**Ethanol precipitation of sugar beet pectins as affected by electrostatic
interactions between counter ions and pectin chains**

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1 **Abstract:** The ethanol precipitation of sugar beet pectins (SBPs) with and without
2 counter ions was investigated. In aqueous solution, when cations bind to SBPs, the
3 negative charges on the pectin chains are reduced and the hydrodynamic radius (R_h)
4 of the whole polymer is decreased. Cation-bound SBP is more prone to precipitation
5 by ethanol because it is less solvated by water molecules. In the absence of cations,
6 partially-ionized SBP interacts extensively with water molecules at the initial
7 precipitation pH (I-pH) range of 3.26–2.83, which makes it difficult to precipitate the
8 pectins by addition of ethanol. However, in the presence of cations, precipitation of
9 SBPs is straightforward due to cation binding and ethanol dehydration. Protonated
10 SBPs and SBP salts were prepared and their conformational features were analyzed by
11 ^{13}C solid-state NMR and X-ray diffraction spectroscopies. The results show that no
12 conformational transition occurs in cation-bound SBP after ethanol precipitation,
13 which indicates that the electrostatic nature of the cation–SBP interaction is important
14 during the precipitation process. A schematic illustration of the cation–SBP
15 electrostatic interaction during the precipitation process is proposed.

16 **Keywords:** Sugar beet pectins; Ethanol precipitation; Electrostatic interactions;
17 Cation.

1. Introduction

Pectins are a family of heterogeneous polysaccharides that are comprised of three types of structural regions: a homogalacturonan (HG), a type I rhamnogalacturonan (RG-I) and a type II rhamnogalacturonan (RG-II) (Voragen, Coenen, Verhoef, & Schols, 2009). Galacturonic acid (GalA) residues act as important constructing units in these regions, particularly in the HG and RG-I regions. Hence, the galacturonic acid residues are the most widely distributed and common structural unit among the diverse glycosyl residues. The carboxyl groups of the galacturonic acid residues can be partially esterified by methanol (Caffall & Mohnen, 2009) and the degree of methylation (DM) depends on the plant source and extraction conditions (Chan & Choo, 2013; Yapo, Robert, Etienne, Wathelet, & Paquot, 2007). Pectins can be classified into two categories: high-methylated-pectins ($DM > 50\%$) and low-methylated-pectins ($DM < 50\%$) (Yapo, Robert, et al., 2007). Chemically, pectins are considered to be polyanions because the carboxyl groups along the backbone can dissociate into ionic form, making pectins negatively charged. For this reason, pectins are electrostatically different from neutral polysaccharides, such as starch and hemicellulose.

The strength of ionization of the pectins is governed by intrinsic factors, such as the galacturonic acid content and the degree of methylation of the carboxyl groups (Ralet, Crépeau, Buchholt, & Thibault, 2003) and by extrinsic factors, such as pH and solvent (Faravash & Ashtiani, 2007; Kalapathy & Proctor, 2001; Smidsrød & Haug, 1967). Among these, the pH of the solution has the largest effect on the extent of ionization

of the carboxyl groups. The carboxyl groups of pectin are fully deprotonated at $\text{pH} \geq 4.5$ and fully protonated at $\text{pH} \leq 2.0$ (Capel, Nicolai, Durand, Boulenguer, & Langendorff, 2006). The tunability of the electronic properties of the pectins by changing the pH of the solution suggests that varying the state of the pectins may result in distinct interactions (i.e. repulsion or aggregation) between the polymer chains. Furthermore, pectins with different electrostatics show behavior differently with cationic compounds under different pH conditions.

Pectins are hydrophilic polysaccharides and interact extensively with water molecules in aqueous solution. Organic precipitants may be used to interrupt these pectin-water interactions in order to separate the pectins from the solution. Ethanol and isopropanol are commonly used to purify pectins from an aqueous mixture (Yapo, Wathelet, & Paquot, 2007). Isopropanol is less polar than ethanol and is therefore used in large-scale production (May, 1990). A high alcohol concentration reduces the solubility of the polymer in solution. A 3:1 v/v ratio of alcohol to water gives the best pectin yield for the cost (Xu, et al., 2014; Yapo, Wathelet, et al., 2007). It should be noted that the pectin precipitation process can vary largely based on how the pectin solution is mixed with the alcohol. When a pectin solution is added to a known volume of alcohol, the initial relative alcohol concentration is high. However, this relative alcohol concentration decreases upon mixing. Alternatively, when alcohol is added to a pectin solution, the relative alcohol concentration increases from 0 to a maximum concentration. The continuous rise in alcohol concentration, in this case, favors a sequential precipitation of pectin fractions with varying tolerances toward

alcohol concentration (Guo, Meng, Zhu, et al., 2016; Karnik, Jung, Hawking, & Wicker, 2016).

Pectins are usually precipitated from water/extract mixtures that contain inorganic sodium, potassium, magnesium and calcium salts (Kamnev, Colina, Rodriguez, Ptitchkina, & Ignatov, 1998). These cations may contribute to the precipitation process by forming ion pairs with the pectins (Smidsrød, et al., 1967). This is likely, as the precipitation process is conducted in solutions with pH 1.5~3.5 (Faravash, et al., 2007; Kalapathy, et al., 2001) where polyanionic pectin chains may bind cations. Since there are cations present in mixtures of pectin extract and water, it is crucial to investigate how the cations impact the alcoholic-precipitation of pectins. The relationship between cations and pectins during the process of alcohol precipitation has, to our knowledge, never been explored.

The aims of the present work are (i) to elucidate the role of Na^+ , K^+ , Ca^{2+} and Mg^{2+} in the precipitation of sugar beet pectins in ethanol-water mixtures and (ii) to study the effects of the binding of these cations on the hydrodynamic properties of SBP. A modified precipitation approach was utilized in which ethanol was gradually added to a pectin solution while stirring. Ethanol was chosen as the precipitation solvent because it is highly miscible with salt solutions. Based on the results, possible precipitation behaviors of the protonated-SBPs and deprotonated-SBPs in the presence or absence of cations are proposed.

2. Materials and methods

2.1. Materials

Sugar beet pectins were extracted from sugar beet pulp (Lvxiang sugar factory) using a heat-acid extraction described in the literature (Guo, Meng, Tang, et al., 2016). Glucan with a molecular weight of 200 kDa was purchased from Sigma-Aldrich Corp. (MO, United States). HPLC-grade chemicals were used in chromatography and reagents for chemical analysis were analytical grade.

2.2. Purification of SBP

Fig. 1 shows the scheme for pectin purification and the preparation of different samples. Generally, 10 g of SBP powder was stirred in 1 L Milli-Q water for 12 h at room temperature. The supernatant was collected by centrifugation (15 min at 5000 g), and subsequently purified by precipitating free proteins by addition of Cu^{2+} (Guo, Meng, Zhu, Zhang, & Yu, 2015). The resulting pectin solution was passed through a cation-exchange column (500 mm $\times$ 30 mm, 001 $\times$ 7 resin, Zhengguang Ltd., Zhejiang, China) at a flow rate of 300 mL/h in order to remove any bound metal ions. Then, the decationized SBP solution was freeze-dried and then air-dried at 45 °C until it was a constant weight. After this purification, a negligible amount of metal ions were present according to high performance cation exchange chromatography (HPCEC) analysis (Supplementary Information Fig. 1). Based on the GalA content (65.8 wt %) and the DM (34.8 %), the purified SBPs had an effective formula weight of 422 per carboxyl group.

2.3. Ethanolic Precipitation

2.3.1. Effects of cations

The polymer concentration used in all precipitation experiments was 5 mequiv $\cdot\text{L}^{-1}$.

The SBP solutions were precipitated with ethanol in the presence of counter ions (Na^+ , K^+ , Mg^{2+} and Ca^{2+}). Each counter ion was added at a known equivalent ratio of cation to carboxyl group in the SBPs before precipitation of the SBPs with ethanol. Ethanol was gradually added to the SBP solution while stirring, and the flow rate of the added ethanol (10 mL/min) was controlled by a constant pump (Longerpump Ltd, Hebei, China). The effect of ethanol on SBP precipitation was evaluated at two concentrations (50% and 67% v/v). The precipitation mixtures were allowed to stand for 6 h to reach an equilibrium state of precipitation, and then centrifuged for 10 min at 12000 g to separate the precipitated pectin gels. After centrifugation, the remaining polysaccharides in the supernatant were analyzed by the phenol–sulfuric acid assay (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Ethanol precipitation experiments were carried out at ~25 °C and effects from temperature variation were not taken into account.

The percentage of un-precipitated pectins (% PNP) was calculated using equation 1:

$$\% \text{ PNP} = 100 - (W_i - W_s) / W_i \times 100 \quad (1)$$

where W_i is the initial weight of the pectins before ethanol precipitation and W_s is the weight of pectins in the supernatant after precipitation.

2.3.2. Precipitations of SBP and glucan as a function of ethanol concentration

Aqueous solutions of SBP and glucan were precipitated by adding ethanol to obtain a final concentration of 0–83.3%. Ethanol precipitation experiments are carried out as described above. Values of precipitation yield were calculated by equation 1, plotted

against ethanol concentration to reflect the kinetic aspects of the precipitation.

2.3.3. Effects of pH

An acidic solution (pH 1.34) containing 5 mequiv·L⁻¹ SBP was prepared by dissolving 2.11 g pectin powders in 1 L of acidified water (0.15% v/v HCl). Aliquots (100 mL) of this solution were diluted appropriately 1–150 fold with the control SBP solution (5 mequiv L⁻¹, pH 3.26). Each of the resulting SBP solutions with varying initial pH were mixed with 3 volumes of ethanol. After letting the solutions sit for a period and centrifugation, the supernatants were subjected to the phenol–sulfuric acid assay.

Given that the obtained precipitation yields of the SBPs from acidic solution were relatively low, attempts were made to recover more of the SBP from the ethanol solution. The supernatant from the precipitation medium with I-pH 1.93 was brought to pH 3.5 using 1 M NaOH. After measuring the volume, the clear supernatant was mixed with a half volume of CaCl₂ ethanol solution (5 mequiv·L⁻¹, 75% v/v ethanol). An excessive amount of Ca²⁺ was used for fully promoting the electrostatic binding between Ca²⁺ and SBP. After standing for 6 h, the pectin precipitates were centrifuged, thoroughly washed with 75% ethanol, recovered in water, and freeze-dried. The fraction obtained by this calcium-involved precipitation method (CIPM) was referred to as SBP-CIPM.

2.3.4. Preparation of protonated and salt forms of SBP

Sugar beet pectins were converted to different salt forms by addition of cations to the SBP solutions and precipitation by ethanol (cation–GalA equivalent ratio of 0.5:1).

A 3:1 volume ratio of ethanol to SBP solution was used to obtain a high recovery yield. The addition of ethanol was conducted identically to the procedure discussed previously. After standing, the precipitated hydrated pectin gels were separated by centrifugation (10 min at 12000 g) and washed with 75% ethanol (gel-ethanol ratio, 1:5, w/v) 5 times to thoroughly remove free cations and until the waste gave a negative response to AgNO₃ (0.1 % w/v). After careful separation of the supernatant with a syringe, the hydrated precipitates were weighed, recovered in Milli-Q water, freeze-dried, and then air-dried at 45 °C until a constant weight was achieved. SBPs precipitated with Na⁺, K⁺, Mg²⁺ and Ca²⁺ salts were prepared and named NaSBP, KSBP, MgSBP and CaSBP, respectively.

SBP in the protonated form was prepared by direct ethanol precipitation of the starting SBP solution at around I-pH 2.0 and following the same wash procedures, and was referred to as PSBP.

2.4. pH measurement

The pH measurements of various SBP solutions were carried out using a pH meter (FiveEasy, Mettler Toledo). Prior to measurement, each tested solution was stirred gently for 10 min and allowed to stand for 10 min to reach a state of equilibrium.

2.5. Hydrodynamic radius (R_h) and electrophoretic mobility (EM)

The hydrodynamic radius (R_h) and electrophoretic mobility (EM) of the SBP solutions were determined using a Nano-Zetasizer (Malvern Ltd., UK). Prior to measurement, each solution was filtered through a 0.45 µm filter. For R_h measurements, a square cell (12 mm O.D.) was employed, whereas a DTS 1060

cuvette was used for EM measurements. All runs were carried out at 25 °C. Data are presented in triplicate $\pm$ SD.

2.6. Chromatograph

The molecular weight distribution of each sample was determined using high-performance size-exclusion chromatography (HPSEC). One hundred μ L of a 0.1% polymer solution was passed through two columns (300 mm $\times$ 7.5 mm), an Ultrahydrogel 2000 and an Ultrahydrogel Linear Column (Waters, Milford, MA), at a flow rate of 0.6 mL/min with 100 mM NaNO₃ as the eluent. Each 0.1% sample solution was filtered through a 0.45 μ m filter before injection onto the columns. The eluent from the column was attached to a 2414 refractive index detector (RI) (Waters). Runs were performed at 35 °C. The content of neutral sugars (NS) and cations were quantified by high-performance anion-exchange chromatography (HPAEC) and high-performance cation-exchange chromatography (HPCEC) as described in the literature (Guo, Meng, Tang, et al., 2016).

2.7. Relative viscosity

Viscosity measurements was performed using an Ubbelohde-capillary viscometer (ID: 0.5 mm) in a 30 ± 0.1 °C water bath. Prior to measurement, the each solution was allowed to stand at 30 °C for 10 min. The relative viscosity was calculated by equation 2 (Fang, et al., 2008):

$$\eta_r = t_s / t_0 \quad (2)$$

where η_r is the relative viscosity, t_s and t_0 are the flow time of the sample and the solvent, respectively.

2.8. Compositional analysis

The galacturonic acid (GalA) content was quantified colorimetrically by the automated *m*-phenylphenol (Blumenkrantz & Asboe-Hansen, 1973). The degree of methylation and acetylation was determined by HPLC (Levigne, Thomas, Ralet, Quemener, & Thibault, 2002). The protein content ($N \times 6.25$) was determined by the Kjeldahl procedure (AOAC, 1990).

2.9. ^{13}C Cross-polarization magic-angle-spinning (CP/MAS) nuclear magnetic resonance (NMR)

Solid-state NMR spectra of various samples were recorded using a Bruker (Germany) AVANCE III HD 400 spectrometer operating at 100.61 kHz with a 4 mm probe. In each ^{13}C CP/MAS acquisition, 1520 scans were collected with a pulse delay of 1.5 s. All measurements were performed at 25 °C.

2.10. X-ray diffraction (XRD)

XRD patterns of the samples were obtained on a Bruker diffractometer (Bruker D8 Advance, Germany) equipped with a Cu anticathode. Each analysis was performed in the 2θ angle range (5–50°) with a step size of 0.04 2θ angle.

2.11. Statistical analysis

Obtained data were statistically evaluated by the one-way analysis of variance (ANOVA). The data were evaluated by the Duncan's posthoc comparison. Means were considered significantly different at $p\text{-value} < 0.05$.

3. Results and discussion

3.1. Binding of cations to SBP in aqueous solution

Fig. 2a shows the change in the pH of SBP solutions with increasing amounts of added cations. There was a decrease in pH after beginning the addition of the cations due to the liberation of protons from carboxyl groups (Dronnet, Renard, Axelos, & Thibault, 1996). The magnitude of Δ pH reflects the degree of cation binding to SBP in aqueous solution. Comparing the Δ pH values between two types of cations reveals that divalent cations had a more pronounced effect on the decrease in pH, and Ca^{2+} was the most effective of all cations tested. Ca^{2+} and Mg^{2+} are more capable of binding to SBP than are K^{+} and Na^{+} because they can simultaneously bind two neighboring carboxyl groups. Although both Ca^{2+} and Mg^{2+} bind carboxyl groups in the same electrostatic way, there is evidence that the SBPs have a greater affinity to Ca^{2+} which may be due to the higher affinity of galacturonic acid units toward Ca^{2+} (Malovíková, Rinaudo, & Milas, 1994). To extensively neutralize the carboxyl groups, a cation concentration up to 20 times that of $-\text{COO}^{-}$ is required, which may be due to the non-stoichiometric nature of the binding model.

In addition to pH measurements, the electrophoretic mobility (EM) of the SBP solutions was analyzed in order to understand the associations between cations and SBP (Fig. 2b). Upon the addition of cations, the EM notably increases from $-1.2 \mu\text{m}\cdot\text{cm}/\text{V}\cdot\text{s}$ to a range of -0.2 and $-0.6 \mu\text{m}\cdot\text{cm}/\text{V}\cdot\text{s}$, depending on the cation/GalA mole ratio. Neutralizing negative charges on the pectin chains reflects that cations bind to SBP in aqueous solution, and shows the polyelectrolyte nature of pectin. This is consistent with the results found from measuring the pH of the solutions.

3.2. Effect of added cations on R_h

The role of the cation on the R_h of pectin molecules was studied using dynamic light scattering (DLS), and the results are shown in Fig. 3. The R_h of SBPs decreases upon increasing the cation concentration, as reported previously (Lima, Soldi, & Borsali, 2009). In contrast, the change in R_h was not statistically significant in the case of glucan. This difference in the change of R_h for SBP versus glucan is most likely due to structural differences of the two with respect to GalA units. Divalent cations were found to be more effective than monovalent cations in reducing the R_h . It should be noted that pectin molecule dimerization via intermolecular bonding is unlikely in the presence of calcium because of the steric hindrance generated by the acetyl groups. Therefore, the trend of the decrease in R_h with increasing amount of added cation is ascribed to the neutralization of intramolecular electrostatic repulsion. Interestingly, there was no significant change in the R_h between solutions with different mole ratios of Ca^{2+} to GalA. However, the reason for this result is not known.

Pectin has been reported to adopt an extended conformation or semi-flexible conformation in aqueous solution (Morris, et al., 2008; Pérez, Mazeau, & Hervé du Penhoat, 2000). The conformational flexibility of pectin depends on chemical features such as the DM and the relative proportion of side chains (RG-I) to backbones (HG) (Morris, Foster, & Harding, 2000; Morris & Ralet, 2012). Electrostatic interactions between pectin chains may lead to a conformational change (Morris, et al., 2000). Considering the results obtained in this work, the binding of cations to SBPs may decrease the R_h , but simultaneously promotes some changes in the conformation of pectin (e.g. molecular flexibility). However, more evidence is required to confirm a

conformational change.

3.3. Effect of cations on relative viscosity

The relative viscosity plotted against the cation/GalA ratio is shown in Figure 4. It has been reported that counter ions can exert an effect on the relative viscosity of polymers by diminishing intramolecular repulsion (Lima, et al., 2009). The relative viscosities of the pectins in this work were also found to be reduced with the addition of cations. This suggests that pectin can act as a polyelectrolyte. When compared to monovalent cations, divalent cations have a more pronounced effect on the reduction of the relative viscosity of the pectins, especially when the mole ratio of cation to GalA ranges from 0.1–2. Results from measuring the relative viscosities, DLSs and EMs of these pectins show that they remain soluble upon binding each examined cations.

3.4. Precipitations of SBP and glucan as a function of ethanol concentration

Fig. 5 shows the precipitation yields of de-cationized SBP and glucan as a function of ethanol concentration. SBP and glucan exhibit different precipitation kinetics. Glucan began to precipitate when the ethanol concentration reached 30% and was almost fully precipitated at ethanol concentrations higher than 67% v/v. However, SBP was much more difficult to precipitate by ethanol although it contained some hydrophobic groups (i.e. acetyl groups). Only 7.2% by weight of SBP was precipitated at an ethanol concentration of 83.3% v/v. This observation indicates that acetyl groups contribute little to decreasing the solubility of SBP in the ethanol solution. Since ethanol can compete with water molecules, the discrepancy in the

precipitation behavior between SBP and glucan can be explained by the structural differences of the charged groups.

By performing a modeling simulation of a polygalacturonic acid chain, Roto and co-authors (2005) have suggested that the pectin chain becomes more rigid when it is negatively charged because it can form more hydrogen bonds with water molecules. In this work, the main component of the SBP (i.e. HG) was moderately charged (Fig. 2b). Due to the negatively charged carboxyl units, the SBP chains are more polar than glucan, and thus favor being hydrated by water molecules based on the principle of 'like dissolves like'. Accordingly, de-methylated SBPs are expected to be more hydrated in ethanol solution because the charge density of the polymer is strengthened.

3.5. Precipitation yield of SBP with cation at various ethanol concentrations

Fig. 6 shows changes in the %PNP as a function of the cation/GalA ratio in 50% and 67% v/v ethanol solutions. At 50% v/v ethanol concentration (Fig. 6a), the addition of monovalent cations had a slight effect on the %PNP, except when the K^+ -GalA ratio was 5:1. On the contrary, addition of Ca^{2+} or Mg^{2+} led to a continuous decrease in pectin solubility. When the cation-GalA ratio was increased from 0.2:1 to 5:1, the precipitation yield of the SBP was reduced by ~80%. This sudden increase in pectin precipitation indicates that the pectin chains are significantly less hydrated. As SBP is fully hydrated in up to 50% ethanol solution (Fig. 5), the precipitation of SBP is believed to be induced by the pectin-cation interactions, probably by neutralizing the electrostatic repulsion between the pectin chains. The ability for different cations

to assist in pectin precipitation follows the order: $\text{Ca}^{2+} > \text{Mg}^{2+} > \text{K}^+ > \text{Na}^+$ (Fig. 6).

When the solution is less than 67% v/v ethanol (Fig. 6b), the precipitation yields upon adding K^+ and Na^+ were comparable, and were less pronounced than those from the addition of Mg^{2+} and Ca^{2+} . A rapid increase in pectin precipitation was observed when the ratio of added cation was increased from 0.5 to 1.0 and 0.2 to 0.5, respectively for Na^+ and K^+ . For Ca^{2+} and Mg^{2+} , a low cation–GalA ratio led to an almost complete precipitation. It was observed that more than 95% of the SBP precipitated out of solution after increasing the cation–GalA ratio to 0.5. Moreover, the differences between the precipitation of SBP solutions containing Ca^{2+} and Mg^{2+} became smaller at a ratio of 67% ethanol. These results indicate that the precipitation of SBP is affected by pectin-cation interactions and the ethanol concentration of the solution.

In ethanol solutions >50% v/v, the dielectric constant is low and cations can form ion pairs with the anionic carboxyl groups on SBP chains. Assuming that the ion pairs are equivalent to protonation in terms of reducing the net charge and polarity of the polymer, SBP may adopt a more flexible conformation during precipitation (Morris, et al., 2000; Noto, et al., 2005).

3.6. Ethanolic precipitation of SBP with I-pH and Ca^{2+}

Ethanol precipitations of SBP with and without Ca^{2+} at various I-pHs were assessed. Fig. 7 shows the percentage of unprecipitated SBP from various 75% v/v ethanol solutions. The precipitation of SBP greatly depends on the I-pH at which SBP is precipitated. At $\text{I-pH} \geq 2.83$, there is no noticeable precipitation of pectin, indicating

that the level of pectin hydration is high. However, the %PNP decreased to 23% at I-pH 2.69, and further decreased to 11.5% at I-pH 2.13. Below this pH, no precipitation occurs. This trend can be partially explained by the decrease in charge density of the pectin backbone as the I-pH is lowered (Guo, Meng, Tang, et al., 2016).

In the presence of Ca^{2+} , the %PNP was low at $\text{I-pH} \leq 2.58$, and the value increased as the I-pH of the solution was lowered below 2.13. At $\text{I-pH} \leq 1.68$ where the pectin chain are completely uncharged (Capel, et al., 2006; Li, Fang, Phillips, & Al-Assaf, 2013), the %PNP of solutions in the presence of and the absence of added Ca^{2+} was not significant ($p < 0.05$), indicating the ineffectiveness of Ca^{2+} . Since the precipitation of ionized SBP occurs over pH range 2.13–3.26, this precipitation yield must be associated with the electrostatic binding between SBP and Ca^{2+} .

It is worth emphasizing that the SBPs used in this work contain heterogenous fractions with different molecular sizes (Fig. 8). Therefore, the precipitation process of SBP under the applied precipitation conditions is most likely a sequential precipitation of different molecular-sized fractions (Guo, Meng, Zhu, et al., 2016; Karnik, et al., 2016). As can be seen from Fig. 7, one fraction of the SBP remained in solution ($\text{I-pH} \leq 2.0$) and could not be recovered even by decreasing the I-pH in the presence of Ca^{2+} . On the other hand, one part of the pectin sample precipitates out of solution in the presence of Ca^{2+} over the I-pH range of 3.26–2.58, which indicates a rather low %PNP (2.6–3.1%). Based on these results, the binding between Ca^{2+} and SBP is likely essential for the precipitation of some components of SBP with specific structures.

In fact, we successfully recovered one fraction of SBP in the supernatant from the ethanol precipitation at I-pH 1.93 by the developed CIPM procedure. This result implies that the binding of calcium to SBP plays a role in the precipitation of SBPs.

3.7. HPSEC

Fig. 8 shows the molecular weight distribution (MWD) chromatograms of samples obtained under various precipitation conditions. NaSBP, CaSBP and PSBP eluted from the solutions from 22 min to 34 min, and their MWDs were similar. There were two peaks with the minor peak assigned to the fraction of pectins with a large molecular size and the major peak 2 assigned to the fraction of pectins having a smaller molecular size. Peaks 1 and 2 were partially overlapped which indicates that the broad MWD is multimodal. In contrast, the SBP-CIPM eluted from 26 min to 35 min and consisted over pectins with smaller molecular sizes. The smaller size of the SBP-CIPM fraction can be interpreted by the acid degradation of pectin chain during extraction. From Fig. 7 and 8, it appears that the binding of Ca^{2+} with a small size fraction leads to the precipitation of SBP-CIPM.

It has previously been shown on standard dextrans and pullulans (Xu, et al., 2014) that polymer chains with larger molecular sizes are precipitated at a lower ethanol concentration. However, in our previous work (Guo, Meng, Zhu, et al., 2016), the sequential precipitation of SBP with increasing ethanol concentrations was not strictly size-dependent. These results suggest that the molecular size and shape of the molecules are decisive factors in the ethanol precipitation of SBP. Considering the results obtained in this work, the strong interactions between protonated SBP-CIPM

and solvent molecules may be due to the smaller molecular size of each chain.

3.8. Pectin recovery and hydrous pectin precipitates

Fig. 9a shows the recoveries of SBP in the presence of added Na^+ or Ca^{2+} in 75% ethanol solution. The addition of Na^+ or Ca^{2+} resulted in a complete recovery of SBP, except when the mole ratio of Na^+ to GalA was 1:1. By relating the pectin recovery to the weight of the pectin precipitates, the degree of hydration of the pectin precipitates can be evaluated. As shown in Fig. 9b, the weight of the precipitated pectin decreased with increasing concentration of Na^+ , and it remained constant within the examined concentration range of Ca^{2+} . The precipitates of NaSBP appeared to be more hydrated than those of CaSBP (Fig. 9c), particularly when the cation–GalA ratio was 1:1 and 2:1. A comparison of the precipitate weights and pectin recovery showed that Ca^{2+} was more effective than Na^+ at interrupting the interactions between SBP chains and solvent molecules at relatively low cation–GalA mole ratios.

3.9. ^{13}C CP/MAS NMR spectroscopy

Fig. 10a displays the ^{13}C CP/MAS NMR spectra of PSBP, NaSBP and CaSBP. All spectra had a carboxyl carbon resonance around 170 ppm, a C-1 resonance centered at 100 ppm and superimposed by weak resonances at 96 ppm, a C-4 resonance around 80 ppm with a shoulder on a sharp peak at 69 ppm, and a resonance around 54 ppm for the methoxy carbon (Larsen, et al., 2011; Renard & Jarvis, 1999). The carbon resonances from the rhamnose and acetyl groups were also detected within 18–22 ppm (Larsen, et al., 2011). Typically, resonances from C-1 and C-4 determine the conformational features (Jarvis & Apperley, 1995; Renard, et al., 1999). To investigate

whether metal cations binding to the carboxyl groups has any effect on the conformation, special attention was therefore given to the resonances at 170, 100 and 80 ppm. However, there were no detectable differences between the C-1 and C-4 resonances across all samples. This indicates that metal cations bound to carboxyl units act analogously to protons and likely adopt similar conformations.

Fig. 10b shows a magnified view of the resonances of the carboxyl carbon. This resonance is sharper in PSBP than in other PSBs. Furthermore, the peak band at 170 ppm in the PSBP sample is nearly a gaussian distribution, while those for CaSBP and NaSBP are non-symmetric and are slightly shifted to higher frequencies. The reason for the narrower peak width in the PSBP sample may be explained by the homogenous state of the carboxyl groups. The peak width for CaSBP was broader than that of NaSBP, which is probably due to the higher degree of cation binding (Table 2).

3.10. X-ray diffraction spectroscopy

Fig. 11 shows the XRD patterns of NaSBP, CaSBP and PSBP. The observed XRD patterns show two broad peaks centered at 12.5° and 21.5°, which indicate that they are amorphous polymers. The increase in peak intensity by PSBP may suggest a more ordered structure compared to the others. It appears that a higher degree of cation binding leads to a weaker XRD peak intensity. It has been previously reported that the dimerization of pectins by calcium leads to the formation of ordered Ca^{2+} -bridged dimers (Ventura, Jammal, & Bianco-Peled, 2013). However, this is not relevant to the current study as Ca^{2+} cannot bridge acetylated sugar beet pectin chains via

intermolecular bonds. It can only bridge through intramolecular electrostatic bonds.

3.11. Chemical features

Chemical features of the resulting SBP salts and two commercial pectin products are given in Table 1. Compared to pectin products from citrus and apple, the SBP samples were structurally different with respect to composition (GalA and neutral sugars) and substitution (degrees of methylation and acetylation).

In terms of the SBP salt samples, the GalA ranged from 65.2% to 67.4%, which is a typical feature in pectic polysaccharides. There are no significant differences across all samples. The NS ranged from 17.7% to 20.3% and galactose (9.9–11.8%) and rhamnose (3.8–4.1%) were found to be the major compositional sugars. The arabinose content ranged from 2.6 % to 3.3%. These values are lower than the values reported in literature (3.5–12.3%) (Yapo, Robert, et al., 2007), and may indicate that these samples have been degraded by the acid at glycosyl linkages of arabinan during extraction. In comparison, the NS of the SBPs were significantly higher ($p < 0.01$), as they are chiefly composed of arabinose and galactose. This is likely due to the loss of arabinan and galactan during ethanol precipitation, as they are highly soluble in ethanol. The DM and degree of acetylation (DA) ranged from $33.1 \pm 1.9\%$ to $34.8 \pm 1.4\%$, and from $21.6 \pm 0.9\%$ to $23.1 \pm 1.2\%$, respectively. Based on these values, all of the pectin salts prepared in this work can be categorized as low-methylated-pectins. In terms of the DM and DA values, there is no significant difference among the samples.

There were no significant differences between the neutral sugar content and the

DM values among the SBP salts, which suggests that no conformational modifications occurred after preparation (Morris, et al., 2000; Morris, et al., 2012).

3.12. Degree of cation binding (DCB)

Table 2 shows the bound cation contents and DCBs of various samples. The bound sodium, potassium, magnesium and calcium contents were $1.3 \pm 0.3\%$, $1.9 \pm 0.4\%$, $1.3 \pm 0.1\%$ and $2.5 \pm 0.2\%$, respectively, for the SBP salt samples. The DCBs of NaSBP, KSBP, MgSBP and CaSBP were $26.0 \pm 4.9\%$, $21.7 \pm 5\%$, $50.5 \pm 2.2\%$, and $56.1 \pm 3.5\%$, respectively. Comparison of the DCBs reveals that the divalent cations are bound more extensively to the carboxylic groups of the polygalacturonates compared to the monovalent ions. These results were consistent with the trend in affinities between these two types of cations and pectins (Fig. 2).

3.13. Role of cation binding on pectin precipitation behavior

From an electrostatic point of view, the binding of cations to a pectin chain neutralizes the negative charge on the pectin chains (Fig. 2). Since there were no conformational changes observed (Fig. 10) among PSBP, NaSBP and CaSBP, the established cation–pectin interactions are considered to be electrostatic. However, the addition of metal cations has a greater effect on the precipitation of SBPs than simply protonating the carboxyl groups, even though protonation neutralizes the overall negative charge more completely. The proposed precipitation behavior of SBPs has been summarized into a schematic illustration (Fig. 12).

In the high I-pH region (> 2.69), pectin chains may be widely distributed in solution. The high polarity of dissociated carboxyl groups allows strong interactions

between the pectin chains and solvent molecules (Fig. 12a). At pH < 2.69, pectins are overall less negatively charged due to the suppression of the carboxylic groups in the galacturonic acid units. As a result, the pectins can get closer together, thereby weakening the interactions generated between the pectin chains and the solvent molecules. However, a fraction of the protonated SBP that consists of low molecular size remains soluble after ethanol addition (Fig. 12b). The high hydration level of this fraction is most likely due to the shorter chain lengths.

The cation-involved precipitation of SBP was more effective than pH-induced precipitation for shorter SBP chains (Fig. 12c–d). In this case, electrostatic bonds between the metal cations and carboxyl groups in aqueous solution can become more ion-pair in nature during ethanol precipitation (Smidsrød, et al., 1967). At equal added metal cation concentrations, divalent cations had a larger impact on precipitation yields.

It has previously been shown that SBPs in solutions containing metal ions such as Na⁺, K⁺, Ca²⁺ and Mg²⁺ are solubilized by acidic extractants from sugar beet pulp (Guo, Meng, Tang, et al., 2016; Kamnev, et al., 1998). Thus, electrostatic interactions between SBP and counter cations are likely to occur prior to precipitation by ethanol. However, in aqueous solution, these interactions are not easily observed because the binding of Na⁺, K⁺, Ca²⁺ and Mg²⁺ to SBP chains is much weaker than the binding between SBP chains and Cu²⁺ or Pb²⁺. The results obtained in the current work further emphasize that the electrostatic relationship between the counter cations and carboxyl groups plays an important role in the precipitation behaviors of SBPs.

4. Conclusion

The precipitation of sugar beet pectins (SBPs) in the presence or absence of counter cations and by the addition of ethanol was investigated. Interactions between added counter cations and the SBPs affected the affinity between SBP and water molecules and facilitated the precipitation of the SBPs. An analysis of the hydrodynamic properties of the precipitated SBPs showed that the cations-pectins electrostatic interaction significantly weakened the hydration degree of SBP, especially when divalent cations were used. The combined effect of added counter cations and ethanol led to a significant increase in the precipitation of negatively-charged SBP. The results of ^{13}C CP/MAS NMR analysis revealed that the interactions between the SBPs and cations during ethanol precipitation are electrostatic nature. Clearly, the electrostatic interaction between ionized carboxyl groups in these pectins and counter cations play a key role in determining the ethanol precipitation behavior of SBPs.

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Table 1. Chemical composition (% w/w), degree of methylation (mol %) and degree of acetylation (mol %) of various pectin samples ^a

Sample ID	SBP	NaSBP	KSBP	MgSBP	CaSBP	Citrus ^b	Apple ^c
GalA	65.8±1.1a	67.4±2.9a	67.3±1.6a	65.2±2.3a	67.2±1.8a	79.2	82
Rhamnose	4±0.1a	3.8 ±.4a	4.1±0.2a	3.9±0.1a	4±0.1a	1.4	0.6
Arabinose	3.3±0.1b	2.7±0.2a	2.7±0.2a	2.6±0.1a	2.6±0.1a	1.1	3.3
Galactose	11.8±0.1b	10.2±0.3a	10.2±0.3a	9.9±0.2a	10.3±0.4a	2.4	5.2
Glucose	0.6±0.1a	0.5±0.1a	0.8±0.2a	0.7±0.1a	0.7±0.1a	n/d ^d	0.9
Xylose	0.6±0.1a	0.6±0.1a	0.6 ± 0a	0.5±0.1a	0.5±0.1a	0.2	0.3
NS	20.3±0.1b	17.8±0.3a	18.4 ± 0.6a	17.7±0.4a	18.1±0.5a	5.1	10.3
DM	34.8±1.4a	33.4±0.9a	33.1±1.9a	34.4±1.2a	34.3±0.8a	72	70–75
DA	22.1±1.2a	22.3±1.6a	22±1.3a	23.1±1.2a	21.6±0.9a	1.4–1.6 ^e	5 ^e

^a Data are means ± standard deviations (n = 3); Mean values in the same row with different letters are significantly different ($p < 0.05$).

^b Axelos and Thibault (1991).

^c Garna, Emaga, Robert, and Paquot (2011).

^d n/d: not determined.

^e Kravtchenko, Voragen and Pilnik (1992).

Table 2. Metal ion content (% w/w) and degree of cation binding (DCB) (mol %) of various pectin samples ^a

	NaSBP	KSBP	MgSBP	CaSBP
Na	1.3±0.3	n/d ^b	n/d	n/d
K	n/d	1.9±0.4	n/d	n/d
Mg	n/d	n/d	1.3±0.1	n/d
Ca	n/d	n/d	n/d	2.5±0.2
% DCB ^c	26±4.9a	21.7±5a	50.5±2.2b	56.1±3.5b

^a Data are means ± standard deviations (n = 3); Mean values in the same row with different letters are significantly different ($p < 0.05$).

^b n/d: not determined.

^c % DCB = mequiv cations / [mequiv GalA × (100 – DM)/100] × 100.

Figure captions:

Fig. 1. Scheme for the ethanol precipitation of protonated SBP and SBP salts.

Fig. 2. Effect of cation binding on the Δ pH (a) and electrophoretic mobility (b) for SBP solution containing $5 \text{ mequiv} \cdot \text{L}^{-1}$.

Fig. 3. Change in R_h as a function of the cation/GalA mole ratio.

Fig. 4. Effect of cation/GalA mole ratio on the relative viscosity of SBP solution.

Fig. 5. Percentages of precipitation yield of SBP and glucan as a function of ethanol concentration.

Fig. 6. Precipitation of SBP at ethanol concentrations 50% v/v (a) and 67% v/v (b) with increasing cation concentrations.

Fig. 7. Percentages of unprecipitated SBP with and without Ca^{2+} at different initial precipitation pHs (I-pHs).

Fig. 8. Molecular weight distributions of PSBP, NaSBP, CaSBP and SBP-CIPM.

Fig. 9. Yields of SBP precipitated with Na^+ and Ca^{2+} (a), weights of their hydrous pectin precipitates (b), and pictures of the hydrous pectin precipitates (c) as a function of the cation/GalA mole ratio.

Fig. 10. ^{13}C CP/MAS NMR spectra of PSBP, NaSBP and CaSBP showing the spectral ranges (a) 0–200 ppm, and (b) 150–190 ppm.

Fig. 11. X-ray diffraction patterns of PSBP, NaSBP, and CaSBP.

Fig. 12. Schematic illustrations of the precipitation process at precipitation I-pH 3.26 and 2.0 (a–b) in the absence of cation ions and involved the participation of monovalent ion (c) and divalent ion (d) during the ethanol

precipitation.

Table captions:

Table 1. Chemical composition (% w/w), degree of methylation (% mol) and degree of acetylation (% mol) of various pectin samples ^a

Table 2. Metal ion content (% w/w) and degree of cation binding (DCB) (% mol) of various pectin samples ^a

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