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In vitro measurement of resistant starch of cooked milled rice and physico-chemical characteristics affecting its formation

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Abstract

Resistant starch (RS) content was determined in 10 *indica* and *japonica* milled rices with different levels of amylose. The effect of microbial growth during starch digestion on the measurement of RS, and the correlation between physico-chemical characteristics and RS contents of milled rice were analysed. Results indicated a significant decrease ($P < 0.01$) in assay values of RS after antibiotics addition, and the markedly decreased sample pH due to fermentation might be the main reason for errors in RS determination. Correlation analyses showed that RS contents of milled rice were closely related to amylose content ($r = 0.75$, $P < 0.05$) and protein content ($r = 0.78$, $P < 0.01$). No significant correlation existed between RS content and some relatively simple physical properties, such as width, shape and elongation ratio of rice grain, which were reported to be good indictors of rate of rice starch digestion, while a significant positive correlation was found between elongation ratio and digestible starch in cooked milled rice ($r = 0.67$, $P < 0.05$).

Keywords: Rice; Resistant starch; Amylose content; Elongation

1. Introduction

Resistant starch (RS) is the sum of starch and starch degradation products that are not absorbed in the small intestine of healthy individuals, due to their resistance to enzyme digestion (Asp, 1992). RS that escapes hydrolysis in the small intestine enters into the large intestine, where it is fermented. Consequently, the metabolites formed during the fermentation process (i.e. short-chain fatty acids) may serve as a main energy source for the colonocytes (butyric acid) (Mortensen & Clausen, 1996), and thus help to maintain colon health, or have beneficial effects on glucose and lipid metabolism (Hylla et al., 1998; Muir et al., 1993; Thorburn, Muir, & Proietto, 1993).

Several direct and indirect methods have been proposed for *in vitro* evaluation of the amount of RS in foods. Direct methods quantify RS in the residues obtained after removing digestible starch (Berry, 1986). Indirect methods determine RS as the difference between total starch and digestible starch (Englyst, Kingman, & Cummings, 1992). Comparing both procedures, Dysseler and Hoffem (1994) concluded that Berry's method was more suitable for nutritional labeling of foodstuffs. This is mainly due to the fact that the values obtained by indirect methods are less accurate, especially for foods with a low RS content, because they accumulate the errors of two experimental determinations (Dysseler & Hoffem, 1994). The original Berry method was further modified by Goñi, García-Diz, Mañas, and Saura-Calixto (1996) for a better simulation of the stomach and intestine physiological conditions. The main

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modifications were the removal of protein and omission of ethanol precipitation and drying during analysis. In addition, starch digestion was performed at pH 6.9 and 37 °C, however, without antimicrobial agent added to avoid bacterial proliferation. As a result, serious fermentation was observed in incubation medium as RS contents of rice were assayed using the method described by Goñi et al. (1996). The problem of microbial proliferation was already considered by several authors (Champ, 1992; McCleary & Monaghan, 2002), who added sodium azide for this purpose, but no information was given about the relevance for antimicrobial agent addition. Currently, it is not clear whether the RS content remains in the residue after enzymatic digestion and might not be affected by microbial growth in the supernatant. Also, it is not clear whether antimicrobial agents result in overestimation of the RS values, mainly in samples that contain low values of RS, such as rice, due to possible inhibitory effects on α-amylase. Experimental data concerning these questions are not available. The present investigation was thus carried out to study the possible impact of microbial growth and antimicrobial agent on the determined levels of RS. Antibiotics and sodium benzoate, as less toxic antimicrobial agents, were employed in this study.

RS is known to be slowly digestible and has evident impact on the rate of starch digestion. Tetens et al. (1997) found thick grains, a low length/width ratio and a high elongation ratio during cooking to be good indictors of low rate of starch digestion from milled rice, aside from a high amylose content. So far, the correlation between RS content and these simple physical characteristics of milled rice has not been investigated. In addition, other physico-chemical characteristics, such as protein and lipid contents, are supposed to influence the formation of RS. Hence, an additional objective of this work was to investigate the relationship between these physico-chemical characteristics and RS contents among the different rice cultivars and to provide information on the mechanism involved in the formation of RS. Leading Chinese commercial and several less studied rice cultivars of the *japonica* and *indica* types with different levels of amylose were employed in the present study.

2. Materials and methods

2.1. Samples

Rice samples used were obtained from the Rice Research Institute, Nanjing Agricultural University. The cultivars studied had high, intermediate, low and very low amylose contents and were of the *indica* type: Xiaoheigu (27.70%), Xiangxian (25.85%), Nanjing11 (25.56%), Nanjing16 (18.84%) and 9311 (15.57%), and *japonica* type: Guihuahuang (21.31%), Gensidao (21.06%), Suzi2 (19.67%), C418 (16.41%) and Huinuo (3.00%). The samples were directly collected from the field and dried at 40 °C to a moisture content of approximately 12%. Rough rice was dehulled in a Satake dehuller (Satake, Tokyo, Japan) and the resulting brown rice milled in a Statake TM-05 grain mill (Satake, Tokyo, Japan). Milled rice was used for all analyses since this is the major form of rice consumed in China.

2.2. Processing methods

Raw rice sample (100 ± 1 mg), which corresponds to 5–7 entire rice grains, depending on the variety, was boiled at 100 °C in tap water for 20 min in a capped tube. The optimum water to rice ratio was 1.2 for *japonica* rice and 1.3 for *indica* rice. Samples were homogenized in 0.2 M KCl–HCl buffer (pH 1.5) using a POLYTRONB homogenizer (Kinematica AG, Littau/Lucerne, Switzerland) with controlled speed.

2.3. Analytical procedures

2.3.1. Resistant starch (RS)

RS was determined by a modified procedure, based on that of Goñi et al. (1996). Duplicate portions (100 mg) of test material were preincubated with a pepsin solution containing 20 mg of pepsin (ref. 7180, Merck; Darmstadt, Germany) for 60 min at 40 °C for protein removal. Then starch was hydrolysed at 37 °C for 16 h after adding an enzyme solution containing 120 mg of α-amylase (ref. A-3176, Sigma; Taufkirchen, Germany). Furthermore, a combination of antibiotics, including ampicillin, kanamycin, streptomycin and tetracycline, was added with a final concentration of 60, 50, 50 and 50 μg/ml, respectively, after 4 h of enzymatic incubation. This modification gave significantly lower assay values of RS as shown later. In addition, for rice samples with and without antibiotics addition, the pH values during starch digestion were recorded using a pHS-3 pH meter (TianDa Co. Ltd., Shanghai, China).

After α-amylase hydrolysis, samples were centrifuged, and the supernatants that were discarded in the procedure of Goñi et al. (1996), were collected for analysis of the digestible starch. The residue (isolated RS) was dispersed in water before adding KOH to a final concentration of 2 M (30 min, room temperature with constant shaking). The suspension was incubated with 80 μl of amyloglucosidase (45 min, 55 °C) from *Aspergillus niger* (ref. A-3042, Sigma). After centrifugation (10 min, 3000 × *g*), glucose concentration in the supernatant was determined using a glucose oxidase–peroxidase kit (Rongsheng; Shanghai, China). Colour absorption was measured at a wavelength of 505 nm using a Beckman DU7400 Nucleic Acid and Protein Analyser (Beckman Coulter, Fullerton, CA, USA) and glucose concentration was converted into starch content by applying the factor 0.9.

2.3.2. Rate of amylolysis

Rate of amylolysis was determined according to Chen (2002) with slight modifications. Sample preparation and

pepsin incubation was carried out as described above. Antimicrobial agents were added before α-amylase (time 0) or after 4 h of incubation. Aliquots of 0.2 ml were withdrawn from each sample at 4 and 16 h, and rapidly transferred to centrifuge tubes containing 2 μl of 10 M NaOH. After centrifugation, 100 μl supernatant, 1.9 ml distilled water and 2 ml 3,5-dinitrosalicylic acid reagent were mixed in a 20-ml screw-topped glass tube. The tubes were boiled for 5 min, cooled and diluted with 16 ml distilled water before measuring the reducing power spectrophotometrically at 520 nm using maltose as reference. Rate of amylolysis was expressed in terms of mg maltose released in each sample at the different incubation times (4 and 16 h). Each sample was analysed in duplicate.

2.3.3. Amylose content, total starch and digestible starch

Amylose content (AC) was determined by iodine colorimetry based on the procedure of Juliano (1985). Four standard samples with amylose at four levels: 1.5%, 9.2%, 17.1% and 26.0%, provided by the China National Rice Research Institute (CNRRI), were used to construct a standard curve. Total starch (TS) was determined enzymatically by the method of Goñi, Garcia-Alonso, and Saura-Calixto (1997). Raw rice samples were ground to pass through a 0.5-mm sieve. Subsequently, 25 mg of ground sample material were dispersed in 6 ml of 2 M KOH and shaken vigorously for 30 min at room temperature. Solubilized starch was then hydrolysed by adding 60 μl of amyloglucosidase from *A. niger* (Sigma). Glucose concentration in the supernatant was analysed as described earlier. Digestible starch (DS) was determined by measuring the reducing power in the supernatant collected in the determination of RS and converted into starch content (conversion factor 0.95), or calculated as the difference between TS and RS according to Goñi et al. (1997). Each cultivar was analysed in duplicate.

2.3.4. Elongation ratio and elongation index

The procedure described by Juliano and Perez (1984) was used for measuring elongation ratio and elongation index. Length and width of milled grains were measured on 10 unbroken milled grains by slide calipers. Elongation ratio (ER) was defined and measured as the ratio of length of 10 sound cooked rice grains to the length of the uncooked sample while elongation index (EI) was calculated as the ratio of length/width ratio of cooked rice grains to that of the uncooked sample. Replicate runs on different dates were not performed due to insufficient samples.

2.3.5. Protein and lipid content

Nitrogen was determined by the Kjeldahl method using a Kjeltec 2300 Autoanalyser (Foss Tecator AB, Hogänäs, Sweden). A factor of 5.95 was used to compute the protein value for rice. Lipid content was determined by the Soxtec method using a Soxtex-Avanti 2050 Total Fat System (Foss Tecator AB, Hogänäs, Sweden). Determinations were performed in duplicate.

2.3.6. Statistical analysis

Standard deviations, standard errors, Pearson correlation coefficients of the means were calculated using the SPSS/PC+ program, version 10. A one-way analysis of variance (ANOVA) followed by Duncan's multiple range test was performed to test the statistical significance of differences in the data.

3. Results and discussion

3.1. Influence of microbial growth and antimicrobial agent on the determination of RS

Fig. 1 shows the pH shift in rice samples during starch digestion by the procedure of Goñi et al. (1996). This pH shift, from the original 6.9 to the final 5.2, usually began at about 7 h of enzymatic incubation. The incubation mixture obviously went sour and turbid after 16 h of incubation at 37 °C, along with a marked decrease in reducing power (Fig. 2). Nevertheless, microbial growth was not observed in rice samples without α-amylase addition, as reflected in the almost constant sample pH and reducing power after the overnight incubation (data not shown). These results indicated that free carbohydrates from amylolysis might be the principal sources of carbon and energy for bacteria. Organic acids (lactic acid, etc.) produced from the degradation of free carbohydrates by microorganisms thus resulted in significant acidification of sample pH.

Antibiotics exhibited weaker inhibitory effects on α-amylase compared with sodium benzoate, as reflected in the significantly higher reducing power in samples with antibiotics after 4 h of incubation when both antimicrobial agents were added before α-amylase (0 h) (Fig. 2). The final reducing power at 16 h increased whenever antibiotics were added (0 h or 4 h), contrary to sodium benzoate, indicating more effective inhibition of fermentation (Figs. 1 and 2). To reduce the inhibitory effects of antibiotics on α-amylase, we

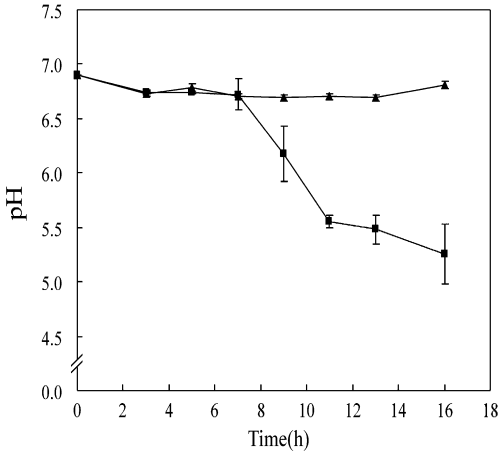

Fig. 1. The effect of antibiotics on sample pH during the overnight incubation. Values are means of duplicate analyses. Standard deviations were indicated. ■, samples without antibiotics; ▲, samples with antibiotics.

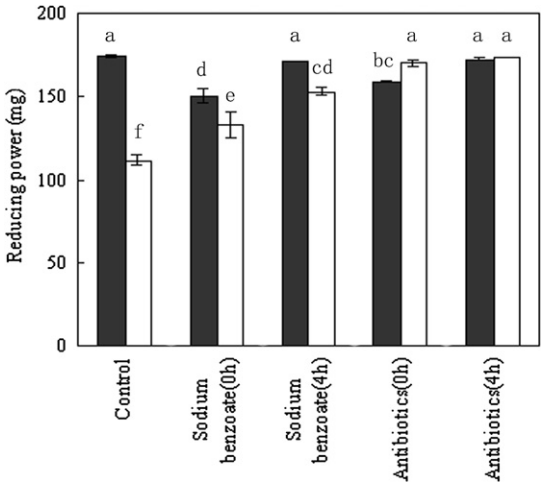

Fig. 2. Effects of sodium benzoate and antibiotics on the hydrolysis of cooked rice samples of the *japonica* cultivar C418. Note that the approximate 90% lactose that α-amylase contains by weight may also be measured as reducing power. Values in parentheses indicate the time sodium benzoate and antibiotics added. Standard deviations are indicated. Closed bars indicate the reducing power of samples after 4 h of incubation and open bars denote the reducing power of samples after 16 h of incubation. Different letters (a–f) on top of the bars denote statistically significant difference ($P < 0.05$).

selected antibiotics addition after 4 h of incubation for further studies. Furthermore, we investigated the possible influence of antibiotics on subsequent amyloglucosidase incubation. Almost no differences in the enzyme activities of amyloglucosidase were observed between samples with and without antibiotics (data not shown).

RS content in cooked milled rice was then assayed with and without antibiotics. The results are given in Table 1. Significant differences were observed between the assay values of RS with and without antibiotics added ($P < 0.01$). However, the amount of RS was much lower in case of antibiotics added, contrary to the foregoing assumption. It is possible that microbial growth might affect the activity of α-amylase and decrease the hydrolysis degree of starch due to acidification of sample pH and possible degradation of α-amylase glycoprotein during incubation. The pH range for pancreatic α-amylase activity is 5.5–8.0, with the pH optimum at 7. McCleary and Monaghan (2002) found that the relative activity and stability of pancreatic α-amylase decreased remarkably with a decreasing pH. Therefore, the significantly decreased sample pH during incubation could reduce hydrolysis rate of starch so that the small amount of residual digestible starch might give major errors in RS determination.

As a check on the precision of the modified assay procedure, RS was determined in six replicate samples of the high amylose *indica* cultivar Xiangxian. The mean value of RS in the material was 1.72% (SE 0.08).

Additionally, digestible starch (DS) can only be calculated as the difference between TS and RS (Table 1, DS_2) in the method of Goñi et al. (1996) due to fermentation in incubation medium. In this study, DS was determined in the supernatant of samples with antibiotics (Table 1, DS_1). When RS was added to DS_1, an underestimation of total starch was observed in the samples tested, in agreement with a previous report (Åkerberg, Liljeberg, Granfeldt, Drews, & Björck, 1998). A possible explanation for this might be that the supernatant contained some of the starch as soluble low molecular weight dextrins, which could not be determined accurately with 3,5-dinitrosalicylic acid reagent.

3.2. Relationship between physico-chemical characteristics and RS contents among the different rice cultivars

Table 2 gives the values for total starch (TS), resistant starch (RS), digestible starch (DS), amylose content (AC), and other physico-chemical parameters such as width, length/width ratio (shape), elongation ratio (ER), elongation index (EI), protein content and lipid content, for each of the cultivars investigated.

Comparing the shape of milled rice, the grains of *indica* cultivars were more slender than those of *japonica* cultivars. The ER or EI tended to be higher in *japonica* cultivars compared with *indica* cultivars. *Indica* cultivars also

Table 1
Effect of antibiotics on resistant starch and digestible starch values of *japonica* and *indica* cooked milled rices[a]

Types	Cultivars	TS (%DM)	Assay procedure with antibiotics			Assay procedure without antibiotics		
			RS (%DM)	DS_1 (%DM)	DS_2 (%DM)	RS (%DM)	DS_1 (%DM)	DS_2 (%DM)
Indica	Xiaoheigu	80.69 ± 0.07	0.93 ± 0.04	74.19 ± 1.29	79.76	1.05 ± 0.01	–	79.64
	Xiangxian	78.89 ± 1.07	1.82 ± 0.08	70.81 ± 0.85	77.07	2.76 ± 0.65	–	76.13
	Nanjing11	84.71 ± 0.45	1.29 ± 0.07	70.27 ± 1.89	83.42	1.41 ± 0.13	–	83.30
	Nanjing16	89.48 ± 0.07	0.47 ± 0.07	81.05 ± 3.62	89.01	–	–	–
	9311	80.67 ± 0.07	0.49 ± 0.08	73.56 ± 4.77	80.18	0.94 ± 0.09	–	79.73
Japonica	Guihuahuang	76.45 ± 0.66	0.76 ± 0.11	71.47 ± 2.28	75.69	1.54 ± 0.24	–	74.91
	Gensidao	83.56 ± 0.34	0.92 ± 0.22	66.65 ± 1.30	82.64	1.73 ± 0.31	–	81.83
	Suzi2	92.98 ± 1.28	0.40 ± 0.02	77.88 ± 0.41	92.58	0.83 ± 0.15		92.15
	C418	82.23 ± 0.25	0.34 ± 0.04	77.57 ± 1.48	81.89	0.77 ± 0.06	–	81.46
	Huinuo	82.27 ± 2.45	0.16 ± 0.02	79.18 ± 4.14	82.11	0.22 ± 0.02	–	82.05

TS, total starch; RS, resistant starch; DS_1, assay values of digestible starch; DS_2, the difference between TS and RS; DM, dry matter.
[a] Values are means of duplicate analyses and expressed as mean ± standard deviation.

Table 2
Resistant starch, digestible starch, and physico-chemical characteristics of *japonica* and *indica* milled rices[a]

Types	Cultivars	TS (%DM)	RS (%DM)	DS (%DM)	AC (%DM)	Width (mm)	Shape	ER	EI	Protein (%DM)	Lipid (%DM)
Indica	Xiaoheigu	80.69 ± 0.07	0.93 ± 0.04	74.19 ± 1.29	27.72 ± 0.14	2.29 ± 0.07	2.47	1.96	1.21	10.17 ± 0.07	0.66 ± 0.09
	Xiangxian	78.89 ± 1.07	1.82 ± 0.08	70.81 ± 0.85	25.85 ± 0.02	2.05 ± 0.11	2.69	1.79	1.09	11.31 ± 0.29	0.66 ± 0.02
	Nanjing11	84.71 ± 0.45	1.29 ± 0.07	70.27 ± 1.89	25.56 ± 0.02	2.54 ± 0.07	2.15	1.82	1.17	12.26 ± 0.20	0.27 ± 0.05
	Nanjing16	89.48 ± 0.07	0.47 ± 0.07	81.05 ± 3.62	18.84 ± 0.02	2.21 ± 0.11	3.23	1.85	1.08	8.29 ± 0.06	0.37 ± 0.02
	9311	80.67 ± 0.07	0.49 ± 0.08	73.56 ± 4.77	15.57 ± 0.08	2.38 ± 0.12	2.69	1.83	1.12	9.86 ± 0.08	0.06 ± 0.00
Japonica	Guihuahuang	76.45 ± 0.66	0.76 ± 0.11	71.47 ± 2.28	21.31 ± 0.05	2.95 ± 0.06	1.70	1.97	1.34	11.06 ± 0.06	0.60 ± 0.01
	Gensidao	83.56 ± 0.34	0.92 ± 0.22	66.65 ± 1.30	21.06 ± 0.02	2.89 ± 0.06	1.55	1.67	1.07	9.97 ± 0.13	0.94 ± 0.10
	Suzi2	92.98 ± 1.28	0.40 ± 0.02	77.88 ± 0.41	19.67 ± 0.18	2.94 ± 0.07	1.57	2.11	1.51	7.76 ± 0.09	0.79 ± 0.04
	C418	82.23 ± 0.25	0.34 ± 0.04	77.57 ± 1.48	16.41 ± 0.08	2.49 ± 0.11	2.40	1.93	1.31	9.02 ± 0.12	0.11 ± 0.01
	Huinuo	82.27 ± 2.45	0.16 ± 0.02	79.18 ± 4.14	3.00 ± 0.02	2.79 ± 0.08	1.66	2.08	1.60	9.05 ± 0.10	0.55 ± 0.10

TS, total starch; RS, resistant starch; DS, digestible starch; AC, amylose content; shape, length/width ratio; ER, elongation ratio; EI, elongation index; DM, dry matter.
[a] Values are means of duplicate analyses and expressed as mean ± standard deviation.

showed a higher protein content than *japonica* cultivars. TS values ranged between 76.45% and 92.98% on a dry matter basis. Two of the selected cultivars, Nanjing16 and Suzi2, stood out with relatively higher TS values (89.48% and 92.98%, respectively). The contents of RS were low in all cultivars tested, while DS contents (corresponding to DS_1 in Table 1) were high. The RS content showed a positive correlation with AC ($r = 0.75$, $P < 0.05$) (Table 3), which was in agreement with the results of Eggum, Juliano, Perez, and Acedo (1993). Among the rice cultivars studied, the waxy cultivar Huinuo had the lowest RS value and the high amylose cultivar Xiangxian contained the highest (Table 2). However, AC was not the only factor determining RS content as reflected in the substantial differences occurring between the high amylose *indica* cultivars. Xiaoheigu, a high amylose *indica* cultivar, had the smallest RS level despite an apparently higher amylose index (27.72%) than those recorded for the other two *indica* cultivars Xiangxian and Nanjing11 (25.85% and 25.56%, respectively). In addition, a statistically significant difference ($P = 0.02$) was observed between the two high amylose cultivars, Xiangxian and Nanjing11, which are almost equal in AC on a dry matter basis (Table 2). This poor correlation between AC and RS content was also observed in previous studies (Frei, Siddhuraju, & Becker, 2003; Walter, da Silva, & Denardin, 2005), and other factors were suggested to affect RS formation, like molecular association between starch components, degree of crystallinity, and other physico-chemical properties that may affect starch gelatinization. In addition, this apparent contradiction between RS and AC was already found in starches isolated from different botanical origins, a fact that might be attributed to putative differences in structures and physico-chemical properties among starches from different species (Vasanthan & Bhatty, 1998).

The Pearson correlation coefficients for the relationship between RS, DS and physico-chemical properties of different rice cultivars are shown in Table 3. RS showed a highly significant correlation with protein content ($r = 0.78$, $P < 0.01$), and a negative correlation was also observed between DS and protein content, however, at lower significance level ($P < 0.05$). It is known that rice grains are rich in a storage protein called rice glutelin or oryzenin (over 80–90% of total proteins) which interacts with starch by binding to amylose and/or amylopectin (Chrastil, 1990). Higher RS formation in rice flour was observed as compared with purified starch where no oryzenin is present (García-Alonso, Jiménez-Escrig, Martín-CarrÓn, Bravo, & Saura-Calixto, 1999). Eerlingen and Delcour (1995) proposed that proteins may have an impact on RS type I (physically inaccessible starch) rather than on RS type III (retrograded starch). Also, the formation of complexes between amylose and lipids upon heating can affect the formation of RS (Czuchajowska, Sievert, & Pomeranz, 1991). Nevertheless, no significant correlation was observed between lipid and RS content in milled rice.

Table 3
Pearson correlation coefficients ($N = 10$) for the relationship between physico-chemical properties, resistant starch and digestible starch of *japonica* and *indica* milled rices

	RS	DS	TS	AC	Width	Shape	ER	EI	Protein	Lipid
RS	1.00	−0.70*	−0.35	0.75*	−0.43	0.18	−0.57	−0.58	0.78**	0.25
DS		1.00	0.48	−0.54	−0.13	0.30	0.67*	0.49	−0.76*	−0.32

RS, resistant starch; DS, digestible starch; TS, total starch; AC, amylose content; shape, length/width ratio; ER, elongation ratio; EI, elongation index.
* and ** indicate significant differences at $P < 0.05$ and $P < 0.01$ probability level, respectively.

The amounts of RS in cooked milled rice showed negative but not significant correlation with ER and EI. Among the three high amylose *indica* cultivars, ER and EI were highest in the cultivar Xiaoheigu and lowest in the cultivar Xiangxian, whereas the cultivar Xiangxian had the highest RS content, followed by the cultivars Nanjing11 and Xiaoheigu. Similarly, the RS of intermediate amylose *japonica* cultivar, Gensidao, with low ER and EI, was relatively higher than that of the *japonica* cultivar Guihuahuang despite similar ACs (21.06% and 21.31%, respectively). In addition, a significant positive correlation was found between ER and DS in cooked milled rice ($r = 0.67$, $P < 0.05$). Tetens et al. (1997) found significant correlations between rate of rice starch digestibility and width, shape and elongation ratio of rice grain, however, in the present study no significant correlation was observed between RS and these physical properties.

4. Conclusions

The method of Goñi et al. (1996) has proven to be a valuable attempt to bring more convenience to the determination of RS content in human food and has been used widely. In this study, we found significant decrease in assay values of RS after antibiotics addition, it is, therefore, necessary to add antimicrobial agents in the basic Goñi procedure.

While no correlation was found between the AC of starches from different botanical origins and their ability to produce RS in previous reports, the current study reveals that RS contents in milled rice can differ significantly despite similar ACs. The result clearly demonstrates that numerous factors, aside from AC, must have an impact on the formation of RS. Protein content has been demonstrated to be one of these factors in the present study. In addition, the elongation was observed to be negatively correlated with RS content and a significant positive correlation was found between ER and DS. More knowledge is needed for a further understanding of the relationship between elongation and protein content and RS formation in milled rice.

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