidizing mechanisms. The peroxygen requires hydrogen perooxide as the oxidizing agent while laccase oxidizes the substrate via indirect 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, Guillert, 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, Sugihaara, 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 methylation 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 (H2O2) 50% (w/w), sodium acetate, trifluoroacetic acid (TFA) 98% (w/w), and monosaccharide standards including: p-galactose, l-arabinose, d-fucose, l-rhamnose monohydrate, and l-galacturonic acid monohydrate were purchased from Sigma–Aldrich (Steinheim, Germany). p-glucose, p-xylene, disodium hydrogen phosphate (Na2HPO4) and anhydrous sodium sulfate (Na2SO4) 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 Novozenymes 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, N2) 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 Na2SO4. The pooled samples were dried by evaporation under N2 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 1100 series column (50 × 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, Vikse-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 H2O2 (for HRP only) at 25 °C. A H2O2 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\(_2\)O\(_2\) concentration in the experimental design for HRP). Since the gelation rate catalyzed by laccase did not reach a maximum (plateau) in the experimental design for HRP (data not shown). The development of storage (G') and loss (G'') modulus 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 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 (10 ± 0.1 μmol g\(^{-1}\)), respectively (Table 2).

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'') modulus 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 (Kuiva 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\(_2\)O\(_2\) catalyzed by HRP relative to the oxidation by direct interaction of oxygen catalyzed via the copper cluster in the laccase enzyme. Addition of H\(_2\)O\(_2\) 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).
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 and 2.0 U mL\(^{-1}\) enzymatically oxidized SBP gels after 20 min of reaction with 0.5 U mL\(^{-1}\) enzyme and with and without EDTA addition (to chelate any Ca\(^{2+}\)) did not produce gelation (data not shown) supporting that the SBP system was resistant of the laccase catalyzed gels versus the HRP catalyzed gels (Fig. 1). The elastic modulus values (Fig. 3A, B) and are in accord with the higher gel strengths obtained for the two replicates.

Fig. 3. Rheological properties of diFA cross-linked sugar beet pectin gels

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 the 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 (900 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
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/H2O2 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 $\sim 500$ 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 to 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 H2O2 also increased the SBP gelation rate significantly ($P < 0.0001$) (Table 3). Furthermore, interactions between temperature and pH, and substrate and H2O2 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 ($75.5 \pm 19$ Pa min$^{-1}$ and $52.8 \pm 2.4$ Pa min$^{-1}$, for HRP and laccase, respectively) being close to the coefficient of the constant ($68.4 \pm 8.3$ Pa min$^{-1}$ and $52.8 \pm 21.2$ Pa min$^{-1}$, respectively). The regression models were as given in Eqs. (1) and (2) for HRP and laccase, respectively.

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

**Rate$_{Lacc}$** = $52.8 + 48.5x_1 + 46.9x_2 + 4.59x_3 + 80.4x_4 + 36.6x_1x_2 + 68.1x_1x_4 + 81.7x_2x_4$ \hspace{1cm} (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
understanding of how the gelation rate responded to different factor combinations (Fig. 4). Although the enzyme dosage \times 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/H2O2 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, pH 4.5) and the pH of 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 H2O2 0.55 mM, in the high temperature 55°C, 55°C 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 H2O2 concentration, the model produced a maximal rate of gelation of \sim 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 H2O2 level is one of the limiting factors for the H2O2 cross-linking as also indicated by the highly significant, positive effect of H2O2 on the gelation rate (Table 3). However, the positive interaction between H2O2 and the SBP substrate concentration (Table 3), and notably the response surface plot (Fig. 4D), also revealed a complex influence of the H2O2 on the gelation rate. Hence, at low H2O2 levels, the HRP catalyzed gelation did not occur at either the low or high substrate levels, but at high H2O2 levels neither did a gelation occur at the low substrate levels (Fig. 4D). This essentially zero gelation at high H2O2 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 H2O2 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 arabinins in which diFA levels tended to decrease at high H2O2: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 \sim 85 Pa min\(^{-1}\) would be achieved at \sim 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. 18), 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 \times 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 \sim 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 \times temperature, and temperature \times 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 O2 solubility decreases with temperature, being e.g. 0.28 mM in water at 25°C vs. \sim 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 H₂O₂; (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 H₂O₂; (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 H₂O₂; (D) H₂O₂ (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 H₂O₂. Light to dark tones represent low to high values.
2.0 U mL\(^{-1}\) HRP, 0.55 mM H\(_2\)O\(_2\), 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\(_2\)O\(_2\) 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.

\[
G'_{10,\text{HRP}} = 133.8 - 4.75x_1 + 42.11x_2 + 1.48x_3 + 34.42x_4 + 40.83x_5 - 35.82x_2x_3 + 26.25x_4x_5
\]  

\[
G'_{10,\text{lacc}} = 230.1 + 80.76x_1 + 44.71x_2 - 2.92x_3 + 137.76x_4 + 55.99x_1x_4 - 30.65x_2x_3 + 34.06x_2x_4
\]
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.
SBP or 4.0% (w/v) SBP catalyzed using either (A) HRP (at 1 mM H2O2) or (B) laccase. The reaction rate of gelation in SBP was catalyzed by the two enzymes, with laccase exhibiting a slower rate compared to HRP (Fig. 1). The observed differences in the rate of gelation and the formation of di-FAs (Fig. 9, Table 4) at similar enzyme dosage of 2.0 U mL\(^{-1}\) were accompanied by an increase in FA levels after 5 min reaction. This result correlated well with the differences in rates of gelation and formation of di-FAs. The data recorded in the present work showed that the profile of di-FAs 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 radical from which the formation of several di-FAs was also higher for laccase catalyzed gelation as the reaction progressed, although no pronounced differences in di-FA levels were found after 20 min of reaction (Table 4). The rapid formation of di-FAs 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 H2O2. 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 H2O2/peroxidase reaction which resulted in the formation of several di-FAs. The data recorded in the present work showed that the profile of di-FAs 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 O2 and the copper cluster in the enzyme while H2O2 can rapidly oxidize the FA and HRP therefore catalyze rapid radical dimerization. An excess amount of H2O2 in the reaction might also affect the reaction by further oxidation to trimers and tetramers which explained the decrease in di-FAs after 6 min reaction (Fig. 9). Despite of their different mechanisms, three main di-FAs were detected for both enzymes: 5,5-8-O-4 and 8,5-Benzofuran-diFA with 8,5-Benzofuran diFA being most dominant contributing approximately 45–47% of the total di-FAs, with 38–40% and 15–17% contributed by 8-O-4-diFA and 5,5-diFA respectively (Table 4).

### 3.6. Formation of di-FAs

The amount of FA decreased after addition of HRP and laccase, respectively, and this decrease was accompanied by an increase in di-FAs (Fig. 9, Table 4). At similar enzyme dosage of 2.0 U mL\(^{-1}\) a slower decrease in FA occurred with laccase catalysis as compared to HRP catalysis during the first few minutes of the reaction and the formation of di-FAs 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 di-FAs was also higher for laccase catalyzed gelation as the reaction progressed, although no pronounced differences in di-FA levels were found after 20 min of reaction (Table 4). The rapid formation of di-FAs 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 H2O2. 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 H2O2/peroxidase reaction which resulted in the formation of several di-FAs. The data recorded in the present work showed that the profile of di-FAs 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 O2 and the copper cluster in the enzyme while H2O2 can rapidly oxidize the FA and HRP therefore catalyze rapid radical dimerization. An excess amount of H2O2 in the reaction might also affect the reaction by further oxidation to trimers and tetramers which explained the decrease in di-FAs after 6 min reaction (Fig. 9).

### Table 4

Profile of di-FAs formation within 20 min reaction after addition of 2 U mL\(^{-1}\) HRP (0.14 mM H2O2) and 2 U mL\(^{-1}\) laccase: 5,5’-8-O-4 and 8,5’-Benzofuran diFA (mg g\(^{-1}\) DM).

<table>
<thead>
<tr>
<th>Reaction time (min)</th>
<th>HRP/H2O2</th>
<th>Laccase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5,5’ (mg g(^{-1}))</td>
<td>8,5’-Benzofuran (mg g(^{-1}))</td>
</tr>
<tr>
<td>0</td>
<td>0.075 ± 0.004</td>
<td>0.159 ± 0.008</td>
</tr>
<tr>
<td>0.5</td>
<td>0.25 ± 0.03</td>
<td>1.27 ± 0.06</td>
</tr>
<tr>
<td>2</td>
<td>0.64 ± 0.03</td>
<td>1.57 ± 0.08</td>
</tr>
<tr>
<td>5</td>
<td>0.57 ± 0.03</td>
<td>1.36 ± 0.07</td>
</tr>
<tr>
<td>10</td>
<td>0.45 ± 0.02</td>
<td>1.28 ± 0.06</td>
</tr>
<tr>
<td>20</td>
<td>0.40 ± 0.02</td>
<td>1.16 ± 0.06</td>
</tr>
</tbody>
</table>

Fig. 8. Correlation between rate of gelation and G’ 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 H2O2) 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 di-FAs formation during oxidative cross-linking of SBP catalyzed by HRP and laccase at 2.0 U mL\(^{-1}\) for both enzymes (0.14 mM H2O2 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.
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. H2O2 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 (1.6–18 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 coupling reaction of ferulic acid. From this investigation, the use of laccase to catalyze such gelation seems to be a better choice than HRP – also because HRP requires H2O2 for the reaction. At lower enzyme dosage and slower rate of gelation, laccase could produce stronger gels than HRP and the use of H2O2 would be avoided, which is considered advantageous for food applications of this technology.

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References


