Next-generation healthy rice

by Prof Chris Blanchard
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Foreword

Rice breeding programs do not have access to an accurate and high-throughput method for ranking the digestibility of germplasm materials. While *in vitro* digestion assays cannot replicate the complex interactions between food and body, the development of a quick *in vitro* assay that can differentiate breeding lines based on digestibility is critical in the development of low-digestibility rice cultivars.

The objective of this project was to develop a rapid *in vitro* digestion assay that can be deployed in the Australian rice breeding program to select germplasm with low digestibility. The effectiveness of the assay was assessed by benchmarking glycaemic index (GI) values of rice varieties against values obtained using the newly developed assay. A diverse set of varieties were also analysed to assess the genetic variability in digestibility.

The main beneficiaries of this research will be Australian rice producers. Having the ability to rapidly screen the digestibility of rice breeding lines will hasten the development of high-quality rice varieties with reduced digestibility. Demand for food with enhanced health properties is increasing, and high-quality rice with a low GI is in high demand. This high demand will translate to a more valuable product that will ultimately increase the profitability of rice production in Australia.

There is also a secondary benefit for consumers of rice with a lower GI. Foods with a low GI are thought to be associated with health benefits that might include better weight management and lower levels of diabetes. If rice with a lower GI is widely adopted, there may be an associated decrease in “lifestyle” diseases.

Initial investigations determined that existing methodologies, including the commercially available automated GI estimation instrument, were not suitable for screening rice breeding lines. Modifications were made to published methodologies to develop a rice-specific technique that was able to rank genotypes based on their digestibility.

Commercial rice genotypes with diverse GI values were successfully differentiated using the *in vitro* protocol. The proportion of starch hydrolysed at 60 minutes (SH-60) during the *in vitro* digestion assay was used to rank the digestibility of the samples. Further investigations with nine rice genotypes indicated that the assay can successfully differentiate genotypes with a wide range of GI values. The technique was then used to evaluate the genetic basis of digestibility by screening a diverse panel of rice varieties.

Training has been completed with NSW DPI technical staff to allow deployment of the method in the Australian rice breeding program. This training consisted of “hands-on” workshops held at Charles Sturt University’s (CSU) National Life Sciences Hub. These workshops further enhanced the method, as indicated in NSW DPI staff feedback.

This research has been made possible through the generous support of AgriFutures Australia. AgriFutures funding supported the work of several postdoctoral scientists working over a total period of 3.5 years. The project also demonstrated the benefit of funding collaborative projects between CSU and NSW DPI. The complementary skills of these two organisations contributed to the success of the project. The project also interacted with several other Australian Research Council (ARC)-funded Functional Grains projects, which resulted in enhanced outcomes for all projects.

The successful deployment of a GI screening tool in the Australian rice breeding program will facilitate the development of higher value rice varieties. The development of higher value rice varieties will enhance the profitability of rice production in Australia and potentially prevent the decline in the Australian rice industry. The current research and development efforts in the Australian rice industry are largely focused on increasing yield and water efficiency. This focus assumes that the
most effective way to maintain the Australian rice industry is through increasing the amount of rice produced per megalitre of water. While these are important areas of research, it is our belief that more effort should be devoted to increasing the value of our rice. Farmers are actually more interested in “dollars per megalitre” than in “tonnes per megalitre” because their choices are based on profitability measures. Given the relatively small research budget that the Australian rice industry has access to, we are more likely to make substantial gains in profitability by increasing the value of our grain than in making gains in yield and water-use efficiency.

The success of this project has also been assisted through collaboration with ARC-funded projects. This collaboration highlights the opportunity that partnerships with other funding partners may offer in achieving the objectives of the Australian rice industry. As the amount of funding available for rice research has decreased due to a shrinking industry, it is important that we look to partner with other funding sources to maintain the level of investment required to sustain the industry.

This report for the AgriFutures Rice Program adds to AgriFutures Australia’s diverse range of over 2,000 research publications. It forms part of AgriFutures Australia’s Growing Profitability arena, which aims to enhance the profitability and sustainability of our levied rural industries.

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Abbreviations

AA: \( \alpha \)-amylase
AMG: amylglucosidase
CSU: Charles Sturt University
FGC: Functional Grains Centre (or Australian Research Council Industrial Transformation Training Centre for Functional Grains)
GI: Glycaemic index
NSW DPI: New South Wales Department of Primary Industries
SH-60: starch hydrolysed at 60 minutes
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Figure 2: Starch digestogram of three rice genotypes. The dotted line denotes 55% of starch hydrolysed.

Figure 3: Box and whisker plot of starch hydrolysed at the 60 min time-point (SH-60) for different rice genotypes. The dotted line denotes 55% of starch hydrolysed.

Figure 4: Correlation plot of five rice genotypes: in vivo GI data vs % starch hydrolysed at 60 min of in vitro digestion (SH-60).

Figure 5: Diverse genotype screening using SH-20 (%): The % starch hydrolysed at 20 min of in vitro digestion (SH-20) of a diverse panel of rice genotypes was assessed with partial replication and expressed as a scatter plot (a), and a histogram (b).
Executive summary

What the report is about

This report outlines the work conducted by CSU and NSW DPI to develop a rapid method for ranking rice genotypes for their starch digestibility properties. The development of a rapid digestibility method is required for the development of low GI rice varieties that will attract a premium in domestic and international markets.

Who is the report targeted at?

The information in this report will be of use to the Australian rice breeding program for the development of low GI rice. The information generated by this project is also of use to rice researchers interested in understanding the factors that affect rice digestibility. The report’s recommendations will be of interest to farmers, consumers, nutritionists, researchers, research funding bodies, policy makers, and all rice industry stakeholders.

Where are the relevant industries located in Australia?

This project has delivered a digestibility method to the Australian rice industry, which is currently predominantly located in southern NSW in association with the Murray, Murrumbidgee and Coleambally irrigation areas. There are currently some efforts to explore a northern rice industry.

In recent years, the volume of rice produced in Australia has declined because less water has been available. More water is being directed to the environment and more profitable crops, such as cotton and perennial plantings.

The outputs of this project are likely to benefit the Australian rice industry by helping to develop new high-value healthy rice varieties. It is hoped that the increased value of these rice varieties may compensate for the overall reduction in rice production in Australia.

While the assay developed in this project was designed specifically for rice, the possibility exists to adapt it for other starch-based grains or products. One possibility would be to adapt the assay so that it could predict the digestibility of feed rations from livestock.

Background

Consumers are becoming more aware of the potential for food to deliver health benefits. One area of interest is in the consumption of foods with a low GI because they are thought to be associated with a number of health benefits. Most rice varieties are considered to have high GI values, making them unattractive to the health-conscious consumers. GI measurement is a time-consuming and expensive procedure that is not suitable for implementation in grain breeding programs. Therefore, to develop low GI grain varieties, rapid and inexpensive methods are needed so that breeding lines can be screened for their digestibility behaviour.

Aims/objectives

The aims of this project were to develop, validate and deploy a high-throughput assay for screening the digestibility of rice genotypes. The assay will help rice breeders develop new high-value rice varieties that have a low GI.
Methods used

The method developed is an in vitro technique that mimics simple starch digestion as a way of predicting GI values. While the method does not provide a GI value, it is accurate enough to rank rice genotypes as either high, medium or low digestibility.

Results/key findings

The objectives of the project have been met. The technique that has been developed has the capacity to screen large numbers of genotypes for their digestion behaviour. The method has been validated by screening 200+ diverse genotypes. NSW DPI training has ensured the method is ready for adoption.

Implications for relevant stakeholders

This project will help deliver new high-value rice varieties that could increase profitability for Australian rice farmers. SunRice, an Australian rice marketer and exporter, will also benefit through the delivery of premium varieties into high-value markets. From the outcomes of the project, consumers will also benefit by having access to healthier rice varieties.

Recommendations

This report recommends the urgent implementation of the newly developed digestibility screening tool in the Australian rice breeding program. This should be done in combination with a market study that clearly identifies the market opportunities for low GI rice products. Further, it is recommended that other opportunities should be explored to increase the value of Australian grain so that rice remains a profitable option for Australian farmers.
Introduction

The incidence of obesity, type 2 diabetes and related chronic diseases is alarmingly high, both in developing and developed countries. About 1.9 billion adults are either overweight or obese, while 382 million people suffer from type 2 diabetes worldwide. To address these global health concerns, individuals and communities are encouraged to adopt a balanced diet and to increase their physical activity. For diet, the availability of healthy food options is vital and requires a population-based approach involving intervention from both food production (e.g. modification of primary produce) and food processing sectors (e.g. reformulation of food products).

The role of dietary components in the prevention of chronic conditions has gained much research attention. Starch is a major component of the human diet and, like other dietary carbohydrates, has been given an energy value of 17 kJ/g (4 kcal/g). However, it is now well understood that a number of different starch fractions exist that are not digested at an equal rate. These fractions have been classified as follows: rapidly digestible starch (RDS) – digested within 20 minutes; slowly digestible starch (SDS) – digested between 20 and 120 minutes; and resistant starch (RS) – digested beyond 120 minutes. Generally, SDS has been shown to elicit a moderate postprandial glycaemic and insulinaemic response. This fraction of starch is considered beneficial for the dietary management of individuals with chronic and metabolic conditions, particularly those with type 2 diabetes and hyperlipidaemia. RS is also considered to be beneficial because it evades digestion in the human gastrointestinal tract and, hence, food high in this starch fraction has a very low digestibility. In addition, RS performs a physiological function similar to dietary fibre because it provides a substrate for the microbial fermentation of short-chain fatty acids (SCFAs) in the human colon.

While the nutritional classification of starch fractions is useful, it cannot accurately represent the intricacies of the digestion process. The GI is a well-established in vivo measure of the postprandial blood glucose-raising potential of carbohydrate-containing foods. Regular consumption of high GI diets has been shown to increase the risk of developing type 2 diabetes. While physiologically informative, there are many practical and logistical limitations to the standard in vivo methods. Thus, in vitro digestion models remain a necessary research tool and allow for better control of key factors important to the digestion process.

Rice (Oryza sativa L.) is the staple crop for billions of people worldwide, with global consumption estimated at 402 million metric tons (milled rice basis) and a global per capita food use of 54.1 kg per person in 2016-17. White rice, otherwise known as “milled” or “polished” rice, is the most common form consumed. It is physically processed to remove the hull and bran layers to reveal the starchy endosperm, contributing to an overall high proportion of starch of up to 90% (dry weight basis). The digestion and intestinal absorption of cooked white rice is complete or near complete in humans. However, the rate of digestion is influenced by several intrinsic (e.g. starch properties, starch-protein interactions and starch-lipid interactions) and extrinsic factors (e.g. hydrothermal treatments and variations of domestic cooking).

The diversity in genes associated with controlling rice digestibility provides the opportunity to select new varieties with reduced digestibility using classical breeding. Rice breeding programs currently use relatively high-throughput but inaccurate proxy measures for GI, such as amylose content and resistant starch levels. While an in vitro digestion system could never replicate the complex interactions between food and body, the development of physiologically relevant and statistically robust in vitro assays is warranted in the rapid screening for low-digestibility phenotypes. There are two main benefits of in vitro digestibility assays: (1) variation in product composition can be studied under the same conditions; and (2) accurately controlled conditions do not give the high variability of in vivo studies, reducing the number of replicates needed for sufficient statistical power. Numerous in vitro digestion methods exist that aim to simulate human carbohydrate digestion and to estimate the likely glycaemic properties of the food. In vitro assays have been used to estimate the digestibility of rice. However, these in vitro methods are not suitable for high-throughput applications to satisfy the quick results turnaround time of rice breeding programs.
Objectives

1. Develop a cost-effective and consistent *in vitro* GI method to select advanced breeding lines for low GI potential for implementation by the Rice Quality component of the Australian Rice Partnership II Project.

2. Due to challenges with importing porcine pancreatin into Australia, there is also a need to develop an alternative digestive method to replace this enzyme with another accessible option.

3. Fast-track development of potential low GI rice varieties by assessing up to 120 advanced breeding lines each year for the duration of this project. (*NB: samples provided by NSW DPI were from a genetic diversity panel.*)

4. Assist with delivering advanced rice breeding lines with low GI in the Australian Rice Partnership II Project.
Methodology

Materials

Sodium hydroxide pellets (NaOH), sodium acetate anhydrous (CH\textsubscript{3}COONa) and hydrochloric acid (HCl) were obtained from Chem-Supply Pty Ltd (Gillman, SA, Australia). Calcium chloride dihydrate (CaCl\textsubscript{2}.2H\textsubscript{2}O) was purchased from Thermo Fisher Scientific. Glacial acetic acid (CH\textsubscript{3}COOH) and magnesium chloride anhydrous (MgCl\textsubscript{2}) were sourced from Sigma-Aldrich (Castle Hill, NSW, Australia). Alpha-amylase (porcine pancreas, 100,000 U/g), α-amylase (Aspergillus niger, 3,260 U/mL), Total Starch Assay Kit and d-Glucose Assay Kit (oxidase/peroxidase, GOPOD format) were sourced from Megazyme International Ireland Ltd (Wicklow, Leinster, Ireland). Stir bars (PTFE, pivot ring, 30 x 6 mm) were purchased from Aim Scientific (Prospect, SA, Australia). Milli-Q quality (Millipore, Bedford, MA, USA) water was used throughout the analysis.

Rice genotypes were provided in paddy form by the NSW DPI, Yanco Agricultural Institute (YAI). These include commercial varieties, such as Doongara, Illabong, Koshibikari, Langi, Opus, Reiziq, Sherpa, Topaz, and YRL127. One glutinous rice genotype (white glutinous rice, Thailand) was purchased from the local grocery store (Wagga Wagga, NSW, Australia). A coded panel of 200+ genetically diverse genotypes were also provided by NSW DPI to validate the assay.

Sample preparation

Australian paddy rice samples were dehulled with the Testing Husker THU 35A and polished using the OnePass Rice Whitening & Caking Machine (Satake Engineering Co., Ltd, Tokyo, Japan) at Yanco Agricultural Institute, NSW DPI. To prepare flour samples, white rice grains were ground with a hammer mill, Laboratory Mill 3100 (Perten Instruments, Kungens Kurva, Sweden), with a 0.5 mm sieve. White rice grains and flour samples were stored in sealed plastic specimen jars at 4 °C and then equilibrated to room temperature for at least 24 h before analysis.

Samples were freshly cooked on the day of starch digestion analysis. Water (5 mL) was added to four (pre-weighed) intact, white rice grains in 150 mL Schott bottles. Bottles were tightly capped and immediately submerged in a vigorously boiling water bath for 30 min to ensure all samples were fully cooked. Sufficiency of cooking was routinely tested by squashing cooked milled grains between two glass slides. The absence of a white core was used as a visual indication of well-cooked grains. Freshly cooked samples were transiently stored in a 60°C water bath, and the digestibility assay was carried out immediately to prevent starch retrogradation.

Moisture content

The moisture content of rice flour samples was determined by automated thermogravimetric analysis (TGA) using a TGA701 Thermogravimetric Analyser (Leco, Michigan, USA). Each sample was heated under air (dry oil-free) atmosphere up to 130 °C, ramped at 10.5 °C per min until a constant weight was obtained. Moisture analyses were conducted in triplicates and the results were averaged to obtain a mean value.

Total starch content

Total starch content of rice flour was determined as glucose released by enzymatic hydrolysis after treatment with 2 M KOH using an assay kit (Megazyme Total Starch Assay Kit, Wicklow, Ireland).
Enzyme optimisation

Doongara rice was generally used in enzyme optimisation assays. This variety was chosen because there is extensive available data relating to its GI. The optimum α-amylase (AA) and amyloglucosidase (AMG) concentrations were determined by monitoring the kinetic profile of starch digestion over 3 h, with varying concentrations of each enzyme and both enzymes combined. For single-enzyme assays, samples were digested with AA alone (0.0001, 0.001, 0.01, 0.1, 1, and 10 U/mL) or AMG alone (0.5, 1, 5, 10, 50, and 100 U/mL). For dual-enzyme assays, samples were digested by adding 1 U/mL AA combined with varying concentrations of AMG (0.5, 1, 5, 10, 50, 100 U/mL). In addition, in another variation to the method, 1 U/mL AA and 5 U/mL AMG were added sequentially (digesting with AA alone, heat inactivating AA at 100 °C, and then adding AMG to aliquot samples) and compared with adding the enzymes simultaneously (digesting with a mixture of AA and AMG together).

In vitro starch digestion assay

Samples were cooked (as described in the sample preparation section) and sample bottles were immediately transferred to a 60 °C water bath. Forty millilitres of sodium acetate buffer (0.2 M, pH 6.0), which had previously been equilibrated to 60 °C, was added to each bottle. All samples were then equilibrated to 37 °C and stirred for exactly 5 min at 200 rpm. At this stage, duplicate aliquots (0.2 mL) were sampled to measure for the presence of intrinsic enzymes in the rice grains; this was considered the 0 min time-point.

An enzyme mixture of 1 U/mL pancreatic α-amylase per 5 U/mL amyloglucosidase was then added; the mixture was further incubated at 37 °C for 3 h with stirring at 200 rpm using a submersible magnetic stirrer that can simultaneously digest up to 15 samples at a time (2mag-USA, MIXdrive 15HT Stirring Drive). Temperature was maintained at 37 °C using a recirculating water bath (2mag-USA, MIXbath S Stainless Steel Bath Tank with Julabo, Corio C Immersion circulator). Digesta in duplicate aliquots (0.2 mL) were sampled from each bottle using a 1 mL micropipette and immediately frozen using liquid nitrogen. To monitor the kinetics of starch hydrolysis, the following sampling time-points were used: 5, 10, 20, 30, 45, 60, 90, 120 and 180 min. For high-throughput screening, sampling was taken only at the 60 min time-point to determine the amount of starch hydrolysed at 60 min (SH-60). A low SH-60 value was defined as ≤ 55% starch hydrolysed.

Digesta samples were heated at 100 °C for 20 min to inactivate enzymes and then centrifuged at 18,928 g for 10 min. The glucose concentration of the supernatant was measured using a n-Glucose Assay kit and a FLUOstar® Omega spectrophotometer (BMG Labtech, Ortenberg, Germany).

In vitro starch digestion screening of a diverse set of genotypes

A diverse set of rice genotypes were screened for their digestibility using the in vitro assay developed here. To improve the throughput of the assay, the level of digestion was recorded only at 20 minutes because the level of digestion at this time-point was found to correlate well with the GI value of control samples.

Statistical analysis

Enzyme kinetic data were automatically plotted and statistically compared by nonlinear regression with the least squares fit function using GraphPad Prism version 7.03. Comparison of means values are denoted by letters, with similar letters signifying no significant difference using Tukey post test (P < 0.05).
Results

A single time-point measurement of starch hydrolysis is an effective method for rapid estimation of digestibility

The efficiency of single and dual-enzyme digestion of cooked rice grains was compared by monitoring the kinetic profile of starch hydrolysed by varying concentrations and addition times of AA and AMG (data not shown). The chosen enzyme concentrations resulted in a SH-60 value which was close to the *in vivo* value commonly reported for this variety (typically 54). For single-enzyme assays, concentrations of 1 U/mL AA and 10 U/mL AMG resulted in SH-60 values of 54 ± 2.6% and 55 ± 0.49%, respectively. For dual-enzyme digestibility, a combination of 1 U/mL AA and 5 U/mL AMG produced an SH-60 value of 51 ± 1.1%.

Assays using 1 U/mL AA and 5 U/mL AMG either alone or in combination were undertaken to elucidate the potential synergistic or antagonistic effects of these enzymes. The kinetic profile of starch digestion was assessed for three enzyme treatments: AA alone; AMG alone; and sequential additions of AA followed by AMG. Nonlinear regression analysis revealed a different curve for each data set (data not shown). Irrespective of the enzyme combination used, all sets of data displayed monophasic digestion behaviour. Digestion with AMG alone was slower than digestion by AA alone or with the AA/AMG combination. SH-60 values for AMG alone, AA alone, and AA/AMG sequentially combined were 37 ± 3.8%, 45 ± 2.9%, and 49 ± 0.6%, respectively.

The kinetic profile of starch digestion in rice using simultaneous addition of AA and AMG was also assessed and compared with digestion by sequential addition of these enzymes (Figure 1). The SH-60 value for cooked grains digested by AA/AMG added simultaneously (54 ± 6.2%) was not statistically different from the digestion by the enzymes added sequentially (60 ± 10.7%). Based on this result, simultaneous addition of AA and AMG was used in the final optimised assay to expedite ease and convenience of the assay.
Rice genotypes with varying GI values can be differentiated using the digestibility assay

The digestibility kinetics of three rice genotypes that typically display widely varying GI values—Doongara, Reiziq and Waxy—were assessed using the in vitro assay to test whether the optimised method is suitable for wider range in GI scores. The kinetic profiles of starch hydrolysis showed a wide variation in the digestion rate and extent of digestion between the three rice genotypes (Figure 2). The starch digestograms showed the same monophasic behaviour; however, different curves for each data set resulted from nonlinear regression analysis. Complete hydrolysis of Waxy rice was observed after 90 min, whereas the hydrolysis of Reiziq and Doongara at the final time-point of the assay (180 min) reached 90 ± 3.7% and 79 ± 4.8%, respectively. Digestion of the rice genotypes at each time-point followed the trend: Waxy > Reiziq > Doongara. In terms of SH-60, Doongara clearly
showed a substantially lower value (52 ± 6.0%) compared to Reiziq (73% ± 5.5%) and Waxy (93% ± 2.0%).

Figure 2: Starch digestogram of three rice genotypes. The dotted line denotes 55% of starch hydrolysed.

The *in vitro* assay differentiated nine rice genotypes based on their digestibility

Nine rice genotypes were digested *in vitro* with sampling at the 60 min time-point to obtain the corresponding SH-60 values (Figure 3). The low GI variety, Doongara, was the least digestible and had a low SH-60 value of 52 ± 6.0%. The most digestible rice among the nine genotypes was the high GI rice, Waxy, with a SH-60 value 93 ± 2.0%. The remainder of the genotypes had medium (55-70%) to high (> 70%) SH-60 values.
Figure 3: Box and whisker plot of starch hydrolysed at the 60 min time-point (SH-60) for different rice genotypes. The dotted line denotes 55% of starch hydrolysed.

**SH-60 is correlated with GI**

Several years of *in vivo* GI data for five rice varieties were provided by SunRice (Leeton, NSW, Australia). An average of these data were used as an indicator of typical GI values. A positive correlation between *in vitro* SH-60 values and an average of the *in vivo* GI values was observed ($r^2 = 0.671$) (Figure 4).
Figure 4: Correlation plot of five rice genotypes: *in vivo* GI data vs % starch hydrolysed at 60 min of *in vitro* digestion (SH-60).

**Digestibility screening of a diverse set of rice genotypes illustrated a wide variation in digestibility levels**

Single time-point measurements for starch digestibility were taken for 275 genetically diverse samples and were reported as starch hydrolysed within 20 min (Figure 5a). Among the 275 rice genotypes, SH-20 values followed a normal distribution (Figure 5b), with values ranging from 29.9 to 77.2 %, an average value of $48.4 \pm 8.6\%$, and a median value of 47.8 %.
Figure 5: Diverse genotype screening using SH-20 (%): The % starch hydrolysed at 20 min of *in vitro* digestion (SH-20) of a diverse panel of rice genotypes was assessed with partial replication and expressed as a scatter plot (a), and a histogram (b).
Discussion

Studies of *in vitro* starch digestion to predict the glycaemic behaviour of carbohydrate-containing foods have been widely reported in the literature. Digestion methods that try to mimic physiological *in vivo* conditions typically include the oral, gastric, and small intestinal phases, and occasionally a large intestinal phase for microbiology studies. Multiple factors are normally considered in the development of these *in vitro* methods, including temperature, pH, transit time, materials, instrumentation, buffer concentration, as well as enzyme and substrate concentrations. The final combination of parameters used in these *in vitro* methods is influenced by the purpose of the assay. The purpose developed here is for use in rice breeding programs. This application requires an assay that does not necessarily exactly replicate the digestion process, but is sufficiently representative to enable the identification of genotypes with a low GI value. Other important factors are the need for the digestibility assay to have a high-throughput format to reduce the cost per assay and to expedite quick turnaround time to guide breeders in selecting lines that should be carried through the next round of breeding cycle.

Controlled conditions during preparation of rice samples (e.g. simulated mastication carried out by mechanical disaggregation with a magnetic stirring) were included in the method to ensure assay reproducibility. However, for the assay to be physiologically relevant, certain aspects of realism were also applied. Rice is always cooked before consumption and the overwhelming majority of people consume rice as grains, rather than flour or starch. All rice varieties in this protocol were cooked to completion using an extended cooking time to overcome the potential effect of variations in cooking time. The substrate concentration and number of grains used (4) was based upon the use of 50 mg available carbohydrates adopted in other *in vitro* assays. Intact (unbroken) grains were used because it more accurately reflects the way that rice is consumed.

Preliminary investigations using a commercially available automated *in vitro* digestibility instrument to rank the digestibility of rice varieties was found to have poor resolution (data not shown). Also, this commercial method was found to be unsuitable as a high-throughput assay due to the limited number of samples that could be analysed in one day. Hence, a custom screening method that quickly scored the digestibility of milled rice grains was developed for use in rice breeding programs. As with most *in vitro* digestion models reported in the literature, the assay reported here uses a static system (maintaining constant substrate-to-enzyme ratios, salt, etc.) maintained at 37 °C, allowing for simplicity and ease of sampling. The same vessels were used during cooking and digestion of samples to prevent sample loss and to improve control of sample temperature. Continuous stirring was used during digestion to achieve homogenous mixing. The assay incubation time of 3 h approximately mimics the time taken for substrates to transit through the small intestine. To inhibit the enzymatic action of the samples withdrawn during the digestion, aliquots were snap frozen in liquid nitrogen.

While the assay reported here aims to simulate intestinal starch digestion, some compromises were made to increase the reliability and to decrease the cost of the assay. Pancreatin (a mixture of proteases, amylases, lipases and ribonucleases) was not included in the assay because enzyme activities of commercial preparations of pancreatin differ by source and grade, introducing batch-to-batch variation and a requirement to recalibrate the assay regularly. Simulated oral phase and gastric phases were also excluded from the *in vitro* digestion. Others have reported that the oral phase by salivary α-amylase has not been found to be necessary when chewing is simulated (in our case, stirring for 5 min). In addition, it was reported that the hydrolysis of cooked rice using a simulated gastric phase (using pepsin in a high pH environment) before simulated intestinal phases (with AA and AMG) was not significantly different from samples hydrolysed only by simulated intestinal digestion with AA and AMG.
During assay development, the digestibility of cooked Doongara rice grains was measured at a wide range of AA and AMG concentrations, individually and combined (enzymes added simultaneously or sequentially). For dual-enzyme digestibility, 1 U/mL AA and 5 U/mL AMG was found to have a similar SH-60 value to the in vivo GI value for Doongara. To ensure complete intestinal digestion of starch (when digestion assessment is by glucose analysis), excess AMG is needed to convert 100% of AA reaction products to glucose. Using excess AMG is beneficial because the measurement of glucose is simplified (no extra step needed to convert products of AA to glucose) and potential inhibitory products of AA (e.g. maltose and maltotriose) are depleted. However, AMG not only converts products of AA to glucose; increasing the AMG activity at a fixed AA activity was shown to increase the rate of in vitro digestion (data not shown).

The potential synergistic and antagonistic effects of AA and AMG were investigated. There is a need to determine the optimal synergistic concentration for AA and AMG because the former cleaves the glucan chain internally, while the latter releases the glucose molecule from the external reducing ends. The action of AA and AMG on native starch granules has been observed to be synergistic via two mechanisms: (1) the AA randomly splits the substrate molecules on the granular surface, providing new non-reducing end groups to AMG; and (2) AMG can “peel” starch molecules from the surface of a granule, exposing newly non-reducing end groups for attack by AA. Evidence of the synergism between AA and AMG has been demonstrated by experiments where the released glucose value is more than twice that observed in the mixed-enzyme system compared to the corresponding value for the digestion by AMG alone. A similar trend was observed here, albeit to a much lesser degree, for cooked rice grains. The digestion curve of AMG alone displays a slower rate compared to AMG digestion after pretreatment with AA (data not shown). These findings are contradictory to the antagonistic action of AA and AMG on cooked maize and potato starch. This suggests that AA is a rate-limiting enzyme during starch digestion for cooked rice, at least for the concentrations we used. Further investigation of starch digestion using AA/AMG (data not shown) revealed a minor antagonistic effect between the enzymes when added simultaneously (likely by inhibitory products of AA); however, this effect was not statistically significant at the SH-60 time-point.

Three commercial rice genotypes with diverse GI values (Doongara, Reiziq and Waxy) were successfully differentiated using the in vitro protocol. Doongara has been demonstrated to have a significantly lower GI than Waxy and other rice varieties, which has been attributed to its high amylase content. The in vitro assay described here demonstrated that the digestion rates of various genotypes were different and had the capacity to clearly identify the low GI variety.

The ultimate goal of this work was to develop an assay that would rank different rice varieties based on starch digestibility. The assay also needed to be suitable for high-throughput screening and, therefore, needed to be rapid, scalable and inexpensive. The results indicate that the proportion of starch hydrolysed at 60 minutes (SH-60) during the in vitro digestion assay can be used as a proxy measure for GI. The classification of SH-60 values follows the same scale as GI values: low SH-60 is less than 55%, intermediate SH-60 is between 55% and 70%, and high SH-60 is greater than 70%. As expected, the low GI variety Doongara had a low SH-60 value of 52 ± 6%, and the Waxy rice had a high SH-60 value of 93 ± 2%. The non-commercial genotype, YRL127, is a low GI breeding line and so, unsurprisingly, its SH-60 value of 59 ± 3% was lower than most of the other varieties. The remainder of the rice varieties had intermediate to high SH-60 values that correlated to their expected GI values. The validity of the in vitro digestibility assay was established by measuring five rice genotypes and comparing the results with an average in vivo GI value from historical data. Although the in vivo GI values and SH-60 values were not derived from the exact same sample set (same genotype grown in different years), the correlation still demonstrated a positive relationship between the resultant data (Figure 4). In the rapid protocol described here, 15 samples can be analysed simultaneously, allowing 60 samples to be easily analysed per day (assuming that 15 samples can be prepared every two hours). In a commercial breeding program, the genotypes identified as having low SH-60 values (for example, YRL127 in Figure 3) can be further analysed using the three-hour in vitro enzyme kinetics protocol to provide a deeper understanding of the digestion profile of those samples.
To further improve the throughput of the assay, the use of a 20-minute digestion time was assessed. It was found that 20 min is a satisfactory digestion time. Using the 20-minute digestion time, a panel of 200+ genetically diverse samples were screened for their digestion properties. The analysis demonstrated a wide variation in digestion properties, suggesting there is an opportunity to make further gains in developing rice varieties with low-digestibility properties.
Implications

The glycaemic index is a nutritional measure of carbohydrate-containing foods, quantifying the potential of these foods to raise postprandial blood glucose levels. As such, GI testing requires human participants, which is expensive and time consuming, and therefore highly impractical for most applications. In the present study, it was demonstrated that the use of a rapid in vitro assay for ranking the digestibility of rice overcomes the financial and time limitations of in vivo testing. The measurement of SH-60 or SH-20 using the in vitro digestibility assay offers a practical, high-throughput tool for rice breeding programs to screen for new low GI varieties that can then be validated by a more detailed analysis, if required.
Recommendations

The assay developed here appears to provide a useful and practical approach to screening genotypes for their digestibility properties. NSW DPI staff have been trained in the technique so it is ready to be used in the Australian rice breeding program. However, before this is done, a clear assessment of what style of low GI rice is required. In the past, amylose levels have been used as a proxy for selecting for low GI rice. However, high amylose levels result in firm cooking rice, which is not always desired. The assay developed here is independent of amylose, hence, the development of low GI rice with low amylose levels is now possible.

The industry is urged to adopt this technology as soon as possible. CSU are happy to provide support for the implementation of this assay; however, as the funding for the Functional Grain Centre will soon end, our capacity to support this activity will soon diminish.
Reference List


Next-generation healthy rice

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