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Ferulated Pectins from Sugar Beet Bioethanol Solids: Extraction, Macromolecular Characteristics, and Enzymatic Gelling Properties

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Abstract: Pectin from sugar beet (*Beta vulgaris* L.) (SBP) was extracted from a sugar beet waste (SBW) registering a 4.4% (*w/w*) yield. SBP presented a weight-average molar mass of 459 kDa, galacturonic acid content of 52.2%, and a low esterification degree (30%). The macromolecular characteristics of SBP revealed a flexible and extended coil chain conformation. The main neutral sugars in SBP were galactose (20.7%), mannose (5.0%), and arabinose (3.60%) while ferulic acid (FA) content was 2.1 μg·mg^{-1} sample. FA remained in the SBP chain mainly in RG I region even after suffering both, industrial processing and harsh weathering conditions. Consequently, SBP formed covalent gels induced by laccase. Covalent cross-linking content (dimers and trimer of FA) was 0.97 mg·g^{-1} SBP. The 8-5′, 5-5′, and 8-*O*-4′ dimers of FA isomers proportions were 75, 17, and 8%, respectively. SBP gels at 4% (*w/v*) registered storage (G′) and loss (G″) moduli final values of 44 and 0.66 Pa, respectively. SBP gels were soft and adhesive according to texture profile analysis. Scanning electron microscopy analysis of SBP lyophilized gels revealed an imperfect honeycomb-like structure with 4.5 ± 1.4 μm average cavities diameter.

Keywords: agro-industrial waste; ferulated polysaccharide; oxidative crosslinking; sugar beet waste; microstructure

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1. Introduction

Sugar beet (*Beta vulgaris* L.) is a resource highly used in industrial processes for sugar production and other products [1]. The beet is known for having a high content of sucrose; as a result, refined sugar, alcoholic beverages, and ethyl alcohol can be produced from it [2]. Some of the sugar beet agro-industrial processing waste products are rich in fiber, protein, and minerals; thus, these are used primarily as cattle and ovine feed [2,3]. Besides this, sugar beet waste (SBW) is a residue with high pectin content (15–30% dry weight basis) [1,4]. The European Union is the world´s leading sugar beet-based sugar producer. In America, sugar beet is not only used for sugar production but also for bioethanol. The United States leads the sugar beet and bioethanol production in America [2,3]. Currently, Mexico is exploring and starting to produce bioethanol from this plant. It is considered an agricultural species capable of adapting to warm areas with high yield potential during winter [5]. As a result, in the last decade, the potential to grow and harvest sugar beet for bioethanol production in the northwest semi-desert regions of Mexico has been explored, generating SBW. For instance, Jimenez-Leon et al. [6] evaluated the productive potential of three sugar beet cultivars; Cadet, Coronado large, and SV MEI in two sowing dates in the experimental field of the University of Sonora (Coordinates: 29°_00′48″ NL, 111°08′07″ WL and 151 MASL (meters above sea level)), under split-plot design. Authors reported

that Coronado's large cultivar sowing in November had the highest tubers production (109.2 t·ha^{-1}) and highest total soluble solids content (19.3 °Brix), while the highest foliage production (25.4 t·ha^{-1}) in dry weight was obtained in Coronado's large cultivar sowing in October. Jimenez-Leon et al. concluded that tuber yields and total soluble solid content obtained under the agroecological conditions of their study are similar to the highest values reported in the literature as a consequence compared to commercial exploitations. Based on the above-mentioned report and a previous one [5], a bioethanol production facility was set in Sonora state, Mexico (Coordinates: 30°42′59″ NL, 112°08′60″ WL and 275 MASL (meters above sea level)). After harvest, beets were milled and fermented; then, filtered and the solids were piled under the sun to dry. The SBW had a granulate appearance and a dark brown-purple color.

In general, SBW contains ferulated pectin (SBP), as previously reported [7–9]. Pectins are structural polysaccharides found in the plant cell wall composed mainly of galacturonic acid (GA) units with high variations in structure, composition, and molecular weight [10]. They contribute to the firmness and structure of plant tissue, bringing mechanical resistance to cell walls. During plant and fruit growth, pectins are important in texture quality [11]. Structurally, this polysaccharide has three main domains as presented in Figure 1: homogalacturonan (HG), rhamnogalacturonan I (RG I), and rhamnogalacturonan II (RG II) [12,13].

HG is a linear region, consisting mainly of D-galacturonic acid units, linked by α-(1-4) glycosidic bonds. The *O*-2 or *O*-3 positions may be acetylated, and the carboxylic group found at the C-6 position may be partially esterified by methyl groups [13]. Based on the proportion of esterified groups distributed along the main chain, an index known as the degree of esterification (DE) has been established. Based on the percentage of DE, pectins are classified as having a high degree of esterification (HDE) and low degree of esterification (LDE); HDE refers to the ratio where more than 50% of the carboxyl groups are esterified, while less than 50% of the carboxyl groups esterified corresponds to LDE [14]. The DE of pectins largely determines the physicochemical and gelling properties of pectins.

RG I is a branched region of the polysaccharides consisting of repeating units [α-D-GalA-1,2-α-L-rhamnose-1,4] n. This region contains a significant amount of neutral sugars, such as arabinose and galactose, which together form arabinan, galactan, and arabinogalactan; attached in position *O*-4 to rhamnose, they are considered ramifications [15,16]. Besides neutral sugars, hydroxycinnamic acids, mainly ferulic acid, may be esterified to arabinans and galactans in RG I of some species from *Chenopodiaceae* [9,17]. The most-reported species containing ferulated pectin is sugar beet (*Beta vulgaris* L.) referred to as SBP. In fact, SBP has ferulic acid (FA) associated with its chemical structure [18–20]. FA groups are ester-linked with pectins mainly on the *O*-2 and *O*-5 position of arabinose residue and *O*-6 of galactose residues in side chains of RG I [9,18,19]. This compound is distributed almost equally between the arabinan and galactan components of the pectin side chains [21]. SBP can form gels driven through an oxidative coupling reaction, mediated by chemical or enzymatic oxidation, and the cross-linking is formed by a carbon- carbon covalent bond between two ferulated phenyl rings given the formation of phenoxy radicals [22]. In general, the gelling ability of pectins enables the use of pectin in the food industry as gelling agents, stabilizers, thickening agents, and emulsifiers [23,24].

RG II region is characterized by being a compound of repeating D-galacturonic acid units linked by α-(1-4) glycosidic bonds, as the base structure. This structure is replaced by L-rhamnose, D-galactose, and other neutral sugars rarely found in nature, such as apiose, 3-*O*-methyl-L-fucose, and 2-*O*-methyl-D-xilose. The RG II region is the most complex of the three structural regions that make up the pectins but is also the region with the highest degree of structural conservation among plant species [15,16,25]

In the present study, an added value product was extracted from an agro-industrial waste (SBW) subject to harsh weathering conditions. Authors hypothesize that the gelling property of SBP will not be adversely affected by the industrial pretreatment and extraction process, and ferulic acid will remain in the RG I region to allow covalent gel-forming

through an oxidative coupling reaction. In particular, FA, dimers of FA (di-FA), and trimers of FA (tri-FA) content before and after SBP gelation using laccase as a crosslinking agent were determined by high-performance liquid chromatography. Then, SBP gelation kinetics was followed by the storage (G′) and loss (G″) modulus obtained from rheological measurements. Finally, texture profile analysis and microstructural characterization of SBP gels were studied by Texture Analyzer and scanning electron microscope, respectively. Here the authors report the potential for SBW, considered regional industrial waste generated in the northwest of Mexico, specifically under Sonoran Desert conditions, as a source of ferulated pectin for the food and non-food industries.

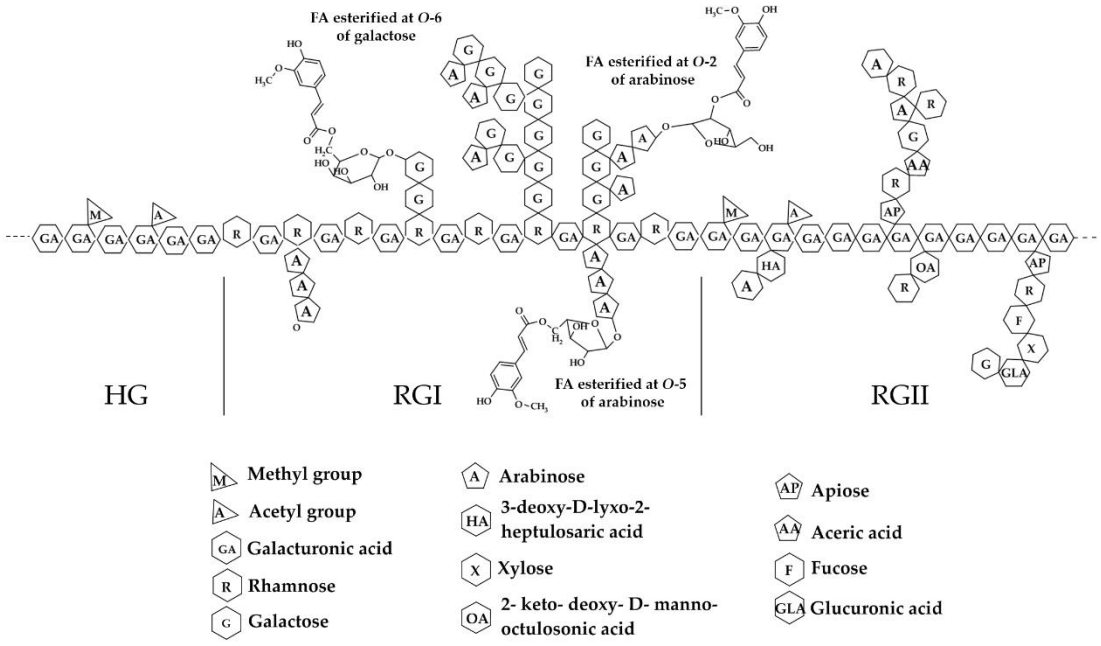

Figure 1. Schematic structure of SBP. Adapted from [25]. Reproduced with permission from Ohlmaier-Delgadillo F. et al., Molecules; published by MDPI, 2021.

2. Materials and Methods

2.1. Industrial Waste

Solid, brown powdered SBW was bought as cattle feed at an ethanol production facility located in Sonora, Mexico (Coordinates: 30°42′59″ NL, 112°08′60″ WL and 275 MASL (meters above sea level)). The sample was used directly without extra processing for pectin extraction. Extraction experiments were carried out between January and March 2019.

2.2. SBW Characterization

Ash content was determined according to the AOAC 4.1.03 (1934) method 934.01. [26]. The Kjeldahl method was used to characterize the protein content, according to the AOAC method 2.057 (1984) [27]. Fat content was determined by the Soxhlet AOAC method 996.06, and total fiber content was determined according to AOAC (1995) method [28].

2.3. Pectin Extraction and Purification

Pectin was extracted from SBW, based on the methodology reported by Li et al. [29] with some modifications. Briefly, 150 g of dried SBW was dispersed in 1.5 L of 0.1 M hydrochloric acid (1:10/*w:v*) and pH was adjusted to 1.5. The mixture was homogenized and heated on a plate with stirring at 85 °C for 2 h and allowed to cool at room temperature. Then, the mixture was centrifuged at 10,000× *g* for 15 min and 25 °C in a centrifuge (Thermo Scientific, USA) to keep the supernatant. SBP was precipitated with ethanol (96%) in a 1:2 (*v/v*) ratio. Next, pectins were centrifuged (10,000× *g* for 10 min and 25 °C) and

then dried overnight by solvent exchange (acetone). SBP was milled in a porcelain mortar until a fine powder was obtained.

The extracted SBP was purified following the procedure described by Yapo et al. [30] with some modifications. Dried pectin was dispersed in Milli-Q water (1:200 *w/w*) for 24 h with stirring; afterward, centrifuged at 10,000× *g* for 15 min and filtered through 3, 1.2, 0.8, and 0.45 µm membrane filters, sequentially. SBP was precipitated by ethanol (96%) under 1:2 (*v/v*) liquid relation and stored at 4 °C. Then, SBP was concentrated by centrifugation and dried by solvent exchange with acetone. Finally, SBP was stored in the dark at room temperature until use [30].

2.4. Pectin Yield

SBP yield was determined by the proportion of the weight of the extracted pectin after drying concerning the original weight of SBW ($g \times 100\ g^{-1}$). Recovery was calculated as follows:

$$\text{SBP yield } (\%\ w \times w^{-1}) = (m_0 \times m^{-1}) \cdot 100 \quad (1)$$

where m_0: dry SBP weight in grams; m: dry SBW weight in grams.

2.5. SBP Characterization

2.5.1. Chemical Composition

Galacturonic acid content was determined following the procedure established by Urias-Orona et al. [31] where 10 mg of SBP were hydrolyzed with 2 N trifluoroacetic acid at 120 °C for 2 h. The hydrolysis reaction was stopped on ice, and the extracts were evaporated under airflow at 40 °C, rinsed twice with 200 µL of water. The evaporated extract was solubilized in 500 µL of water. Mannitol was used as an internal standard. Samples were filtered through 0.45 µm (Whatman) and analyzed by high-performance liquid chromatography (HPLC, Varian Prostar 210 within a Refractive Index Detector Prostar 350) using a MetaCarb H Plus column (Agilent, Santa Clara, CA, USA; 7.8 × 300 mm). The mobile phase was 0.001 N sulfuric acid (H_2SO_4), and the elution rate was 0.4 $mL \cdot min^{-1}$ at 65 °C.

Sugar composition was determined according to Blakeney et al. [32] with some modifications. Accordingly, 20 mg of SBP were hydrolyzed with 3 N H_2SO_4 for 2 h at 100 °C, and derivatives were separated and quantified by gas chromatography (PerkinElmer, Clarus 580) using a high-performance capillary column (Elite 225, PerkinElmer, 30 mL × 0.32 mm ID × 0.15 µm film thickness). Neutral sugar standards were L-rhamnose (99%, Sigma-Aldrich, St. Louis, MO, USA), L-arabinose (99%, Sigma-Aldrich, St. Louis, MO, USA), D-xylose (99%, Sigma-Aldrich, St. Louis, MO, USA), D-mannose (99%, Sigma-Aldrich, St. Louis, MO, USA), D-galactose (99%, Sigma-Aldrich, St. Louis, MO, USA), and D-glucose (99%, Sigma-Aldrich, St. Louis, MO, USA). Inositol (99%, Sigma-Aldrich, St. Louis, MO, USA) was used as an internal standard. Samples were filtered through a 0.45 µm membrane prior to injection.

FA, dimers of FA (di-FA), and trimers of FA (tri-FA) content were analyzed using high-performance liquid chromatography. First, 100 mg of SBP were saponified using 2 N NaOH, later acidified with 4N HCl. Finally, they were extracted with diethyl ether. Samples were injected in an HPLC (Waters, e2695), equipped with an Alltima C18 column (Alltech, Deerfield, IL, USA; 250 × 2.6 mm), previously filtered through 0.45 µm membrane [33].

Residual soluble protein content in SBP was determined according to the Bradford method [34]; and ash, according to the AOAC 4.1.03 (2000) method 934.01 [26].

2.5.2. Macromolecular Characteristics

The weight-average molar mass (Mw), number-average molar mass (Mn), polydispersity index (PDI, $Mw \cdot Mn^{-1}$), radius of gyration (RG), hydrodynamic radius (Rh), intrinsic viscosity ([*n*], $mL \cdot g^{-1}$), and the Mark-Houwink-Saturada constants (α, K) were determined by the combination of multi-angle laser light scattering and size- exclusion chromatography using SEC- MALLS equipment (Wyatt Technology, Santa Barbara, CA, USA), following the methodology described by Yang et al. [35] with some modifications. The ASTRA 6.1 software was used. The mobile phase was sodium azide 0.02% ($w \cdot v^{-1}$) and sodium

nitrate 50 mM. The sample was prepared as follows, 5 mg of SBP were dissolved in 1 mL of the mobile phase, to ensure the dispersion, the sample was vortexed for 10 s and then, set at 80 °C for 1 h. After, the sample was tempered to room temperature and centrifuged at 15,000×·*g* for 10 min. Finally, the sample was filtered through a 0.45 µm membrane before injection. The specific refractive index increment ($dn{\cdot}dc^{-1}$) value of 0.148 $mL{\cdot}g^{-1}$ was used [36]. The characteristic ratio (C∞) and persistence length (q) were calculated as previously described [37] using the following equations:

$$q = (C\infty + 1) \times (I_0 \times 2^{-1}) \quad (2)$$

$$C\infty = 6 \times RG^2 \times (M_0 \times {I_0}^{-2}) \times Mw \quad (3)$$

where C∞ is the characteristic ratio, I_0 = 0.45 nm (length of α-D-galacturonic acid residue) [38], M_0 = 194 $g{\cdot}mol^{-1}$ (molar mass of anhydro-galacturonic acid residue) [39], and Mw the molar mass of SBP.

The degree of methylation (DM) and acetylation (DA) of SBP were determined following the technique proposed by Levigne et al. [40]. Briefly, methanol and acetic acid were produced by saponification of pectin with 1 M NaOH at 4 °C for 2 h. Isopropanol was used as an internal standard. Samples were centrifuged for 10 min at 8000× *g* and 25 °C. Also, supernatants were neutralized through a Maxiclean IC-H device (S*Pure, Singapore) before injection. Methanol, acetic acid, and isopropanol were quantified by HPLC Varian 500 within a Refractive Index Detector on a C18 column (Superspher 100 RP-18 endcapped, Merk KGaA, 250 × 4 mm). Elution was carried out with 4 mM H_2SO_4 at 0.7 $mL{\cdot}min^{-1}$ and 25 °C [40].

2.5.3. Infrared Spectroscopic Analysis

Fourier Transform-Infrared Spectroscopy (FTIR) analysis was performed on a NICOLET IS-50 spectrometer (ThermoScientific ™, Waltham, MA, USA) with an attenuated refractive detector. Spectrum scan was achieved from 4000 cm^{-1} to 400 cm^{-1}. Samples were dried before being processed and absorbance vs. wavelength was reported in graphs [41]. FTIR spectrums of SBP and low methylated commercial citrus pectin (Grinsted® pectin LC950 Danisco) were analyzed and used as the control.

2.6. SBP Gel Preparation

SBP gels were prepared using SBP dispersion at 4% (*w/v*) in 0.1 M sodium acetate buffer, pH = 5.5, at 25 °C, as previously described by Ohlmaier-Delgadillo F. et al. [25]. Laccase from *Trametes versicolor* (Sigma-Aldrich, St. Louis, MO, USA) 24 $units{\cdot}mg^{-1}$ FA was dispersed in sodium acetate buffer (pH = 5.5) and added to SBP as cross-linking agent. The gelling process was set to 80 min at 25 °C.

2.7. FA, di-FA, and tri-FA Content of SBP Gel

Ferulic acid (FA), di-FA, and tri-FA contents of SBP gels were analyzed using high-performance liquid chromatography after (80 min) laccase exposure as previously described. In the saponification step, 50 mg of SBP gel were used [42].

2.8. Rheological Measurements

The formation of SBP gel was followed using a low deformation strain-controlled rheometer (Discovery HR-2 rheometer, TA instruments) in oscillatory mode, as reported previously [43]. Cold (4 °C) solutions of 4% (*w/v*) SBP were mixed with laccase (24 $units{\cdot}mg^{-1}$ FA) and immediately placed in the cone and plate geometry (5.0 cm in diameter, 0.04 rad in cone angle) maintained at 4 °C. SBP gelation kinetics were monitored at 25 °C for 80 min by following the storage (G′) and loss (G″) modulus. All measurements were carried out at a frequency of 0.25 Hz and 5% strain (linearity range of viscoelastic behavior). The mechanical behavior of SBP gel was obtained by frequency (0.01 Hz to 10 Hz, 5% strain) and strain (1% to 10%, 0.25 Hz) sweep at the end of the network formation at 25 °C.

2.9. Texture Profile Analysis

Additionally, 6 mL of 4% (*w/v*) SBP gels were fresh and directly prepared in a 50 mL glass flask with 55 mm height and 35 mm of internal diameter. Texture profile analysis (TPA) was made in a TA.XTA Texture Analyzer (Texture Analyzer Stable Micro Systems, Surrey, UK) within a cylindrical plunger (diameter 25 mm). SBP gels were deformed by compression at a constant speed of 1.0 mm·s^{-1} to 4 mm from the gel surface. Eight parameters were used to describe texture: hardness, fracturability, adhesiveness, springiness, cohesiveness, gumminess, chewiness, and resilience [31,43].

2.10. Scanning Electron Microscopy

Surface characterization of SBP gels was performed using a scanning electron microscope (SEM) JEOL54 10LV equipped with an INCA dispersive X-ray detector system (Oxford Instruments) and operated at a voltage of 20 kV. Samples were flash-frozen with liquid nitrogen and then lyophilized, later coated with gold before being observed under a high vacuum using the secondary electron detector according to Morales-Burgos et al. [44]. The average porous diameter was determined from SEM images using ImageJ software.

2.11. Statistical Analysis

All measurements were performed by triplicate, and the coefficients of variation were lower than 5%. Texture measurements were done six times, and the coefficients of variation were lower than 10%. All results were expressed as mean values and standard deviation, expressing descriptive statistics. To determine significant differences between the respective values, a one-way analysis of variance (ANOVA) was performed, and averages were compared using the Tuckey–Kramer test with a $p \leq 0.05$. The statistic software NCSS 12 was used.

3. Results and Discussion

3.1. SBW Characterization

Table 1 shows the constituents of SBW on a dry matter basis. The recovery percentage of SBP from waste was 4.4 ± 0.1% (w SBP· w^{-1} SBPW). This value is not only higher than those reported by Müller et al. [45] for two different SBWs (2.8–3.2%); but also lower than those reported by Phatak et al. [46] for SBP (19.3–24.7%), who studied the effect of changing extraction parameters conditions, as hydrolysis solution, pH, temperature and hydrolysis time [46]. Several authors agree that the extraction conditions have important effects on the quantity, as well as the quality of the extracted pectins. Among the main parameters, we find the temperature, pH, extraction time, and the nature of the acid [47]. Additionally, SBW studied in this work is stored outdoor with no protection against weather conditions, reaching up to 45 °C and 10 UV index in summertime. Plus, previous industrial processes of SBW might affect pectin content and extraction yield as well. SBW was found with low fiber content, representing 44 ± 3%. Low fiber content is desired to get higher yields of pectin extraction [45]. As revealed by Zieminski et al. [48], SBW with high amounts of fiber (close to 85%) affects biogas yield production in an industrial approach. Protein content in SBW was 9.5 ± 0.3% dw (dried weight), a value comparable to some previously reported for similar materials in research papers, 8.2% [49], 11.3% [50], and 10.3% [48]. SBW is usually used as an ovine feed due to its great amount of proteins, and in Mexico, this represents the main economical usage of SBW [2,3]. Fat content for SBW was 0.67%, and this result is also compared to 0.9% reported for Turquois et al. [49], who defined the waste as a low-fat waste. Ash content represents 12.2% of the total weight; this value is quite comparable to the value reported by Turquois et al. [41], for a raw SBW, corresponding to 13.3%, but highly contrasting to 1.2% reported by Ziemiński et al. [48] for a raw SBW used for biogas production. It is well documented that the physical and chemical properties of SBW present variations, among others, caused mainly by industrial processing conditions [46,49,50]. As early discussed, in Mexico, SBW is used for ovine feed with no extra usage; however, pectin extraction from SBW represented an alternative usage of industrial solids with important

pectin yield (4.4%); furthermore, SBW recovered after pectin extraction could be used as ovine feed with some process adaptations, but further research is needed to test such an argument.

Table 1. Pectin yield and SBW composition (dry matter basis).

Content	% (*w*/*w*)
Pectin yield	4.4 ± 0.1
Fiber	44 ± 3
Protein	9.5 ± 0.3
Fat	0.67 ± 0.02
Ash	12.2 ± 0.2

Values are presented as means ± standard deviations of triplicates (n = 3).

3.2. SBP Characterization

3.2.1. Chemical Composition

The chemical composition of SBP is presented in Table 2. Galacturonic acid content was found to be 52.2% of SBP´s total monomers. Our value is higher than 46.5% reported by Turquois et al. [49] who obtained pectins from a similar industrial waste and under comparable extraction conditions. Furthermore, Pi et al. [51] extracted SBP with 66.2% of GalA content, higher than GalA content found in this work. It seems that not only industrial process conditions but also sugar yield (in our case) are relevant to determine pectin characteristics. As mentioned above, differences in industrial processes might induce changes in the chemical structure of pectins extracted, such as galacturonic acid content.

Table 2. Chemical composition of SBP.

Component	Value
Galacturonic acid §	52.2 ± 1.6
Rhamnose §	1.50 ± 0.02
Arabinose §	3.60 ± 0.04
Xylose §	1.20 ± 0.02
Mannose §	5.0 ± 0.04
Galactose §	20.7 ± 0.4
Glucose §	12.3 ± 0.2
Ferulic acid ¶	2.1 ± 0.1
Ferulic acid dimers ¶	0.22 ± 0.02
8-5′ ¶	0.060 ± 0.003
8-5′benzo ¶	0.030 ± 0.003
8-*O*-4′ ¶	0.09 ± 0.01
5-5′ ¶	0.040 ± 0.004
Protein §	2.4 ± 0.1
Ash §	1.0 ± 0.1

Values are presented as means ± standard deviations (n = 3). § Results are expressed g·100 g^{-1} SBP dry matter. ¶ Phenolics are expressed in mg·g^{-1} SBP dry matter.

The total sugar content of SBP is also shown in Table 2. As presented, arabinose, mannose, and galactose are the main neutral sugars found in SBP. Additionally, some glucose was found in the sample. The more representative neutral sugar amount was galactose with 20.7%, followed by mannose (5%), arabinose (3.6%), rhamnose (1.5%), and xylose (1.2%). As discussed previously, arabinose, and galactose are crucial neutral sugars where FA is commonly found esterified to rhamnose in RG I structural region for SBP. Neutral sugar content for our SBP differed from some SBP reported previously, Phatak et al. [46] found lesser amounts of arabinose, galactose, and rhamnose; 1.6, 5.7, and 0.94%, respectively. In contrast, Turquois et al. [49] found a higher percentage of arabinose (9.6%), less galactose (3.1%), and less rhamnose (0.7%) for their SBP. In recent work, Pi et al. [51] found less content of galactose (7.1%), and a higher amount of arabinose (5%)

and rhamnose (3.6%). Based on information previously presented authors aimed that neutral sugar content in SBP is affected not only by industrial processing and the pectin extraction process but also by waste storage. Particularly, the SBW used in this study is stored outdoors without extra protection from the climate and temperature changes, as a result, this could deteriorate polysaccharide chemical structure, obtaining less neutral sugar content as observed in rhamnose content, which is related to RG I degradation. As a counterpart, we can infer that the glucose content of SBP can be related to galacturonic acid percentage. While low glucose content is related to high industrial yield and high glucose content, to low industrial yield; sugar beet undergoing aggressive chemical treatment for high glucose yield can result in low glucose SBW with low galacturonic acid percentage SBP content as observed by Chen et al. [4] and Turquois et al. [49]. In contrast, SBP in this study showed both high glucose and high galacturonic acid percentage content, which suggests the industrial ethanol process is an opportunity for optimization.

FA content is also shown in Table 2. As presented, our SBP had an FA content of 2.1 $mg \cdot g^{-1}$ of pectin. Certainly, the FA content is higher than some values reported previously for a comparable SBP: 1.9 $mg \cdot g^{-1}$ of pectin reported by Chen et al. [4], 0.68 $mg \cdot g^{-1}$ of pectin reported by Phatak et al. [46], 0.28 $mg \cdot g^{-1}$ of pectin reported by Pi et al. [51], and 0.38 $mg \cdot g^{-1}$ of pectin reported by Pacheco et al. [52]. This fact may be correlated with the low content of neutral sugars in those studies, which correlated with the lower amount of FA in SBP. All these references, determined less galactose and arabinose neutral sugar, as compared to SBP reported in this work. As previously described, FA groups are ester-linked with pectins mainly on the *O*-2 and *O*-5 position of arabinose residue and *O*-6 of galactose residues in side chains of RG I [9,18,19]. Furthermore, our research group in a recent study [41] observed that FA content in pectin extracted from three fresh sugar beet (*Beta vulgaris* L.) cultivars grown under similar desertic conditions, geographic location, and extraction conditions to the SBW used in this study, were higher than 2.1 $mg \cdot g^{-1}$ of pectin (FA_1 = 3.5, FA_2 = 4.7, and FA_3 = 5.5 $mg \cdot g^{-1}$ of pectin); thus, the chemical composition of the vegetable matrix was modified during the bioethanol production process and possibly due to the outdoor storage conditions of SBW. By changing the storage conditions of SBW to indoor and more controlled conditions, the FA content of SBP could be made higher, but more studies to confirm this idea must be conducted.

The total feruloyl dimers content is also presented in Table 2. The values range between 0.03 to 0.09 $mg \cdot g^{-1}$ of pectin. From the total dimers of FA studied, the 8-5´, 8-5´benzo, 8-*O*-4´, and 5-5´ dimers were detected in SBP, with the dimers 8-*O*-4´ and 8-5´ being most prevalent with about 40.9% and 27.3% of the total dimers of FA, respectively. In addition, minor percentages of 5-5´and 8-5´benzo dimers of FA were also detected, with approximately 18.2% and 13.6%, respectively. It is well documented that dimers 8-5´ and 8-*O*-4´ are the major dimers formed after oxidation reactions of FA monomers, in sugar beet pectins [21,53].

Protein content in SBP was found lower than various values found in the literature, 6% [49], 10.4% [52], and 6.7% [51]. Certainly, protein can enhance the emulsifier properties of pectins.

3.2.2. Macromolecular Characteristics

The macromolecular characteristics of SBP are presented in Table 3. The Mw of SBP was 459 kDa; which is superior to other values reported in the literature. Phatak et al. [46] found Mw values of 35 kDa, 44.7 kDa, and 39.8 kDa for pectins extracted from a similar industrial waste sample. Also, Yapo et al. [30] reported an Mw rounding of 90 kDa for a similar pectin. The higher Mw registered for SBP in the present study can be related not only to SBW characteristics but also to extraction conditions. The Mn of SBP was 94 kDa, the relationship between Mw and Mn brings up the PDI value, which is a measure of the width of molecular weight distribution. The PDI value for SBP was 4.9. A high PDI value indicates the presence of high Mw fractions; the larger the PDI, the broader the molecular weight [54]. In addition, the RG, Rh, and [η] of SBP were 45 nm, 14.3 nm, and

77.6 mL·g^{-1}, respectively. Fishman et al. [55] studied the conformational structure of SBP extracted from fresh sugar beet root by microwave-assisted flash-extraction under various conditions (temperature, pressure, and time). In their work, RG and [η] values ranged from 35 nm to 51 nm and 300 mL·g^{-1} to 430 mL·g^{-1}, respectively. The RG value obtained in the present study, corresponding to 45 nm, matched in the range reported by Fishman et al., however [η] is lower. A low [η] value can describe fairly rigid molecular chains as in this case [56]. Some other polysaccharides have presented a low [η] caused by its highly branched structure, similarly to SBPs, which are known for having highly branched structural regions (RG I and RG II) [57]. In a recent study, Hotchkiss et al. [36] reported the macromolecular characteristics of blueberry pomace pectin obtained by microwave-assisted extraction under two process conditions (condition 1: pH = 2, 3 min, 80 °C; condition 2: pH= 1, 10 min, 120 °C). In their study, RG, Rh, and [η] values ranged from 40 nm to 48.9 nm, 52 nm to 63.3 nm, and 37 mL·g^{-1} to 280 mL·g^{-1}, respectively. As observed, RG and [η] found in the present study are similar to those reported by Hotchkiss et al. however, Rh is lower. From the previous information, we can infer that macromolecular characteristics of pectins will change based on vegetable or row material source and extraction process and conditions. For instance, pH (acid medium) and temperature (80 °C) of extraction conditions reported by Hotchkiss et al. were similar to our research work, yet extraction time and extraction process were distinct. As a consequence of the above-mentioned similarities in extraction conditions, RG and [η] values could be comparable. Furthermore, C∞ and q values were calculated for SBP. The persistence length represents the average projection of the end-to-end distance vector and indicates the distance through which the longitudinal axis of a chain can be considered as linear, therefore giving information on the chain stiffness described by characteristic ratio [58]. The values calculated for q and C∞ were 5.9 nm and 25.4 (dimensionless unit), respectively. These values are similar to those reported by Cros et el. [58], q = 6 nm and C∞ = 57 (dimensionless unit) for citric pectin with a similar degree of methylation (40%) to the SBP studied in the present work. In particular, both q values are too close, indicating that pectin chain flexibility is similar and higher than other citrus pectins with a lower degree of methylation (28%) with q = 7.5 nm, as studied by Cross et al. In addition, Cros et al. expressed that C∞ = 57 (dimensionless unit) describes an extended polysaccharide chain, this value is quite a bit higher than C∞ = 25.4 (dimensionless unit) as determined in the present work, consequently, we can assume that SBP chains are extended. High C∞ values indicate "unperturbed" chains; therefore, HG chains adopt extended conformations having a low probabilities of folding, a feature that may be largely attributed to the axial-axial glycosidic linkage [58]. SBP Mark–Houwink–Sakurada constants were obtained, α = 0.45 and K = 0.32. Values of α and K can be used to study the molecular conformation of SBP in sodium nitrate (50 mM) dispersion. It has been established that the exponent α usually lies in the range of 0.5–0.8 for linear random coil polysaccharides and increased with increasing chain stiffness [59]. The constant α is an indicator of interaction between polysaccharide and solvent, a low α value indicates a poor solvent, and a high α value indicates a good solvent. In our case, SBP presented a value lower than 0.5, indicating that sodium nitrate was probably not the best solvent option for SBP, and a sodium chloride solution can be a good alternative to increase the α value as observed in previous works [59–62]. In contrast, the K value obtained in this study was higher compared to some others obtained using a sodium chloride solution as pectin solvent such as K = 0.0234 [59]. High K values describe a polysaccharide with expanded coil conformation [56].

Table 3. Macromolecular characteristics of SBP.

Component	Value
Mw (kDa)	459 ± 3
Mn (kDa)	94 ± 6
PDI (Mw·Mn^{-1})	4.9 ± 0.3
RG (nm)	45 ± 5
Rh (nm)	14.3 ± 1.5
[η] (mL·g^{-1})	77.6 ± 0.1
C∞	25.4
q (nm)	5.9
Mark-Houwink-Sakurada α	0.45 ± 0.01
Mark-Houwink-Sakurada K	0.32 ± 0.04

Mw, weight-average molar mass. Mn, number-average molar mass. PDI, polydispersity index. RG, radius of gyration. Rh, hydrodynamic radius. [η], intrinsic viscosity. C∞, characteristic ratio. q, persistence length.

As a counterpart, the degrees of methylation and acetylation of SBP were 30% and 13%, respectively. Therefore, SBP is classified as a low methylated and low acetylated pectin. In a previous report, Turquois et al. [49] stated that extraction conditions were the main aspects leading to the chemical composition of extracted pectins. Accordingly, the authors, low methylated and acetylated pectins are obtained only after alkaline extraction. Hence, a high degree of methylation and acetylation is caused by acidic conditions. Interestingly, this work shows some other factors affecting the chemical composition of extracted pectins, mainly obtained from the industrial waste matrix, as previous treatments during industrial process and storage conditions (indoor, outdoor). As revealed by Yapo et al. [30], it is possible to extract low methylated (14.4%) and acetylated (3.1%) SBP from SBW under acidic conditions, under similar conditions. Also, Gómez et al. [63], and Pi et al. [51] recovered low methylation and low acetylation degree pectins from similar SBW. In recent work, Lara-Espinoza et al. [41] observed that pectin extracted from three fresh sugar beet (*Beta vulgaris* L.) cultivars grown under similar desertic conditions, geographic location, and extraction conditions than SBW used in this study have a high degree of methylation (up to 50%), supporting the fact that previous industrial processes suffering through vegetal matrix and storage condition will affect pectin characteristics extracted from SBW. The degree of methylation was also confirmed by estimation, analyzing the FT-IR spectrums of SBP based on Urias-Orona et al. [31]. The degree of methylation was 37%, supporting the result obtained by HPLC. On the other hand, low methylated and ferulated pectin may combine two gelation mechanisms enabling interesting new applications.

3.3. Infrared Spectroscopic Analysis

Figure 2 shows the FT-IR spectrum of (a) SBP and (b) low methylated commercial citrus pectin (CCP). Chemical and structural information was collected from spectra and some differences were highlighted. Wide absorption bands at 3343 cm^{-1} and 3245 cm^{-1}, corresponding to stretching vibration of hydrogen-bonded O-H groups of galacturonic acid, are presented for SBP and CCP, respectively. In addition, a characteristic stretching signal of O-CH_3 from methyl esters of galacturonic acids is found to be proximally 2920 cm^{-1} for SBP and 2946 cm^{-1} for CCP. Other common signals found in pectin spectra are those related to the degree of methylation; regularly, both signals are located side by side proximally to 1700 and 1600 cm^{-1}. In Figure 2, bands at 1721 cm^{-1} (Figure 2a) and 1735 cm^{-1} (Figure 2b) are associated with the vibrations of esterified carboxyl groups or ester carbonyl (C=O). Besides this, bands located at 1600 cm^{-1} (Figure 2a) and (Figure 2b) 1598 cm^{-1} correspond to free carboxyl groups or carboxylate ion (COO^-) [3,64]. Stretching vibrations commonly observed on second derivative spectra (inserted in (Figure 2a) at 1520 cm^{-1} (C-C aromatic ring) and 1049 cm^{-1} (-OCH_3) are assigned to FA content. The region from 700 to 1500 cm^{-1} is the fingerprinting region for pectins [1,65]. Within this region, a small signal at 1410 cm^{-1} (Figures 2a and 3b) can be attributed to the symmetric stretching vibrations of the COO^- functional group of amino acid side chains, free fatty acids, or other derivative

compounds carried as an effect of the extraction process [1]. An absorption band located at 1232 cm^{-1} is assigned to the C-CO bending vibration of pectin polysaccharides. C-O-C vibration of glycosidic linkages of the pectin backbone structure is observed at 1136 cm^{-1} (Figure 2a) and 1151 (Figure 2b) cm^{-1}. An important band located at 1001 cm^{-1} (Figure 2a) and 1014 cm^{-1} (Figure 2b) are attributable to C-O stretching of side groups in C-O-C glycosidic linkages. In addition, an absorption band characteristic of α-linkage in pectin polysaccharides is identified by the weak peak at 811 cm^{-1} in the anomeric region of the spectra (950–750 cm^{-1}) [1,3,66].

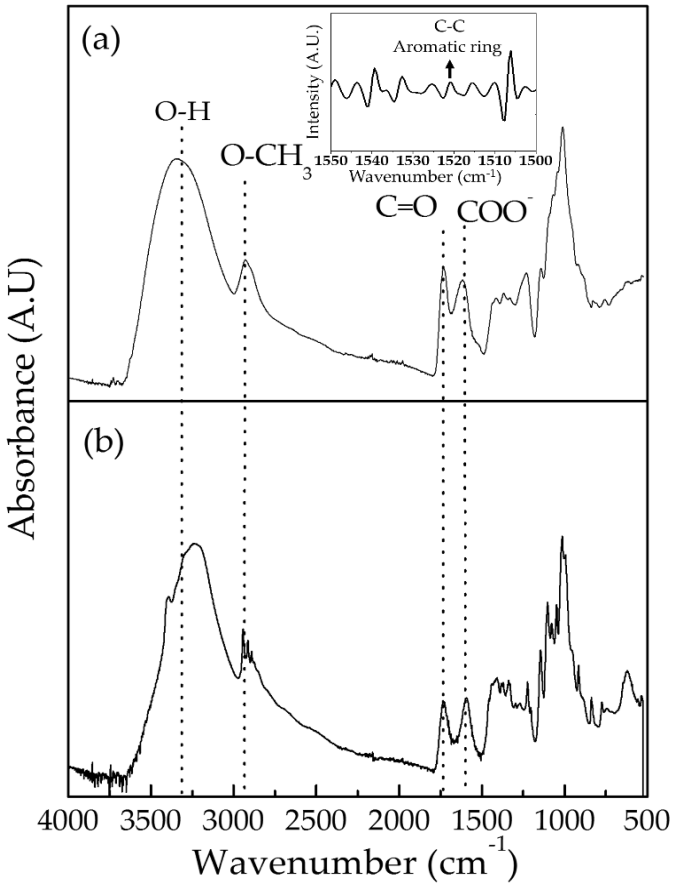

Figure 2. FT–IR spectra for SBP (**a**) and commercial citrus pectin (**b**).

3.4. SBP Gels

3.4.1. Covalent Cross-Linking

FA, di-FA, and tri-FA were detected in SBP gels induced by laccase as a cross-linking agent. FA dimerization promoted the formation of three di-FA isomers (8,5′, 8-*O*-4′ and 5,5′) and one tri-FA (4-*O*-8′,5′-5″-dehydrotriferulic acid) in SBP gels, commonly found in pectin gels [25,42]. Table 4 shows FA, di-FA, and tri-FA contents in SBP dispersion at 0 and 80 min of laccase action. After SBP gelation, 53% of the initial FA content was oxidized resulting in the formation of di-FA and tri-FA participating in the 3D network development. The 8-5′, 5-5′, and 8-*O*-4′ structures represented 75, 17, and 8% of the total di-FA in the SBP gel, respectively. The same FA dehydrodimers were found in SBP before gelation, however, the major dehydrodimers found in SBP before gelation were 8-*O*-4′ with approximately 40.9%, 8,5′ with about 27.3%, and 5-5′with 18.2% of the total di-FA. Notably, the thermodynamic stability of 8-5′ dehydrodimer contributed to increasing this di-FA after the gelation step using laccase as an enzymatic free radical generating agent [66]. The increase in the 8-5′di-FA isoform is quite relevant for the potential application of SBP gels in the food industry since scientific evidence has demonstrated the contribution

of this isoform to major elasticity of ferulated polysaccharide hydrogels as pectins and arabinoxylans, caused by crosslinking between polysaccharide chains [9,41,44,66,67].

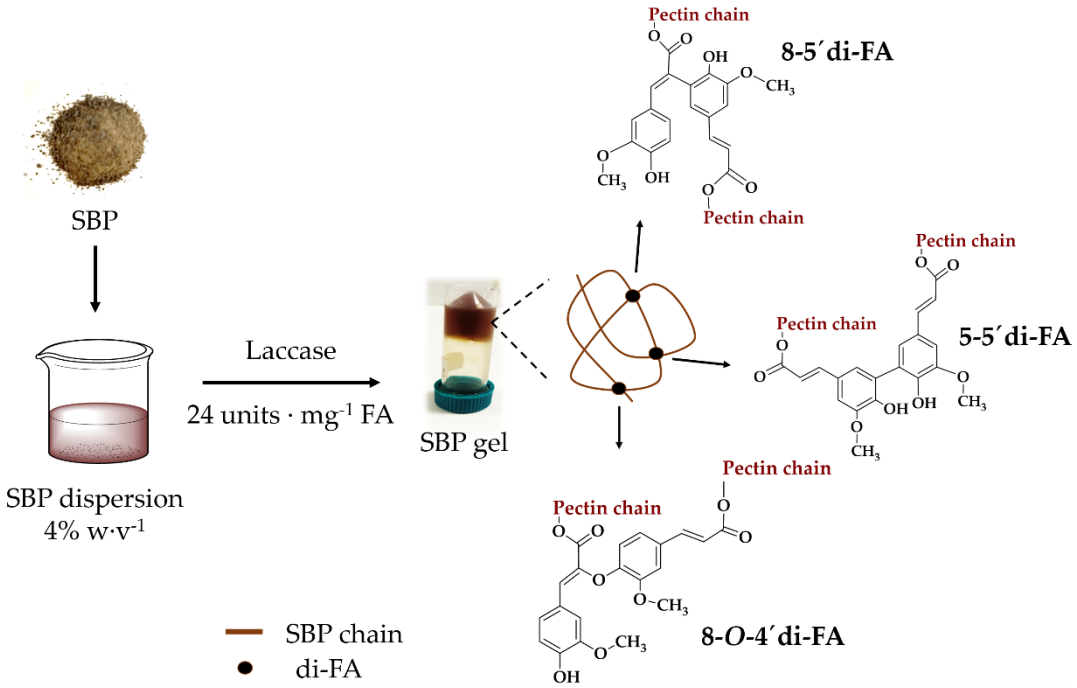

Figure 3. Schematic steps to SBP gel preparation and di-FA isomers found in SBP gel.

Table 4. Ferulic acid (FA), dimers of FA (di-FA), and trimer of FA (tri-FA) contents in SBP dispersion before (0 min) and after (80 min) enzymatic gelation.

Time	FA	di-FA	tri-FA
(min)	(mg·g^{-1} SBP Dry Matter)		
0	2.1 ± 0.1	0.22 ± 0.02	nd
80	0.97 ± 0.02	0.36 ± 0.03	0.13 ± 0.01

Values are presented as means ± standard deviations (n = 3). nd = non detected.

SBP gel preparation steps and di-FA isomers found in SBP gels after laccase action are schematized in Figure 3.

3.4.2. Rheology

The gelation of SBP was studied by dynamic mode rheological analysis. Figure 4a shows the evolution of storage (G′) and loss (G″) moduli as a function of time for 4% (*w/v*) SBP exposed to laccase. As observed, values increased rapidly in the first 10 min for both moduli, G′ and G″, indicating the ability of SBP gel to resist deformation during measurement evolution, in other words, the increase of elasticity. The elasticity of SBP gel increased over time caused by the formation of new covalent bonds between FA residues found in SBP chemical structure and laccase action. In fact, FA dehydrodimers are formed between contiguous polysaccharide chains leading to gel formation.

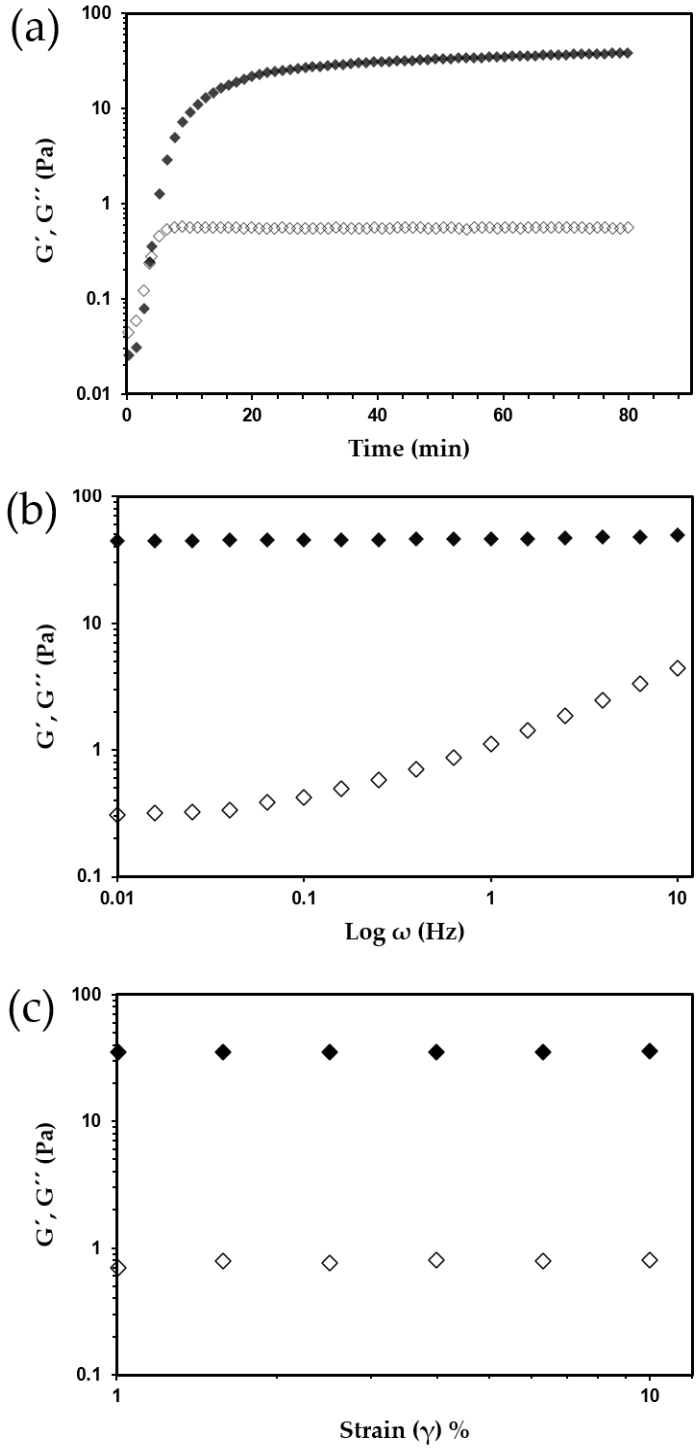

Figure 4. (**a**) Rheological kinetics of 4% (*w/v*) SBP during laccase induced cross-linking; (**b**) Mechanical spectrum and (**c**) Strain sweep of 4% (*w/v*) SBP gel. Gelation was induced with laccase (24 units·mg^{-1} FA), G′♦, G″ ◊.

After the first 10 min, the evolution process of G′ and G″ becomes slower. As a result, asymptotic behavior was reached and SBP gel was completely formed. G′ and G″ values were 44 and 0.6 Pa, respectively. In addition, gelation time was determined by the cross-over among G′ and G″ (G′ = G″), where G′ reaches higher values than G″; in this study, it was observed at ~5 min.

Tan δ = 0.014 ($G'' \cdot G'^{-1}$) [66] defines the overall behavior of SBP gel, predominating the elasticity than viscosity in the new covalently tridimensional network This behavior has also been observed in arabinoxylans gels [66,68] and previous ferulated SBP gels [69].

The mechanical spectrum (Figure 4b) and strain sweep (Figure 4c) of 4% (*w/v*) SBP gel after 80 min of laccase exposure were typical of solid-like materials with a linear G′ independent of frequency and strain, also G″ smaller than G′ and dependent of frequency [70].

The rheological analysis demonstrates gel formation, because of dehydrodimer formation caused by the oxidative coupling of FA triggered by laccase action, the was affected SBP while being extracted. No pretreatment was required to gel.

3.4.3. Texture Profile Analysis

Table 5 presents the texture profile analysis (TPA) for SBP gels formed by laccase action as an oxidative crosslinking agent. TPA results are correlated with previous texture profiles retrieved from SBP hydrogels constituted by pectins directly collected from the vegetable (sugar beet) due to limited information found in the literature regarding SBP extracted from SBW.

Table 5. Texture profile analysis of SBP gels.

Index	Value
Hardness (N)	3.0 ± 0.1
Fracturability (N)	1.6 ± 0.2
Adhesiveness (N)	1.3 ± 0.2
Springiness (mm)	0.98 ± 0.02
Cohesiveness (%)	0.32 ± 0.04
Gumminess (N)	1.0 ± 0.2
Chewiness (mJ)	1.0 ± 0.2

Values are presented as means ± standard deviations (n = 3).

Hardness is defined as the force required to compress 10% of gel. Then, for SBP gels hardness was 3.0 N. Norsker et al. [71] reported 11.2 and 10 N as hardness values for sugar beet pectin gels, meaning that our SBP gel is more sensitive to compression, thus softer. Additionally, Chen et al. [72] reported 1.99 N for hardness; actually, this parameter is highly related to morphological aspects; as a result, the three-dimensional morphology of SBP gels had a higher value of hardness compared to non-three-dimensional structures [72].

As mentioned by Norsker et al. [71] fracturability is highly related to hardness value, and even though neither Norsker et al. [71] nor Chen et al. [72] reported this value. In this study, fracturability was 1.6 N representing the force needed for the gel to crack, related to SBP chain-breaking caused by the applied force.

Our adhesiveness values (1.3 N) were also higher than those reported by Chen et al. [72], probably porosity contributes to this parameter to some extent. On the other hand, springiness for our gel was lower than the reported value by Chen et al. [72]. Springiness is the distance recording to compress 10% of the total mass, therefore the higher the hardness, the less the springiness.

Cohesiveness (0.32%), gumminess (1.0 N), and chewiness (1.0 mJ) values were similar comparing SBP gels and gels reported on Chen et al. [72]. On the other hand, gumminess is a property to be cohesive and sticky, defined as the energy required to disintegrate a semisolid food to a state of readiness for swallowing [73]. On the other hand, chewiness is defined as the energy required to masticate a solid food product [72,73]. At this point, morphological characteristics of the gel appear not to affect either of these textural parameters drastically.

3.4.4. Morphological Analysis

A three-dimensional network of SBP gels (4% *w/v*) were prepared through covalent cross-linking of FA upon oxidation by enzymatic free radical-generating agents. Therefore, the microstructure and network structure of gels are studied in various magnifications and

multiple images. Figure 5a shows a 200 magnification image of SBP gel and evidence of the presence of cavities and irregular surfaces. Also, at this magnification, we can assume that cavity shapes are well defined. In the same way, from Figure 5b we can ensure the presence of cavities in SBP gels. At 500 magnification, there is evidence for the presence of thin walls. This fact implies that the oxidative gelation process initiated by laccase promotes an organized cross-linking process. Figure 5c shows the organization of cavities, in an imperfect honeycomb-like structure.

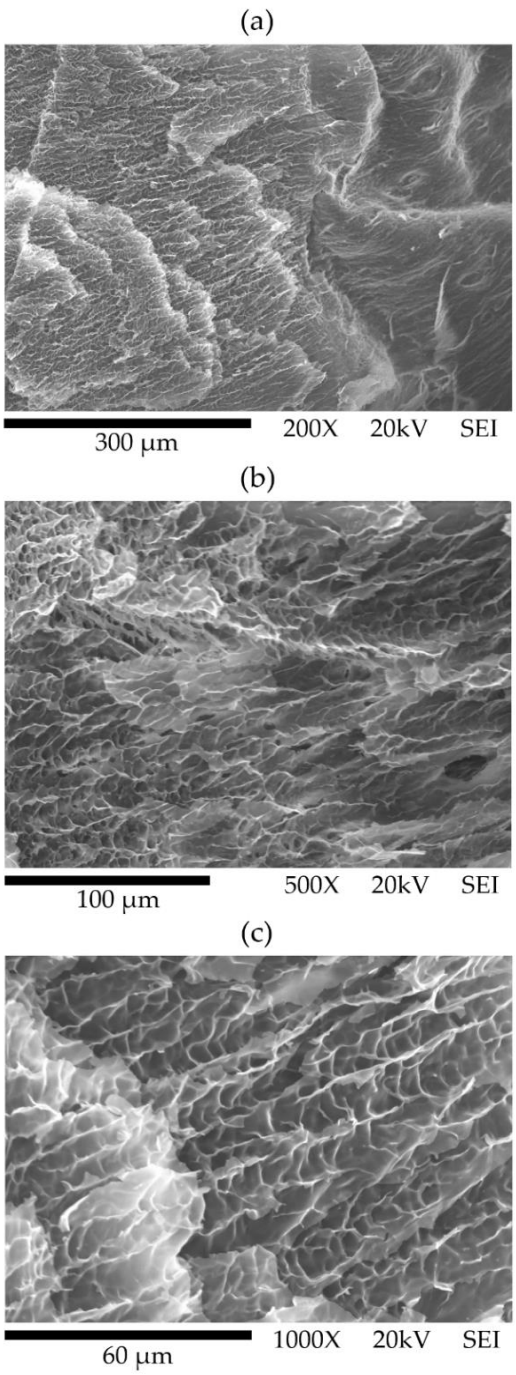

Figure 5. SEM micrographs of SBP gel at various magnifications (**a**) 200X magnification, (**b**) 500X magnification, and (**c**) 1000X magnification.

As far as the authors reviewed, this is the first report on morphological analysis for hydrogels composed of SBP extracted from SBW. However, recently some studies presented

SEM micrographs of hydrogels composed of pectins extracted directly from whole sugar beet vegetables. Certainly, the microstructure observed for SBP gels in this work contrasts those previously reported. To show that, Chen et al. [72] described their sugar beet pectin gels as non-three-dimensional structures, almost a solid filled gel with sphere-like pores. Considering the chemical composition of pectins and some critical aspects for gelling properties like FA content, polysaccharide molecular weight, and enzyme as a gelling agent are different, morphological characteristics of the resulting gel reflect them accurately.

As expected, the industrial processes used in the food industry modify the chemical and physicochemical composition of the organic matter used to produce food products. However, the foregoing does not limit the possibility of finding a utility for agro-industrial waste obtained after industrial processes, as has been shown in this work. The chemical and physicochemical characteristics of SBP reported in our research are comparable to SBPs extracted directly from the plant, which have been previously reported. Therefore, we have demonstrated that SBPs extracted from an agro-industrial residue have not lost the ability to form covalent gels by oxidative coupling reaction of FA triggered by laccase action. Even more important, the previous industrial processes undergone by sugar beet waste did not alter the typical imperfect honeycomb microstructure of SBP covalent gels due to laccase action. Hence, a comparable texture profile of SBP gels is maintained according to SBP gels previously reported in the literature. The fact that the SBP gels reported in this research maintained a three-dimensional structure opens a range of possibilities of potential applications for this polysaccharide, including use in the food industry as a protection and entrapment matrix for nutraceutical products, as well in the design of controlled release systems for drugs and biomolecules in the biomedicine and pharmaceutical areas.

4. Conclusions

SBP from the industrial waste of sugar beet bioethanol production was extracted successfully. SBP compositional and macromolecular characteristics were in the range reported for other ferulated pectins in the literature. Particularly, macromolecular characterization of SBP described a flexible and extended coil conformation with low probabilities of folding. SBP form 4% (*w/v*) covalent gels with G′ and G″ values of 44 and 0.66 Pa, respectively. As described above, SBPs extracted from an agro-industrial residue have not lost the ability to form covalent gels by oxidative coupling reaction of ferulic acid triggered by laccase action. Texture profile analysis exhibits a soft texture for SBP gels. Lyophilized SBP gels present an imperfect honeycomb-like structure with an average cavity diameter of 4.5 μm. SBP could be applied as a food additive to texturize or stabilize food products, such as jams and gummies, where soft food products are desired. Covalently cross-linked SBP gel could be an attractive food additive due to its stability to changes in temperature and pH.

Author Contributions: F.O.-D. performed the experiments and writing the paper. A.R.-C. conceived and designed the experiments, contributed to the discussion of the results, and edited the paper. E.C.-M. help in the design of the experiments and contributed to the discussion of the results. Y.L.L.-F. and M.A.I.-O. analyzed the data and contributed to the discussion of the results. C.L.-E. contributed to the discussion of results. J.A.M.-E. and J.A.S.-V. help in the design of experiments. All authors have read and agreed to the published version of the manuscript.

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