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# Physicochemical properties of starches and proteins in alkali-treated mungbean and cassava starch granules

Kamolwan Israkarn[a], Nantarat Na Nakornpanom[b], Parichat Hongsprabhas[a,*]

[a] Department of Food Science and Technology, Faculty of Agro-Industry, Kasetsart University, 50 Ngam Wong Wan Road, Chatuchak, Bangkok 10900, Thailand
[b] Department of Food Science and Nutrition, Faculty of Agricultural Product Innovation and Technology, Srinakharinwirot University, 114 Sukhumvit 23, Bangkok 10110, Thailand

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ABSTRACT

This study explored the influences of envelope integrity of cooked starch granules on physicochemical and thermophysical properties of mungbean and cassava starches. Alkali treatment was used to selectively leach amylose from the amorphous region of both starches and partially fragmented starch molecules into lower-molecular-weight polymers. It was found that despite the loss of 40% of the original content of amylose, both mungbean and cassava starches retained similar crystallinities, gelatinization temperature ranges, and pasting profiles compared to the native starches. However, the loss of granule-bound starch synthases during alkali treatment and subsequent alkali cooking in excess water played significant roles in determining granular disintegration. The alterations in envelope integrity due to the negative charge repulsion among polymers within the envelope of swollen granules, and the fragmentation of starch molecules, were responsible for the alterations in thermophysical properties of mungbean and cassava starches cooked under alkaline conditions.



## 1. Introduction

The structure-forming process of starch paste and gel is a multi-step process which occurs in the presence of excess water. The process involves phase transition from a semi-crystalline structure into a disordered hydrated state and the swelling of starch granules, followed by leaching of (mainly) linear chain amylose and the packing of swollen granules. This results in a structure of granular starch particles embedded in a continuous amylose matrix, a so-called close-packing structure, particularly at high starch concentration. Such a composite structure is dominated by swollen granules in a continuous amylose matrix (Hermansson & Svegmark, 1996). Starch gel characteristics are thus mainly governed by the volume fraction of the dispersed swollen granules, their rigidity and surface interactions. The integrity of the envelope encasing swollen granules is therefore responsible for the degree of amylose/amylopectin leaching, as well as the capacity of the swollen granules to dissipate force during shearing or compressing (BeMiller, 2011; Debet & Gidley, 2006, 2007; Hongsprabhas & Israkarn, 2008; Hongsprabhas, Israkarn, & Rattanawattanaprakit, 2007).

Phase separation in the continuous phase may also play an important role in determining the properties of starch pastes and gels. The microscopic phase separation involves demixing of macromolecular amylose and amylopectin after the granular structure disintegrates. As a consequence, amylopectin usually forms the continuous phase due to its higher mass and volume ratio compared to amylose and is responsible for starch paste characteristics (Hermansson & Svegmark, 1996).

Our previous investigation showed that the gelatinization temperatures of native mungbean starch (N-MB) and native cassava starch (N-CS) granules were lowered during alkali cooking. This resulted in drastic leaching of starch constituents out of the granules and disintegration of swollen starch granules (Israkarn, Hongsprabhas, & Hongsprabhas, 2007). As a consequence, the mechanical and thermophysical properties of demixed amylose–amylopectin paste and gel were entirely different from those governed by a close-packed structure.

Alkali treatment or nixtamalization has been used in starch processing to remove the pericarp of cereal grains. For example, alkali soaking of maize in NaOH or $Ca(OH)_2$ is part of tortilla and taco shell production (Flores-Morales, Jiménez-Estrada, & Mora-Escobedo, 2012; Mestres, Colonna, & Buleon, 1988; Toro-Vazquez & Gómez-Aldapa, 2001). In Asia, yellow alkali noodles and certain desserts are made under alkaline conditions to give

* Corresponding author. Tel.: +66 2 562 5043; fax: +66 2 562 5021.
*E-mail addresses:* parichat.h@ku.ac.th, paree_h@yahoo.com (P. Hongsprabhas).

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a soft but cohesive texture (Karim et al., 2008). Starch extraction, as well as starch modification processes – etherification, esterification and crosslinking – are often performed under alkaline conditions. Sui, Shah, and BeMiller (2011) recently reported that subjecting a starch to the conditions of derivatization at pH 11.2 or 11.3 using 1 M NaOH without added reagent could result in greater changes to pasting profiles and gelatization temperature ranges.

Nevertheless, the current understanding of the effects of alkali cooking on starch properties is limited mainly to maize grain, flour and starch. Most research work has been carried out on the traditional nixtamalization or alkali cooking of maize with $Ca(OH)_2$ during the production of tortillas. Industrial nixtamalization of maize was shown to have both melting and annealing effects on maize starch (Mestres et al., 1988; Toro-Vazquez & Gómez-Aldapa, 2001). It was also found that nixtamalization of maize resulted in an increase in crystallinity of the starch fraction, alteration of the protein structure, and enhancement of protein–starch–lipid interactions (Flores-Morales et al., 2012).

Recently, Karim et al. (2008) found alterations of sago starch characteristics after alkali treatment. They reported that modification of sago starch by alkaline soaking at 30 °C for 30 d, followed by neutralization to pH 7 and oven-drying at 40 °C for 3 d, increased the swelling power and solubility of the starch. Amylose leaching, endodepolymerization, and oxidation of starch molecules could occur during soaking, resulting in lower peak viscosity and reduced breakdown of modified sago starch compared with the native starch.

Drastic changes of starch granule characteristics and in the content of constituents leached from the granules (particularly amylose and granule-bound proteins) during alkali cooking of N-MB and N-CS have been reported by our group (Hongsprabhas & Israkarn, 2008; Hongsprabhas et al., 2007). Both the —OH groups on starch molecules, which are ionized at alkaline pH above 12.5 (Suortti, Gorenstein, & Roger, 1998), and the negatively charged granule-bound proteins are likely involved in the formation of the envelope encasing starch contents within the swollen granules (BeMiller, 2011; Debet & Gidley, 2006, 2007). Nevertheless, the influence of alkali treatment on the physicochemical changes of starch molecules and proteins within the treated granules has received little attention. The characteristics of alkali-treated granules thus merit further investigation.

We hypothesized that the granular integrity of swollen granules during cooking under alkaline pH (pH ~ 11) was determined by the viscoelastic properties of the starch granule envelope. Disintegration of this envelope, particularly by charge repulsion, could enhance disintegration of the swollen granules. The objectives of this study were therefore to investigate the influences of alkali treatment on the characteristics of dried starch granules and subsequent cooking of the alkali-treated starches. The insights on the alterations of physicochemical characteristics of starch and protein fractions during alkali cooking may help in better controlling the textural properties of demixed amylose–amylopectin–protein composites.

## 2. Materials and methods

### 2.1. Materials

Food-grade mungbean starch ("Pine" brand; Sithinan, Bangkok, Thailand) and cassava starch ("Fish" brand; E.T.C. International Trading, Bangkok, Thailand) were purchased from a local supermarket. All chemicals used were of reagent grade.

### 2.2. Methods

#### 2.2.1. Preparation of alkali-treated starches

Native mungbean starch (N-MB) and native cassava starch (N-CS) were soaked in 100 mM NaOH solution for 18 h at a concentration of 25% (w/v). During the first 3 h of soaking, starch suspensions were stirred using a magnetic stirrer at 25 °C, and then left for 15 h. After that, the supernatant liquid was discarded.

The alkali-treated starches were prepared using two washing regimes. Starches in the first group, designated as AN-MB and AN-CS, were prepared by washing the alkali-treated starches with distilled water until the alkali-treated starches were neutralized to pH 7.0. Starch in the second group, designated as A-MB, was prepared by washing the alkali-treated starch once with distilled water, resulting in alkali-treated starch having high pH (pH ~ 11) after drying. All modified starches were dried at 40 °C in a hot-air oven (model 400; Memmert, Schwabach, Germany) for 18 h until the final moisture content was about 10%. Then they were ground, sifted through a 100-mesh sieve, sealed in plastic bags and stored in a freezer at −20 °C.

#### 2.2.2. Chemical composition

N-MB, N-CS, AN-MB and AN-CS starches were analyzed for moisture (AOAC, 2000), protein (AOAC, 2000), phosphorus (AOAC, 2000) and amylose (Chrastil, 1987) contents, using potato amylose as a standard.

#### 2.2.3. Thermal properties

The thermal properties of native and treated starches were determined by differential scanning calorimetry (DSC). Briefly, a Pyris 1 DSC (PerkinElmer, Waltham, MA, USA) was used to characterize the thermal properties of a 12% (w/w) slurry of starch in distilled water. Each suspension was incubated at 25 °C for 24 h in a stainless steel pan and hermetically sealed prior to measurement. The samples were heated at a rate of 5 °C/min from 25 to 95 °C to determine the transition temperature and enthalpy of gelatinization. The transition temperatures reported were the onset ($T_o$), peak ($T_p$) and end ($T_e$) temperatures of the gelatinization endotherm, and the gelatinization temperature range ($\Delta T$, or $T_e - T_o$). The enthalpy of gelatinization ($\Delta H$) was estimated by integrating the area between the thermogram and a baseline connecting the points of onset and end temperatures, and was expressed in J/g starch (dry basis).

#### 2.2.4. Crystallinity

The native and modified starches were equilibrated at 25 °C and 86% relative humidity (RH) in KCl for 18 d before being examined by wide-angle X-ray diffractometry (XRD). Analyses were conducted using an JDX-3530 X-ray diffractometer (JEOL, Tokyo, Japan) and Jade software version 6.5 for data collection. The samples were analyzed between 3° and 50° $2\theta$, at a step angle of 0.02°, a count time of 1 s, tube voltage of 30 kV, and tube current of 40 mA. The % relative crystallinity (% RC) of the samples was calculated as the proportion of crystalline area to total area multiplied by 100.

#### 2.2.5. Pasting characteristics

A rapid visco analyzer (RVA) (Newport Scientific, Warriewood, NSW, Australia) was used to characterize pasting properties of native and treated starches. The starch suspensions (12%, w/v) were prepared in individual canisters using distilled water as a dispersant. Each starch suspension (25 mL) was held at 50 °C for 1 min, heated from 50 to 95 °C at a rate of 13 °C/min, held at 95 °C for 2.50 min, cooled to 50 °C at a rate of 13 °C/min, and finally kept at 50 °C at 160 rpm for 1.24 min. Pasting characteristics were described using amylograms, and included pasting temperature

(the temperature at which starch granules begin to swell and gelatinize due to water uptake), peak time (the time at which the viscosity peaks), peak viscosity (the maximum viscosity developed soon after the end of the heating cycle), holding strength (the viscosity after holding at 95 °C for 2.50 min), and breakdown (the viscosity difference between peak viscosity and holding strength). Non-linear curve-fitting was used to differentiate the behavior of starch during water absorption and swelling of paste under shear, using Boltzmann sigmoidal curve-fitting during heating from 50 to 95 °C up to peak viscosity, as shown in the following equation (Israkarn et al., 2007; Love, Piguet-Ruinet, & Teyss, 2008): $\eta_t = \eta_{t_0} + \frac{\eta_{t_{end}} - \eta_{t_0}}{1+e^{(t_{half}-t)/slope}}$

Viscosity data during the heating stage were fitted from time zero ($t_0$, at 50 °C) to the time that peak viscosity was achieved ($t_{end}$, at 95 °C). This equation describes the viscosity increase ($\eta_t$) as a function of time ($t$) when the temperature was increased during the heating stage. The viscosity varied from time zero ($t_0$) to the viscosity at the terminal time selected ($t_{end}$), at which the maximum value of the sigmoidal curve was achieved and designated as fitted peak viscosity. Half-time ($t_{half}$) is the time at which the viscosity of each curve is halfway between $t_0$ and $t_{end}$. *Slope* describes the steepness of the curve, with a larger value denoting a shallower curve.

### 2.2.6. Microstructure

One mL sample suspensions, 0.5% (w/v), were prepared in distilled water and shaken vigorously, then stained with 1 μL of Lugol's iodine solution and incubated for 5 min unless stated otherwise. Each sample was then loaded into a slide and observed under a light microscope (Axio Imager M1; Carl Zeiss, Jena, Germany) with and without a polarizing filter in order to study the influence of alkali treatment on the birefringence of starch granules and films. Images were acquired using Image-Pro Plus software version 6.0 (Media Cybernetics, Rockville, MD, USA).

Confocal laser scanning microscopy (CLSM) (Axio Imager; Carl Zeiss) was used to investigate protein distribution in starch granules. One mL sample suspensions were prepared in distilled water or 100 mM NaOH at a concentration of 0.5% (w/v) and shaken vigorously. A solution of rhodamine B (0.01% in 95% ethanol) was added to starch suspensions. After incubation for 5 min, each sample was loaded into a slide well and observed for the location of fluorescent-labeled protein. A HeNe laser with an excitation wavelength of 543 nm was used. CLSM digital images were acquired using the LSM 5 PASCAL program (Carl Zeiss).

### 2.2.7. Molecular weight of granule-bound proteins in mungbean and cassava starches

Granule-bound proteins (GBPs) were extracted using three types of solvent. Starch powder (0.25 g) was suspended in 0.5 mL of 2% SDS, 0.5 mL of 2% SDS + 8 M urea, or 0.5 mL of 2% SDS + 8 M urea + 5 mM dithiothreitol (DTT), and incubated at 25 °C for 15 min, using methods modified from those described by Hamaker, Griffin, and Moldenhauer (1991) and Dennett, Schofield, Roake, Howes, and Chin (2009). The samples were mixed with 0.5 mL of dissociating sample buffer: 0.5 M Tris–HCl (pH 6.8), 10% glycerol, with or without 5% 2-mercaptoethanol, and 1% (w/v) bromophenol blue. The solutions were placed in a boiling water bath for 30 s to avoid gel formation, and then centrifuged at 2153 × *g* (Spectrafuge™ 16M; Labnet International, Woodbridge, NJ, USA) for 5 min to remove insoluble material.

The molecular weight (MW) profiles of proteins of the native and treated starches were determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) in a 4% stacking gel and a 15% separating gel using a Mini-PROTEAN® II cell (Bio-Rad Laboratories, Hercules, CA, USA). The separation was performed at a constant current of 25 mA for 50 min. Continuous buffer systems, containing 0.375 M Tris–HCl (pH 8.8) and 0.1% SDS for the separating gel, and 0.125 M Tris–HCl (pH 6.8) and 0.1% SDS for the stacking gel, were used. The running buffer consisted of 0.124 M Tris, 0.959 M glycine and 0.1% SDS (pH 8.3) diluted with distilled water five times before use. Ten microliter sample solutions were loaded into each well. Gel slabs were fixed and stained simultaneously using stain solution (40% methanol, 10% acetic acid and 0.1% Bio-Rad Coomassie Brilliant Blue R-250) for 30 min, and then destained with Bio-Rad Coomassie Brilliant Blue R-250 destain solution for 5 h, with 2–3 changes of destain solution. The MW of each band was determined using full-range rainbow molecular weight markers, MW ~ 10–250 kDa (RPN 8000; Amersham Biosciences UK, Buckinghamshire, UK) as the MW standards.

### 2.2.8. Molecular weight of starch molecules from high-performance size-exclusion chromatography (HPSEC)

An aliquot containing 20 mg of N-MB, N-CS, A-MB or A-CS dispersed in 5 mL of 90% dimethyl sulfoxide (DMSO) in deionized water was prepared and boiled in a boiling water bath for 1 h. The solutions were then stirred at room temperature (25–30 °C) for 48 h, and EtOH was added to precipitate starch molecules. Precipitated starches were obtained by centrifugation at 5000 × *g* for 15 min using a Sigma laboratory centrifuge (6K15; Sartorius, Göttingen, Germany). The precipitation step was performed in triplicate. The precipitated starches were dried in a vacuum drying oven at 32 °C (Binder, Tuttlingen, Germany) and kept in a sealed tube prior to analysis within 1 wk.

The precipitated starches were dissolved in 10 mL of deionized water by heating in a boiling water bath with constant stirring for 1 h. The hot starch dispersions were filtered through a nylon membrane (Vertipure™ nylon syringe filter 5 μm, 25 mm diameter; Vertical Chromatography, Bangkok, Thailand) and injected into individual vials (2 mL clear vial, Shimadzu caps, PTFE/silicone septa; National Scientific, Rockwood, TN, USA). A 100 μL sample of starch suspension was auto-injected (SIL-20A HT autosampler; Shimadzu, Kyoto, Japan) into a high-performance liquid chromatography (HPLC) (LC-20A; Shimadzu) or high-performance size-exclusion chromatography (HPSEC) system. The HPSEC system was equipped with a refractive index (RI) detector (RID-10A; Shimadzu) set at 40 °C, a guard column (Shodex™ OHpak SB-G; Showa Denko, Tokyo, Japan), and two analytical columns (Shodex OHpak SB-806M HQ and SB-804 HQ; Showa Denko). The column oven (CTO-20A; Shimadzu) was set at 55 °C. Deionized water was used as a mobile phase. The sample flow rate was 0.6 mL/min.

Five mg of pullulan standards (weight-average molecular weight ($\overline{Mw}$) of $6.20 \times 10^3$ (P-5), $2.17 \times 10^4$ (P-20), $1.13 \times 10^5$ (P-100), $3.66 \times 10^5$ (P-400), $8.05 \times 10^5$ (P-800); Showa Denko) were dispersed in 5 mL of distilled water and allowed to dissolve at room temperature (25 °C) with constant stirring. The clear standard pullulan solutions were then filtered into individual vials and auto-injected into the HPSEC system. The standard curve was plotted, and the given linear equation was used to calculate the MWs of samples.

### 2.2.9. Statistical analysis

The experiments were carried out in two separate trials. Each trial was run in duplicate. The data were analyzed by a paired *t*-test or analysis of variance (ANOVA) at a 95% significance level, where appropriate. Significant differences among mean values from ANOVA were determined by Duncan's multiple range tests. All statistical analyses were performed using SPSS software version 12. Non-linear curve-fitting was performed using GraphPad Prism software version 5.00 (trial).

**Table 1**
Composition of mungbean starch and cassava starch before and after soaking in alkaline solution. N-MB, native mungbean starch; AN-MB, alkali-treated and neutralized mungbean starch; N-CS, native cassava starch; and AN-CS, alkali-treated and neutralized cassava starch.

| Type of starch | Moisture content (%) | Amylose content (% w.b.) | Protein content (% w.b.)* | Phosphorus content (% w.b.) |
|---|---|---|---|---|
| N-MB | 11.96[a] | 35.44[a] | 0.10 | 0.012[a] |
| AN-MB | 12.60[a] | 27.03[b] | ND | 0.008[b] |
| N-CS | 12.91[a] | 24.66[c] | 0.09 | 0.008[b] |
| AN-CS | 13.56[a] | 13.57[d] | ND | 0.005[c] |

Means in the same column followed by different superscripts are significantly different ($P < 0.05$).
* Nitrogen factor = 6.25; ND, not detected.

## 3. Results and discussion

Alkali treatment using 100 mM NaOH, which increased the pH to around 12, ionized some of the —OH groups of starch molecules (Suortti et al., 1998). This resulted in the macromolecules becoming negatively charged for both starch and protein fractions in starch granules. The ionic repulsion between negative charges could disrupt the H-bonds between starch molecules in the amorphous region. Therefore the starch molecules, particularly in the amorphous region, could dissolve during soaking and washing at room temperature. Table 1 shows that the amylose contents in AN-MB and AN-CS were drastically decreased from those in N-MB and N-CS, respectively, after alkali treatment followed by neutralization.

Not only amylose, but proteins and phosphorus were also reduced after alkali treatment and neutralization (Table 1). The protein contents in AN-MB and AN-CS were so low they could not be detected by the Kjeldahl method. Phosphorus may be lost during the modification since it is attached to glucose units as starch phosphate monoester (Jane, Kasemsuwan, & Chen, 1996). The loss of amylose from starch granules may account for the reduction in phosphorus content.

The amylopectin in AN-CS retained its A-type crystalline structure, with % relative crystallinity (% RC) similar to that of N-CS (Table 2). Moreover, both N-CS and AN-CS had a similar gelatinization temperature range, enthalpy of gelatinization, and RVA pasting characteristics ($P \geq 0.05$) despite the drastic reduction of amylose content from 24.7% to 13.6% (Table 1). Alkali treatment followed by neutralization before drying at 40 °C did not change the pasting properties of AN-CS compared to the native starch (N-CS). The reduction in amylose also did not affect the pasting properties of AN-CS. This suggested that swollen granules of cassava starch played a more important role in determining the RVA pasting profile than did the leached amylose. Similar behavior of swollen granules of mungbean starch was also reported previously (Hongsprabhas & Israkarn, 2008).

Alkali treatment followed by neutralization (AN-MB) did not change the crystalline type or thermal properties of MB (Table 3). However, AN-MB showed a slight increase in % RC ($P < 0.05$). The increase in % RC after alkali treatment and drying might be due to the drastic decrease of amylose in the amorphous region of mungbean starch, as well as the rearrangement of the double helix of amylopectin during drying in a hot-air oven at 40 °C.

A-MB, which was not neutralized prior to drying, however, had a % RC similar to that of N-MB ($P \geq 0.05$). This suggested that the residual negative charges on starch polymers inhibited their rearrangement during drying. The gelatinization temperature of A-MB showed a slight increase compared to that of N-MB ($P < 0.05$). However, the temperature range ($\Delta T$), as well as the energy used for the breakdown of crystalline structure ($\Delta H$), was not significantly affected by alkali treatment ($P \geq 0.05$).

Modification by alkali treatment and drying without the neutralization step resulted in the starch suspension having a pH of 10.74 after A-MB was dispersed in water prior to RVA pasting (Table 4). Table 4 shows that A-MB had higher pasting temperature, lower holding strength and higher degree of breakdown compared with N-MB ($P < 0.05$). This result corroborated the gelatinization characteristics of A-MB in comparison with N-MB, as reported in Table 3. The reduction in holding strength of A-MB may arise from the influence of alkaline pH of 10.74 in the starch suspension during pasting. A reduction in holding strength and an increase in breakdown values of starch suspension heated in alkaline solution were also observed in our previous report (Israkarn et al., 2007). This was most likely due to the charge repulsion of residual ionized starch

**Table 2**
Physical characteristics of native cassava starch (N-CS) and alkali-neutralized cassava starch (AN-CS).[ns].

| Characteristics | N-CS | AN-CS |
|---|---|---|
| X-ray diffraction | | |
| Type | A | A |
| Relative crystallinity (%) | 15.77 | 15.96 |
| Gelatinization | | |
| $T_o$ (°C) | 59.1 | 59.2 |
| $T_p$ (°C) | 65.9 | 65.7 |
| $T_e$ (°C) | 71.1 | 74.0 |
| $\Delta T$ (°C) | 12.1 | 14.8 |
| $\Delta H$ (J/g (d.b.)) | 20.0 | 22.2 |
| RVA pasting profile | | |
| Pasting temperature (°C) | 64.9 | 63.7 |
| Peak time (min) | 3.80 | 3.85 |
| Peak viscosity (Pa s) | 5.46 | 5.09 |
| Holding strength (Pa s) | 1.87 | 1.94 |
| Breakdown (Pa s) | 3.59 | 3.15 |

**Table 3**
Physical characteristics of native mungbean starch (N-MB), alkali-treated and neutralized mungbean starch (AN-MB) and alkali-treated mungbean starch without neutralization (A-MB).

| Characteristics | N-MB | AN-MB | A-MB |
|---|---|---|---|
| X-ray diffraction | | | |
| Type | A | A | A |
| Relative crystallinity (%) | 13.97[b] | 15.21[a] | 13.19[b] |
| Gelatinization | | | |
| $T_o$ (°C) | 69.6[b] | 71.1[ab] | 72.3[a] |
| $T_p$ (°C) | 72.3[b] | 73.7[ab] | 75.3[a] |
| $T_e$ (°C) | 75.4[b] | 76.4[b] | 78.6[a] |
| $\Delta T$ (°C) | 5.9[a] | 5.3[a] | 6.3[a] |
| $\Delta H$ (J/g (d.b.)) | 15.9[a] | 13.1[a] | 16.8[a] |

Means in the same row followed by different superscripts are significantly different ($P < 0.05$).

**Table 4**
Pasting characteristics of native mungbean starch (N-MB) and alkali-treated mungbean starch without neutralization (A-MB).

| Characteristics | N-MB | A-MB |
|---|---|---|
| pH of starch suspensions | 5.79[b] | 10.74[a] |
| RVA pasting profile | | |
| Pasting temperature (°C) | 77.3[b] | 81.4[a] |
| Peak time (min) | 4.47[a] | 4.38[a] |
| Peak viscosity (Pa s) | 8.27[a] | 7.28[a] |
| Holding strength (Pa s) | 5.18[a] | 2.33[b] |
| Breakdown (Pa s) | 3.09[b] | 4.95[a] |
| Boltzmann sigmoidal curve-fitting during heating from 50 to 95 °C, up to peak viscosity | | |
| Fitted peak viscosity (Pa s) | 8.09[a] | 7.16[a] |
| Half-time (s) | 216[b] | 233[a] |
| $R^2$ | 0.9977 | 0.9983 |
| Standard deviation of residuals (Pa s) | 0.14 | 0.09 |

Means in the same row followed by different superscripts are significantly different ($P < 0.05$).

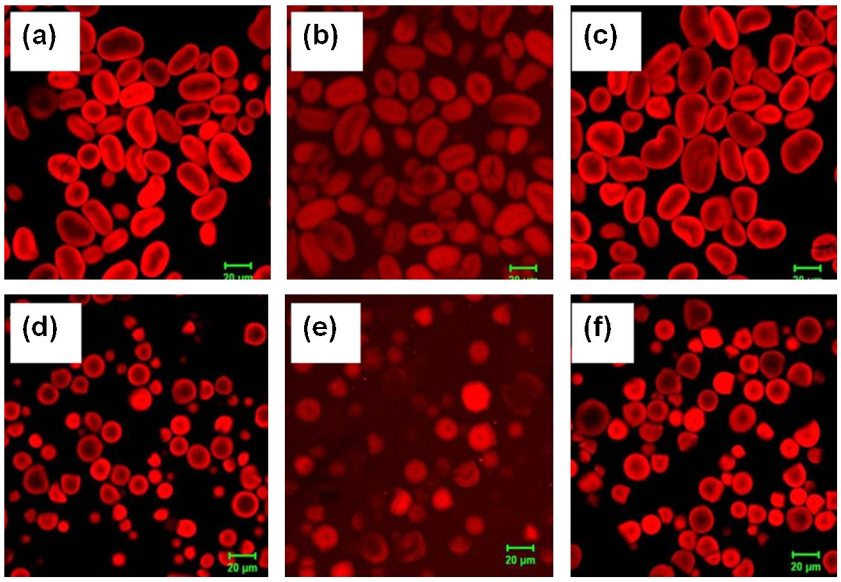


**Fig. 1.** CLSM of granule-bound proteins in mungbean and cassavava starches: (a) native mungbean starch (N-MB); (b) mungbean starch soaked in 100 mM NaOH; (c) alkali-treated and neutralized mungbean starch (AN-MB); (d) native cassava starch (N-CS); (e) cassava starch soaked in 100 mM NaOH; and (f) alkali-treated and neutralized cassava starch (AN-CS). Bars = 20 μm.

molecules, which enhanced the disintegration of swollen starch granules under shear during cooking.

Non-linear curve-fitting was used to differentiate the behavior of N-MB and A-MB during water absorption and swelling (Israkarn et al., 2007; Love et al., 2008). The viscosity alteration of the starch pastes was composed of two consecutive phenomena. The lag phase at the beginning of heating included water absorption by starch granules at a temperature below the pasting temperature where starch granules retained their granular size. The second phase involved a dramatic increase of viscosity due to granular swelling and friction among the swollen granules under shear.

Although the times required for N-MB and A-MB to reach peak viscosity were not significantly different ($P \geq 0.05$), the viscosity behavior of starch granules before the paste reached peak viscosity could be differentiated. The half-time ($t_{half}$) was the time at which the viscosity of each curve was halfway between time zero and the time at which the maximum viscosity was reached. It was found that the half-time of A-MB starch was significantly increased, which indicated that the A-MB starch granules needed more time to swell than did N-MB. This was possibly due to the higher gelatinization temperature range of A-MB compared with that of N-MB (Table 3).

It is most likely that the differences in granular swelling and paste viscosity between N-MB and A-MB were due to the integrity of granular structure. Results in Tables 2 and 3 indicate that modification and drying did not alter the crystalline structure of starch granules, and the extensive loss of amylose apparently did not affect pasting characteristics of the modified starches. Nonetheless, other constituents, particularly granule-bound proteins (GBPs) in starch granules, may play important roles in determining the granular integrity during pasting of the modified starches. Phase separation between starch and protein fractions in the presence of excess water is likely responsible for the integrity of swollen granules (BeMiller, 2011; Debet & Gidley, 2006, 2007; Hongsprabhas & Israkarn, 2008; Hongsprabhas et al., 2007; Israkarn et al., 2007).

Although Kjeldahl analysis could not detect the presence of GBPs after modification since the method was not sensitive enough, CLSM was used to assess the existence of GBPs. Fig. 1 illustrates the fluorescent signals of GBPs in N-MB (Fig. 1a) and N-CS (Fig. 1d) when starch granules were stained with rhodamine B. CLSM revealed that protein was solubilized in the aqueous phase during soaking of MB and CS in 100 mM NaOH (Fig. 1b and e, respectively). Although there was no noticeable change in the intensity of the signal showing protein fractions in modified starches – i.e. AN-MB (Fig. 1c) and AN-CS (Fig. 1f), compared with N-MB (Fig. 1a) and N-CS (Fig. 1d) – CLSM results indicate that the partial loss of protein from treated starches occurred as a result of soaking in alkaline solution and subsequent washing with distilled water.

The GBPs of N-MB and N-CS were further characterized for their MWs by SDS-PAGE, as shown in Fig. 2. We were not able to characterize the GBPs from AN-MB and AN-CS due to their extremely low concentrations in the treated granules. The proteins in N-MB and N-CS investigated in this study were likely granule-bound starch synthases (Baldwin, 2001; Ko, Dong, Hsieh, & Kuo, 2009). The MW of the extracted protein investigated in the present study was around 52 kDa for both N-MB and N-CS, which was in accordance with the MW of 58 kDa determined by Ko et al. (2009) by MALDI-TOF mass spectrometry of the starch synthase I, the major enzyme in mungbean, cowpea and maize starches.

In the present study, we also found that using 2% SDS alone could not extract GBPs from either starch (Fig. 2, lane 1). However, using a combination of 2% SDS + 8 M urea could extract GBPs from N-MB granules (shown as a protein band in lane 2) but not from N-CS granules. The intensity of 52 kDa bands of N-MB and N-CS were higher when DTT was included in the extraction solvent (Fig. 2, lane 3). It was postulated that the GBPs from both N-MB and N-CS were embedded in a semi-crystalline structure and that the proteins were tightly bound together via disulfide bonds.

High-performance size-exclusion chromatography (HPSEC) was applied to study the effect of alkali treatments on MW distribution of starch molecules in N-MB and A-MB starches. The weight-average MW ($\overline{Mw}$) of each fraction was calculated from the retention time (RT) at the peak, using a linear equation from the standard curve. The number of molecules in each fraction was the area under each fraction peak divided by the total area under the peaks. The results from HPSEC (Fig. 3 and Table 5) revealed that alkali treatment followed by drying at 40 °C caused alterations in the MW distribution of A-MB compared with that of N-MB.

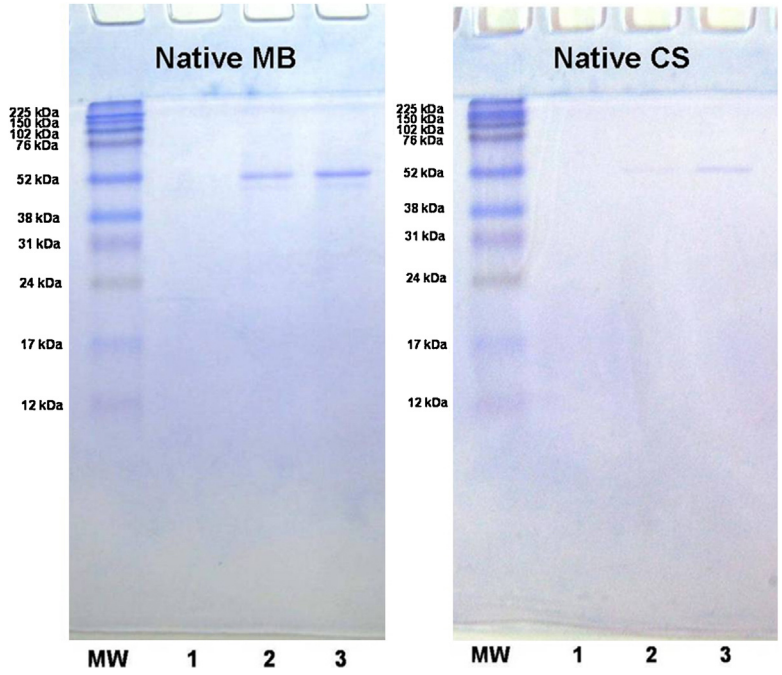


**Fig. 2.** SDS-PAGE of granule-bound proteins (GBPs) of native mungbean starch (N-MB) and native cassava starch (N-CS) extracted by different extraction buffers. Lane 1: 2% SDS; lane 2: 2% SDS + 8 M urea; and lane 3: 2% SDS + 8 M urea + 5 mM DTT.

**Table 5**
Effect of alkali modification on molecular size of native mungbean (N-MB) and alkali-treated mungbean (A-MB) starches, reported as weight-average molecular weight ($\overline{Mw}$) (g/mol) and amount reported as area (%) of each fraction.

| Sample | I | | II | | III | | IV | |
|---|---|---|---|---|---|---|---|---|
| | $\overline{Mw}$ (g/mol) | Area (%) | $\overline{Mw}$ (g/mol) | Area (%) | $\overline{Mw}$ (g/mol) | Area (%) | $\overline{Mw}^{ns}$ (g/mol) | Area (%) |
| N-MB | $4.1 \times 10^{7b}$ | $57.3^{a}$ | $7.8 \times 10^{6b}$ | $9.8^{b}$ | $4.8 \times 10^{6a}$ | $9.9^{b}$ | $4.5 \times 10^{5}$ | $23.1^{a}$ |
| A-MB | $4.3 \times 10^{7a}$ | $41.0^{b}$ | $1.2 \times 10^{7a}$ | $15.8^{a}$ | $3.1 \times 10^{6b}$ | $25.5^{a}$ | $4.3 \times 10^{5}$ | $17.6^{b}$ |

Means in the same column followed by different superscript are significantly different ($P < 0.05$). ns = no significant difference ($P > 0.05$).

The number of starch molecules in fractions II and III, which were intermediate MW molecules, was significantly increased after alkali modification. The amylopectin and amylose molecules in fractions I and IV, however, were significantly reduced (Table 5). This was probably because of the depolymerization of amylopectin and amylose during the alkali treatment process used in this study (Jackson, Choto-Owen, Waniska, & Roony, 1988; Karim et al., 2008).

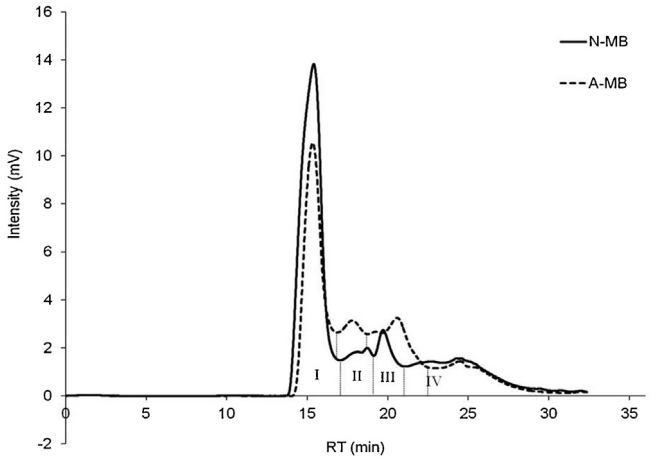


**Fig. 3.** Chromatograms of soluble N-MB and A-MB starches separated by HPSEC.

## 4. Conclusions

This study revealed that alkali treatment reduced amylose and granule-bound starch synthases in uncooked starch granules, but it did not affect the crystallinity of starch granules. The influence of alkali on thermophysical properties was enhanced when starch granules were cooked under alkaline conditions. This was due to the fragmentation of both amylose and amylopectin, as well as the solubilization of granule-bound starch synthases. As a result, the involvement of macromolecules in maintaining envelope integrity was lost, leading to the reduction in holding strength and increase in breakdown viscosities of starch during cooking under alkaline conditions. The results also suggested that the selective leaching of amylose, mainly from the amorphous region in uncooked starch granules, did not strongly affect the physicochemical and thermophysical characteristics of the alkali-treated and neutralized starches (AN-MB and AN-CS). Alkali cooking, however, further solubilized proteins and fragmented starch molecules, which led to the ease in disintegration of swollen mungbean and cassava starch granules during cooking.

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