Enhancing Enzymatic Resistance of Green Saba Banana Flour by Pullulanase Debranching and Autoclave-Cooling Treatment

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ABSTRACT Flour or starch with low digestibility has long known to demonstrate beneficial physiological effects to human health. Various methods had been employed to enhance the indigestible starch content of the starch/flour from various botanical origin. In the present study, green Saba banana flour was subjected to pullulanase debranching and autoclave-cooling cycles with the aim to investigate the influence on its resistance toward digestion. These two treatments in general resulted in greater enzymatic resistance of the flour but affected the digestion profile of the flour in different way. Debranching produced more amylose and hence promoted formation of highest resistant starch (RS) in the flour ($p<0.05$). On the other hand, autoclave-cooling treatment, either alone or in combination with pullulanase debranching were predominantly effective in enhancement of SDS content ($p<0.05$). When combined with autoclave-cooling treatment, debranched resultant RS was partially degraded and converted to SDS. Even though the enzymatic resistance of green Saba banana flour was improved by the treatments employed, the glycaemic index (GI) of the modified flours was still considerably high. Future work to further increase the enzymatic resistance of green Saba banana flour, with the aim to lower the GI is still necessary.

KEYWORDS: Green banana flour; Pullulanase; Autoclave-cooling cycle; Resistant starch; Glycaemic index
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INTRODUCTION

Green banana has attracted increasing interest of many researchers in recent years due to its great potential as a healthy food ingredient that can be obtained from inexpensive raw materials (Yee *et al*., 2021). The health benefits of green banana lie in its high content of low digestible starch or resistant starch (RS) which were reported by most of the previous investigations (Kaur *et al*., 2020). There are five types of RS: RS_1 (physically inaccessible starch); RS_2 (native starch granules); RS_3 (retrograded starch); RS_4 (chemically modified starch) and RS_5 (Amylose-lipid complexes). In raw form, green banana contains large amount of RS_2, whereby the crystalline structure of the granules prevents the enzyme digestion of the starch (Nugent, 2005). The starch granules are densely packed with more perfect crystalline structure that is highly resistant to pancreatic amylase (Englyst *et al*., 1992). Most of the RS_2 will be destroyed follows by improved digestibility upon cooking (gelatinization). Nonetheless, cooling of gelatinised starch causes retrogradation (slow recrystallisation of starch component) and formation of another type of resistant starch, RS_3. RS_3 has advantage over RS_2 in term of its thermally stability and allows for a relatively higher incorporation level without causing a detrimental effect on the quality of certain food products (Haralampu, 2000).

RS_3 formation can be accomplished by thermal or enzymatic modifications, as well as a combination of both (Morales-Medina *et al*., 2014). In thermal treatment, starch granules are gelatinised to destroy the crystalline structure of amylose molecules. Slow cooling that follows will allow retrogradation or reassociated of liner amylose chains by creating new double helices, which are stabilized by hydrogen bones. The resultant crystals prevent accessibility of α-amylase to the glycosidic bonds, hence creating resistance to digestion. Since formation of RS_3 is mainly by linear

amylose, debranching enzymes (i.e. pullulanase and iso-amylase) can be employed to hydrolyse the α-1,6 glucosidic bonds of highly branched amylopectin to release more linear glucan chains to further promote RS_3 formation in various starches, including green banana starch (Li *et al*., 2019; Reddy *et al*., 2017; Morales-Medina *et al*., 2014; Vatanasuchart *et al*., 2010). By combining autoclaving and debranching, increment of resistant starch content in banana starch and black chickpea starch was observed (Demirkesen-Bicak *et al*., 2018; Gonázlez-Soto *et al*., 2004).

RS behaves like dietary fibre that escapes digestion in the small-intestine and will be fermented as a prebiotic in the colon to support the beneficial gut microbiota. The positive physiological benefits of RS including weight management, prevention of gastrointestinal diseases, treatment of hyperglycemia and hypercholesterolemia, and enhancement of mineral absorption (Reddy *et al*., 2017). It is also reported to influence health by direct immune interactions (Lépine *et al*., 2018).

Saba banana (ABB triploid hybrid) is a cooking banana widely available in Sabah. Due to its low price, abundancy, short shelf-life and limited industry application (Lee *et al*., 2021), converting the flour into a high value-added product will diversify the applications of the crop and promote the sustainability of the sector. This study was aimed to enhance the enzymatic resistance of Saba green banana flour. The effects of pullulanase debranching and autoclave-cooling cycle on the digestibility of green Saba banana flour were investigated in the current study.

METHODOLOGY

Materials

Matured Saba banana (*Musa acuminata x Musa balbisiana,* AAB triploid hybrid) with total green peel was purchased from a market in Kota Kinabalu, Sabah, Malaysia. Green banana was immediately processed upon arrival in the laboratory. All chemicals used were of reagent grade or USP grade. The chemicals were used as received without any further purification. Distilled water was used throughout the experiment.

Preparation of Green Banana Flour

Freshly received green banana was peeled, sliced into pieces with 2 mm thickness and immediately soaked in 0.5% (w/v) citric acid for 10 min before subjecting to convection air drying at 50°C for 24 h. After drying, the chips were ground into flour and sieved through 60-mesh screen. The powder was kept in an air-tight container until further use.

Experimental Design

Two independent variables of this study were pullulanase debranching and autoclave-cooling cycle. 2 x 2 factorial design was used, which consisted of two levels of autoclave-cooling cycle (1 cycle and 3 cycles); and two levels of debranching treatment (un-debranched and debranched). A total of four modified flours were investigated. The abbreviations use to denote the samples are UN1 and UN3 for un-debranched flour subjected to 1 and 3 autoclave-cooling cycles respectively; as well as PU1 and PU3 for pullulanase debranched flour subjected to 1 and 3 autoclave-cooling cycles respectively. Unmodified green banana (denoted as UN) flour and pullulanase debranched flour (denoted as PU) were used as controls of experiment.

Debranching by Pullulanase

10% (w/w) of flour slurry was first heated for 15 min in a 90 °C water bath and then cooled down to 50 °C before adding with 74 npun pullulanase (Sigma e2412)/g banana flour. One nupn (new

pullunanase unit novo) is defined as the amount of enzyme that releases one µmol glucose per minute under standard conditions (Liao & Hung, 2015). The mixture was incubated with continuous stirring at 50 °C for 24 h and the enzyme reaction was deactivated by heating in boiling water bath for 30 min. After that, the mixture was oven-dried (40 °C for 24 h), cooled and ground (60-mesh sieve). Debranched flour was kept in air-tight container until further use. For dual-modified flour, after inactivation of enzyme activity, the flour mixture was directly subjected to the autoclave-cooling treatment without drying.

Autoclave-Cooling Treatment

This treatment was carried out according to Liao and Hung (2015). 10% (w/w) of flour slurry was pregelatinized in water bath (90 °C) for 15 min before subjecting to autoclave (Autoclave SX-500, Tomy, Japan) at 121 °C for 30 min. After that, the suspension was chilled at 4 °C for 24 h and centrifuged at 4000 x g for 15 min before oven-dried and ground to pass through 60-mesh sieve. The autoclave-cooling cycle was repeated whenever necessary. Modified flour was kept in air-tight container until further use.

Determination of Moisture Content and Amylose Content

Moisture content of the flour samples was determined at 105 °C using gravimetric method. Apparent amylose content was determined using a colorimetric iodine affinity procedure with slight modification (Khoomtong & Noomhorm, 2015). The absorbance was measured at 620 nm (Lambda 25 UV/Vis Spectrohphotometer, Perkin Elmer) against a reagent blank as the reference. Standard curve was prepared using amylose from potato starch (Sigma, A0512, USA) ($y = 0.0103x + 0.0256$, $R^2 = 0.9928$).

Determination of In-Vitro Digestibility

Determination of *in vitro* digestibility based on the fractions of rapidly digestible starch (RDS), slowly digestible starch (SDS) and resistant starch (RS) in the flour samples was performed according to the method of Ng *et al.* (2018). Glucose oxidase peroxidase (GOPOD) assay kit (K-GLUC, Megazyme international Ireland Ltd.) was used.

Determination of Glycaemic Index

Glycaemix index (GI) of the flour was determined using the method of Goñi *et al*. (1997). The hydrolysis index (HI) was calculated based on the starch hydrolysis curve (0 – 3 h) and glucose content was determined using GOPOD reagent. White bread was used as the reference food. Glycaemic index was estimated by the following equation.

$$GI = 39.71 + 0.549HI \quad (1)$$

Statistical Analysis

Triplicate determinations were performed for each analysis and the results reported were the mean values. One-way ANOVA was conducted using SPSS (Statistical Package for Social Sciences) version 24, and Tukey test was performed to separate the means with confidence level of 95%.

RESULT AND DISCUSSION

Moisture Content and Amylose Content

Table 1 shows the moisture content and amylose content of Saba banana flour and its modified counterparts. The moisture content of the samples was slightly different as the drying was based on

duration without controlling the final moisture content. The drying was aimed to reduce the water activity of the samples for stable storage (Khoozani *et al.*, 2019) and standardisation of final moisture content was not necessary. Since all the modified flour were prepared using native flour (UN), they were subjected to two times of oven-drying, and thus mostly contained lower moisture content. PU however reported higher moisture content than UN ($p < 0.05$), which could be attributed to water was added for gelatinisation during sample preparation.

Table 1. Moisture content and amylose content of unmodified and modified Saba green banana flour.

Sample	Moisture content (%)	Amylose content (%)
UN	10.36 ± 0.26^{b}	19.01 ± 1.18^{b}
PU	11.32 ± 0.16^{a}	22.90 ± 0.15^{a}
UN1	9.13 ± 0.03^{c}	18.32 ± 0.96^{b}
UN3	8.64 ± 0.08^{cd}	10.03 ± 1.51^{c}
PU1	9.05 ± 0.13^{c}	7.21 ± 0.66^{d}
PU3	8.31 ± 0.29^{d}	9.34 ± 0.29^{cd}

UN – unmodified green banana flour; PU – pullulanase debranched flour. 1 and 3 denotes 1 and 3 autoclave-cooling cycle, respectively.
Means with different superscript letters within the same column indicate significant difference ($p < 0.05$).

The amylose content of green Saba banana flour obtained in this study falls within the range of amylose content of several varieties of banana starch (19.32 – 26.35%) reported by Utrilla-Coello *et al.* (2014). After debranching, amylose content of banana flour was increased ($p < 0.05$), in corroboration with previous findings (Babu & Parimalavalli, 2018; Reddy *et al.*, 2017). Increasing the autoclave-cooling cycle further decreased the amylose content, implying severe deterioration of amylose crystallites by the treatment. Combining debranching and autoclave-cooling treatments resulted loss of more amylose in the flour. It is postulated that some of the amylose-like molecules produced by pullulanase were destroyed in the subsequent autoclave-cooling treatment.

In Vitro Digestibility and Glycaemic Index

The *in vitro* digestibility of the flour was measured by determining the starch fractions based on the rate of digestibility. Starch fraction that was digested within 20 min is classified as rapidly digestible starch (RDS), whereas the fraction digested between 20 to 120 min and the undigested fraction within 120 min were classified as slowly digestible starch (SDS) and resistant starch (RS), respectively (Englyst *et al.*, 1992). Without modification, UN in its raw form comprised of densely packed crystals (RS_2) hence contributing to the highest RS among all tested samples ($p < 0.05$). Since all the modification methods used involved gelatinisation, UN was subjected to the similar gelatinisation condition for comparison purpose. The digestibility of UN was remarkably increased (72.46% of RDS) after gelatinisation, whereby the starch granules were disintegrated and became more vulnerable to enzymatic attack. The improved digestibility directly increased the glucose liberated as shown in the higher glycaemic index (GI) ($p < 0.05$).

Substantially change of digestion profile in UN1 and UN3 suggested autoclave-cooling treatment promoted more amylopectin retrogradation as indicated by the enhanced SDS content and reduced RS content ($p < 0.05$). It was reported that amylopectin retrogradation mostly leads to slowly digestible structures, and the fine structure of Musa amylopectin has been reported to possess an outstanding ability to form SDS upon retrogradation (Roman *et al.*, 2019; Martinez *et al.*, 2018). Increasing autoclave-cooling cycles seemed to significantly elevate the SDS content but diminished the RS content. Comparatively, UN3 contained highest SDS yet lowest RS among all flour samples ($p < 0.05$). SDS also contributes to beneficial health benefits for individuals with diabetes, obesity, or

cardiovascular diseases (Li *et al*., 2019). It promotes satiety by down-regulating appetite-stimulating neuropeptide gens and improves blood glucose control (Hasek *et al*., 2018; Miao *et al*., 2015; Engyst *et al*., 1992).

Table 2. In vitro digestibility and glycaemic index for unmodified and modified Saba banana flour.

Sample	RDS (%)	SDS (%)	RS (%)	GI
UN	7.66 ± 0.88^{d}	8.46 ± 2.80^{c}	83.87 ± 1.91^{a}	47.48 ± 0.08^{d}
UN (gelatinised)	72.46 ± 2.32^{a}	7.73 ± 0.78^{c}	19.81 ± 2.79^{c}	85.14 ± 1.58^{b}
PU	44.76 ± 4.85^{c}	13.45 ± 4.71^{c}	41.80 ± 0.52^{b}	70.83 ± 1.28^{c}
UN1	57.48 ± 3.60^{b}	29.84 ± 0.82^{b}	12.69 ± 3.49^{d}	85.60 ± 5.65^{ab}
UN3	56.86 ± 0.34^{b}	42.11 ± 1.14^{a}	1.04 ± 1.37^{e}	92.02 ± 1.34^{a}
PU1	58.84 ± 0.48^{b}	27.20 ± 1.24^{b}	13.96 ± 0.77^{d}	85.16 ± 0.87^{b}
PU3	46.92 ± 2.00^{c}	38.42 ± 0.87^{a}	14.66 ± 1.47^{cd}	85.22 ± 0.22^{b}

UN – unmodified green banana flour; PU – pullulanase debranched flour. 1 and 3 denotes 1 and 3 autoclave-cooling cycle, respectively.
Means with different superscript letters within the same column indicate significant difference ($p < 0.05$).

Enzymatic debranching with pullulanse gave rise to linear glucans that were more readily reassociate in PU and thus achieved highest RS content and lowest GI ($p < 0.05$). The high amylose content (Table 1) was responsible for the formation of RS_3 in PU. Banana starch contains long outer amylopectin α-1,6-linked side chains that is desirable to produce RS_3 (Lehmann *et al.*, 2002). The chain length with degree of polymerization (DP) of about 20 is optimal for high RS_3 formation (Schmiedl *et al*., 2000). Combining with autoclave-cooling cycle on the other hand reduced the RS content ($p < 0.05$). This observation agrees well with the declined amylose content shown in Table 1. SDS contents in both PU1 and PU3 were found higher than PU ($p < 0.05$). Results suggest that RS_3 formed in debranched banana flour (PU) could not withstand the thermal degradation by autoclave; in which extensive dissociation of double-helical crystallites took place by the elevated temperature, making the starch chains more susceptible to amylolysis. Besides, part of the RS in PU1 and PU3 might have been converting to SDS, resulting higher GI in these samples. The digestibility of PU3 was relatively lower than PU1 with lower RDS content and higher SDS content ($p < 0.05$); nonetheless, insignificant difference was found in the GI for these two samples.

Pullulanase debranching and autoclave-cooling treatment created different digestion profiles in green Saba banana flour. These treatments brought about reduction of RDS content ($p < 0.05$), but the effects on SDS and RS content varied in corresponding with the treatment used. Enhancement of RS content was obtained by pullulanase debranching, whilst the autoclave-cooling treatment, either alone or in combination with pullulanase debranching were predominantly on the enhancement of SDS content. It is worth noting that irrespective of the variation in digestion profile, all the flour samples (unmodified and modified) are still classified as high GI food (GI > 70) (Eyinla *et al*., 2021), except PU which was reported to have GI value close to medium GI food.

Scanning Electron Microscopy

Figure 1a shows that the native Saba banana starch granules are characterized by smooth surface, with regular rod like and oval shapes, in agreement with previous findings (Reddy *et al*., 2017; Utrilla-Coello *et al*., 2014). After debranching, the granular structures were collapsed and irregularly shaped structures with bigger size were formed (Figure 1b). Similar observation was reported in pullulanse-treated black chickpea starch (Demirkesen-Bicak *et al*., 2018). Pre-treatment gelatinization and debranching by pullulanase caused crystallite melting and total loss of granular structure. Aggregation of linear starch fragment after retrogradation (Vatanasuchart *et al*., 2010) contributed to the formation of new structure.

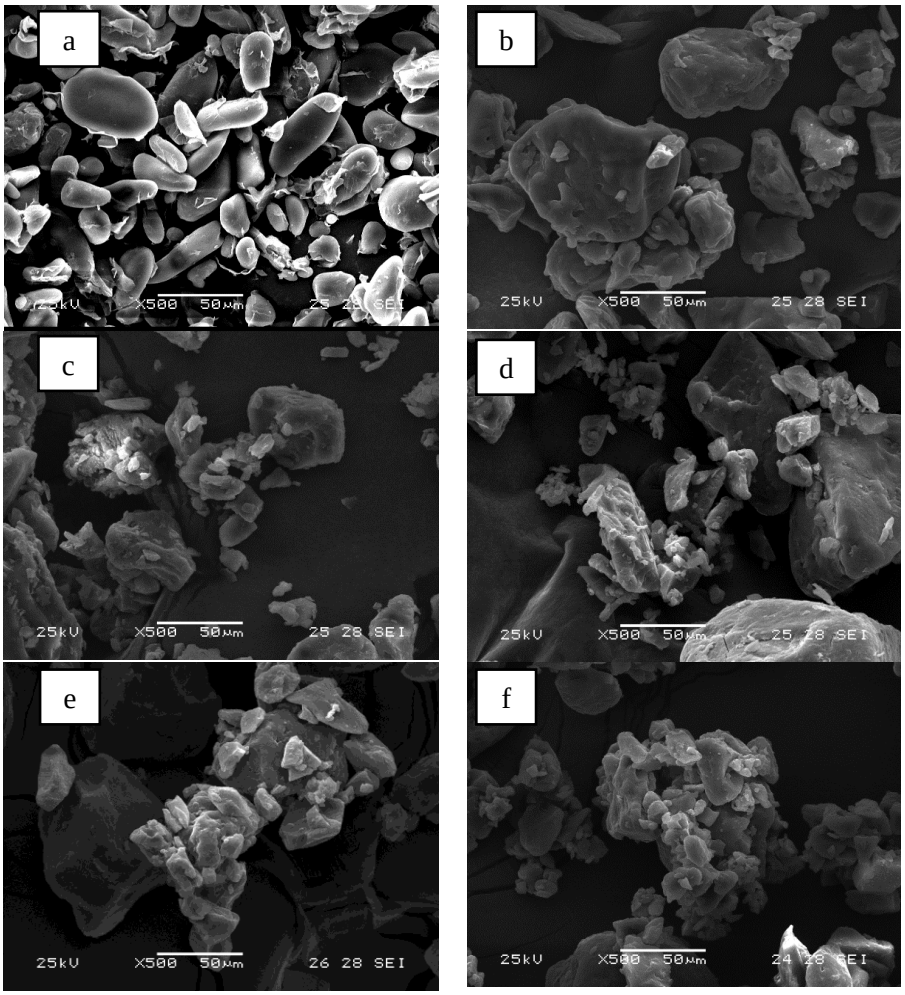

Figure 1. Scanning electron micrographs for (a) UN, unmodified Saba banana starch; (b) PU, debranched banana starch; (c) UN1, 1-cycle autoclaved-cooled banana starch; (d) UN3, 3-cycle autoclaved-cooled banana starch; (e) PU1, debranched + 1-cycle autoclaved-cooled banana starch; and (d) PU3, debranched + 3-cycle autoclaved-cooled banana starch at 500 x magnification.

The morphology of all the other modified samples (Figure 1c, 1d, 1e and 1f) resembled that of PU; except they contained relatively higher number of smaller particles as compared to Figure 1b. Autoclaving at high temperature is expected to cause higher degree of cellular disruption; producing more smaller starch fragments that were leached out, solubilized in the paste, and later participated in the realignment during retrogradation.

CONCLUSION

Results indicated that pullulanase debranching and autoclave-cooling treatment used in the current study could significantly enhance the enzymatic resistance of green Saba banana flour. The regulating effect however differs in corresponding with the treatment used. Pullulanase debranching was the only treatment that could effectively increase the RS content in green Saba banana flour, whereas other treatments were predominantly resulting increment of SDS content. Though enzymatic resistance of the flours was improved, the GIs were considerably high. Future work looking into further enhancement of enzymatic resistance of green Saba banana flour is still necessary to ensure production of ingredient with more promising health benefits for food industry application.

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