Effects of natural starch-phosphate monoester content on the multi-scale structures of potato starches

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ABSTRACT

Starch phosphate content (SPC) is an important structural parameter affecting the functionality of tuber starches, but how different phosphate groups affect starch structures is still unknown. In this study, four potato starches with different SPC were selected as models to investigate the effects of SPC on their multi-scale structures. A higher SPC is related to more long amylopectin (AP) chains with a degree of polymerization (DP) > 24, fewer short AP chains with DP ≤ 24, lower double helical content and crystallinity, higher long period distance, and lower scattering intensity (I max), indicating that potato starch with a higher SPC is less ordered and has a more flexible nano-lamellar structure. Notably, two types of phosphate monoesters, namely C-3 (C3P) and C-6 phosphate monoesters (C6P), were both significantly positively correlated with the average chain lengths (ACL) of AP chains with DP 25–36 (fb2) and negatively correlated with I max; however, only C3P was significantly positively correlated with ACL of AP chains with DP 13–24 (fb1) and negatively correlated with the absorbance ratio at 1047/1016 cm⁻¹. C3P was the main reason for the disordered structure of starches with high SPC. These findings can be helpful for potato breeding to generate different functionality by controlling SPC.

Abbreviations: AC, amylose content analyzed by iodine method; ACL X, average chain lengths (DP) of fraction X; AM, amylose; AP, amylopectin; AP1, short amylopectin chains (DP 6–36) analyzed by SEC; AP2, long amylopectin chains (DP 37–100) analyzed by SEC; ATR-FTIR, attenuated Total Reflectance-Fourier Transform Infrared; C3P, C-3 phosphate monoesters; C6P, C-6 phosphate monoesters; D, Bragg lamellar repeat distance; d a, thickness of amorphous lamellae; d c, thickness of crystalline lamellae; DMSO, dimethyl sulfoxide; DP, degree of polymerization; HPAEC-PAD, high-performance anion exchange chromatography-pulsed amperometric detection; fa, amylopectin chains (DP 6–12) analyzed by HPAEC-PAD; fb1, amylopectin chains (DP 13–24) analyzed by HPAEC-PAD; fb2, amylopectin chains (DP 25–36) analyzed by HPAEC-PAD; fb3, amylopectin chains (DP >36) analyzed by HPAEC-PAD; HPPS, high phosphate potato starch; I max, peak scattering intensity; LPPS, low phosphate potato starch; NMR, nuclear magnetic resonance; NMS, normal maize starch; NPS, normal potato starch; SEC, size-exclusion chromatography; SEM, scanning electron microscopy; SPC, starch phosphate content; WMS, waxy maize starch; WPS, waxy potato starch.

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1. Introduction

Potato (Solanum tuberosum L.) is one of the most important staple crops worldwide and plays a significant role in global food security (Blennow et al., 2020). Starch is the major component in potato tubers, crops worldwide and plays a significant role in global food security from previous data. The amylose content (AC) was analyzed by the iodine complexation method (Carciofi et al., 2012).

2. Materials and methods

To reveal how SPC affects the multi-scale structures of starch and to test whether the C3P and C6P have different functionality on starch structures, four native types of potato starches with different SPC (3–74 nmol/mg starch), C3P (1–22 nmol/mg starch) and C6P (2–52 nmol/mg starch) were selected as model systems. As these four starches also contain 2–30 % of AC, two types of non-phosphorylated maize starch with different AC (0.1 % and 30.5 %) were used as controls to test whether this parameter exert specific functionalities related to phosphorylated starch systems. The multi-scale structures of these six types of starches were analyzed, including their molecular, helical, crystalline, and amorphous regions (M ventilating effects on the multi-scale structures and provide a foundation for breeding and engineering elite potato varieties with desirable functionality by new breeding techniques. Few studies have been reported that C6P is preferentially located in the amorphous regions of the starch granules, whereas C3P is equally distributed among the semi-crystalline and amorphous regions (Blennow, Bay-Smidt, Olsen, & Møller, 2000; Engelsen et al., 2003). Force-field modeling further indicated that the C6P units might fit into a natural void in the double helices of amylopectin without inflicting steric problems, while C3P might exert a significant strain or make local defects in the crystalline AP sections by inducing a major shift in the conformational equilibrium of the glucosidic linkage, thereby destabilizing the helical structure (Hansen et al., 2009). Therefore, we hypothesized that C3P and C6P have different functionality on multi-scale structures of starches, and they are as important as AC in affecting starch structures.

To reveal how SPC affects the multi-scale structures of starch and to test whether the C3P and C6P have different functionality on starch structures, four native types of potato starches with different SPC (3–74 nmol/mg starch), C3P (1–22 nmol/mg starch) and C6P (2–52 nmol/mg starch) were selected as model systems. As these four starches also contain 2–30 % of AC, two types of non-phosphorylated maize starch with different AC (0.1 % and 30.5 %) were used as controls to test whether this parameter exert specific functionalities related to phosphorylated starch systems. The multi-scale structures of these six types of starches were analyzed, including their molecular, helical, crystalline, and amorphous regions (M ventilating effects on the multi-scale structures and provide a foundation for breeding and engineering elite potato varieties with desirable functionality by new breeding techniques. Few studies have been reported that C6P is preferentially located in the amorphous regions of the starch granules, whereas C3P is equally distributed among the semi-crystalline and amorphous regions (Blennow, Bay-Smidt, Olsen, & Møller, 2000; Engelsen et al., 2003). Force-field modeling further indicated that the C6P units might fit into a natural void in the double helices of amylopectin without inflicting steric problems, while C3P might exert a significant strain or make local defects in the crystalline AP sections by inducing a major shift in the conformational equilibrium of the glucosidic linkage, thereby destabilizing the helical structure (Hansen et al., 2009). Therefore, we hypothesized that C3P and C6P have different functionality on multi-scale structures of starches, and they are as important as AC in affecting starch structures.

2. Materials and methods

2.1. Materials

Four potato tuber and two maize kernel starches with different SPC and AC were selected (Table 1). The four potato starches were extracted

Table 1 Sample code, phosphate and amyllose contents of potato and maize starch samples.

<table>
<thead>
<tr>
<th>Starch varieties</th>
<th>Sample code</th>
<th>SPC (nmol/mg starch)</th>
<th>C3P (nmol/mg starch)</th>
<th>C6P (nmol/mg starch)</th>
<th>AC (%)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waxy potato (Kuras)</td>
<td>WPS</td>
<td>25.0</td>
<td>3.7</td>
<td>21.3</td>
<td>1.9</td>
<td>(Kozlov et al., 2007; Wikman, Blennow, &amp; Bertoft, 2013)</td>
</tr>
<tr>
<td>Normal potato (Dianella)</td>
<td>NPS</td>
<td>24.0</td>
<td>9.9</td>
<td>14.1</td>
<td>19.8</td>
<td>(Kozlov et al., 2007; Wikman et al., 2013)</td>
</tr>
<tr>
<td>Low phosphate potato</td>
<td>LPPS</td>
<td>3.1</td>
<td>0.9</td>
<td>2.2</td>
<td>28.2</td>
<td>(Wikman et al., 2011)</td>
</tr>
<tr>
<td>High phosphate potato</td>
<td>HPPS</td>
<td>73.8</td>
<td>21.8</td>
<td>52.0</td>
<td>29.6</td>
<td>(Wikman et al., 2011)</td>
</tr>
<tr>
<td>Waxy maize</td>
<td>WMS</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.05</td>
<td>(Zhong, Tian, et al., 2021)</td>
</tr>
<tr>
<td>Normal maize</td>
<td>NMS</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>30.7</td>
<td>(Zhong, Krarathurana, et al., 2021)</td>
</tr>
</tbody>
</table>

* The starch phosphate content (SPC), C-3 phosphate monoester content (C3P), and C-6 phosphate monoester content (C6P) in the four potato starches are referred from previous data. The amylose content (AC) was analyzed by the iodine complexation method (Carciofi et al., 2012).

1. n.d. = not detectable.
from Dianella wild type (normal potato starch, NPS), Dianella RNAi GWD1 line (asGWD, low phosphate potato starch, LPPS), Dianella dual RNAi starch branching enzyme I and II line (asSBE, high phosphate potato starch, HPPS), and Kuras RNAi GBSS line (waxy potato starch, WPS) as described before (Blennow et al., 2005; Kozlov et al., 2007). Isoamylase (EC 3.2.1.68, E-ISAMY, 200 units/mL) and pullulanase (E-PULBL, 1000 units/mL) were bought from Megazyme (K-TSTA, Megazyme, Co. Wicklow, Ireland). All other chemicals used in this study were of analytical grade.

2.2. Size-exclusion chromatography (SEC)

Chain length distributions of debranched starch samples were analyzed using a size exclusion chromatography (SEC)-triple detector array (SEC-TDA) system (Viscotek, Malvern, UK), as previously described (Zhong, Keeratiburana, et al., 2021). The SEC was equipped with tandem GS-520HQ/GS-320HQ Shodex columns attached to a TDA302 detector array. The detailed method is described in the supplemental information.

2.3. High-performance anion exchange chromatography-pulsed amperometric detection (HPAEC-PAD)

Debranched starch samples were prepared as described in the Section 2.2 and references therein and analyzed using an HPAEC-PAD system (Dionex, Sunnyvale, CA, USA). A 40 μL of samples (5 mg/mL) was injected into a CarboPac PA-200 column at a flow rate of 0.4 mL/min. The chain length, defined as the degree of polymerization (DP) between 1 and 83, was integrated, and the detector response was corrected (Blennow, Bay-Smidt, Wischmann, Olsen, & Møller, 1998).

2.4. Solid-state 13C nuclear magnetic resonance (NMR) spectroscopy

13C NMR spectroscopy was performed at a 13C frequency of 150.9 MHz using a Bruker AV-600 spectrometer as previously described with slight modifications (Tan, Planagan, Halley, Whittaker, & Gidley, 2007; Zhong, Li, et al., 2021). The detailed method is described in the supplemental information. The raw NMR spectra were resolved into amorphous and ordered sub-spectra by subtracting the spectra of amorphous references at about 85 ppm, and the individual peaks in the ordered sub-spectra were fitted using Topspin software. The single and double helices are found at 102–103 ppm and 99–101 ppm, respectively, in the C1 region of ordered NMR sub-spectra, so the ratios of the peak area for single helix and double helix to the total areas are relative contents of single and double helix, respectively, and the relative content of the amorphous region was calculated by the subtraction of total relative contents of single and double helix helices from 100% (Tan et al., 2007).

2.5. Proton nuclear magnetic resonance (1H NMR) spectroscopy

The degree of branching of starch was determined using one dimensional 1H NMR spectra acquired on 600 MHz NMR spectrometers (Bruker Avance III; Bruker Biospin, Rheinstetten, Germany), following a previous protocol (Zhong, Keeratiburana, et al., 2021) with some modifications. The detailed method is described in the supplemental information.

2.6. Small-angle X-ray scattering (SAXS)

SAXS was performed at the Shanghai synchrotron facility as described before (Kuang et al., 2017). Normalized 1D correlation function and SAXS scattering curve fitting were adopted to obtain a series of SAXS parameters, including Bragg lamellar repeat distance (D), peak scattering intensity (I_{max}), thickness of crystalline (d_c), amorphous lamellae (d_a) and long period distance (d_{ac}) (Xu, Blennow, Li, Chen, & Liu, 2020).

2.7. Wide-angle X-ray scattering (WAXS)

A Nano-inXider instrument (Xenocs SAS, Grenoble, France) equipped with a Cu K source with a 1.54 Å wavelength and a two-detector setup was used to analyze the crystalline granule structures (Zhong, Li, et al., 2021). Briefly, samples equilibrated at ~90 % relative humidity were sealed in 5–7 μm mica films and then analyzed. The radially averaged intensity I was given as a function of the scattering angle 2θ in the angular range of 5°–35° after subtraction from the mica background. The total relative crystallinity was calculated as the ratio of the crystalline peak area to the total diffraction area using PeakFit software (Version 4.0, Systat Software Inc., San Jose, CA, USA). The ratio of the peak at 20° 2θ was the V-type relative crystallinity, while the relative crystallinity of A-type for maize starches or the B-type for potato...
starches was calculated by the subtraction of V-type crystallinity from the total relative crystallinity.

2.8. Fourier Transform InfraRed (FTIR) spectroscopy

The short-range order structure of the starch granules was estimated using a Bomem MB100 FTIR spectrometer (ABB-Bomem, Quebec, Canada) equipped with an attenuated total reflectance (ATR) single reflectance cell with a diamond crystal. The starch powder samples were scanned 64 times over the range of 4000–6000 cm⁻¹ at a resolution of 8 cm⁻¹ against air. The ratio of absorbance at 1047 cm⁻¹ to that at 1016 cm⁻¹ was calculated as previously described (Wang, Wang, Guo, Liu, 2017).

2.9. Scanning electron microscopy (SEM)

The granular morphology was analyzed using a Field Emission Scanning Electron Microscope (FE-SEM) (FEI Quanta 200). Starch samples were fixed, sputter-coated with gold, and images were taken at an acceleration voltage of 10 kV at 2000× magnification.

2.10. Statistical analysis

In the case of SAXS, 13C NMR, and 1H NMR, one measurement was performed, while other experiments were performed at least in triplicates, and the results were expressed as means ± standard deviations. Statistically significant differences (p < 0.05) were analyzed by Analysis of Variance (ANOVA) followed by Duncan’s test using SPSS 25.0 software (SPSS, Inc. Chicago, IL, USA). Normalized data were used to perform the principal component analysis (PCA) with the factoextra package in R (http://www.sthda.com/), and the mean values used for the correlation analysis. Flow diagram of the experimental design in this study is shown in Fig. 1.

3. Results and discussion

3.1. Effects of starch phosphate monoester on the glucan molecular structures

Chain length distribution (CLD) profiles (Fig. 2) of debranched starch samples examined by SEC showed three main components: short AP chains (AP1, DP 6–36), long AP chains (AP2, DP > 37–100), and AM chains (DP > 100) (Zhong, Bertoft, Li, Blennow, & Liu, 2020). The peak positions, average chain lengths (ACLs), and relative contents (RCs) of these three starch components (Table 2) were analyzed to investigate the effects of the SPC on the structural features of debranched starches.

The comparison between WPS and NPS, and between WMS and NMS in these structural parameters, demonstrated that increased AC resulted in a lower RCAP1 and RCAP2 in both maize and potato starch systems but had no effect on the ACLs of AP chains. The comparison of LPPS and HPPS showed that increased SPC was associated with increased PeakAP and ACLAP1, and decreased PeakAP2 and ACLAM in the potato starch system.

Detailed chain length distributions and fine structural parameters of AP molecules as determined by HPAEC-PAD (Zhong, Li, et al., 2021)
demonstrated that typical AP CLD profiles (Fig. 3 and Table 3) can be divided into four categories: fa (DP 6–12), fb1 (DP 13–24), fb2 (DP 25–36), and fb3 (DP >36) chains (Hanashiro, Abe, & Hizukuri, 1996). The fa, fb1, and fb2 chains are mainly located in the crystalline nanolamellae, while fb3 chains have been suggested to be oriented in the amorphous nano-lamellae (Bertoft, 2017). Increasing AC in both potato (comparing WPS and NPS) and maize starch (comparing WMS and NMS) systems had no significant effect on the ACLs and RCs of the AP fraction. However, an elevated SPC in potato starch systems with a similar AC was found, as expected, that increased AC in both potato (comparing WPS and NPS) and maize starch systems (comparing WMS and NMS) was related to a decreased α-1,6 ratio (Table 4), as AM chains are mostly linear and with only a few branches. Similar α-1,6 ratio of HPPS (3.6) and LPPS (3.5) and amylose contents (Table 1, ~29%) were found even though it is well documented that the AP in HPPS has longer chains (less branching, Table 3). As a consequence, the AM fraction in the LPPS must have somewhat fewer branches (more linear) than AM in the HPPS. AM is not normally considered to be phosphorylated, but the AM fraction of HPPS is probably so-called “amylose-like” material with relatively more branches than normal AM, which can be somewhat phosphorylated (Zhong, Qu, et al., 2022).

<table>
<thead>
<tr>
<th>Starch varieties</th>
<th>αCLfa (DP)</th>
<th>αCLfb1 (DP)</th>
<th>αCLfb2 (DP)</th>
<th>αCLfb3 (DP)</th>
<th>ACLal (DP)</th>
<th>ACLab (DP)</th>
<th>ACRL (DP)</th>
<th>RCfa (%)</th>
<th>RCfb1 (%)</th>
<th>RCfb2 (%)</th>
<th>RCfb3 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WPS</td>
<td>10.1 ± 0.8</td>
<td>18.0 ± 0.9</td>
<td>29.5 ± 0.4</td>
<td>53.2 ± 0.1</td>
<td>29.0 ± 0.3</td>
<td>12.4 ± 0.2</td>
<td>41.5 ± 0.9</td>
<td>17.1 ± 0.4</td>
<td>28.5 ± 0.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NPS</td>
<td>10.1 ± 0.8</td>
<td>18.1 ± 0.9</td>
<td>29.5 ± 0.1</td>
<td>53.9 ± 0.1</td>
<td>29.2 ± 0.4</td>
<td>11.7 ± 0.4</td>
<td>42.2 ± 1.2</td>
<td>17.5 ± 0.5</td>
<td>28.1 ± 1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPPS</td>
<td>10.2 ± 0.1</td>
<td>18.0 ± 0.9</td>
<td>29.4 ± 0.2</td>
<td>53.6 ± 0.4</td>
<td>28.2 ± 0.5</td>
<td>13.3 ± 0.2</td>
<td>43.0 ± 0.2</td>
<td>17.3 ± 1.0</td>
<td>26.1 ± 1.4</td>
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<tr>
<td>HPPS</td>
<td>10.1 ± 0.8</td>
<td>18.3 ± 0.9</td>
<td>29.7 ± 0.1</td>
<td>53.9 ± 0.4</td>
<td>30.2 ± 1.1</td>
<td>9.8 ± 0.5</td>
<td>38.8 ± 2.0</td>
<td>21.3 ± 0.2</td>
<td>29.2 ± 2.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WMS</td>
<td>10.3 ± 0.8</td>
<td>17.9 ± 0.9</td>
<td>29.5 ± 0.1</td>
<td>49.6 ± 0.2</td>
<td>25.2 ± 0.2</td>
<td>15.2 ± 0.2</td>
<td>45.8 ± 0.4</td>
<td>18.6 ± 0.4</td>
<td>20.0 ± 0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NMS</td>
<td>10.3 ± 0.8</td>
<td>17.9 ± 0.9</td>
<td>29.5 ± 0.1</td>
<td>48.9 ± 0.5</td>
<td>24.7 ± 0.7</td>
<td>16.0 ± 0.7</td>
<td>46.4 ± 1.3</td>
<td>18.1 ± 0.8</td>
<td>19.2 ± 1.0</td>
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</table>

Table 4
α-1,6 ratio, the relative contents of double-helix (DH), single-helix (SH) and amorphous region (AR) derived from solid state NMR data, A/B/V type crystalline polymers derived from WAXS, and FTIR ratios of potato and maize starches.*

<table>
<thead>
<tr>
<th>Starch varieties</th>
<th>αCL (%)</th>
<th>NMR</th>
<th>WAXS</th>
<th>FTIR Ratio at 1047/1016 cm⁻¹</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>DH</td>
<td>SH</td>
<td>AR</td>
</tr>
<tr>
<td>WPS</td>
<td>6.3</td>
<td>34.0</td>
<td>n.d.</td>
<td>66.0</td>
</tr>
<tr>
<td>NPS</td>
<td>4.0</td>
<td>30.4</td>
<td>16</td>
<td>68.0</td>
</tr>
<tr>
<td>LPPS</td>
<td>3.6</td>
<td>37.2</td>
<td>3.8</td>
<td>59.0</td>
</tr>
<tr>
<td>HPPS</td>
<td>3.5</td>
<td>31.1</td>
<td>1.7</td>
<td>67.0</td>
</tr>
<tr>
<td>WMS</td>
<td>7.3</td>
<td>36.0</td>
<td>n.d.</td>
<td>64.0</td>
</tr>
<tr>
<td>NMS</td>
<td>5.2</td>
<td>31.1</td>
<td>3.9</td>
<td>65.0</td>
</tr>
</tbody>
</table>

* Values with different uppercase letters in the same column are significantly different at p < 0.05.

Fig. 4. The raw 13C NMR spectra (A) and ordered sub-spectra (B) of starch samples.
3.2. Effects of starch phosphate monoester on the helical structures

The helical structures, including the relative contents of single (SH), double helices (DH), and amorphous regions (AR) analyzed by solid-state $^{13}$C NMR (Fig. 4 and Table 4) showed that increased AC in both potato (comparing WPS and NPS) and maize starches (comparing WMS and NMS) decreased the relative DH content, and concomitant increased both SH and AR content. This is consistent with a previous study (Zhong, Li, et al., 2021). Amylose can form DH in the crystalline lamellae, which is interpreted as crystalline defects, whereas amylpectin side chains form ordered DH (Koroteeva et al., 2007). Interestingly, LPPS showed higher relative contents of DH and SH than HPPS, indicating that starch with lower SPC forms more DH and SH. This is likely attributed to the fact that phosphate monoesters exert a significant strain or induce local defects in the crystalline AP section to prevent helical formation (Hansen et al., 2009).

3.3. Effects of starch phosphate monoester on the crystalline and lamellar structures

WAXS profiles showed that all potato starches had a typical B-type allomorph characterized by major reflections at 2$\theta$ of 5.8°, 15.0°, 16.9°, 22.1°, and 24.2° (Fig. 5B). The two maize starches had A-type allomorphs with strong reflections at 2$\theta$ of 15.2°, 17.2°, 18.2°, and 23.1°, consistent with previous data (Varatharajan et al., 2011; Zhong, Liu, et al., 2020). The total relative crystallinities of the six starch types varied in different starch samples, from 26 % to 41 % (Table 4). Increasing AC in both potato (comparing WPS and NPS) and maize (comparing WMS and NMS) starch systems resulted in lower amounts of B-type and A-type crystals, respectively, due to the effect of AM on generating crystal defects (Bertoft, 2017). However, LPPS showed significantly higher B-type crystallinity than HPPS, corresponding to the higher relative contents of DH in LPPS, as mentioned in Section 3.2.

ATR-FTIR was used to investigate the structural ordering of the surface layer of the starch granules (Liu, Li, Li, Zhang, & Li, 2021). The deconvoluted ATR-FTIR spectra of the different starch samples (Fig. 5IC), and the ratios of absorbance at 1047/1016 cm$^{-1}$, were calculated to indicate the degree of the short-range order structure related to molecular and rotational vibrations of the starch matrix (Table 4) (Cozzolino, Roumeliotis, & Eglinton, 2014; Liu et al., 2021; Van Soest, Tournois, de Wit, & Vliegenthart, 1995). Increasing AC in potato (comparing WPS and NPS) and maize (comparing WMS and NMS) starch systems, and enhancing SPC from LPPS to HPPS both resulted in lower 1047/1016 cm$^{-1}$ ratios, suggesting that both AC and SPC had a disordering effect on the granular surfaces.

SAXS profiles (Fig. 5DA) displayed the typical scattering peak around the $q$ value of 0.06–0.07 Å$^{-1}$, demonstrating the presence of a 9–10 nm semi-crystalline lamellar structure in all starch samples. The 1D correlation function (Fig. 5LB) was used to obtain the Bragg lamellar repeat distance ($d$), peak intensity ($I_{max}$), the thickness of crystalline lamella ($d_c$), the thickness of amorphous lamella ($d_a$), and long period distance ($d_{ac}$) (Kuang et al., 2017), as shown in Table 5. Increasing AC in both maize starch (comparing WMS and NMS) and potato starch (comparing WPS and NPS) systems predominantly increased the $d_c$, $d_a$, $D$, and $d_{ac}$. Our previous study on rice starch also demonstrated that AC was positively correlated with both $D$ and $d_c$ (Zhong, Li, et al., 2021). In addition, HPPS showed higher $d_a$, $D$, and $d_{ac}$ than those of LPPS, suggesting that phosphate groups may contribute to a more flexible lamellar structure. In addition, increased SPC (comparing LPPS and HPPS) reduced the $I_{max}$ by as much as three-fold, indicating a disordering effect of phosphate monoesters on the lamellar structure.

### Table 5

<table>
<thead>
<tr>
<th>Starch varieties</th>
<th>$D$ (nm)</th>
<th>$I_{max}$ (a.u.)</th>
<th>$d_a$ (nm)</th>
<th>$d_c$ (nm)</th>
<th>$d_{ac}$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WPS</td>
<td>9.80</td>
<td>2170</td>
<td>2.94</td>
<td>6.88</td>
<td>9.82</td>
</tr>
<tr>
<td>NPS</td>
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<td>2050</td>
<td>3.09</td>
<td>7.02</td>
<td>10.12</td>
</tr>
<tr>
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<td>2490</td>
<td>2.98</td>
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<td>9.92</td>
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<tr>
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<td>851</td>
<td>3.02</td>
<td>7.10</td>
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<tr>
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<td>1060</td>
<td>3.02</td>
<td>6.89</td>
<td>9.91</td>
</tr>
<tr>
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<td>1550</td>
<td>3.06</td>
<td>6.96</td>
<td>10.02</td>
</tr>
</tbody>
</table>

$^a$ $D$: Bragg lamellar repeat distance; $I_{max}$: peak intensity; $d_a$: thickness of crystalline lamella; $d_c$: amorphous lamella; $d_{ac}$: long period distance.

![Fig. 5. Principal component analysis (PCA) of structural parameters of four potato starches.](image-url)

(SPC: starch phosphate content; C3P: C-3 phosphate monoester content; C6P: C-6 phosphate monoester content; AC: amylose content; PeakQ: the DP of the peak of the fraction X; ALC: average chain lengths (DP) of fraction X; RCF: relative amount of fraction X; TC: total crystallinity; B-type: B-type crystalline polymorph; V-type: V-type crystalline polymorph; SH: relative amounts of single helices; DH: relative amounts of double helices; AR: relative contents of amorphous region; $D$: Bragg lamellar repeat distance; $I_{max}$: peak intensity; $d_a$: thickness of amorphous lamella; $d_c$: thickness of crystalline lamella; $d_{ac}$: long period distance.)
3.4. Effects of starch phosphate monoester on the granular structures

SEM images (Fig. S3) displayed ellipsoidal or round granules for all potato starches. Some irregular starch granules originated from the fusion of several smaller granules were observed for the HPPS; fusion is probably due to the high AC, as these granules have also been found in high amylose maize starches (Girling, Koch, & Blennow, 2006). Nodular protrusions at the granular surface were found in a few granules of WPS, NPS, and LPPS, likely indicating the irregular packing of the starch molecules in these regions (Blennow et al., 2003). The two types of maize starch showed either round or irregular shapes, which is in line with previous findings (Zhang et al., 2014).

3.5. Effects of starch phosphate monoesters on the multi-scale structures of potato starches — principal component analysis (PCA) and correlation analysis

Based on the above structural results, a higher AC is related to decreased double helical content, crystallinity and lamellar structures of phosphorylated and non-phosphorylated starch systems. In addition, the effects of AC on the multi-scale structures of starch granules have already been widely documented (Kozlov et al., 2007; Li, Dhital, Gilbert, & Gidley, 2020; Zhong, Liu, et al., 2020), hence this part mainly discusses the effects of SPC and phosphate distributions on the starch structural traits using PCA and correlation analysis.

PCA analysis, where the two main principal components (PC1 and PC2) explained 83% of the total variability, was conducted to interpret general effects of C6P, C3P, and SPC (Fig. 5). The four starches were grouped into four clusters, among which WPS was clustered with the α-1,6 ratio and relative content of AP1 chains (RCAP1); NPS was clustered with average chain length of β3 chains (ACLβ3, ACLAM, and PeakAM); LPPS was grouped with high PeakAP2, RCfb1, and ACLβ2, whereas C3P was clustered with RCfb2 and ACLβ1 chains. The β2 and β1 AP chains function as the major and second connector chains, respectively, between the double helix and backbone of AP chains (Zhong, Bertoft, et al., 2020; Zhong, Li, et al., 2021). Hence, these data suggested that both C6P and C3P might be predominately located at the connector chains between the backbone and double helical matrix.

Consistent with the PCA results, correlation analysis (Fig. 6) showed that C3P and C6P were significantly positively correlated with ACLβ2 and ACLAM, but negatively correlated with RCfb. These results underpin that longer AP chains are highly suitable for accommodating phosphate groups, an effect attributed to the preferred catalytic activity of GWD1 and GWD3 on long AP chains (Mikkelsen et al., 2004). Phosphate monoesters might be preferably located far away from the branching points (normally more than nine glucosyl residues) (Wikman et al., 2011), and thus longer connectors are more likely to accommodate a higher number of phosphate monoesters. It is also worth mentioning that both C3P and C6P contents were negatively correlated with the scattering intensity (I_0max), underlining the disordering effect of phosphate monoesters on lamellar structures, consistent with previous studies (Blennow & Engelsen, 2010; Kozlov et al., 2007). Interestingly, only the C3P content showed a significant negative correlation with the 1047/1016 cm⁻¹ ratio as analyzed by FTIR, emphasizing the effect of C3P on disturbing the structures of starch granules in external regions, as also supported by force field models (Hansen et al., 2009). It was reported that phosphate groups exist in both the internal and external regions of potato starch granules (Girling et al., 2006), and the present study emphasized that C3P might be mainly located in the external regions of starch granules. AC, one of the most important structural traits affecting the starch structure (Lin, Guo, Huang, et al., 2016; Lin, Guo, Zhao, et al., 2016; Zhong, Liu, et al., 2020), was also considered in the correlation analysis, and AC was found to be negatively correlated with RCAP1 and the α-1,6 ratio, and positively correlated with ACLAP2 and RCfb, as shown in Fig. 6, consistent with previous reports (Cheetham & Tiao, 1997; Zhong, Liu, et al., 2020). However, AC had no significant correlation with RC of fa, fb1, and fb2-three components of AP1, due to that RCAP1 was calculated based on the total contents of AP and AM, while fa, fb1, and fb2 were calculated based on the total contents of only AP fraction. Compared to AC, SPC, C3P, and C6P had a stronger correlation with more structural parameters at molecular and lamellar structural levels, suggesting that SPC is an additional critical factor determining the structural traits of potato starches, even though its content is much lower than the AC.

3.6. Proposed schematic molecular structures of potato starches with different SPC and similar AC

As shown in Table 4, HPPS had a lower relative crystallinity, contents
of single and double helices and $I_{\text{max}}$ than LPPS, despite having a similar AC level (27.1 % cf. 31.2 % with no significant difference, Table 3). As a consequence, HPPS showed a more disordered matrix with crystal defects. This is probably attributed to its high SPC and/or long AP chains. For AP chains, the $\text{ACL} \left( \rho \right)$ has a positive correlation with crystallinity and the relative content of the double helices in rice starches (Zhong, Li, et al., 2021). HPPS exhibited a significantly higher $\text{ACL} \left( \rho \right)$, i.e., more chains involved in the formation of double helices and potentially in crystalline order. It was therefore expected that HPPS is more ordered than LPPS, however, this was not the case. Hence, the disordered structure of HPPS might have been attributed to its high SPC. As discussed in Section 3.5, both C3P and C6P are suggested to be mainly located at the connector chains between backbone chains and double helices (Blennow, Engelsen, Munck, & Moller, 2000). However, phosphorylation can also take place in double-helical segments at both the C-3 and C-6 positions, in which the C6P aligns well in the groove of the double helix, whereas C3P is more exposed and even strains the double helix by being incompatible with its conformation (Blennow & Engelsen, 2010; Hansen et al., 2009). A weak negative correlation between the C3P and double helix content (Fig. 6) further implies that C3P can induce local defects during the double helix formation (Blennow & Engelsen, 2010; Hansen et al., 2009). Therefore, we suggest that a high content of C3P was the main reason for the disordered structure of HPPS, probably by preventing the double helical formation and disorienting the parallel alignment of the double-helical lamellae. This is the first study providing experimental data to support the functional differences of C3P and C6P in affecting the structural ordering of potato starches, and these findings will be helpful for potato breeding efforts that aim to control the phosphate group distributions to produce customized functional starch varieties. To support these efforts, future studies should provide a comprehensive understanding of how C3P, C6P or how the resulting structural differences caused by these starch phosphate monoesters affect the functionality of the potato starches.

4. Conclusion

The effects of starch phosphate monoesters, namely C3P and C6P on the multi-scale structures of potato starches were studied using four types of potato starches with different SPC as models. The structural results suggest that a higher SPC is related to smaller amylopectin (AP) molecular size, more long AP chains with DP > 24 and fewer short AP chains with DP ≤ 24; moreover, higher SPC correlates with lower amounts of double helices and crystallinity, as well as higher long period distance, and a lower scattering intensity ($I_{\text{max}}$), indicating less structural ordering and a more flexible lamellar structure of potato starch with higher SPC. Correlation analysis suggested that C3P were significantly negatively correlated with $I_{\text{max}}$ and short-range structural ordering at the starch granule surface (1047/1016 cm$^{-1}$), while C6P was only negatively correlated with the $I_{\text{max}}$. These results emphasized the disturbing effect of C3P on the structural ordering of potato starches, especially in the exterior part, probably by preventing the helical formation and disorienting the parallel alignment of the double-helical lamellae. This is the first study providing experimental data to support the functional differences of C3P and C6P in affecting the structural ordering of potato starches, and these findings will be helpful for potato breeding efforts that aim to control the phosphate group distributions to produce customized functional starch varieties. To support these efforts, future studies should provide a comprehensive understanding of how C3P, C6P or how the resulting structural differences caused by these starch phosphate monoesters affect the functionality of the potato starches.

CRediT authorship contribution statement

Li Ding: Conceptualization, Investigation, Methodology, Software, Writing – original draft. Wenxin Liang: Conceptualization, Investigation, Methodology, Software. Jianzhou Qu: Conceptualization, Investigation, Methodology, Software. Staffan Persson: Writing – review & editing. Xingxun Liu: Methodology, Software. Klaus Herburger: Methodology, Writing – review & editing. Jacob Judas Kain Kirkensgaard: Methodology, Writing – review & editing. Bezkod Khakimov: Methodology, Writing – review & editing. Kasper Enemark-Rasmussen: Methodology, Writing – review & editing. Andreas Blennow: Resources, Conceptualization, Supervision, Writing – review & editing. Yuyue Zhong: Resources, Conceptualization, Supervision, Writing – review & editing.
Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data can be found online at https://doi.org/10.1016/j.carbpol.2023.120740.

References


