Valorizing guava (*Psidium guajava* L.) seeds through germination-induced carbohydrate changes

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Abstract Guava seeds are produced as a waste product by the guava processing industry. Their high carbohydrate contents may suit the carbohydrate needs of the feed sector but their high dietary fiber content limits their feed value. The feed values of fruit seeds can be improved through germination, which involves the mobilization of nutrients through seed enzymes and alters the seed carbohydrate composition. The changes of selected carbohydrates in guava (*Psidium guajava* L.) seeds brought by germination to those in red bean (*Vigna angularis*) and winter wheat (*Triticum aestivum* L.) were compared. The contents of soluble carbohydrates, digestible starch, resistant starch and cellulose in the seeds were determined. The radial diffusion method was used to detect carbohydrate-degrading enzymes in the seed extracts. Guava seeds were rich in cellulose (402.2 mg/g), which decreased progressively during germination, probably through the action of cellulase. Winter wheat contained the highest starch content (412.2 mg/g) and also distinct quantities of α -amylase and cellulase. The starch contents of all the seeds decreased, but the soluble carbohydrate contents in red beans and guava seeds increased significantly by the end of germination, suggesting the transient oversupply of reserve metabolites. The content of hydrolyzed polysaccharides increased in the germinated seeds with detectable amounts of cellulose-degrading enzymes present, indicating improved value as feed. Further research is warranted to

explore the potential of guava seeds as a source of low-cost animal feed supplements.

Keywords Carbohydrate · Enzymes · Germination · Guava seed · Red bean · Winter wheat

Abbreviations

ANOVA Analysis of variance
CMC Carboxymethyl cellulose
GFG Glucose, galactose and fructose
GOPOD Glucose oxidase/peroxidase

Introduction

Guava is a popular tropical fruit that is rich in dietary fiber and exhibits high antioxidant capacity (Jiménez-Escrig, et al. 2001). Global production of guava fruits reached 6.2 million metric tons in 2012 (USAID-ACCESO 2014) with India, Pakistan and Brazil as the top three producers. Fresh guava spoils rapidly and is widely processed into shelf life-stable products for export trade. The mass processing of fruits and vegetables generates a great amount of waste and by-products such as peel, pomace and seeds. About 80 kg waste per metric ton of fresh fruits is produced in guava processing (Souza et al. 2016). Brazil is the greatest producer of red guava fruits, which produce 4–30% of their weight in wastes. These guava wastes comprise 15–50% seeds (Mantovani et al. 2004). The composition of guava seeds governs their use. Seeds intrinsically contain nutrient reserves for the development of the plant embryo, and thus are rich in carbohydrates, lipids and proteins. Guava seeds contain mainly 53.6–67.7% dietary fiber, 10.5–16% fat, 7.9–9.6% protein and 0.9–1.2% ash (Chek Zaini et al. 2010; El-Din

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and Yassen 1997; Nicanor et al. 2001; Prasad and Azeemoddin 1994). The minerals zinc, iron, potassium, phosphorus and manganese are also present in significant amounts in guava seed meal (Uchôa-thomaz et al. 2014). Guava seed oil is rich in oleic and linoleic acids, which are essential fatty acids. Additionally, the protein extracted from guava seeds consists of all essential amino acid except lysine (Nicanor et al. 2001). Plant seeds can also be a source of bioactive compounds. Guava seeds contain high levels of yellow flavonoids, coumarin and resveratrol (Ribeiro da Silva et al. 2014), as well as phytic acid (Chang et al. 2014).

The high dietary fiber content and the presence of anti-nutritional compounds are the main constraints to using guava seeds as feed. Dietary fiber-rich feeds often have lower digestibility. Feed enzyme inclusion and pretreatments like heating, autoclaving, fermentation and germination have been found to improve the digestibility of certain legumes and cereals (Chitra et al. 1996; Mäkinen et al. 2013). Similar approaches are being developed to increase the feed value of many underutilized agricultural by-products (Wadhwa and Bakshi 2013) to cope with the rising demand for food and animal feed. Dietary carbohydrates are metabolized as structural and energy storage materials. The high content of dietary carbohydrates in seeds may suit the energy needs of the feed industry. Our previous work showed that germination significantly reduced the contents of some anti-nutritional compounds in guava seeds (Chang et al. 2014). Germination can mobilize the nutrient reserves in seeds through seed enzymes including carbohydrate-degrading enzymes (Bhushan et al. 2013; Nirmala et al. 2000). Changes in seed carbohydrates can improve the feed value of the seed itself. Staple foods for humans and animals in the form of seeds include rice, wheat, soybean, mung bean and other common grains and beans whose nutrient compositions are well-established (USDA 2014). Little research has been carried out on the different carbohydrates in guava seeds and there have been no reports on guava seed enzymes or the changes in seed carbohydrates induced by germination.

We carried out this study to determine whether germination could induce carbohydrate profile changes in guava seeds and to evaluate their feed value. We also attempted to detect carbohydrate-degrading enzymes in the fruit seeds in comparison with a cereal and a legume. To achieve these objectives, we analyzed the contents of selected carbohydrates in seeds of guava (*Psidium guajava* L.), red bean (*Vigna angularis*) and winter wheat (*Triticum aestivum* L.) germinated for different times. The seed extracts were then screened for cellulase and α -amylase to explore their potential as a source of enzymes.

Materials and methods

Seed samples

Fresh guava seed cores were obtained from a local fresh-cut fruit supplier in Kampar, Malaysia. The seed cores were placed in a blender (*Cucina* Model 7727/60; Philips DAP, Drachten, Netherlands), excess water was added, and they were blended using the pulse mode. The blended material was then separated as described by Chang et al. (2014). Red beans and winter wheat were bought from a local Tesco hypermarket and were kept at 4 °C prior to further treatments.

Germination treatments

The seeds from each species were weighed and divided into five equal portions. The seeds were washed and soaked in distilled water at ~25 °C for 8 h to end seed dormancy. The drained seeds were surface sterilized using 1% sodium hypochlorite solution and rinsed with distilled water. The seeds were then germinated at ~25 °C on a layer of sterilized wet cotton in covered 9 cm-diameter Petri dishes under normal daylight. The winter wheat grains were germinated for 0, 2, 6, 24 and 30 h; the red beans for 0, 6, 24, 30 and 50 h; and the guava seeds for 0, 6, 24, 48 and 72 h. We set the maximum germination period for each type of seed based on the time frame before any obvious seedling growth. The non-germinated and germinated seeds were frozen in liquid nitrogen, freeze-dried (Scanvac-Cool Safe 55-4, Labogene, Lynge, Denmark) and stored in airtight containers at 4 °C before further analysis.

Carbohydrate extraction

The soluble carbohydrate was extracted as described by Chow and Landhausser (2004). Ground seeds (150 mg) were added to 10 ml ethanol (80% v/v) and then incubated at 90 °C for 10 min. The mixture was centrifuged using a CT15RT centrifuge at 14,000×g (Techcomp Ltd., Hong Kong) for 30 min. We decanted the supernatant and the remaining seed residue was extracted another two times, using 10 ml ethanol (80% v/v) each time. All supernatants were combined to determine the total soluble carbohydrate content and the residue was retained to determine its starch content.

Total soluble carbohydrate content

The total soluble carbohydrate content in the seeds was determined based on the phenol-sulfuric acid method (Chow and Landhausser 2004). Working in the dark,

phenol (2% w/v) and concentrated sulfuric acid (H_2SO_4) were added to a suitably diluted seed extract at a ratio of 2:5:1. The total volume of the mixture was 4 ml. The mixture was reacted for 10 min, and the absorbance at 490 nm was recorded using a Genesys 20 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The total soluble carbohydrate content was estimated based on a standard curve constructed using a mixture of glucose, galactose and fructose (GFG) (150 mg/ml, at a ratio of 1:1:1) over a concentration range of 25–150 $\mu\text{g/ml}$.

Digestible starch content

The seed residue remaining after soluble carbohydrate extraction was dried at 70 °C in an UNB 400 oven (Mettler GmBh, Schwabach, Germany) overnight. The digestible starch content was determined using an enzymatic-colorimetric method (Bruno et al. 2013). The dried sample residue was reacted with α -amylase (300 U) and amyloglucosidase (300 U) at 37 °C in a WNB 22 shaking incubator (Mettler) for 20 h. Then, an equal volume of ethanol (95%) was added before it was centrifuged at 14,000 $\times g$ for 10 min. After decanting the supernatant, the residue was extracted twice with ethanol (50%). The residue was kept to determine its resistant starch content. The combined supernatants were mixed with ethanol (50%) and amyloglucosidase (300 U) at a ratio of 10:10:1 and incubated at 50 °C for 20 min. After further reaction with glucose oxidase/peroxidase (GOPOD) reagent at a ratio of 1:5 at 50 °C for 20 min, the absorbance of the mixture at 510 nm was recorded.

Resistant starch content

The seed residue was mixed with 2 ml of potassium hydroxide (2 M) and incubated in an ice bath for 20 min. The mixture was further reacted with sodium acetate buffer (1.2 M, pH 3.8) and amyloglucosidase at 50 °C in a shaking water bath for 20 min before centrifugation. The supernatant was mixed with an equal amount of sodium acetate buffer (1.2 M) and further reacted with GOPOD as described in the previous section. The absorbance of the mixture at 510 nm was recorded. Both the digestible starch and resistant starch contents were estimated using a calibration curve of glucose over a concentration range of 50–400 mg/ml. A factor of 0.9 was used in the calculation (Bruno et al. 2013).

Cellulose content

The cellulose content of the seeds was determined as described by Updegraff (1969). Six milliliters of acetic nitric reagent was added to 100-mg ground seed sample and the mixture was incubated at 95 °C for 30 min. The

residue obtained was then dried at 100 °C using an UNB 400 oven (Mettler) overnight. The dried residue was hydrolyzed with 10 ml H_2SO_4 (67% v/v) at ~25 °C for 2 h until fully dissolved. This sample was diluted with distilled water and kept in an ice bath for 10 min. Anthrone reagent was added at a ratio of 2:1 and mixed slowly. The mixture was then incubated at 95 °C for 15 min, cooled and kept at room temperature for 5 min. The absorbance of the mixture at 620 nm was recorded. The cellulose content was estimated using a calibration curve of cellulose over a concentration range of 20–100 $\mu\text{g/ml}$.

Detection of enzymes

The ground seed sample was mixed with sodium acetate buffer (50 mM, pH 5.6) at a ratio of 1:10 before centrifugation at 14,000 $\times g$ and 4 °C for 1 h. The supernatant collected was the crude seed extract. Agar solutions containing 1% starch and 1% carboxymethyl cellulose (CMC) were used to detect α -amylase and cellulase, respectively, as described by Colombatto et al. (2003). One-hundred microliters of crude seed extract was dispensed into a 1 cm-diameter well within the autoclaved and solidified agar. Commercial enzymes as positive controls and extraction buffer as a negative control were dispensed into the other wells. The agar plates were incubated at ~25 °C (starch agar) or 37 °C (CMC agar) for 24 h. Iodine solution (1% w/v) was used to stain the starch agar and Gram's iodine solution (1% w/v) was used for the CMC agar. The presence of a particular enzyme was indicated by the hydrolyzed substrate forming a clear zone.

Experimental design and data analysis

Seed germination was carried out twice. For each batch of seeds, the samples were analyzed twice. Thus, each value reported was the mean of quadruplicate measurements. For the enzyme screening test, the values reported were means of triplicates from a mixture of two batches of seed. We used the statistical package SPSS version 16.0 for Windows (SPSS Inc., Chicago, IL, USA) for data analysis. An independent sample *t* test was used mainly to compare the means of two samples while one-way analysis of variance (ANOVA) and Tukey's multiple comparison test were used for most analysis to assess the significance of differences between mean values at a probability level of $P = 0.05$.

Results and discussion

The radicle took 30 h to appear in winter wheat, 50 h in red beans, and more than 72 h in guava seeds. The sizes of the winter wheat grains and red beans increased slightly but no

Fig. 1 Total soluble carbohydrate content in guava seeds (filled triangle), red beans (filled circle) and winter wheat (open diamond) as a function of germination time. Values represent mean $\pm$ SD ($n = 4$). Means with different letters within the same type of seed are significantly different ($P < 0.05$)

change in size were observed in the guava seeds. This may indirectly reflect variations in the cellular components that form the outer coats of these seeds. Guava seeds have a rather hard coat and thus it took more time for the radicles to protrude from the endosperm.

Carbohydrate contents in non-germinated seeds

Guava seeds contained mainly cellulose (402.2 mg/g) while winter wheat and red bean were rich in digestible starch (412.2 and 264.3 mg/g respectively). Starch and resistant starch are composed of amylose and amylopectin. These complex carbohydrates vary in content and ratio depending on their source (Labaneiah and Luh 1981). Resistant starch is the part of the starch that resists α -amylase action (Annison and Topping 1994) and has prebiotic activity that benefits human and animals (Slavin 2013). Seeds with higher resistant starch content are expected to possess higher feed value. Guava seeds had the lowest resistant starch and soluble carbohydrate contents despite their high cellulose content. Red beans contained the highest resistant starch (6.6 mg/g) and soluble

carbohydrate (45.6 mg/g) contents while winter wheat contained less soluble carbohydrates compared with red beans. Soluble carbohydrates in seeds probably comprise a mixture of glucose, sucrose, sucrosyl oligosaccharides or complex carbohydrates like fructans, β -glucans and arabinoxylans (Ziegler 1995). Such materials can be utilized and incorporated into animal feeds as a source of carbohydrates. Non-digestible soluble carbohydrates like sucrosyl oligosaccharides and fructans are prebiotics that support the growth of probiotic strains in the host's gastrointestinal tract (Slavin 2013). Commercial prebiotics interact with probiotic strains and exert immune-stimulating effects on the host. The incorporation of commercial prebiotics into animal feed can reduce the use of in-feed antibiotics (M'Sadeq et al. 2015). In the present study, the cellulose contents in winter wheat grains and red beans were within the range of 2–5%, in agreement with other studies (Mendu et al. 2011). Cellulose is indigestible for most livestock except for ruminants. From a nutritional viewpoint, the low amount of cellulose and high content of soluble carbohydrates make both winter wheat grains and red beans better feed materials than guava seeds.

Fig. 2 Digestible starch content in guava seeds (filled triangle), red beans (filled circle) and winter wheat (open diamond) as a function of germination time. Values represent mean $\pm$ SD ($n = 4$). Means with different letters within the same type of seed are significantly different ($P < 0.05$)

Germination-induced changes in total soluble carbohydrate content

Germination induced a continuous increase in the total soluble carbohydrate content in guava seeds and red beans, with the maximum value being attained at the end of germination (Fig. 1). This increase in soluble carbohydrate content is evidence of nutrients being mobilized rather than utilized immediately in the germinating seeds (Martin-Cabrejas et al. 2008). The mobilization of complex insoluble carbohydrates to soluble carbohydrates in the germinating seeds probably increases the carbohydrate digestibility. Nonetheless, the germinating winter wheat showed a slight increase in total soluble carbohydrate to 26.8 mg/g after 2 h, followed by a continual drop to 20.5 mg/g after 30 h, suggesting early mobilization of the complex carbohydrate reserve followed by its metabolization (Ziegler 1995). This physiological phenomenon may not have favorable effects on the feed value.

Germination-induced changes in starch content

Germination decreased amount of digestible starch in all the seeds studied: from 1.5 to 0.8 mg/g in guava seeds; from 412.2 to 340.6 mg/g in winter wheat; and from 264.3 to 178.5 mg/g in red beans (Fig. 2). These findings agreed with physiological changes observed in other germinating

seeds (Bhushan and Gupta 2008; Swieca. et al. 2013). The starch reserves of seed were probably converted to soluble carbohydrates or other metabolites. These physiological changes require the action of specialized enzymes (Ziegler 1995). This observation also correlates with the continual increase in total soluble carbohydrates in red beans and guava seeds (Fig. 2). This suggested that the mobilization of the less reactive polymeric reserves to simple sugars and oligosaccharides occurred a faster rate than their metabolization. Soluble carbohydrates accumulate simultaneously with starch breakdown in red beans and guava seeds, implying a transient oversupply of reserve metabolites. Thus, a greater increase in feed value would be expected for both germinated red beans and guava seeds than for germinated winter wheat. If we assume a material's prebiotic activity score contributes to its feed value, this observation agrees with the results obtained in another study. We found that both guava seeds and red beans showed greater increase in their prebiotic activity scores (5.3- and 2.2-fold, respectively) after germination compared with winter wheat (Chang and Tan 2016).

Figure 3 shows that germination decreased resistant starch content in both winter wheat and red beans, by 40% and 30.5%, respectively. The content of amylose and amylopectin is the main intrinsic factor that affects resistant starch content (Perera et al. 2010). Germination

Fig. 3 Resistant starch content in guava seeds (filled triangle), red beans (filled circle) and winter wheat (open diamond) as a function of germination time. Values represent mean $\pm$ SD ($n = 4$). Means with different letters within the same type of seed are significantly different ($P < 0.05$)

reduces the polymeric carbohydrate chain length (Xu et al. 2012) or progressively decreases the ratio of amylose to amylopectin (Labaneiah and Luh 1981), which may have contributed to the decrease in resistant starch content. The resistant starch content of guava seeds remained constant after 72 h germination. This suggests that the seed enzymes involved in germination had a minimal effect on the resistant starch in guava seeds.

Germination-induced changes in cellulose content

The cellulose content in guava seeds tended to decrease as germination progressed (Fig. 4). Guava seeds, which had the highest cellulose content, mobilized and used both cellulose and digestible starch (Fig. 2) during germination (in agreement with the detectable cellulase activity, as discussed in the following section). Starch and cellulose were not restored during germination. A decrease in cellulose content means an increase in feed value through carbohydrate digestibility. In contrast, the cellulose content fluctuated in red beans but remained constant in germinating winter wheat. This observation may have been caused by cellulose reforming in the new seedlings.

Enzymes present in seeds

Most of the crude extracts of the seeds showed the presence of the enzymes tested. The winter wheat crude extract produced the largest clear zones on the two types of agar (Table 1). Thus, α -amylase and cellulase were present in the winter wheat extract. The winter wheat extract hydrolyzed starch agar in a similar way to commercial α -amylase (positive control). However, red beans and guava seeds showed no sign of *in vitro* α -amylase activity, despite showing continuous starch breakdown (Fig. 2). Ziegler (1995) noted a similar disconnect between starch mobilization and α -amylase activity in other seeds. The mobilized seed reserves produce degraded phytate and proteinaceous substances that may inhibit the *in vitro* α -amylase. Additionally, the hydrolyzed seed starch provides a large amount of metabolizable sugars that limit the action of amylase (Ziegler 1995). This explanation agrees with the transient oversupply of reserve metabolites that we observed and discussed in both red beans and guava seeds in the previous section. Both the guava seed and red bean extracts produced clear zones on CMC agar, although the sizes of the zones were slightly smaller than that of winter wheat extract (Table 1). Cellulase was present in both the

Fig. 4 Cellulose content in guava seeds (filled triangle), red beans (filled circle) and winter wheat (open diamond) as a function of germination time. Values represent mean $\pm$ SD ($n = 4$). Means with different letters within the same type of seed are significantly different ($P < 0.05$)

Table 1 Clear zone diameters on starch agar and carboxymethyl cellulose (CMC) agar

Sample	Diameter of clear zone (cm)*	
	Starch agar	CMC agar
Positive control- α -amylase	3.3	3.1
Non-germinated		
Winter wheat extract	2.78 $\pm$ 0.1	2.63 ^a $\pm$ 0.12
Red bean extract	No clear zone	1.95 ^b $\pm$ 0.15
Guava seed extract	No clear zone	2.13 ^b $\pm$ 0.15
Germinated		
Winter wheat extract	2.9 $\pm$ 0.1	2.47 ^a $\pm$ 0.23
Red bean extract	No clear zone	1.97 ^b $\pm$ 0.12
Guava seed extract	No clear zone	2.2 ^b $\pm$ 0.26

* Values represent mean $\pm$ SD ($n = 3$). No significant difference was observed between non-germinated and germinated seed extracts (for the same type of seed) at $P < 0.05$. Means with different letters within a column are significantly different ($P < 0.05$)

guava seed and red bean extracts. The main roles of cellulase in seeds include digesting the seed cell walls and producing a supply of carbohydrates to support seedling growth. The dissolution of the cell wall enables radicles to emerge from the germinating seeds. In addition, cellulases initiate cell wall degradation before other enzymes become involved (Boswell 1941). Cellulases are included among

the feed enzymes that improve the digestibility of nutrients, thereby reducing the cost of feed (Chang and Woo 2016). The diameters of the clear zones observed for the non-germinated seed extracts were not significantly different to those of the germinated seed extracts. Because the crude seed extract showed detectable cellulase activity, we assume that whole guava seeds (non-germinated or germinated) contain cellulases and are suitable to incorporate into feed. However, further investigation on the cellulases and other cell-wall degrading enzymes present in guava seeds is warranted.

Conclusion

Guava seeds were rich in cellulose while both red beans and winter wheat contained mainly digestible starch. The high amount of cellulose in guava seeds may limit their inclusion in animal diets. By the end of germination, the starch content was decreased in all seeds, but the soluble carbohydrate content in red beans and guava seeds was increased significantly, suggesting a transient oversupply of reserve metabolites. These changes may improve the feed value of the seeds through increased carbohydrate digestibility and prebiotic activity. The germinating guava seeds also showed the progressive breakdown of cellulose with detectable cellulase activity. These results warrant

further research to explore the potential of guava seeds as a source of low-cost animal feed supplements.

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