Controlled Germination to Enhance the Functional Properties of Rice

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Abstract

The production of prebiotic oligosaccharides during germination of rice has been investigated. Germination of waxy (RD6) and non-waxy (RD17) rice was compared by evaluating the total reducing sugars, free amino nitrogen, pH and enzyme activities over a 7-day period. An increment of amylolytic enzymes and chemical changes were observed in both varieties. RD6 showed higher levels of total reducing sugars and also higher amylolytic activities including α -amylase and α -glucosidase, which reached maximum values at the third day of germination. However, the amount of free amino nitrogen in RD6 was lower than in RD17. Sugar analysis indicate that RD6 produces higher concentration of sugars and oligosaccharides during germination. Based on these results, germinated RD6 at different times was used to produce malted rice syrup through mashing. After saccharification, the malted rice syrup contained different concentrations of sugars and oligosaccharides, particularly isomaltose, panose and isomaltotriose, which have been reported to have prebiotic properties.

Keywords rice, germination, saccharification, prebiotic, malto-oligosaccharides, isomalto-oligosaccharides

Introduction

Cereal foods in various forms are an essential component of the daily diet. Rice is one of the most consumed cereals worldwide. Nutritionally it is an important source of carbohydrates, vitamin B6, zinc and copper but it contains the lowest protein and dietary fibre content among cereals [1].

In principle, rice can be modified by germination to improve its functionality. Starch represents the main reserve compound in the rice grains [2]. This polysaccharide is stored mainly in the endosperm where it is hydrolyzed during germination to provide soluble sugars to the germinating seedling [3]. Starch degradation is a complex biochemical process, which is modulated by both hormonal and metabolic regulation [4]. A set of enzymes are needed to carry out the starch breakdown: α -amylase, β -amylase, debranching enzyme, and α -glucosidase [5]. Both α -glucosidase and α -amylase are able to degrade native starch granules, but the later enzyme plays the main role in this process and it is the key enzyme in starch hydrolysis.

During the saccharification process, other enzymes contained in malted rice are also activated to break down starch and release more sugars and oligosaccharides, especially isomalto-oligosaccharides which are potentially prebiotic [5,6].

Starch oligosaccharides, which represent the fragments of the original polysaccharide, are composed of α -D-glucopyranosyl units linked by α -1,4 and/or α -1,6 bonds.

Oligosaccharides containing only α -1,4 glucosidic linkages are called malto-oligosaccharides, while those containing both α -1,4 and α -1,6 glucosidic linkages are called branched-oligosaccharides or isomalto-oligosaccharides [8].

Prebiotic oligosaccharides can be used as functional food ingredients to provide useful modifications to the physiochemical properties of foods. It has been reported that these oligosaccharides have various physiological functions and improve the intestinal microflora by selective proliferation of bifidobacteria [7]. They also stimulate mineral absorption, have anticancerogenic potential, and reduce both plasma cholesterol and blood glucose levels [9]. Among these sugars, isomalto-oligosaccharides (IMO) has received a considerable interest as prebiotic in recent years. IMO have demonstrated their usefulness in normalizing bowel movement, increasing stool bulk, colon microbial activity, as stimulate growth of *Bifidobacterium* and *Lactobacillus* and systemic immunity [10-12].

In the present work, controlled germinations of two varieties of rice were performed to monitor the different enzyme activities and the chemical changes. The modified malted rice was also used to produce rice syrup with different sugar and oligosaccharide contents.

Materials and methods

Malted rice preparation

Two cultivars of Thai rice (*Oryza sativa* L.), RD6 (waxy rice) and RD17 (non-waxy rice), were used in these experiments. These two varieties (RD6 and RD7) were obtained from Seed Center in Phatumthani province and Srisaket province, Thailand, respectively. RD6, which is a glutinous rice, widely grown in the northeast of Thailand. It is a commercial rice cultivar represented in the Thai rice export market [12]. It contains amylose 0.1-0.3% [13-16]. RD17 contains amylose 28-30% and it is widely grown in central part of Thailand [17]. It is also one of Thai commercial rice cultivar [18]. Both varieties were washed and steeped separately in distilled water for 24 h. After soaking, 500 g of seeds were placed in Petri dishes containing filter paper moistened with sterile water and maintained at 30°C under aerobic conditions [19]. 2.5 ml of water were added to the Petri dishes every day to avoid drying and maintain the moisture content. Samples were taken every day over a 7-day period.

Preparation of malted rice powder

After germination, the seeds were dried in an oven (LTE, UK at 50°C for 24 h. Then, the roots and shoots were removed and the remaining portion of the paddy was dehusked using a laboratory dehusker (THU35A, Satake, Japan). The malted rice was then ground using a hammer mill (Type 120, Falling number, Sweden) and sieved through a 355 µm screen.

Preparation of malted rice syrup

The mashing process was modified by following the method of Okafor and Iwouno (1990) as well as Ayernor and Ocloo (2007). 200 g (dry weight) of ground malted rice were mixed with water contained 30 ppm Ca^{2+} (CaCl_2 dissolved in water) and adjusted to 2 l. The mixture was adjusted to pH 6 by using lactic acid. The slurry was initially mashed at 50°C and allowed to stand for 30 min. The supernatant was decanted and the remained flour was heated until it gelatinized at 88°C. The supernatant was returned to the cooled and gelatinized slurry, giving an overall temperature of 62°C. The mash was held at this temperature for 60 min. The pH of the mash was tested and adjusted to 5.6 by adding a few drops of lactic acid. One-half of the mash was withdrawn, boiled and returned to the main mash and the temperature increased to between 69 and 71°C. The mixture was held at this temperature for 60 min. The mash was cooled and filtered using funnel and folded Whatman No. 1 filter paper. The filtered solution was finally boiled for 60 min to yield the malt rice syrup.

Measure of Total Reducing Sugar (TRS) and Free Amino Nitrogen (FAN)

The samples of malted rice and rice syrup were diluted with distilled water and analyzed for TRS and FAN following the methods of Miller [21] and Lie [22] respectively.

Enzyme extracted solution

One gram of malted rice was suspended in 10 ml of 0.2 % calcium chloride solution, mixed in vortex mixer for 1 min, and centrifuged at 3000 rpm (Minifuge T, Heraeus, Germany) for 10 min. The supernatant was used to measure the enzyme activity.

Determination of amylolytic enzyme and α -amylase

The amylolytic activity was assayed using the Terashima method [23] after crude extraction of malted rice. 0.5 ml of the supernatant was added to 0.5 ml of a 1% soluble starch solution in 0.05 M acetate buffer. The sample was incubated at 60°C for 5 min and the increase of reducing sugars was measured [21]. One unit of the enzyme activity (U) is defined as the amount of enzyme required to liberate 1 μ mol of maltose per min.

The α -Amylase activity was measured following the increase of reducing sugars with time. 0.5 ml of the supernatant solution was added to 0.5 ml of a 1% soluble starch solution in 0.05 M acetate buffer. The mixture was incubated at 70°C for 15 min in order to inactivate β -amylase, debranching enzyme, and α -glucosidase [24]. One unit of α -Amylase activity (U) is then defined as the amount of enzymes required to liberate 1 μ mol of maltose per min.

Determination of α -glucosidase

The α -glucosidase activity was determined using a modified method of McCue and Shetty [25]. A standard reaction solution is prepared by mixing 0.1 ml of 9 mM p-nitrophenol α -D-glucopyranoside and 0.8 ml of 200 mM sodium of acetate buffer at pH 4.6 in a glass tube. The tubes were pre-incubated at 50°C for 5 min before addition of 0.1 ml of the enzyme extract. The reaction tubes were then incubated for a further 30 min. The enzymatic hydrolysis was stopped by addition of 1 ml of 100 mM sodium

carbonate, and the samples were clarified by centrifugation at 13,500 rpm at room temperature for 5 min. The released p-nitrophenol in each sample was determined by measuring the absorbance at 400 nm compared with the blank. A standard curve was established using pure p-nitrophenol dissolved in sodium acetate buffer. One unit of α -glucosidase activity is defined as the amount of enzyme that releases 1 μ mol of p-nitrophenol per min at pH 4.6 and 50°C under assay conditions.

Determination of sugars by High Performance Liquid Chromatography (HPLC)

The samples of sugars and oligosaccharides were diluted and analyzed by HPLC. The system has a two solvent delivery module (model 210 Varian, UK), an auto sampler (model 410 Varian, UK) and an evaporative light scattering detector (ELSD) (model PL-ELS 2100 Simadzu, UK). The column was a spherisorb 5 μ m NH₂ (200x4.6 mm, Phenomenex, UK). The injection volume was 20 μ l, and the flow rate 1.2 ml/min. For complete separation of the sugars, a mobile phase A (acetonitrile) and a mobile phase B (deionised water) were used in gradient system. The gradient system was 80% of A initially, decreased to 50% in 30 min, increased again to 80% in 5 min and then maintained at 80% for 5 min (the total cycle time was 40 min). The ELSD was set to measure at the evaporator temperature of 90°C, nebulizer temperature of 50°C and gas flow rate of 1.6 ml/min.

Results

Chemical changes during germination of non-waxy rice RD17 and waxy rice RD6

This experiment was performed to investigate the chemical changes during the germination of rice varieties RD17 and RD6. These results are shown in Figure 1.

Additions of 5 ml of water per kg of rice were done every day in order to compensate for evaporation and to maintain the moisture between 30 and 70%. During the germination of RD17 it was found that the pH decreased slightly from 6.4 to 5.9. TRS and FAN concentrations increased and reached a maximum at day 4 and 5 respectively to then slightly decrease. The amylolytic activity reached a peak value of 72.7 U/g at the fifth day, whereas α -amylase and α -glucosidase reached a maximum of 26.8 and 14.3 U/g respectively at the third day of germination.

During the germination of waxy rice RD6 the pH decreased slightly from 6.6 to 5.6. The amount of TRS and FAN increased and reached maximum values of 64.8 and 0.3mg/g respectively at the third day of germination. α -Amylase and α -glucosidase activities were maxima at day 3, while the highest amylolytic activity was observed at day 5.

HPLC was used to analyze the composition of sugars and oligosaccharides in the malted rice over a 7-day period (figure 2). The amount of sugars and oligosaccharides increases from the beginning of germination for both varieties but in a different way.

Concentrations of glucose, maltose and maltotriose reached a maximum in the third day of germination in both cases (58.9, 25.4 and 1.8 mg/g for RD6, and 47.3, 18.2 and 0.23 mg/g for RD17, respectively).

The rest of the sugars exhibit a maximum at different days depending of the variety. For RD6 the maximum concentrations of maltotetraose, maltopentaose, maltohexaose and maltoheptaose are always obtained later in the germination process and at 5, 6, 6 and 5 days respectively. Maxima for the same sugars in RD17 were obtained at 2, 4, 3 and 4 days respectively.

Chemical composition of malted rice syrups

Since results in figures 1 and 2 show that malted waxy rice RD6 contains higher levels of sugars, oligosaccharides and amylolytic enzymes (including α -amylase and α -glucosidase), this variety was used to produce a malted rice syrup through mashing. In order to stop the germination process, samples of rice were dried at 50°C for 24 h. The final moisture content was 10-11%. The dried malted RD6 was then milled and mashed with water for 3 h to reactivate the enzymes and continue the starch hydrolysis. Starch is further degraded into sugars and oligosaccharides during mashing, and saccharification produces a sweet malted rice syrup.

The concentration of TRS and FAN obtained from syrups produced with mated rice at different stages of germination is shown in figure 3. The maximum TRS concentration

(108.2 g/l) is obtained from the sample of day 3, while the maximum FAN concentration (18.9 mg/l) is obtained from the sample of day 1.

Figure 4 shows the sugar concentration in the RD6 syrups produced from malted rice with different degrees of germination. The maximum glucose and maltose extraction takes place in samples of day 3 and remains more or less stationary from then on. The maximum concentrations of the prebiotic oligosaccharides isomaltose, isomaltotriose and panose are observed from samples of day 5 and 6. The concentration of the rest of oligosaccharides measured in the syrup (maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose) also increased with the germination time though in a different manner.

Discussion

To activate germination, rice was initially soaked in water to increase kernel moisture. Takahashi [26] reported that the water requirement for germination was dependent on the cultivar and the dormancy period. Hence, both varieties, RD17 and RD6, were soaked for 24 h to obtain a similar moisture content of approximately 27%. The functions of the steep water include initiation of cell elongation, respiration, secretory activity of the embryo and activation of enzymes [27]. Generally, malting must provide enough water to allow germination, but not too much. The grains will actually show a reduction in germination vigour if exposed to an excess of water. For this reason, a

small amount of water was added every day (0.5 ml/100 g seeds) over the 7-day germination period in order to maintain moisture and prevent dehydration. The moisture content was kept between 30-70%.

In this experiment, aerobic conditions were maintained throughout germination. Although it has been reported that rice seeds can germinate and grow at much lower oxygen concentrations than many other plants, gaseous concentrations below 0.3% retard germination, decrease growth, and reduce the root/shoot ratio [28].

Temperature is one of the main factors affecting germination and could have had an important role in the development of sugars [29]. For temperatures between 27 and 37 °C, the majority of germination (90-97%) takes place during the first 48 h. The germination rate drops sharply for lower temperatures [30]. As correspondingly reported by Cruz and Milach [31], temperatures below 15°C prevent or reduce rice germination at the early stage.

It has been reported that during the germination of rice the protease activity increases within the first 2-3 days and decreases from them on [32]. The FAN profile shown in figure 1 reflects this fact. FAN inceases during the first days of germination due to the proteolitic activity to then decrease when rootlets and shoots in the grain begin to grow. Changes in FAN and metabolic activities could also be related to the change in pH as reported by Magalhães and Huber [33]. Another possible cause for this decrease in that the presence of phenolic acids [32] and/or phytic acid and tannins [33,34] may act as inhibitors of the enzymatic activity in the germination process.

269
270 The differences observed between RD6 and RD17 could be due to their different protein
271 content and protease activity [37,38].

272
273 The amylose:amylopectin ratio in rice starch not only affects its chemical and physical
274 characteristics but also the enzymatic hydrolysis developed during the germination
275 stages. Amylose consists of unbranched chains of poly-[(1→4)- α -D-glucopyranose] and
276 is strongly associated with many polar substances, including some lipids, to form
277 crystalline complexes. In Amylopectin the α -(1→4)-linked chains are extensively
278 branched through α -(1→6)-linkages and the macromolecule has a ramified structure
279 [39]. While amylose molecules have a single reducing and non-reducing glucose end,
280 amylopectin has a reducing end with numerous non-reducing glucose residues in its
281 branches.

282
283 α -amylase is the main enzyme responsible for the starch hydrolysis while α -glucosidase
284 is involved in transglucosylation reactions for the production of isomalto-
285 oligosaccharides. The activity of these enzymes increases during the first 3 days of
286 germination to then decrease steadily. This is also reflected in the TRS profile where
287 after three days the concentration in the grain also decreases due to the formation of the
288 new plant. As a whole the amylolytic activity increases till day 5. The differences
289 observed between waxy (RD6) and non-waxy rice (RD17) could be due to the different
290 amylose/amylopectin ratio.

291
292 Briggs et al [39] reported that during germination α -amylase attacks α -(1→4) linkages at

random locations within the starch chain. The hydrolysis slows down near the chain ends and stops at α -(1 $\rightarrow$ 6) branches. This enzyme acting on its own is able to degrade starch into a complex mixture of sugars including glucose, maltose, maltotriose and a wide range of dextrans, some of which containing α -(1 $\rightarrow$ 6) link branches. α -Glucosidase is able to hydrolyse α -(1 $\rightarrow$ 4) or α -(1 $\rightarrow$ 6) linkages, and release molecules of glucose from the non-reducing end. This enzyme is also able to transfer sugar moieties or groups of sugar residues from one compound to another with the formation of a similar or a distinct type of linkage. Thus, a α -(1 $\rightarrow$ 4) link in a chain might be broken and the separated end could be joined to the same or a different chain via either an α -(1 $\rightarrow$ 4) or α -(1 $\rightarrow$ 6) link. The product of this hydrolysis could be of maltose, isomaltose, panose, isomaltose or long chains of oligosaccharides.

Figure 2 shows the evolution of all the sugars measured in the grain during the germination of the two rice varieties. The evolution of the glucose and maltose concentrations are very similar in both varieties and the maxima reached after three days of germination are of the same order of magnitude. After three days the concentration decreases, which suggests these sugars are used in the formation roots and shoots.

The profiles for the other oligosaccharides is complex, which reflects the complexity of the enzymatic paths taking place. A major difference between the two varieties is the order of magnitude of the oligosaccharides produced. The waxy variety (RD6) produces oligosaccharide concentrations approximately 10-fold when compared to RD17, which is probably due to the higher levels of amylopectin. Most of these oligosaccharides reach

a maximum concentration later on in the germination process, but in some cases (e.g. maltotriose and isomaltotriose in RD17) a maximum concentration is maintain, which indicates that these sugars are not used for plant formation in this particular variety.

The enzymatic activity generated by germination is maintained during the drying and milling of the grains. These amylolytic enzymes continue to act during mashing resulting in an increase of total reducing sugars in the syrup [40]. At temperatures of approximately 50°C the proteolytic enzymes also get activated causing the breakdown of proteins into aminoacids [41], which is clearly observed in figure 3. Clear differences in FAN and TRS concentrations were observed in the germinating grain. During mashing the amylolytic and protelytic enzymes can move freely in the liquid medium and have enough time for full starch and protein hydrolysis. The decrease of FAN in the syrups after day 3 could be due aminoacid utilisation in root and shoot development.

Figure 4 shows that the malted rice syrups obtained from day 3 of germination contain the highest concentrations of maltose and glucose. No significant changes in these sugars are observer in syrups produced from rice with higher degrees of germination. It could be suggested that after three days the existing amylolytic enzymes are able to hydrolyse the gelatinised starch to produce the maximum glucose and maltose concentration.

As RD6 waxy rice starch contains higher levels of amylopectin, the branched chains can be hydrolysed by amylolytic enzymes to produce molecues of malto-oligosaccharides and isomalto-oligosaccharides (branched chain oligosaccharides). Transglucosylation

by α -glucosidase might also take place during mashing [42]. α -glucosidase could catalyse both the hydrolysis of α -D-glucosyl-oligosaccharides and transfer the glucosyl group to 6-OH of glucose. The transfer of glucosyl to D-glucose yields isomaltose, and to maltose yields panose.

The oligosaccharides produced in the syrup will depend on the composition of the rice (amylose:amylopectin ratio), the types of enzymes present and the temperature strategy during mashing. These interactions are complex and make it difficult to justify the development of oligosaccharides during mashing. What seems clear is that higher oligosaccharides are produced from syrups of malted rice with higher degrees of germination. It should also be noted that the prebiotic oligosaccharides (panose, isomaltose and isomaltotriose) are produced from syrups from highly germinated rice (though in small amounts).

Traditionally, malting and mashing strategies have been developed to maximise sugar production for later fermentation like in the brewing process. Results in this work suggest that different malting strategies, maybe with longer germination times, could be designed to maximise the production of prebiotic oligosaccharides in the grain or in the syrup. These naturally developed prebiotics could then be used as functional food ingredients.

In addition to the functional properties of non-digestible carbohydrate like prebiotics, the phenolic acids content and α -1-6 amylase activity should also be studied. The rice-

based product could then be tested *in vitro* using batch fermentation vessels inoculated with faecal slurries. The use of animal models and human trials [43] could also be considered.

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FIGURE CAPTIONS

Figure 1. Evolution of pH, FAN, TRS, amylolytic activity (AA), α -amylase (Am) and α -glucosidase (GI) in RD17 (●) and RD6 (○) during germination at 30°C. Error bars are the confidence intervals ($\alpha=0.05$, $n=3$).

Figure 2. Evolution of glucose, maltose, maltotriose, isomaltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose in malted RD17 (left) and RD6 (right) during germination. Error bars are the confidence intervals ($\alpha=0.05$, $n=3$).

Figure 3. Concentrations of TRS (●) and FAN (○) in RD6 malted rice syrup with different degrees of germination. Error bars are the confidence intervals ($\alpha=0.05$, $n=3$).

Figure 4. Concentrations of glucose, maltose, isomaltose, panose, isomaltotriose, maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose in RD6 malted rice syrup after mashing. Error bars are the confidence intervals ($\alpha=0.05$, $n=3$).

FIGURE 1

FIGURE 2

FIGURE 3

FIGURE 4