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How Pullulanase Affects Resistant Starch and Antioxidant Activity in Purple Sweet Potato Powder?

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ABSTRACT

Purple sweet potato (PSP) serves as a potential source for dual functionalities of resistant starch (RS) and antioxidants. This study aims to evaluate the effects of pullulanase enzyme on these functionalities. Results showed that the incorporation of pullulanase into PSP powder could significantly increase the RS content from 3.06 g/100g to 7.11 g/100g. However, total anthocyanin content and DPPH radical scavenging activity reduced significantly, due to the interference from RS fragments on anthocyanins. Securing both functionalities (RS and antioxidant) within the same sample is seemingly impossible. A compromise between RS and antioxidant properties in coloured, starchy plant powders is recommended.

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Introduction

Resistant starch (RS) was first mentioned by Englyst et al. (1982) to describe a small portion of starch that survives exhaustive amylolytic digestion. To date, RS has been classified into five types, with RS 3 being the most extensively studied and represents the retrograded amylose formed upon cooling of moist-heated starch. RS is gaining limelight as a functional food ingredient due to its outstanding health benefits which include source of dietary fiber and prebiotics, as well as regulation of cholesterol and blood sugar level (Sajilata et al., 2006; Raigond et al., 2015).

During food processing and cooking, the contents of RS 2 and 3 reduces significantly. Use of debranching enzymes, such as pullulanase, can re-increase the RS content in starchy material, particularly that of RS 3. Pullulanase catalyzes the hydrolysis of α-1,6 glycosidic bonds in amylopectins to produce a large amount of linear amylose chains, which then participate in crystal formation by chain elongation and folding. These newly formed crystals are more perfect and firmer than the crystals of native starch granules, hence they resist digestion and form RS (Miles et al., 1985).

Purple sweet potato (PSP) is gaining popularity due to its nutrient-dense character, vibrant color and versatility in cooking. PSP is rich in anthocyanin, primarily peonidins and cyanidins, that are responsible for the deep purplish flesh and strong antioxidant activity. To the authors' best knowledge, no work has been concurrently reported on the RS content and antioxidant properties in PSP, left alone the relationship between RS content and antioxidant

properties, even though both functionalities are extensively studied on a separate basis. Thus, current study is conducted to evaluate the effects of pullulanase on RS and the resulting RS-antioxidant relationship, using PSP as a food model, owing to its starchy and anthocyanin-rich features.

Material and Methods

Material

Unblemished, medium-size PSP was locally purchased from Federal Agricultural Marketing Authority (FAMA), Malaysia. Resistant starch assay kit was bought from Megazyme International (Bray, Ireland). Trolox and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) were obtained from Merck (Darmstadt, Germany). Pullulanase, a debranching enzyme, with an activity of 1350 NPUN/g was purchased from Novozyme (Bagsværd, Denmark). One Pullulanase Unit Novo (PUN) is defined as the amount of enzyme, which under standard conditions, hydrolyzes pullulan, liberating carbohydrate with reducing power equivalent to 1 µmole glucose per minute.

Preparation of Purple Sweet Potato (PSP) Powder

Unpeeled tubers were washed, cut into cubes of approx. 3 cm (l) × 3 cm (w) × 1.5 cm (h) and steamed for 30 min in a stainless steel cooker. Cooked tubers were mashed into puree followed by debranching steps according to Madzlan et al. (2012) with modifications. About 100 g of PSP puree was treated with pullulanase enzyme at different concentrations (0, 0.25, 0.50, 0.75, 1.00, 1.50, 2.00%, v/w) to produce different enzyme activities of 0, 405, 810, 1215, 1620, 2430, 3240 NPUN/100 g slurry, respectively. All samples were incubated with continuous stirring at 60°C for 1, 2, 8 and 24 h. At the end of hydrolysis, pullulanase was inactivated at 100°C min. Upon cooling, samples were fed onto a steam-heated double drum dryer (R. Simon Dryers Ltd., Nottingham, England) at steam pressure = 3 bars and drum speed = 2 rpm. All conditions for preparation of PSP powder were optimized from the previous study (data not shown). The drum-dried flakes were ground, sieved and stored at -20°C for further analysis.

Resistant Starch (RS) Analysis

RS analysis was performed according to the method from Megazyme International (2011) with some modifications. PSP powder (0.1 g) was mixed with 4.0 mL of pancreatic α-amylase (10 mg/mL) which contained 3 U/mL amyloglucosidase. The mixture was stirred to form a homogenous solution and added with 4.0 mL of absolute ethanol, then continuously shaken for 16 h in a warm water bath (37°C) and centrifuged at 1500 *g* for 10 min at the end of incubation. The pellet (residue) was washed with 50% ethanol, resuspended in 2.0 mL of 2 M KOH and continuously stirred in an ice bath for 20 min, followed by the addition of 8 mL of sodium acetate buffer (1.2 M, pH 3.8) and 0.1 mL of amyloglucosidase (3300 U/mL). The mixture was then incubated at 50°C for 30 min and centrifuged at 1500 *g* for 10 min. The supernatant (0.1 mL) was mixed with 3.0 mL of glucose oxidase-peroxidase (GOPOD) reagent and kept at 50°C for 20 min. A blank (0.1 mL of 100 mM sodium acetate buffer pH 4.5 + 3.0 mL of GOPOD) and glucose standard (0.1 mL of 1 mg/mL D-glucose + 3.0 mL of GOPOD), were incubated concurrently. All absorbance was measured at λ = 510 nm.

Determination of Total Anthocyanin Content (TAC)

TAC was measured according to Burgos et al. (2013) and Kim et al. (2011) with some modifications. One gram of PSP powder was added into 15.0 mL of methanol and 1M HCl pre-mixed at 75:25 ratio, then stirred in the dark for 3 h and centrifuged at 3000 *g*. The absorbance was measured at λ = 535 nm. TAC was calculated as follows:

$$\text{TAC} = \frac{\text{A} \times \text{Mw} \times \text{DF}}{(\varepsilon \times \text{W})} \times 100 \quad (1)$$

Where,

A = absorbance
Mw = molecular weight of cyanidin-3-glucoside chloride ($C_{21}H_{21}ClO_{11}$, 484.84 Da)
DF = dilution factor
ε = molar absorptivity (34300)
W = sample weight (g).

Determination of DPPH Radical Scavenging Activity

The scavenging activity of PSP powder was carried out according to Brand-Williams et al. (1995) with some modifications. Briefly, 0.1 g PSP powder was mixed with 3.0 mL methanol, stand for 5 min and centrifuged at 3000 *g* for 5 min. The supernatant (0.1 mL) was mixed with 3.9 mL of 80 µM methanolic DPPH solution. Absorbance was measured at λ = 517 nm after 3 h. Trolox (0-500 µM) standard curve was plotted to express the results in µmol Trolox Equivalents per gram of sample (µmol TE/g).

Statistical Analysis

Analysis of variance (ANOVA) was performed followed by Tukey's test to identify significant differences at $P<0.05$ using Minitab® 14 Statistical Software (Minitab Inc, State College, Pennsylvania, USA). Results were expressed as means ± standard deviations from triplicate measurements.

Results and discussion

Effects of Pullulanase Hydrolysis Time on RS Content

The RS content for PSP powder over 24-hour hydrolysis at different pullulanase concentrations is depicted in Table 1. At any enzyme concentration, the RS content increased significantly over time, then reached plateau from 8 h to 24 h. The initial RS increment in all samples is due to the encounter between pullulanase enzyme and PSP starch molecules whereby active cleavage of α-1,6 glycosidic bonds in amylopectins constantly occurs (Vasanthan and Bhatty, 1998). This debranching process then turns the bulky, highly-branched amylopectins into linear amylose chains, allowing a better chance of alignment and aggregation to form perfect crystalline structure for RS 3 formation (Surendra Babu and Parimalavalli, 2018).

In contrast, RS content reached plateau towards the end of the hydrolysis. This is due to the excessive cutting of linear amylose by amylase enzyme found naturally in PSP. It is reported that sweet potato contains an abundant

amount of native amylase that are heat stable and denature only above 75°C (Walter et al., 1975; McGee, 2004). The pullulanase-hydrolysis temperature used in this study is 60°C. Thus, native PSP amylase remains active at this temperature and, upon long hour of hydrolysis, cleaves a large proportion of linear amylose (formed from amylopectin debranching by pullulanase) into shorter chains, forming fractions with low degree of polymerization (DP<100) that contain high concentration of short fragments that are without dimensions critical for crystal structure formation, therefore does not further increase the RS yield (Eerlingen and Delcour, 1995).

Another interesting observation was made for sample treated with 0% pullulanase whereby RS content increased over time, from 3.06 at 0 h to 4.32 g/100g at 24 h despite the fact that it was free from pullulanase. This is due to the presence of native amylase in PSP which contributes towards the gradual cutting of amylose chains into fragments that readily form crystal structure required for RS 3 formation.

Table 1 Resistant starch content (g/100g) of PSP powder treated with 0 to 2% (v/w) pullulanase at different hydrolysis times

Hydrolysis Time (h)	RS content (g/100g)						
	Pullulanase concentration (%)						
	0	0.25	0.5	0.75	1	1.5	2
0	3.06^{b}±0.01	3.06^{b}±0.01	3.06^{b}±0.01	3.06^{b}±0.01	3.06^{a}±0.01	3.06^{a}±0.01	3.06^{a}±0.01
1	2.16^{a}±0.09	1.95^{a}±0.08	2.57^{a}±0.03	2.74^{a}±0.03	3.13^{a}±0.04	3.35^{b}±0.16	3.35^{b}±0.15
2	3.89^{c}±0.16	3.52^{c}±0.14	4.63^{c}±0.19	4.95^{c}±0.20	5.65^{b}±0.23	6.04^{c}±0.20	6.05^{c}±0.19
8	4.57^{d}±0.13	4.14^{d}±0.18	5.44^{d}±0.21	5.81^{d}±0.23	6.64^{c}±0.26	7.09^{d}±0.24	7.11^{d}±0.24
24	4.32^{d}±0.21	3.91^{d}±0.19	5.14^{d}±0.13	5.49^{d}±0.14	6.27^{c}±0.16	6.70^{d}±0.31	6.71^{d}±0.31

Note: Each value is reported as mean ± SD from triplicates; Different letters within the same column indicate significant difference at P≤0.05

Table 2 Resistant starch (RS), total anthocyanin (TAC) and DPPH radical scavenging activity of pullulanase-treated PSP powder at 60°C for 8 h.

Pullulanase Concentration (% v/w)	PSP properties		
	RS (g/100 g)	TAC (mg/100 g)	DPPH (µmol TE/g)
0.00	4.57^{c}±0.13	629.18^{a}±5.89	5.58^{a}±0.26
0.25	4.14^{c}±0.18	574.35^{b}±7.02	5.19^{a}±0.19
0.50	5.44^{b}±0.21	575.85^{b}±16.79	5.20^{a}±0.19
0.75	5.81^{b}±0.23	585.91^{b}±14.14	5.21^{a}±0.26
1.00	6.64^{b}±0.26	565.32^{b}±5.04	5.11^{a}±0.21
1.50	7.09^{a}±0.24	489.14^{c}±6.70	4.42^{b}±0.13
2.00	7.11^{a}±0.24	459.32^{d}±7.88	4.15^{b}±0.21

Note: Each value is reported as mean ± SD from triplicates; Different letters within the same column indicate significant difference at P≤0.05

Effects of Pullulanase Concentration on RS Content and Antioxidant Properties

The RS content in all samples reached their peaks at 8 h and reduced insignificantly thereafter, according to Table 1. Based on these observations, antioxidant property evaluations were focused on 8 h samples at various pullulanase concentrations (Table 2). It is observed that DPPH value reduced when total anthocyanin content (TAC) decreased. This is in line with the fact that anthocyanin acts as antioxidant that scavenges DPPH radicals and contributes towards the overall antioxidant activity, thus a low TAC would result in a low DPPH value.

In contrast, TAC and DPPH both display a negative correlation with RS, whereby higher RS result in lower antioxidant capacity. This is supported by the findings from Mulinacci et al. (2008) who observed a low TAC value accompanied by high RS content in blue-flesh potato. When pullulanase increases from 0% to 2%, the RS content increases concurrently. This high amount of RS is very likely to encounter with anthocyanin molecules to form new compounds that interfere with the antioxidant activity. Free anthocyanins are unstable and tend to undergo acetylation with organic acids (Alcalde-Eon et al., 2006) or precipitate with macromolecules such as proteins, tannins and polysaccharides (He et al., 2012). RS, as a polysaccharide, is likely to react with anthocyanins to form new, bulky compounds, causing unfavourable structural alteration that leads to poor antioxidant capacity.

The RS-Antioxidant Relationship

Based on Tables 1 and 2, starch hydrolysis using pullulanase could increase the amount of RS in purple sweet potato powder. Higher pullulanase concentration, along with longer hydrolysis time, produces a larger amount of RS. However, these samples show a negative correlation with RS content. When RS is high, the antioxidant content (TAC) and antioxidant activity (DPPH) are low, and *vice versa*. This finding provides better insights on the RS-antioxidant relationship for colored, starchy plant materials and shows that the antioxidant capacity is adversely affected for samples high in RS.

Conclusions

Current findings demonstrate that starch hydrolysis using pullulanase could increase the amount of RS in purple sweet potato, but further RS formation would lead to a decrease in antioxidant activity, due to the undesirable interference from RS fragments on native anthocyanin molecules, which forms an extensive, bulky matrix with poor antioxidative functionality. Securing high RS and

antioxidant activity within the same sample is seemingly impossible, thus a compromise between RS and antioxidant in coloured, starchy plant materials is recommended. Further evaluation on the RS-antioxidant interaction mechanism is strongly proposed.

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