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Extraction and identification of phospholipids from whole grain kabog millet flour and predictive effects on starch binding and retrogradation

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ABSTRACT

Kabog millet, an indigenous gluten-free cereal grain from the Philippines, is considered an ecotype of *Panicum miliaceum* L. (proso millet). Phospholipids (PLs) from kabog millet could improve the texture of kabog millet food products, such as breads and cookies, by their interaction with starch. Besides their influence on baking properties of flours, PLs have been studied for their health effects. No information exists regarding the PL content of kabog millet. In our study, we analysed the content and identity of PLs from whole grain kabog millet flour. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and lysophosphatidylcholine (LPC) were found to be present in whole grain kabog millet flour. To test PL-starch interactions, rice, maize, and potato starches, and commercial amylopectin were mixed with commercial soy PC and the viscosity was measured with a rheometer. Models of PL-amylose/amylopectin complexes were deduced from rheology, iodine capacity, and isothermal titration calorimetry (ITC) experiments. PLs can bind with starch and inhibit its retrogradation by increasing the viscosity after gelatinisation. In summary, whole grain kabog millet flour contains PC, PE, PI, and LPC, which can potentially affect the retrogradation of baked products made from kabog millet flour.

1. Introduction

Phospholipids (PLs), including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and lysophosphatidylcholine (LPC), are abundant in soybeans, eggs, and dairy products (Liu et al., 2013). They have a role in controlling the progression of hepatic diseases (Gundermann, Kuenker, Kuntz, & Droździk, 2011). Starch-lipid complexes are widespread in wheat, rice, and millets. Around 50% of total free fatty acids are bound to starch in non-waxy rice (Morrison, Mann, & Coventry, 1975; Liu et al., 2013) and many different fatty acids can bind to wheat starch (Tang & Copeland, 2007). Lipids in wheat flour can modify the starch granules during bread baking to prolong the storage of bread as well as to improve their final texture (Pareyt, Finnie, Putseys, & Delcour, 2011). Since the addition of PLs can inhibit the retrogradation of starch, it can improve the firmness of baking products and stabilise their sensory values (Siswoyo, Mukoyama, & Morita, 2000).

Kabog millet, an ancient cereal grain from the Philippines, is considered an ecotype of *Panicum miliaceum* L. (proso millet) (Narciso & Nyström, 2020). Kabog millet, which has a higher total protein content than rice, is an ideal protein source. The content and the type of essential amino acids are complementary between kabog millet and rice. Adding kabog millet flour into some rice products, such as noodles, can enrich the essential amino acid content of these food products and might also affect the retrogradation of rice starch. Kabog millet also contains carotenoids, tocopherols, and phenolic acids, which have significant antioxidant properties (Narciso & Nyström, 2020). Kabog millet flour could be mixed with wheat flour and other flour types, and the PLs and antioxidant compounds from kabog millet could have a potential effect on the storage time of these flours and inhibit their retrogradation in baked products. Since kabog millet does not contain gluten, it could be a potential source of PLs and other bioactive nutrients in gluten-free food products. Research on PLs in kabog millet and other ancient grains is important and can serve as a reference study for future food development of whole grain foods.

Lipids, including PLs, are adsorbed by cereal starch granules into their surface layer (Vasanthan & Hoover, 1992; Liu et al., 2013). Raman spectroscopy studies showed that the non-polar head of monostearin can

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be adsorbed on a left-handed single amylose helix to form amylose-lipid complexes. The polar head of the lipid faces the water and is not adsorbed (Carlson, Larsson, Dinh-Nguyen, & Krog, 1979; Putseys, Lamberts, & Delcour, 2010). The binding between amylose and lipids is reversible and amylose-lipid complexes can be separated by heating (Biliaderis, Page, Slade, & Sirett, 1985; Brouwer, 2017). Amylose-lipid levels could be low at high temperatures. PLs could show similar surface properties as other lipids regarding amylose binding: PLs can be adsorbed inside the amylose and inhibit its retrogradation (Liu et al., 2013). Since crystalline amylopectin has a similar structure to amylose (Bonechi et al., 2017), amylopectin could possibly bind to PLs. The binding between amylopectin and PLs needs further research.

The amylose and amylopectin in starch may have different affinities to PLs. PLs in starch-PL complexes can protect the structure of starch during the pasting period and increase the pasting viscosity (Singh, Dartois, & Kaur, 2010). PLs can also promote the formation of hydrogen bonds in the retrogradation and setback periods (Ratnayake & Jackson, 2008). The viscosity of pure starch during gelatinisation decreases significantly after gelatinisation peak and setback during the pasting period (Wang, Li, Copeland, Niu, & Wang, 2015). Thus, changes in the viscosity of amylose and amylopectin during gelatinisation can indicate the level of starch-PL complex formation. Retrogradation is one of the reasons for the firmness of baked products such as bread (Siswoyo et al., 2000). PLs can bind with starch and modify its gelatinisation and retrogradation properties, including an increase in viscosity of starch during setback periods (Liu et al., 2013). Rheological properties can be measured to identify the complex formation between starch and lipids (Shah, Zhang, Hamaker, & Campanella, 2011). The modified gelatinisation and retrogradation of starch can be analysed by a rheometer or rapid viscosity analyser in viscosity curves (Gelders, Goesaert, & Delcour, 2006).

The iodine capacity of amylose and amylopectin, which is the content of adsorbed iodine, is relative to their structural integrity (Rundle, Foster, & Baldwin, 1944; Tang & Copeland, 2007). Starch-PL complexes with a higher binding affinity have a higher iodine capacity. In gelatinisation, amylose mixed with PLs can exhibit a higher iodine capacity than pure amylose at low temperatures and a lower iodine capacity than pure amylose above a certain temperature. The reason is that heating can break the amylose-PL complexes, effectively separating the amylose from the PLs (Biliaderis et al., 1985; Brouwer, 2017). Likewise, the effects of heating on amylopectin-PL complexes and the conditions under which amylopectin-PL complexes form can be tested by iodine-starch binding. The iodine capacity of amylopectin can indicate the binding conditions and heating effects on amylopectin-PL complexation.

This research aims to identify and quantify the major species of PLs including lysophospholipids (LPLs) in whole grain kabog millet flour through thin-layer chromatography (TLC) and normal-phase high-performance liquid chromatography (NP-HPLC), as well as to discover the potential ability of PLs in inhibiting the retrogradation of various starches through rheology and iodine capacity experiments. It is envisioned that this study will add to the knowledge on nutritional and functional value of kabog millet and provide a model for amylose or amylopectin interactions with PLs that can be applied in general to PL-fortified starch-based products.

2. Materials and methods

2.1. Chemicals

The thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) standards were obtained commercially: **standard 1**: soy PC (20%) L-α-phosphatidylcholine, 20% (soy), Soy Total Lipid Extract granular (Sigma-Aldrich, USA); **standard 2**: phospholipid mixture for HPLC from *Glycine max* (soybean) (Sigma-Aldrich, USA); **standard 3**: L-α-lysophosphatidylcholine from soybean, ≥98.0% (10 mg phospholipid per mL chloroform, TLC) (Sigma-Aldrich, USA). Kabog millet samples were purchased from two farmer sources from Cebu, Philippines: source 1 (Mrs. Rosaflor Estenzo) and source 2 (Mr. Nolito Ares) (Narciso & Nyström, 2020). Rice starch samples (starch content 89.9%; amylose content 21.3%; amylopectin content 68.6%), maize starch samples (starch content 88.7%; amylose content 24.2%; amylopectin content 64.5%), and potato starch samples (starch content 81.9%; amylose content 23.1%; amylopectin content 58.8%) were purchased from local supermarkets in Zürich, Switzerland. Two starch samples were purchased commercially: high-amylose maize starch (amylose content 68%) (Megazyme, Ireland) and maize amylopectin (amylopectin content 99.9%) (Sigma-Aldrich, Switzerland). Lugol solution (1.007 g/mL at 20°C) was purchased from Sigma-Aldrich, Switzerland. Ultrapure water was from MilliQ system. All the solvents were obtained from Sigma-Aldrich as HPLC grade.

2.2. Extraction methods

The flours of whole grain kabog millet from two farmer sources were heated at 175°C for 30 min for lipase inactivation and stored in a vacuum desiccator (Caboni, Menotta, & Lercker, 1996; Avalli & Contarini, 2005). The first step was cold extraction to remove non-starch lipids. Flour samples (5 g) were mixed with 15 mL ethanol/diethyl ether/water (2:2:1 v/v) and sonicated for 15 min at room temperature. The upper liquid was collected under nitrogen gas after centrifugation (5 min, 3100×*g*, and 21°C). After repeating the cold extraction three times, hot extraction was performed. The residue samples were mixed with water-saturated *n*-butanol in boiling water bath for 3 h and centrifuged thereafter. After the samples were cooled to 20°C, the extracted liquid was removed. The extracted liquid samples were dried under nitrogen gas and dissolved in chloroform (0.5 mL). The extracted samples were purified by solid-phase extraction (SPE) (Fauland et al., 2013). The chromatography-silica (LC-SI) cartridge was activated by 3 mL *n*-hexane and the TLC plate (Silica G TLC plates w/UV254 + 366, Sorbtech, USA) was utilised. The dissolved samples were added to the LC-SI cartridge. The non-polar lipids were eluted with 4 mL *n*-hexane/diethyl ether (8:2 v/v), 4 mL *n*-hexane/diethyl ether (1:1 v/v). The PLs were eluted with 2 mL methanol and 4 mL chloroform/methanol/water (3:5:2 v/v). The purified samples were dried under nitrogen gas for further experiments.

2.3. TLC and NP-HPLC

The PLs and LPC were separated using a silica gel plate (Silica G TLC Plates w/UV254 + 366, Sorbtech, USA) and iodine vapour (Sigma-Aldrich, Switzerland) for visualisation. The single acidic solvent system (chloroform/acetone/methanol/acetic acid/water, 30:12:6:9:3 v/v) was used, and the TLC was performed in a TLC tank (17.5 × 16.0 × 6.2 cm). The samples from two different extractions (hot and cold) were dissolved in 1 mL chloroform/methanol (2:1 v/v) and applied as PLs dots (10 μL) at the start line.

Three PL standard samples (S1, S2, and S3) for TLC were applied (10 μL each) following the same procedure. S1 was **standard 1** dissolved in chloroform/methanol (2:1 v/v) at 0.5 mg/mL. S2 was pure **standard 2**. S3 was **standard 3** diluted 10 times (see Section 2.1). The plate was exposed in a tank with saturated iodine vapour for visualisation and the spots were marked with a pencil.

The HPLC system (Agilent 1200 Series Gradient HPLC System, Agilent, USA) was equipped with a binary pump and ultraviolet (UV) detector. Standard samples (1, 2, and 3) were prepared with different dilutions as reference. All recovered samples, which were dried under nitrogen gas, were suspended in 1 mL chloroform/methanol (2:1 v/v) and stored at −20°C prior to HPLC analysis. The redissolved PLs samples were filtered (13 mm Syringe Filter, Nylon 66, 1.0 μm, pk.100, BGB, USA) and sealed in vials. The blank sample only contained chloroform/methanol (2:1 v/v) solution for reference. All of the prepared kabog millet samples, standard samples, and the blank sample were analysed using a normal-phase HPLC with diol-bonded silica column in HILIC

mode (Luna 5 μm HILIC 200 Å, LC Column 150 × 3 mm, Phenomenex, Switzerland), with the flow rate of the mobile phase at 300 μL/min, injection volume of 10 μL, and column temperature at 40°C. Mobile phase A was 10 mM ammonium formate and 0.1% (v/v) formic acid in aqueous solution and mobile phase B was 10 mM ammonium formate and 0.1% (v/v) formic acid in water-ACN (95:5 v/v) solution. The binary gradient system was 100% B at the start; 90% B at 40 min; 70% B at 40.5 min; 70% at 50 min; 100% B at 50.5 min and 100% B at 60 min. Comparing with the standard samples, the area and the retention time of the peaks in all curves from the HPLC chromatogram were calculated and detected. A series of peak areas with different standard concentration were used as references for quantifying the PLs concentration.

2.4. Iodine capacity tests

Amylose-water slurry (40 μg/mL) was prepared with high amylose maize starch (amylose content 68%) as well as amylopectin-water slurry (80 μg/mL) was prepared with pure amylopectin (Jarvis & Walker, 1993). PL-water emulsion (10 μg/mL) was prepared with **standard 1** (see Section 2.1) with 10 min sonication. All of the starch-water slurry (5 mL) were mixed with 5 mL of the prepared PL-water emulsion. Controlled samples without PLs were 5 mL of the prepared amylose-water slurry (40 μg/mL) diluted with water to 10 mL, and 5 mL amylopectin-water slurry (80 μg/mL) diluted with water to 10 mL. All of the samples were shaken and treated at different temperatures: at room temperature, 50°C, 70°C, and 90°C. When all of the samples had cooled down at room temperature, 200 μL Lugol solution was added to all of the amylose and amylopectin samples. The PL-water emulsions (5 μg/mL) and pure water samples were prepared for the spectrophotometer test as reference. The wavelengths of the spectrophotometer (Agilent Cary 100 UV–Vis Spectrophotometer, Agilent, Switzerland) were 630 nm for the iodine-amylose complex and 548 nm for the iodine-amylopectin complex (Jarvis & Walker, 1993). The time, from mixing the iodine agent with the starch up to the absorbance reading, was consistent for each sample. The light absorbance values of each sample were collected for comparison and further analysis. Absorbance differences (ΔAbs) between pure starch-iodine and starch-iodine with PLs were calculated as follows:

$$\Delta Abs = Abs_{Starch-PLs} - Abs_{Starch\ only} \quad (1)$$

2.5. Isothermal titration calorimetry

The binding between amylose or amylopectin and PLs in water was measured by MicroCal PEAQ-ITC (Malvern Panalytical Ltd, Switzerland) at 25°C. The stirring speed of 860 rpm and reference power of 10 mcal/s were used. Amylose-water solution (1 mg/mL) was prepared with high amylose maize starch (amylose content 68%), as well as amylopectin-water solution (1 mg/mL) was prepared with pure amylopectin. PLs-water emulsion (20 mg/mL) was prepared with **standard 1** (Soy PC) (see Section 2.1). The sample cell (280 μL) contained amylose or amylopectin solution, the reference cell contained MilliQ water, and the injection syringe (40 μL) contained PLs emulsion. PLs emulsion (3 μL) was titrated into the sample cell every 3 min with a total of 13 injections. The resulting titration curves of amylose and amylopectin were corrected for ligand-free buffer interactions and analysed.

2.6. Rheometer studies

The starch samples used in the rheology studies were rice, maize, and potato starch, and pure amylopectin (1.8 g each added to 18 mL water). The starch samples were mixed with 200 mg **standard 1** (soy PC) (see Section 2.1) until PL granules were completely suspended in the aqueous mixture. Control samples consisted of the starch and water mixture without PLs. All of these samples were performed on a rheometer (MCR 92, Anton Paar Switzerland AG, Switzerland) with their typical method of starch tests (shear rate 160 rpm), which used the ICC 162 standard. Analysis of each of the starch samples was performed in triplicates and the gelatinisation and retrogradation data as well as viscosity curves from the rheometer were collected and compared. Gelatinisation temperature (GT) is reported as the temperature at the peak of viscosity. Changes in peak viscosity (PV) and final viscosity (FV) due to the PLs adding were calculated as follows (Tang & Copeland, 2007):

$$\Delta PV\ (\%) = \left(PV_{Starch-PLs} - PV_{Starch\ only}\right) \div PV_{Starch\ only} \times 100 \quad (2)$$

$$\Delta FV\ (\%) = \left(FV_{Starch-PLs} - FV_{Starch\ only}\right) \div FV_{Starch\ only} \times 100 \quad (3)$$

2.7. Statistical analysis

All experiments were carried out in triplicates unless otherwise stated, and data were reported as mean ± standard deviation. The means in Tables were evaluated using a one-way ANOVA and Tukey's HSD test ($p < 0.05$) (https://astatsa.com/OneWay_Anova_with_TukeyHSD/).

3. Results and discussion

3.1. Quantification of PLs from kabog millet

The schematic diagram of the TLC plate showing the different phospholipid classes from kabog millet and the standard PLs is shown in Fig. 1. The PLs were determined by their Rf values in the TLC pattern. Comparing with the Rf values under the same conditions (Rivnay, 1984), the various PLs detected in the current TLC were LPC with Rf 0.19, PI with Rf 0.23, PC with Rf 0.3, PE with Rf 0.43, and PA with Rf 0.73. There were some overlaps between LPC and PI in two hot-extracted samples. Thus, LPC could be extracted mainly by the hot extraction method. According to the TLC pattern, PC, PE, PI, and LPC were detected in cold-extracted samples and hot-extracted samples. PA was not detected in both samples using cold and hot extraction processes. Some smaller dots had a similar Rf value as PC in hot extraction; however, PC was not detected in hot extraction by HPLC. These dots could overlap with other lipid compounds, which have the similar Rf value as PC.

After the TLC identification of whole grain kabog millet PLs, NP-HPLC was performed to quantify the PLs from kabog millet that were extracted using cold and hot extraction processes. The order of every peak of PLs and their lyso types has been detected by HPLC-MS (Jiang, Ma, Song, Lai, & Cheong, 2018) and the same conditions (mobile phases and HILIC column) were applied in our study. Table 1 showed that the highest component of PL from kabog millet was PE followed by LPC, PC and PI. PI and PC had a similar percentages in kabog millet source 2.

PI and PC did not display a significant difference in Tukey's HSD analysis for both kabog millet sources. Cold extraction can extract more PC than hot extraction, while hot extraction can extract more LPC than cold extraction. Cold-extracted samples contained PE, PI, and PC, while hot-extracted samples contained a high amount of LPC (Table 1). Non-starch lipids in kabog millet samples could be extracted more effectively by cold extraction. LPC, which is existing in cereal starch as a starch lipid, could be extracted by hot extraction (Morrison et al., 1975). Whole grain kabog millet flour from source 1 had a higher total PLs than whole grain kabog millet flour from source 2 (Table 1). Whole grain kabog millet flour from sources 1 and 2 had similar amount of PI and PC (Table 1). PE is the highest PL component in both two sources 1 and 2 followed by LPC (Table 1). Different PL profiles and concentrations from two sources of kabog millet were detected potentially due to the different growing conditions under which the two samples were cultivated.

3.2. Iodine-starch complexes with or without PLs binding

The light adsorptions of iodine-amylose and iodine-amylopectin can

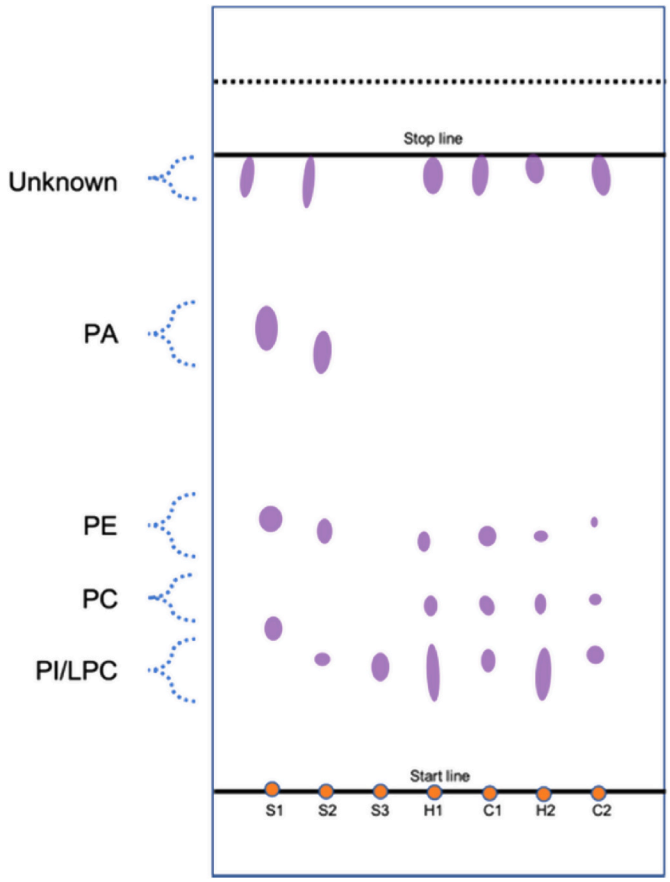

Fig. 1. Schematic diagram of the TLC plate showing the phospholipid class compositions from kabog millet and the standard samples as visualized with iodine vapour. S1: standard sample 1 (soy PC (20%) L-α-phosphatidylcholine, 20% soy); S2: standard sample 2 (phospholipid mixture for HPLC from *Glycine max* (soybean)); S3: standard sample 3 (L-α-lyso-phosphatidylcholine from soybean, ≥98.0%); C1 and C2: kabog millet samples from source 1 and source 2 with cold extraction; H1 and H2: kabog millet samples from source 1 and source 2 with hot extraction. Purple and orange spots indicate iodine spots after visualisation and initial sample spots on the starting line, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Amount of each PL components and total amount of PLs (μg/g) in two kabog millet sources (1 and 2) analysed by NP-HPLC.

Sample	Method	PE	PI	PC	LPC	
Source 1	Cold extraction	150 ± 20[a]	40 ± 10[a]	70 ± 10[a]	0	
Source 2		140 ± 30[a]	40 ± 30[a]	50 ± 20[a]	0	
Source 1	Hot extraction	190 ± 30[a]	10 ± 00[a]	0	220 ± 90[a]	
Source 2		100 ± 20[b]	20 ± 00[a]	0	130 ± 40[b]	
Sample		Total PE	Total PI	Total PC	Total LPC	Total amount
Source 1		340 ± 40[a]	50 ± 10[a]	70 ± 10[a]	220 ± 90[a]	690 ± 100
Source 2		240 ± 40[b]	60 ± 30[a]	50 ± 20[a]	130 ± 40[b]	480 ± 20

Data is expressed as Mean ± SD (n = 3). The different superscript letters represent significance at $p < 0.05$ using Tukey's HSD.

Table 2
Visible light absorbance of iodine-amylose (Am) at 630 nm and iodine-amylopectin (Ap) at 548 nm with or without PLs at different temperatures and their absorbance differences (ΔAbs).

Sample	Room temperature	50°C	70°C	95°C
Abs_{Am}	0.0248 ± 0.0009[a]	0.0264 ± 0.0003[a]	0.0290 ± 0.0011[a]	0.0756 ± 0.0024[a]
$Abs_{Am\text{-}PLs}$	0.0265 ± 0.0016[a]	0.0272 ± 0.0024[a]	0.0310 ± 0.0011[a]	0.0662 ± 0.0024[b]
ΔAbs_{Am}	0.0018	0.0008	0.0020	−0.0093
Abs_{Ap}	0.0643 ± 0.0057[a]	0.0582 ± 0.0057[a]	0.1273 ± 0.0018[a]	0.2141 ± 0.0010[a]
$Abs_{Ap\text{-}PLs}$	0.0685 ± 0.0056[a]	0.0589 ± 0.0023[a]	0.1298 ± 0.0043[a]	0.2230 ± 0.0005[b]
ΔAbs_{Ap}	0.0042	0.0007	0.0025	0.0072

Data is expressed as Mean ± SD (n = 3). The different superscript letters represent significance at $p < 0.05$ using Tukey's HSD.

indicate the affinity of PLs binding. PLs-water emulsion had light absorbances of 0.0015 at 630 nm and 0.0020 at 548 nm. Table 2 showed the light absorbances of iodine-amylose and iodine-amylopectin complexes with or without PLs at room temperature, 50°C, 70°C, and 95°C, from which the absorbance of PLs-water emulsion has been subtracted.

Iodine can react with PLs at the double bond site, which cannot affect the visible light absorptions at 630 nm and 548 nm. PLs can bind to amylose or amylopectin and protect their structure in the aqueous phase (Biliaderis et al., 1985; Brouwer, 2017). Starch, which has a strong affinity with PLs, could present a higher visible light absorbance. The higher ΔAbs value could show a higher affinity between starch samples and PLs. Amylose samples exhibited higher ΔAbs values at room temperature and at 70°C than at 95°C (Table 2). Amylopectin samples exhibited the highest ΔAbs values at 95°C, and the lowest ΔAbs values at 50°C (Table 2). PLs increased the iodine capacity of amylose at lower temperature. This effect was retarded by increasing temperature. This result in which the iodine capacity of the amylose-PL complexes changes with heating is consistent with the results of previous studies (Biliaderis et al., 1985). The affinity between amylose and PLs is higher at lower temperatures than at higher temperatures (Biliaderis et al., 1985; Brouwer, 2017). PLs presented a different modification of iodine capacity to amylopectin upon heating. PLs increased the iodine capacity of amylopectin at 95°C and did not modify its iodine capacity at lower temperatures. PLs could not have a strong affinity with amylopectin at low temperatures (lower than 95°C). At 95°C, PLs could display a stronger affinity with amylopectin than at 50°C and 70°C. Amylopectin showed different PLs binding properties to amylose.

3.3. Isothermal titration calorimetry measurements

After the iodine capacity tests, the binding between amylose- or amylopectin-water solution and PLs emulsion was measured. Amylopectin did not have any binding to PLs. The resulting titration curve of amylose-PLs is shown in Fig. 2. Amylopectin did not show any significant binding to PLs in the aqueous phase at 25°C, while amylose showed weak binding ($K_d = 8.3 \times 10^{-5}$) to PLs in the aqueous phase under similar conditions. Due to the modeling of amylose-lipids, the non-polar head of PLs can be adsorbed in an amylose helix, which can be inhibited by heating (Carlson et al., 1979; Putseys et al., 2010). The natural form of PLs in the aqueous phase is micellar, which could inhibit the release of free PLs. PLs can also be released from the amylose-PLs complexes to form micelles by changing the temperature. The affinity between amylose and PLs was weak by ITC measurements.

3.4. Viscosity features of starch with or without PLs samples

After the ITC tests, the gelatinisation processes of different starches with and without PLs addition were monitored by rheometer. According

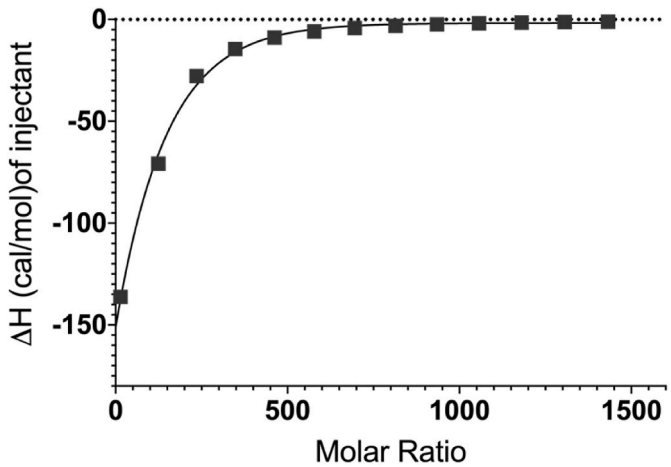

Fig. 2. ITC profile of amylose-water solution (1 mg/mL, 6.8 × 10^{-7} mM) and PLs-water emulsion (20 mg/mL, 5.1 mM) at 25°C.

to Fig. 3 and Table 3, rice starch presented a consistent gelatinisation temperature (GT) at 95°C, which was not modified by PLs. Rice starch with PLs had a slightly higher viscosity from the initial heating to the setback period, and a significantly higher final viscosity (3660 cP) than rice starch without PLs (2350 cP). Addition of PLs resulted in a decrease in GT of maize starch from 93°C to 78°C and an increase in its peak viscosity from 2380 cP to 2930 cP. The viscosity of maize starch was increased by the addition of PLs in the retrogradation and setback period. The presence of PLs decreased the peak viscosity of potato starch from 10000 cP at GT (71°C) to 4960 cP at GT (75°C). The peak viscosity of potato starch in the presence of PLs was not striking throughout the entire gelatinisation process. The final viscosity of potato starch was not modified by the addition of PLs. Pure amylopectin without PLs exhibited GT (77°C) and peak viscosity (3550 cP) that were not modified by PLs. However, the addition of PLs led to an increase in the viscosity during the retrogradation and setback periods. The final viscosity of amylopectin was increased by the addition of PLs from 1250 cP to 1600 cP. The starch, which adsorbs or binds more PLs, can present a higher final viscosity because PLs can protect their helical structures and promote an increase in the viscosity during the setback periods.

ΔPV values showed the changes in viscosity of the starch samples viscosity at GT and ΔFV values showed the changes in viscosity of the starch samples at the pasting period. Rice starch exhibited the highest ΔPV and ΔFV values followed by maize starch, amylopectin, and potato starch (Table 4). Rice starch showed the highest GT and Ap/Am ratio, followed by maize and potato starch (Table 4). GT, ΔPV, and ΔFV values of each sample showed a strong correlation. According to the results of the iodine capacity experiments and ITC measurements, amylose has a significant affinity with PLs at lower temperatures and amylopectin cannot bind to PLs before GT. Because heating can break the binding of amylose-PLs, the level of amylopectin-PLs binding could dominate ΔFV values. Amylopectin with GT at 76°C showed a ΔFV at 28% and the samples, which had higher GTs, showed higher ΔFV values. Potato starch with GT at 77°C (lower than Ap) did not show a significant change of FV (ΔFV at 4%).

According to the results of the rheometer, PLs can increase the viscosity of rice starch, maize starch, and amylopectin in the retrogradation and setback periods. The binding of PLs to amylose dominates at lower temperature, while the binding of PLs to amylopectin occurs at temperatures higher than a critical GT. Thus, the affinity of the PL-amylose complex dominates the viscosity, starting from the initial period to GT. Amylopectin granule contains two amorphous regions with branched linkages (α-1,6-glycosidic bond) and crystalline regions with chain linkages (α-1,4-glycosidic bonds). According to the structure of amylopectin (Bonechi et al., 2017; Patterson, Emes, & Tetlow, 2017), the

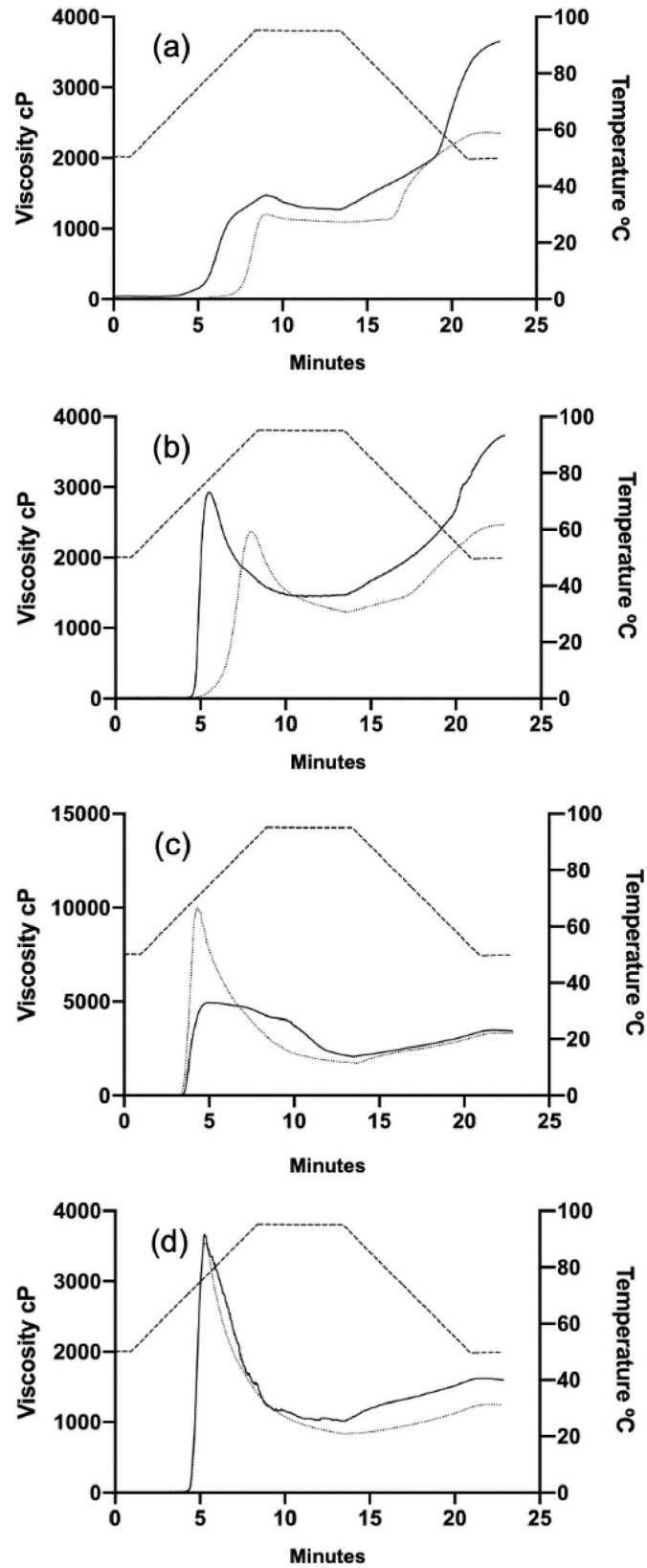

Fig. 3. Viscosity curves of (a) Rice starch and rice starch with PLs (200 mg); (b) Maize starch and maize starch with PLs (200 mg); (c) Potato starch and potato starch with PLs (200 mg); (d) Amylopectin and amylopectin with PLs (200 mg). Dashed lines indicate the temperature profile; light dotted lines indicate pure starch samples, while black solid lines indicate starch-PL mixtures.

Table 3
Gelatinisation properties of rice, maize, potato starches and amylopectin (Ap) with or without PLs addition.

Samples	Peak viscosity (cP)	Peak time (minutes)	Gelatinisation temperature (°C)	Final viscosity (cP)
Rice	1200 ± 230[a]	9±0[a]	95±0[a]	2350 ± 390[a]
Rice-PLs	1480 ± 27[a]	9±0[a]	92±5[a]	3660 ± 230[b]
Maize	2380 ± 190[a]	8±0[a]	93±0[a]	2460 ± 190[a]
Maize-PLs	2930 ± 360[a]	6±0[b]	78±0[b]	3730 ± 300[b]
Potato	10000 ± 560[a]	4±0[a]	71±0[a]	3300 ± 270[a]
Potato-PLs	4960 ± 140[b]	5±0[b]	75±0[b]	3430 ± 26[a]
Ap	3550 ± 21[a]	5±0[a]	77±0[a]	1250±9[a]
Ap-PLs	3680 ± 17[a]	5±0[a]	76±0[a]	1600 ± 42[a]

Data is expressed as Mean ± SD (n = 3). The different superscript letters represent significance at $p < 0.05$ using Tukey's HSD.

Table 4
Amylose (Am) percentage, amylopectin (Ap) percentage, ratio of amylopectin and amylose, ΔPV, ΔFV and gelatinisation temperature (GT) of each starch sample.

Samples	Am (%)	Ap (%)	Ratio (Ap/Am)	ΔPV (%)	ΔFV (%)	GT (°C)
Potato	23.1	58.8	2.6	−50.4	3.9	74.6
Amylopectin	0	99.9		3.7	28.3	76.5
Maize	24.2	64.5	2.7	18.9	51.6	77.9
Rice	21.3	68.6	3.2	23.1	55.3	91.8

Ratio was calculated as follows: Ratio = amylopectin content/amylose content. The GT values of pure starch and amylopectin were modified by PLs addition.

crystalline regions of amylopectin could also have the similar helical space (cavity) to amylose.

Addition of PLs decreased the peak viscosity of potato starch at GT (70°C) and did not change its final viscosity, exhibiting a lower ΔFV value (Table 4). Since amyloid chains in crystalline regions (helical 3-dimensional space) are closed (lowest inner energy) at a lower temperature, the inner helical 3-dimensional (3D) space of crystalline regions is not sufficient for PL adsorption. Potato starch with a high percentage of amylopectin did not bind to PLs. The GT of potato starch was 70°C which could be too low to open the crystalline regions. In the macroscopic view, the viscosity changes of starch samples can indicate the affinity between PLs and starch. PLs could be adsorbed inside by amylose from the initial stage of gelatinisation and adsorbed by amylopectin after GT. The critical GT of PL-amylopectin binding could be higher than 75°C (74.6°C) and lower than 77°C (76.5°C). PLs can bind to crystalline amylopectin whose GT is higher than a critical GT value.

Amylose can bind PLs at room temperature and heating can decrease the affinity of amylose-PLs. The polar head of PLs faces the aqueous phase and is not captured by the helical space of amylose (cavity) (Biliaderis et al., 1985; Brouwer, 2017). In a molecular view, the crystalline regions of amylopectin can supply the potential helical space (cavity) for PLs binding. However, amorphous regions with branched linkages (α-1,6-glycosidic bond), which could not provide enough helical 3D space to water and PLs, could stop this binding because the polar head of PLs should face the aqueous phase at low temperatures. The crystalline regions of amylopectin could capture PLs when it expands until a helical critical 3D space is formed after reaching a critical GT (Fig. 4). At room temperature (or before the GT), crystalline regions of amylopectin could not capture PLs because the inner affinity within the α-1,6-glycosidic bonds in the crystalline regions can stop the PLs adsorption in the helical space (cavity) of the amylopectin. When amylopectin expands completely above a critical GT, PLs could bind with the helical space (cavity) of its crystalline regions and inhibit the retrogradation. After cooling down, PLs could be captured by amylopectin in the retrogradation period (Fig. 4).

4. Conclusion

This study reported the major PLs in two sources of kabog millet including PE, PI, PC, and LPC. PLs have a stronger affinity with amylose at low temperatures (lower than 70°C) and a weak affinity at high temperatures. PLs can bind to amylopectin and inhibit the retrogradation after heating up to a critical GT. By studying the effects of PLs on retrogradation and gelatinisation of starch through iodine capacity, ITC, or rheology measurements, the properties of flours containing PLs can be roughly predicted. Based on our results, PLs addition can increase the final viscosity of starch or flour so it can inhibit the retrogradation in baking processing and improve its softness in final food products.

Author contributions

Boxu Hao: Conceptualization, data curation, formal analysis, investigation, validation, visualisation, writing – original draft; Joan Oñate Narciso: Conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, supervision, validation, writing – review and editing; Laura Nyström: Conceptualization, methodology, project administration, resources, software, supervision, writing – review and editing.

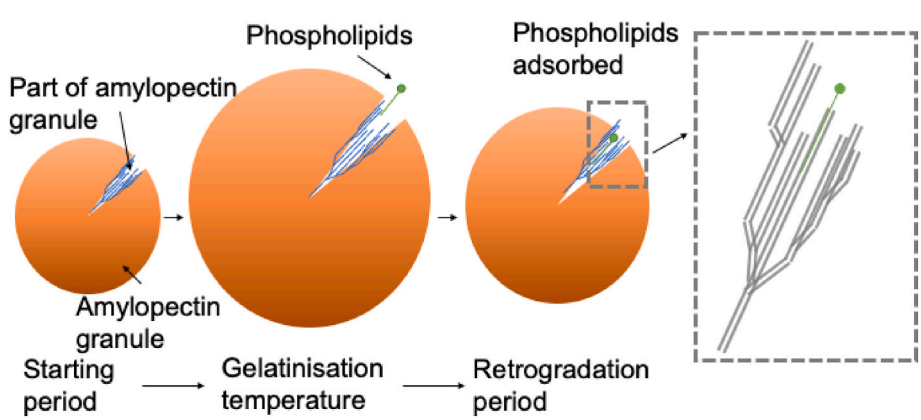

Fig. 4. The forming of PL-amylopectin complexes during gelatinisation. The crystalline regions of amylopectin can bind PLs during gelatinisation. The polar head of PLs could locate outside the helix of crystalline regions and face to aqueous phase.

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Declaration of competing interest

None.

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