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In vitro Digestibility of Hydroxypropylated and Cross-linked Waxy and Non-waxy Rice Starches

In vitro digestibility of hydroxypropylated and cross-linked waxy and non-waxy rice starches was investigated to find the proper resistant starch (RS) assaying method for chemically modified starches. RS and total dietary fiber (TDF) content of hydroxypropylated and cross-linked waxy and non-waxy rice starches were measured using the approved AOAC RS assay procedure (AOAC method 2002.02) and the AOAC TDF assay procedure (AOAC method 985.29). Hydroxypropylation did not alter the RS content of waxy and non-waxy rice starches (less than 1% of RS). Cross-linking also did not change the RS content of waxy and non-waxy rice starches (less than 1% of RS). It is interesting to note that non-RS content decreased with increasing hydroxypropylation (97-80%) and cross-linking (99-95%) in both waxy and non-waxy rice starches. This indicates that some fraction of RS in hydroxypropylated and cross-linked waxy and non-waxy rice starches cannot be measured using approved AOAC RS and TDF assay procedures. Therefore, the RS and TDF assay procedures performed in this study are not appropriate to determine the RS content of chemically modified starch. Further investigation is needed to develop a method to determine the RS content of chemically modified starch.

Keywords: *In vitro* digestibility; Resistant starch; Total dietary fiber; Hydroxypropylated starch; Cross-linked starch

1 Introduction

For nutritional purposes, food starches can be classified as either glycemic or resistant starches. Glycemic starches are degraded into glucose by enzymes in the digestive tract and can be further categorized as either rapidly digestible starch or slowly digestible starch [1]. Resistant starch (RS) escapes from digestion in the small intestine but is fermented in the large intestine by bacterial microflora [2-4]. RS is subdivided into four categories depending on the cause of resistance: RS 1 includes physically enclosed starches, such as partially milled grains; RS 2 includes un-gelatinized, intact starch granules; RS 3 includes retrograded starches; and RS 4 encompasses chemically modified starches with reduced susceptibility to digestion [5, 6].

RS is included in the category of dietary fiber because non-digestible starch like fiber may show physiological health benefits in humans [7]. Consumption of RS has been associated with reduced post-prandial glycemic and insulinemic responses, which may have benefits in the management of diabetes [8]. It is also associated with a decrease in the levels of cholesterol and triglycerides. RS may improve cardiovascular health [9]. Other effects of RS consumption are increases in excretion frequency and fecal bulk, which is important in preventing constipation, a decrease in the production of toxic and mutagenic compounds that might promote the formation of cancer cells, and a decrease in colonic pH and ammonia levels, which promotes colonic health [10–13]. RS escapes from digestion and then is fermented in the colon to produce short-chain fatty acids, mainly butyrate, which exerts antineoplastic effects [14].

Native starches from various plant sources have their own unique properties. To the extent possible, these inherent characteristics are exploited by food processors to meet specific needs. Native starches, however, lack the versatility to function adequately in the entire range of food products currently available in the marketplace. The diversity of the modern food industry and the enormous variety of food products require that starches tolerate a wide range of processing techniques as well as various distribution, storage, and final preparation conditions. These demands can be satisfied through modifying native starches by chemical methods [15]. Significant amounts of commercial starch are modified by cross-linking or substitution reactions. Chemical modification of starch,

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especially cross-linking, has been known to produce resistant starch type 4 [16-18]. The extent of *in vitro* digestibility is highly dependent on the type and degree of chemical modification [2, 19].

Many RS analysis methods are suggested and many researchers are trying to set a standard of RS analysis through comparing various methods. In these studies, mostly RS 2, RS 3 and/or food products are used as a sample [20-22]. Chemical modification, especially cross-linking has long been to known to inhibit *in vitro* digestibility of starch [2, 16-19]. Increasing the degree of cross-linking may be expected to inhibit the entrance of amylase into the starch granule thus decrease the susceptibility of amylase [2, 23]. Recently, as the interest in RS 4 is increasing, RS content analysis of chemically modified starches was studied by some researchers [2, 24]. It has been reported that the pancreatin-gravimetric method is more suitable for determination of RS content in chemically modified starch than the AOAC TDF method because the pancreatin-gravimetric method provide more exhaustive digestion of RS 4 starch than the AOAC TDF method [24]. However, the exact RS assaying method for chemically modified starch is not approved yet. Therefore, the objective of this study was to investigate the proper RS assaying method for chemically modified starch by determining the resistant starch and total dietary fiber fraction of chemically modified waxy and non-waxy rice starches using several methods.

2 Materials and Methods

2.1 Materials

Waxy and non-waxy rice were purchased in a local market. Enzymes for resistant starch assay and total dietary fiber assay for the determination of RS content were purchased from Megazyme International Ireland Ltd. (Bray Co., Wicklow, Ireland). Phosphorous oxychloride ($POCl_3$) and propylene oxide (PO) were obtained from Daejung Chemicals & Metals Co. Ltd. (Gyonggi-do, Korea) and Yakuri Pure Chemicals Co. Ltd. (Osaka, Japan), respectively.

2.2 Isolation of starches

The isolation of waxy and non-waxy rice starches from polished waxy and non-waxy rice kernels was performed with the following procedure as described by *Baik* et al. with some modification [25]. The rice was soaked in distilled water for 8 h and ground in two volumes of 0.4% (w/v) NaOH solution in a Waring blender (51 BL 31, Torrington, CT, USA) for 3 min. The rice suspension was passed through a 100-mesh sieve and allowed to stand at 4°C for 24 h. The supernatant was removed, and two volumes of 0.4% (w/v) aqueous NaOH solution were added under stirring, and then the solution was kept at 4°C for 24 h. This process was repeated until the protein was completely removed. The resulting sediment was washed with distilled water until neutral pH was achieved. The isolated starch was dried at 25°C and ground to pass through an 80-mesh sieve. All starch samples were stored at 4°C before use.

The yield of starch was calculated from the weights of raw rice and prepared rice starch. The proximate analyses of both native waxy and non-waxy rice starches were performed according to the standard methods described in the AOAC [26].

2.3 Preparation of chemically modified starches

Cross-linking was carried out using $POCl_3$ as described by *Zheng* et al. with some modification [27]. Isolated rice starch was suspended in distilled water to make 20% solid content and 8.0% of Na_2SO_4 (based on dry weight of starch) was slowly added to the starch slurry with mild stirring. The pH was then adjusted to 11.5 using 1.0 M aqueous NaOH solution. $POCl_3$ (0.005%, 0.01%, 0.02%, 0.04%, 0.06% based on dry weight of non-waxy rice starch, and 0.002%, 0.004%, 0.006%, 0.008% based on dry weight of waxy rice starch) was added to the starch slurry with a microsyringe, and the cross-linking reaction was allowed to proceed for 2 h with stirring. Afterwards the pH of the starch slurry was lowered to 5.5 using 1.0 M aqueous HCl. The temperature of the starch slurry was maintained at 45°C during all procedures. The slightly acidic starch slurry was centrifuged at $1{,}500 \times g$ for 20 min, and the supernatant was decanted. The precipitate was re-suspended in distilled water and centrifuged five times at $1{,}500 \times g$ for 20 min. After centrifugation, the precipitated cross-linked rice starch was dried at 25°C for 12 h and then the dried starch was ground to pass through 80-mesh sieve and kept in a deep freezer.

Hydroxypropylation was carried out using propylene oxide (PO) according to the method of *Wootton* and *Manatsathit* with some modification [28]. Isolated rice starch was suspended in distilled water to make 20% solid content, and 8.0% of Na_2SO_4 (based on dry weight of starch) was slowly added to the starch slurry with mild stirring. The pH was then adjusted to 11.5 using 1.0 M aqueous NaOH solution. PO (2%, 4%, 6%, 8%, 10%, 12% based on dry weight of non-waxy rice starch, and 3%, 6%, 9%, 12% based on dry weight of waxy rice starch) was added to the starch slurry, and the slurry was hydroxypropylated for 20 h with stirring. After the hydroxypropylation reaction, the pH of the starch slurry was lowered to 5.5 using 1.0 M aqueous HCl solution. The temperature of the starch slurry was maintained at 45°C during all procedures. The slightly acidic starch slurry was centrifuged at 1,500 × *g* for 20 min, and the supernatant was decanted. The precipitate was re-suspended in distilled water and centrifuged five times at 1,500 × g for 20 min. After centrifugation, the precipitated hydroxypropylated rice starch was dried at 25°C for 12 h, and then the dried starch was ground to pass through a 80-mesh sieve and kept in a deep-freezer.

2.4 Determination of the hydroxypropyl group and degree of substitution

The hydroxypropyl group content in the hydroxypropylated starches was determined according to the procedure described by *Johnson* [29]. Hydroxypropylated starch (0.1 g) and 25 mL of 0.5 M aqueous sulfuric acid were added into a 100-mL volumetric flask. A sample of unmodified control starch was prepared in the same manner. The flasks were placed in a boiling water bath and heated until the samples had been dissolved. Then the contents were cooled and diluted to 100 mL with water. Aliquots of the solutions (1 mL) were added to 25-mL graduated test tubes with glass stoppers, and 8 mL of concentrated sulfuric acid were added to each. The tubes were placed in a boiling water bath for exactly 3 min. Immediately afterwards the tubes were transferred to an ice bath until the solution had cooled down, and 0.6 mL of ninhydrin reagent was added. Then the tubes were placed in a 25°C water bath for 100 min. The volume in each tube was adjusted to 25 mL with concentrated sulfuric acid and mixed by inverting the tubes several times. Portions of the solution were immediately transferred to 1-cm cells and after exactly 5 min, the absorbance at 590 nm was measured, using the control starch blank as the reference. A calibration curve was prepared with propylene glycol. The factor 0.7763 was applied to convert micrograms of the glycol to hydroxypropyl group equivalent. The degree of substitution was calculated by the following equation.

$$\text{Degree of substitution} = 162\ \text{HP\%}/(5800 - 58\ \text{HP\%})$$

2.5 Determination of relative degree of cross-linking

The relative degree of cross-linking of the cross-linked starches was determined from the viscosity values, according to the method of *Kaur* et al. [30]. The peak viscosity of the starch samples were recorded using a Rapid Visco Analyser (RVA-3D, Newport Scientific Pty. Ltd, Warriewood, Australia). The starch slurry was heated from 50 to 95°C at 12°C/min, and was then held at 95°C for 2 min. Afterwards the paste was cooled to 50°C at 12°C/min and finally kept at 50°C for 2 min. The relative degree of cross-linking was calculated as follows:

$$\text{Relative degree of cross-linking} = (A - B)/A \times 100$$

where A is the peak viscosity in rapid viscosity units of the control sample (0% $POCl_3$), and B is the peak viscosity of the cross-linked starch.

2.6 Determination of RS and TDF content

RS content and TDF content of cross-linked or hydroxypropylated rice starches were measured using the following two methods. The RS assay procedure used in this study is based on the method of *Englyst* and *Berry* with some modification (AOAC Method 2002.02, AACC Method 32-40). In this method, starch is hydrolyzed with pancreatic α-amylase and amyloglucosidase. Then the sample is divided into soluble and insoluble starch fractions. The soluble starch fraction is incubated with glucose oxidase/peroxidase reagent (GOPOD) to determine the glucose content. Non-resistant starch content is determined using absorbance at 510 nm. The insoluble starch fraction is dissolved in KOH solution, incubated with GOPOD reagent after reaction with amyloglucosidase, and absorbance at 510 nm is measured to determine the resistant starch content. This RS assay procedure is summarized in Fig. 1. The TDF assay procedure used in this study is based on the method of *Lee* and *Prosky* (AOAC Method 985.29, AACC Method 32-05). In this method, starch is consecutively hydrolyzed with heat-stable α-amylase, protease, and amyloglucosidase. The hydrolyzed sample is then filtered and dried until a constant weight is obtained. TDF contents is calculated from the weight of the residue. A summary of the TDF assay procedure is shown in Fig. 2.

The main differences in these two procedures are the enzymes and the determination method of the insoluble starch fraction. The RS assay procedure uses pancreatic α-amylase. Insoluble and soluble starch fractions are measured by chemically using GOPOD reagent and a UV spectrophotometer. On the other hand, the TDF assay procedure uses heat-stable α-amylase and protease. The insoluble starch fraction is determined gravimetrically.

2.7 Statistical analysis

Duplicated samples were used in each experiment, and each sample was measured two times. All statistical

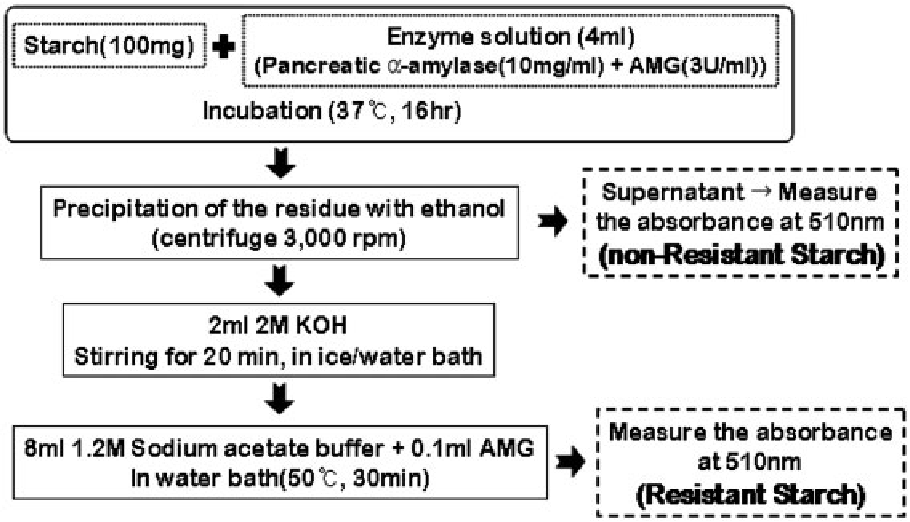

Fig. 1. Summary of the RS assay procedure (AOAC method 2002.02).

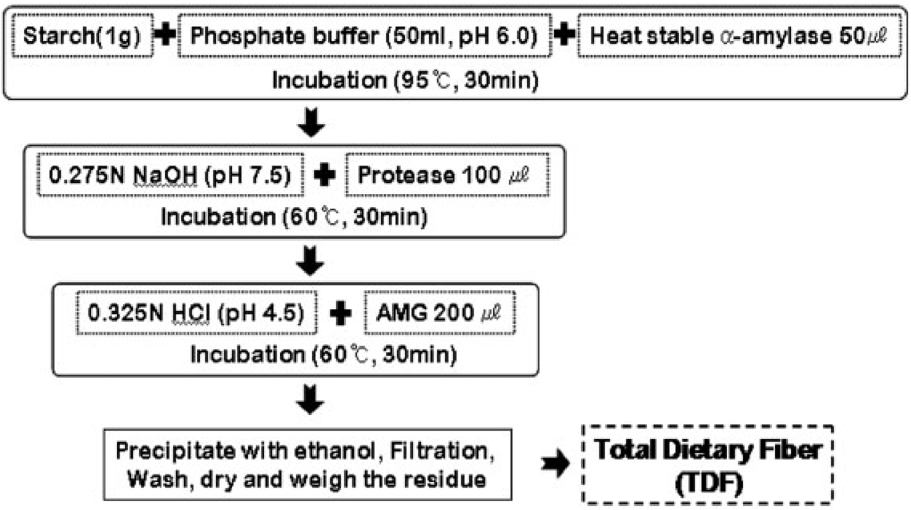

Fig. 2. Summary of the TDF assay procedure (AOAC method 985.29).

significant tests were performed by Duncan's multiple range test using SAS software (version 8.02, SAS Institute, Inc, Cary, NC, USA) at a 95% confidence level.

3 Results and Discussion

3.1 Yield and proximate analysis of native waxy and non-waxy starches

The yields of waxy and non-waxy rice starches were 80.0±1.6% and 82.0±0.9%, respectively. Native waxy rice starch consists of 8.2±0.4% moisture, 0.6±0.1% protein, 0.2±0.03% fat, 0.29±0.01% ash, and native non-waxy rice starch is made up of 9.8±0.02% moisture, 0.6±0.1% protein, 0.2±0.04% fat, 0.3±0.1 ash, respectively.

3.2 RS content measured by the RS assay procedure

The RS and non-RS content of hydroxypropylated and cross-linked waxy and non-waxy rice starches measured by the RS assay procedure are shown in Tab. 1. The RS contents of all samples are less than 0.4%, indicating no distinctive difference between samples. It has been reported that rice starch contains a lower percentage of resistant starch than other starches [31]. The content of resistant starch in rice starch measured by the traditional method is always below 3% [28]. *Janzen* reported that cross-linking potato starch with 0.05 and 0.1% $POCl_3$ had no influence on *in vitro* digestion of the granular product with pancreatic α-amylase as determined by the weight of residue after digestion [17]. *Björck* et al. studied *in vivo* digestibility of chemically modified potato starch and

Tab. 1. RS content (%) of hydroxypropylated and cross-linked waxy and non-waxy rice starches measured by the RS assay procedure.

Non-waxy rice starch			Waxy rice starch		
Sample	RS	Non-RS	Sample	RS	Non-RS
Native	0.21 ± 0.12^{a}	96.94 ± 1.14^{a}	Native	0.14 ± 0.06^{b}	99.20 ± 0.42^{a}
PO 2%	0.13 ± 0.01^{a}	95.33 ± 0.33^{a}	PO 3%	0.26 ± 0.01^{ab}	98.35 ± 0.95^{ab}
PO 4%	0.13 ± 0.01^{a}	90.76 ± 2.37^{bc}	PO 6%	0.30 ± 0.01^{ab}	98.57 ± 0.55^{ab}
PO 6%	0.23 ± 0.14^{a}	88.36 ± 0.23^{c}	PO 9%	0.32 ± 0.16^{a}	95.42 ± 1.23^{d}
PO 8%	0.13 ± 0.03^{a}	83.88 ± 1.17^{d}	PO 12%	0.29 ± 0.03^{ab}	96.36 ± 0.29^{cd}
PO10%	0.11 ± 0.02^{a}	79.97 ± 0.25^{de}			
PO 12%	0.16 ± 0.08^{a}	77.60 ± 0.36^{e}			
$POCl_3$ 0.005%	0.17 ± 0.08^{a}	92.67 ± 3.51^{ab}	$POCl_3$ 0.002%	0.24 ± 0.06^{ab}	98.23 ± 0.18^{ab}
$POCl_3$ 0.01%	0.20 ± 0.02^{a}	93.24 ± 1.96^{ab}	$POCl_3$ 0.004%	0.22 ± 0.04^{ab}	97.79 ± 1.16^{abc}
$POCl_3$ 0.02%	0.18 ± 0.03^{a}	93.03 ± 0.80^{ab}	$POCl_3$ 0.006%	0.27 ± 0.01^{ab}	97.53 ± 0.12^{bc}
$POCl_3$ 0.04%	0.16 ± 0.01^{a}	95.60 ± 3.23^{a}	$POCl_3$ 0.008%	0.15 ± 0.01^{b}	97.53 ± 0.60^{bc}
$POCl_3$ 0.06%	0.23 ± 0.05^{a}	94.80 ± 0.48^{ab}			

* Values followed by the same letter in the same column are not significantly different ($p < 0.05$).

suggested that cross-linking with phosphate at levels used commercially only had a minor effect on *in vivo* digestibility [32]. These studies indicate that a low level of cross-linking has very little impact on starch digestibility and/or RS formation. Similarly, in this study, the $POCl_3$ levels of cross-linked waxy and non-waxy rice starches were lower than 0.06%, resulting in no influence on the formation of the resistant starch fraction.

On the other hand, total starch content, i.e., the sum of RS and non-RS content of hydroxypropylated and cross-linked waxy and non-waxy rice starches, did not reach 100%, indicating that some part of RS or non-RS in hydroxypropylated and cross-linked waxy and non-waxy rice starches could not be measured. This result suggests that the RS assay procedure used in this study is not appropriate for hydroxypropylated and cross-linked waxy and non-waxy rice starches. Moreover, in the RS assay procedure, the insoluble fraction after digestion with pancreatic α-amylase and amyloglucosidase did not dissolve well in the KOH solution, indicating that measurement of the RS content may not have been correct. Additionally, starches are heated or gelatinized before enzyme digestion to increase their susceptibility to the enzyme. The RS assay procedure used in this study, however, does not include a heating or gelatinization step. This may be one reason why the sum of RS and non-RS fractions results in less than 100% total starch content.

3.3 Estimated RS content using an indirect method

The RS content can be estimated from the non-RS content by an indirect method. Since the sum of resistant starch and non-resistant starch is the amount of total starch, we can estimate the RS content from the non-RS content and total starch [6]. The non-RS content was measured by the RS assay procedure, and then the RS content was estimated using the following indirect method:

Estimated RS = total starch − non-RS (measured by the RS assay procedure)

The estimated RS contents of chemically modified waxy and non-waxy rice starches are shown in Tab. 2. The estimated RS content of hydroxypropylated waxy and non-waxy rice starches increased with increasing PO levels. It has been reported that the RS content of hydroxypropylated tapioca starches increased with increasing degrees of substitution because the bulky derivative groups sterically hinder the formation of the enzyme-substrate complex [33]. It is interesting to note that hydroxypropylated non-waxy rice starches showed a dramatic increase of RS content with increasing degrees of hydroxypropylation. On the other hand, hydroxypropylated waxy rice starches also showed a gradual increase with increasing degrees of hydroxypropylation but relatively lower amount of estimated RS than hydroxypropylated non-waxy rice starches. There is a big

Tab. 2. Estimated RS content (%) of hydroxypropylated and cross-linked waxy and non-waxy rice starches using an indirect method.

Non-waxy rice starch			Waxy rice starch		
Sample	Non-RS	Estimated RS	Sample	Non-RS	Estimated RS
Native	96.94 ± 1.14^{a}	3.06^{e}	Native	99.20 ± 0.42^{a}	0.80^{d}
PO 2% (0.0352)1)	95.33 ± 0.33^{a}	4.67^{e}	PO 3% (0.0139)1)	98.35 ± 0.95^{ab}	1.65^{cd}
PO 4% (0.0698)1)	90.76 ± 2.37^{bc}	9.24^{cd}	PO 6% (0.0469)1)	98.57 ± 0.55^{ab}	1.43^{cd}
PO 6% (0.0853)1)	88.36 ± 0.23^{c}	11.64^{c}	PO 9% (0.0807)1)	95.42 ± 1.23^{d}	4.58^{a}
PO 8% (0.1282)1)	83.88 ± 1.17^{d}	16.12^{b}	PO 12% (0.1153)1)	96.36 ± 0.29^{cd}	3.63^{ab}
PO10% (0.1682)1)	79.97 ± 0.25^{de}	20.03^{ab}			
PO 12% (0.1851)1)	77.60 ± 0.36^{e}	22.40^{a}			
$POCl_3$ 0.005% (24.69)2)	92.67 ± 3.51^{ab}	4.85^{de}	$POCl_3$ 0.002% (ND)3)	98.23 ± 0.18^{ab}	1.77^{cd}
$POCl_3$ 0.01% (23.48)2)	93.24 ± 1.96^{ab}	6.76^{de}	$POCl_3$ 0.004% (ND)3)	97.79 ± 1.16^{abc}	2.21^{bcd}
$POCl_3$ 0.02% (25.19)2)	93.03 ± 0.80^{ab}	6.97^{de}	$POCl_3$ 0.006% (ND)3)	97.53 ± 0.12^{bc}	2.47^{bc}
$POCl_3$ 0.04% (22.95)2)	95.60 ± 3.23^{a}	4.40^{e}	$POCl_3$ 0.008% (ND)3)	97.53 ± 0.60^{bc}	2.47^{bc}
$POCl_3$ 0.06% (23.09)2)	94.80 ± 0.48^{ab}	5.20^{de}			

* Values followed by the same letter in the same column are not significantly different ($p < 0.05$).
1) Degree of substitution.
2) Relative degree of cross-linking.
3) Relative degree of cross-linking was not detectable.

difference between waxy and non-waxy rice starches. Waxy and non-waxy rice starches have different amylose/amylopectin ratios, resulting in different ratios of amorphous/crystalline regions. In previous studies [34, 35], X-ray diffraction patterns and relative crystallinities of both hydroxypropylated waxy and non-waxy rice starches showed no changes compared to their native counterparts. These results indicated that hydroxypropylation occurred only in the amorphous region of the starch granule.

Cross-linked non-waxy rice starch revealed relatively higher RS content than cross-linked waxy rice starch, possibly due to the lower level of cross-linking reagent used with the waxy rice starch (0.002−0.008% of $POCl_3$) than with the non-waxy rice starch (0.005−0.06% of $POCl_3$) (Tab. 2). In previous reports [36, 37], waxy rice starch showed higher relative crystallinity than non-waxy rice starch, indicating that waxy rice starch has a relatively small amorphous region. Therefore, the relatively smaller amorphous region of waxy rice starch may not allow the penetration and reaction of cross-linking reagent, resulting in relatively lower RS content in cross-linked waxy rice starch. It is interesting to note that the RS content of both cross-linked waxy and non-waxy rice starches were not significantly different, regardless of the $POCl_3$ level (Tab. 2). Many studies have reported that the cross-linking reaction increases the RS level and that the digestibility of cross-linked starch is highly dependent on the type and degree of chemical modification [2, 16–19]. The relative degree of cross-linking can be estimated by using Rapid Viscosity Analysis. From those results, it was found that cross-linked non-waxy rice starches at different $POCl_3$ levels (0.005−0.06%) showed a similar relative degree of cross-linking (Tab. 2). On the other hand, for waxy rice starches cross-linked with $POCl_3$ we were not able to estimate the relative degree of cross-linking because of relatively higher peak viscosity than native waxy rice starch. These results indicated that the degree of cross-linking of both waxy and non-waxy rice starches was not great enough to restrict the swelling of the granules. Moreover, the pasting properties of cross-linked waxy rice starches were similar to those of substituted waxy rice starches. When waxy rice starches were cross-linked with $POCl_3$ in this experiment, the used levels of $POCl_3$ were relatively low. Therefore, phosphate monoesters were predominantly formed and only a small fraction of di-starch phosphate ester. This may be the reason why both cross-linked waxy and non-waxy rice starches showed similar RS content even at different $POCl_3$ levels.

3.4 TDF contents measured by the TDF assay procedure

The TDF contents of hydroxypropylated and cross-linked waxy rice starches are shown in Tab. 3. Although the TDF assay procedure used different enzymes and a different

Tab. 3. Comparison of RS and TDF content (%) of hydroxypropylated and cross-linked waxy rice starch.*

Waxy rice starch	TDF[1]	RS[2]	Estimated RS[3]
Native	0.39±0.06^{g}	0.14±0.06^{g}	0.80±0.42^{efg}
PO 3%	0.23±0.12^{g}	0.26±0.01^{g}	1.65±0.95^{cde}
PO 6%	0.54±0.03^{fg}	0.30±0.01^{g}	1.43±0.55^{def}
PO 9%	0.63±0.07^{fg}	0.32±0.16^{g}	4.58±1.23^{a}
PO 12%	0.64±0.07^{fg}	0.29±0.03^{g}	3.63±0.29^{b}
POCl$_3$ 0.002%	0.37±0.20^{g}	0.24±0.06^{g}	1.77±0.18^{cd}
POCl$_3$ 0.004%	0.40±0.14^{g}	0.22±0.04^{g}	2.21±1.16^{cd}
POCl$_3$ 0.006%	0.37±0.04^{g}	0.27±0.01^{g}	2.47±0.12^{c}
POCl$_3$ 0.008%	0.48±0.05^{g}	0.15±0.01^{g}	2.47±0.60^{c}

* Values followed by the same letter are not significantly different ($p<0.05$).

[1] Measured by total dietary fiber assay procedure (AOAC method 985.29).

[2] Measured by resistant starch assay procedure (AOAC method 2002.02).

[3] Estimated RS content were measured by an indirect method.

determination method for the insoluble starch fraction, the RS and TDF content of native and chemically modified starches were not significantly different at the 95% confidence level. This result indicates that current RS and TDF assay procedures used in this study are not appropriate for hydroxypropylated and cross-linked waxy and non-waxy rice starches. It is well known that only cross-linked starches have characteristics typical of RS type 4. On the other hand, hydroxypropylated starch or substituted starches do not have RS characteristics in general except highly substituted starches. Normally, cross-linking occurs in two steps. The first step is the substitution reaction, in which the phosphate group of POCl$_3$ interacts with one hydroxyl group of a glucose unit in an amorphous region of the starch granule. The second step is the cross-linking of the substituted phosphate group with another hydroxyl group from a different glucose unit in an amorphous region of the starch granule. It is clear that both steps occur in the amorphous region of the starch granule. Waxy rice starch has a relatively small amorphous region compared to non-waxy rice starch because waxy rice starch has a higher relative crystallinity. This relatively smaller amorphous region may result in less frequent cross-linking reactions, and therefore, most of the starch granules are only phosphorylated and not cross-linked. An alternative explanation is that the relatively low level of POCl$_3$ used in this paper does not allow enough cross-linking in waxy rice starch.

4 Conclusions

When RS and TDF contents of hydroxypropylated and cross-linked waxy and non-waxy rice starches were measured by approved RS and TDF assay procedures, neither RS nor TDF contents of the chemically modified starches were significantly different from that of native starch at the 95% confidence level. On the other hand, estimated RS content of cross-linked and hydroxypropylated waxy and non-waxy rice starches measured by an indirect method showed a significant difference between native and chemically modified starches. Therefore, these results suggest that the approved RS and TDF assay procedures are not appropriate to determine the RS and TDF content of hydroxypropylated and cross-linked waxy and non-waxy rice starches. When RS content was determined for the control sample (43% RS content, RS type 3) included in the RS assay kit, the RS and non-RS content of the control sample were 44.3 ± 1.6% and 57.5 ± 2.5%, respectively. This result indicates that these approved RS and TDF assay methods would be suitable for RS types other than RS type 4. Recently, various *in vitro* RS assaying methods were used in RS determination. However, the measured RS contents were not consistent among the RS assaying methods for the same sample, especially for chemically modified starch or RS type 4. Therefore, further investigation is needed to develop appropriate RS determination methods for type 4 resistant starches.

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