Replacement of Acetate with Ammonium Buffer to Determine Apparent Amylose Content of Milled Rice[1]

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ABSTRACT

An improved iodine colorimetric method based on ammonium-buffered pH 9 solution (NH_4Cl-KI) is reported. The method produces blue color and measures apparent amylose contents (AC) of nonwaxy rice, using potato amylose standard, and results in values similar to those observed when using differential scanning calorimetry (DSC), based on the exotherm of amylose-lipid complex formation, which has no amylopectin and lipid interference. Using the iodine colorimetric method, waxy rice had AC values of 2–3%, while undefatted, milled rice samples had AC values similar to those observed using the DSC method, probably because interference from amylopectin-iodine and amylose-lipid complexes cancelled each other. Using the iodine-NH_4I method, AC values for five nonwaxy rice varieties were identical to DSC values, while AC values for the iodine colorimetric method with acetate buffer were not identical to DSC values when amylose alone was used as the standard. Using an amylose-waxy rice standard, the ammonium buffer method resulted in 4 nonwaxy rice varieties with AC values identical to DSC values. Iodine-NH_4I resulted in 14 AC values identical to DSC values, and AACC International Approved Method 61-03.01 resulted in 7 of 16 rice samples with AC values identical to DSC values. AC determined using DSC and iodine colorimetry, both based on amylose helical complexes, were higher than AC (true amylose plus long-chain amylopectin) determined using high-performance size-exclusion chromatography (HPSEC) of debranched starch and amylopectin. The ammonium buffer method produced a blue color, AC values for nonwaxy rice that were similar to those for the DSC method, and allowed use of undefatted rice samples and an amylose standard alone without waxy rice (amylopectin).

The amylose-iodine colorimetric method of Williams et al. (23) used to determine the apparent amylose content (AC) of milled rice frequently produces unstable blue color due to incomplete HCl neutralization to pH 10.5 of the NaOH used to disperse the milled rice. A simplified assay was introduced in 1971 (14) that uses pH 4.5–4.8 acetate buffer to neutralize the solution and produce a stable color. A potato amylose-waxy rice (amylopectin) mixture was later used as a standard instead of amylose alone to reduce amylopectin-iodine interference, and defatted rice checks were used to correct for amylose-lipid interference. This method was approved as AACC International Approved Method 61-03.01 (1,17). Recent modifications to this method were tentatively proposed by the International Network for Quality Rice (INQR) based at the International Rice Research Institute (IRRI) through a project conducted in collaboration with the International Organisation for Standardisation (ISO) and AACC International, including that color be read at 720 nm instead of 620 nm to minimize amylopectin-iodine interference (9) and to obtain a better defined apparent amylose standard based on high-performance size-exclusion chromatography (HPSEC) of debranched rice starch (3,9). However, the issues of high interference with amylopectin and lipid and greenish color due to excess iodine were not addressed.

The differential scanning calorimetric (DSC) method of measuring AC based on the exotherm of amylose-lipid helical V complex formation has no interference from amylopectin and lipid (19) and is a suitable, but less frequently used, reference method. It is based on the formation of a helical amylose V complex with lipid rather than with iodine. Contents of apparent and true amylose and long-chain amylopectin of rice starch have been measured using HPSEC of isoamylase-debranched starch and amylopectin (3,10–13).

Duldulao and Bergonio (8) of the Philippines Rice Research Institute (PhilRice) Central Experiment Station (CES) recently developed an AC assay using 0.2% iodine and 3.5% NH_4I reagent to simultaneously neutralize and develop iodine blue color at alkaline pH with less interference from lipid. It appears that ammonium, which has a pK_a of 9.24, has a buffering effect at alkaline pH.

The current study compares the use of iodine colorimetry with ammonium buffer at pH 9 (NH_4Cl-KI) for routine analysis of AC with other iodine colorimetric methods based on AC values obtained using the DSC method, which is based on the exotherm of amylose-lipid helical complex formation.

Materials and Methods

Grain Quality Evaluation. Samples of waxy and low- and intermediate-AC rough rice varieties were obtained from PhilRice Los Baños, PhilRice CES, and the University of the Philippines Los Baños. High-AC samples, including the IR36 *amylose extender* (*ae*) mutant, were obtained from IRRI. Samples were selected based on their differences in apparent and true amylose, long-chain amylopectin, degree of polymerization (DP) < 24 glucose units of debranched amylopectin fraction (based on gelatinization temperature [GT]), and amount of DP 25–35 debranched amylopectin fraction (IR36 *ae*) (4,22).

The *Waxy* gene alleles of the varieties were determined from seedlings at the two-leaf stage. DNA was extracted from the leaves using a modified cetyltrimethylammonium bromide method (2). Polymerase chain reaction (PCR) was performed as described previously (6), and the PCR products were separated by polyacrylamide gel electrophoresis exactly as described by Bergman et al. (6). The G/T single nucleotide polymorphism at the 5′-leader intron 1 splice site was measured as described by Ayres et al. (5).

Rough rice was dehulled (Satake THU 35 dehuller) and milled (McGill No. 2 laboratory mill). GT was estimated based on the alkali spreading value (18). Milled rice was ground in a cyclone

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mill (Udy Corp.) with 60-mesh sieve. Moisture content was estimated based on weight lost from 2–3 g of rice flour due to heating at 130°C for 1 hr. Crude protein was measured using the Kjeldahl method and an N factor of 5.95. The starch content of milled rice was estimated as 98.5 – protein content (% db), because, on average, starch and protein together constitute 98.5% of milled rice dry matter.

Iodine-Amylose Colorimetric Assays for Apparent AC. Undefatted waxy and nonwaxy milled rice flour (100 mg) was wetted with 1.0 mL of 95% ethanol and swirled carefully to disperse clumps. Then, ethanol-wetted flour was dispersed in 1 *N* NaOH (9.0 mL) in a 100 mL volumetric flask and let stand overnight. The solution was made up to 100 mL with distilled water and mixed, and a 5 mL aliquot (0.09 *N* NaOH) was placed in a 100 mL volumetric flask with ≈50 mL of distilled water. Next, 1.0 mL of 0.9 *N* NH_4Cl was added, followed by 2 mL of 0.15% iodine in 1.5% KI, and the solution was made up to volume with distilled water to obtain a stable deep-blue color with the least amount of interference from amylopectin (waxy starch produces a greenish tinge). Color was read at 620 nm within 20–60 min, and its stability and pH were measured. AC was calculated from standard curves based on potato amylose V (Avebe) alone (0, 5, 15, 25, and 35 mg/100 mL of 0.09 *N* NaOH) (Fig 1A) and potato amylose V mixed with waxy rice IR29 (1,17) (total including potato amylose of 70 mg/100 mL of 0.09 *N* NaOH). Acids (0.1 *N* acetic acid, 0.1 *N* HCl, and 0.1 *M* NaH_2PO_4) were also tested for partial acidification to pH 9.

AC was also determined using the acetic acid method (pH 4.5–4.8) (14) with potato amylose alone as the standard and AACCI Approved Method 61-03.01 (1,17) with amylose-waxy rice as the standard. Absorbance at 620 nm was read 20 min after mixing.

The iodine-NH_4I method of Duldulao and Bergonio (8) was applied to 16 samples at PhilRice CES. After dispersion of the alkaline solution of milled rice, 2 mL of 0.2% iodine in 3.5% NH_4I was added to a 5 mL aliquot of the 0.09 *N* NaOH rice dispersion without prior acid neutralization and made up to 100 mL with distilled water (8). Color was read at 620 nm 20 min after mixing. Calibration was performed using both potato amylose alone and potato amylose-waxy rice as standards.

DSC Analysis of Amylose-Lipid Complex. DSC analysis was performed using an analyzer (Perkin-Elmer DSC 7) at the Centre International de la Recherche Agronomique pour le Developpement, UMR Qualisud, in Montpellier, France (19). Each 11 mg sample was weighed accurately in 70 µL medium-pressure stainless-steel pans using a 0.0l mg precision balance (Sartorius). Next, 50 µL of 2% L-α-lysophosphatidylcholine aqueous solution from egg yolk (Sigma-Aldrich) was added directly to the sample (to ensure complete amylose-fatty acid complex formation), and the pan was hermetically sealed. The pan was stored for 1 hr before use. Each sample pan was placed at 35°C in a sample cell, whereas an empty stainless-steel pan was placed in the reference cell. The temperature was raised to 160°C at 15 degrees Celsius/min and held at this temperature for 2 min. The temperature was then decreased to 60°C at 10 degrees Celsius/min. The complete cycle of heating, cooling, and stabilization lasted 25 min. The exotherm of complex formation, measured during cooling between 91–92 and 65°C was 28.1 ± 0.3 J/g for potato amylose. AC was calculated from the ratio of the exotherm measured for the sample to that of pure amylose and expressed as percent milled rice dry weight. Pure amylopectin from waxy rice did not show any exotherm during cooling in the presence of lysolecithin.

Determination of AC, Long-Chain Amylopectin, and True Amylose in Starch Using HPSEC. Milled rice flour (20 g) was air-mailed to Tsukuba, Japan, and starch was prepared from 10 g of rice flour using a 0.1% NaOH solution (3). Amylopectin was purified from 1 g of starch sample as described by Aoki et al. (3).

For debranching, 10 mg of rice starch or amylopectin was suspended in 200 µL of 250 m*M* NaOH and 720 µL of distilled water and boiled at 100°C for 10 min. After cooling, the solution was added to 6.4 µL of acetic acid, 80 µL of 500 m*M* sodium acetate (pH 4.0), and 5 µL (≈300 U) of *Pseudomonas amyloderamosa* isoamylase (Hayashibara Biochemical Lab) and incubated for 2 hr at 40°C. The debranched starch and amylopectin were filtered through a 3 mm diameter, 0.45 µm cellulose acetate membrane (Advantec Toyo Kaisha, Ltd.), and 100 µL of the filtrate was subjected to fractionation on an HPSEC system according to Aoki et al. (3).

Debranched starch and amylopectin were analyzed using an HPSEC system equipped with a refractive index detector (Alliance 2695 with 2414, Nihon Waters KK) and a 7.8 × 300 mm column (TSK-GEL G3000PWXL, Tosoh Corporation) at 40°C (3). Elution was performed with 100 m*M* Na_2HPO_4 and 50 m*M* NaH_2PO_4 (pH 8.6) at a flow rate of 0.5 mL/min. Separation produced three peaks: the first fraction corresponded to apparent amylose (including long-chain amylopectin), and the second and third fractions corresponded to shorter amylopectin chains. AC was calculated as the ratio of the peak area of fraction 1 to that of the total peak area for all three fractions of debranched starch × 100. Long-chain amylopectin content was calculated as the ratio of the area of fraction 1 to that of the total fractions of debranched amylopectin × 100. Long-chain amylopectin content in starch was calculated as fraction $1_{amylopectin}$ × [(1 – fraction 1_{starch})/(1 – fraction $1_{amylopectin}$)] × 100. True amylose content of starch was calculated as fraction 1_{starch} – long-chain amylopectin in starch. Least significant difference (LSD; $P = 5\%$) was 1.4% for AC, 0.6% for long-chain amylopectin, and 2.1% for true amylose.

Analysis of Variance. AC data obtained were subjected to analysis of variance using statistical software (SAS System for Windows, version 9.0, SAS Institute Inc.). AC mean values for the same sample for the various methods tested were compared using the LSD test at a 5% significance level.

Results and Discussion

The rice samples had the expected *Waxy* gene alleles: T (Wx^b) in waxy and low-AC rice varieties and G (Wx^a) in intermediate- and high-AC rice varieties (5) (Table I). Waxy and low-AC rice varieties had the *Waxy* gene alleles 17T, 18T, and 20T; intermediate-AC rice varieties had 17G and 20G; and high-AC rice varieties and IR36 *ae* had 11G and 10G.

The method of Williams et al. (23) was followed to determine AC, except for the pH of the NaOH solution used to disperse rice; pH was carefully adjusted with 1 mL of 0.9 *N* NH_4Cl buffer to produce a stable amylose-iodine blue complex when 2.0 mL of 0.15% iodine in 1.5% KI was added and made up to volume with distilled water. A 1.0 mL volume of 0.9 *N* NH_4Cl provided the most stable color and a low AC value for waxy rice at pH 9. Volumes of 0.1 *N* NH_4Cl smaller than 6 mL resulted in unstable color. Reduction of iodine solution from 2.0 mL of 0.2% iodine in 2% KI to 2.0 mL of 0.15% iodine in 1.5% KI removed the blackish blue color of IR36 *ae*. Solutions with low levels of amylose standard tended to be greenish due to excess iodine; however, most nonwaxy rice dispersions were blue, while waxy rice

dispersions were light brown (Fig. 1A and B). Blue color absorbance at 620 nm was stable for 20–60 min after mixing and started to decrease after 60 min. Thus, absorbance should be read within 20–60 min of mixing. The use of 6 mL of 0.1 *N* NH_4Cl and 2 mL of 0.2% iodine in 2% KI produced lower AC values for waxy rice but an AC value of 31% for IR36 *ae* (data not shown); results were not as reproducible as with the higher NH_4Cl addition. Use of NaH_2PO_4, HCl, and acetic acid to neutralize the solution to pH 9, instead of NH_4Cl, produced variable results for optimum acid volume and variable color from blue to blue-green (data not shown). Variability was overcome by the use of ammonium buffer. Borate and glycine buffers were not effective.

Amylose values for iodine colorimetry using buffers with acid pH were compared with values for AACCI Approved Method 61-03.01 (1) using amylose alone (14) or amylose-waxy rice (17) as the standard; iodine colorimetry with ammonium buffer using amylose alone or amylose-waxy rice (all using iodine-KI); iodine colorimetry with iodine-NH_4I (8) using amylose alone or amylose-waxy rice; and HPSEC of debranched starch and amylopectin (3) for 16 selected milled rice samples, using DSC of the exotherm of amylose-lipid complex formation (19) as the standard method.

The iodine colorimetric methods all correlated significantly with the AC values for DSC (r = 0.98**–0.99**) (** indicates correlation is significant at P = 1%). Among the amylose-iodine complex methods tested, the use of NH_4Cl buffer with added iodine-KI produced AC values that were closest to the DSC values for nonwaxy rice when using amylose alone as the standard, except for the 2–3% AC obtained for waxy rice and the higher AC obtained for IR36 *ae* when using iodine colorimetry (Table I). For the nonwaxy rice varieties (including IR36 *ae*), 14 of 15 had identical AC values for both methods. Only the low-AC line had a higher AC value for the ammonium buffer method than for DSC (Table I). Longer chains (>24 glucose units) of amylopectin did not form a complex with lipid because the AC value for DSC of waxy Improved Malagkit Sungsong 2 was 0%. Presumably, residual lipid interference with 3% AC (defatting milled rice flour increases AC by 2–3% [17]) cancelled the 2–3% amylopectin-iodine interference for nonwaxy rice when using the ammonium buffer method provided the degree of milling of the samples was similar. Validation of the method at PhilRice CES using the same 16 samples verified the reproducibility of the method: 1.1–31.4% AC (mean 17.3 ± 0.2%) (data not shown). Addition of waxy rice to the standard produced lower values for the ammonium buffer method than with the amylose standard alone (Table I). Only 2 of 16 samples used in the amylose-waxy rice standard method produced results identical to those of DSC. Use of less NH_4Cl (6 mL of 0.1 *N* or 0.6 mmol NH_4^+) increased the AC of IR36 *ae*, and only 10 samples had AC values that were identical to DSC values (data not shown).

The method using 2 mL of 0.2% iodine and 3.5% NH_4I with the amylose standard alone produced higher AC values than the same method using ammonium buffer (Table I). AC values were 3% for waxy rice, and only 5 of the 16 rice varieties had AC values identical to those from the DSC method. When using the amylose-waxy rice standard, 14 of 16 samples had AC values identical to those from the DSC method (Table I). Only low-AC varieties and NSIC Rc134 and IR42, which had low GT, had lower AC values. AC values for iodine-NH_4I with amylose-waxy rice as the standard were similar to AC values for ammonium buffer with amylose standard alone but required the use of waxy rice in the standard curve.

Table I. Comparison of *Waxy* gene alleles with the G/T single nucleotide polymorphism (SNP) and apparent amylose contents (AC) of milled rice samples determined by differential scanning calorimetry (DSC) of amylose-lipid complex formation or iodine colorimetry with ammonium or acetate buffer, using potato amylose alone or an amylose-waxy rice mixture as the standard

		Apparent AC[a] (% of Milled Rice [dw])							
			Iodine Colorimetry						
			Ammonium Buffer						
			NH_4Cl-KI		NH_4I		Acetate KI Buffer		
Variety	$(CT)_n$ G/T SNP	DSC Lipid Complex Formation	Amylose	Amylose-Waxy Rice	Amylose	Amylose-Waxy Rice	Amylose	Amylose-Waxy Rice	LSD (5%)
Improved Malagkit Sungsong 2	17T	0.0cd	2.8b	–0.6d	3.2b	0.7c	5.4a	–0.4d	0.8
Low-AC line	–	6.4c	7.2b	4.0e	9.2a	7.4b	9.6a	5.4d	0.7
Kasturi	17T	10.2cd	10.8bc	8.8d	12.5ab	10.4cd	13.4a	9.8cd	1.8
IR2071-137-5	20T	10.4b	10.3bc	7.3d	11.6b	9.4c	13.3a	9.7c	1.4
IR24	18T	11.2c	11.2c	9.0d	14.3ab	12.2c	15.7a	12.6bc	2.0
Sinandomeng	17T	13.0bc	13.0bc	9.8d	14.6ab	12.5c	16.0a	13.0bc	1.6
IR64	17G	17.4c	17.3c	15.0d	18.9b	17.0c	21.1a	19.6b	1.2
PSB Rc14	17G	19.2c	18.9c	14.2d	21.0b	19.2c	22.4a	21.2ab	1.2
PSB Rc12	20G	21.1b	20.6b	16.4c	23.0a	21.3b	24.0a	23.4a	1.4
NSIC Rc134	17G	21.7bcd	21.1cd	15.0e	22.4ab	20.6d	23.0a	22.0abc	1.3
NSIC Rc138	17G	22.2c	21.7c	17.2d	23.8b	22.1c	24.8a	24.4ab	0.7
IR36	11G	20.8c	19.7cd	18.2d	22.6b	20.9c	24.6a	23.9ab	1.5
IR8	11G	23.6abc	22.4c	17.0d	24.5a	22.8bc	24.6a	23.9ab	1.2
IR32	11G	23.6cd	22.2d	17.2e	25.8ab	24.2bc	27.0a	27.0a	1.8
IR42	10G	24.3bc	23.2c	16.3d	24.7b	23.0c	29.0a	29.6a	1.4
IR36 *ae*	11G	28.6c	29.4c	23.6d	34.3ab	32.6b	35.0ab	36.6a	2.7
Mean		17.1	17.0	13.0	19.2	17.3	20.6	18.9	1.4
No. of AC values identical to DSC		16	14	2	5	14	0	7	

[a] Values in the same row followed by the same letter are not statistically different at P = 5% based on least significant difference (LSD).

When using a buffer with acid pH, AC values were highest with amylose alone as the standard (Table I; Fig. 1C). All AC values for 14 nonwaxy samples were significantly higher than those for DSC (Table I). Addition of waxy rice to the standard reduced the AC values of waxy and low-AC samples, but not those of the higher AC samples. Only 1 of 16 rice samples (IR8) had an AC value identical to that from the DSC method. In contrast, previous results (17) showed that high-AC samples also had lower AC values when amylose-waxy rice was used as the standard compared with amylose alone. All four high-AC rice varieties (IR36, IR8, IR32, and IR42) showed unexpectedly low AC values (<25%) with use of DSC and iodine colorimetry, similar to intermediate-AC rice varieties, except for IR32 and IR42 with use of AACCI Approved Method 61-03.01 with acetate buffer and amylose-waxy rice as the standard (Table I).

Mestres et al. (19) previously reported that AC (starch basis) determined by DSC was 19% for sample I (IR24), 29% for sample II (IR64), and 33% for sample III (IR74, high AC), while AC determined by iodine colorimetry was 19% for IR24, 29% for IR64, and 33% for IR74. In contrast, in the current study lower AC values were obtained using DSC and iodine colorimetry: 12% for IR24, 19% for IR64, and 23–27% for high-AC rice varieties (Table II) despite their normal *Waxy* gene allele (Table I). At 28.1 ± 0.3 J/g the potato starch-lipid complex formation energy value in the current study was similar to the 28.5 J/g reported by Mestres et al. (19). The difference in values raises the question of whether the lower AC values observed in the current study might have been due to higher ambient temperatures during grain development (20). The DSC energy value for Phil-Rice Avebe potato amylose was 28.3 ± 0.1 J/g.

Because the high-AC samples had typical *Waxy* gene alleles, the lower values obtained were consistent with the reduction in

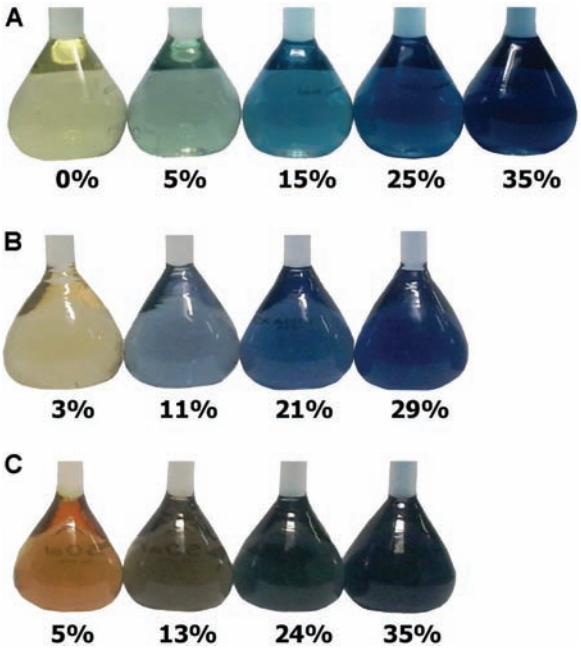

Fig. 1. Iodine blue complex for potato amylose (**A**) and milled rice varieties differing in apparent amylose content (AC) in ammonium buffer (**B**) and for the same milled rice varieties in acetate buffer (**C**). Values below the 50 mL volumetric flasks indicate AC on a milled rice dry basis (with potato amylose standard) for, from left to right, Improved Malagkit Sungsong 2, Kasturi, PSB Rc12, and IR36 *amylose extender* mutant.

Table II. Gelatinization temperature (GT) type; apparent amylose content (AC) determined using differential scanning calorimetry (DSC) of amylose-lipid complex formation or iodine colorimetry with ammonium buffer; and AC, long-chain amylopectin (LC Amp), true amylose, and starch fractions (Fr) 2 and 3 determined using high-performance size-exclusion chromatography (HPSEC) of 16 rice varieties (all on a starch basis)[a]

		AC (% of Starch)		HPSEC Method (% of Debranched Starch)					
				Fraction 1					
Variety	GT Type	DSC Lipid Complex	Iodine Color (NH_4^+, pH 9)	AC	LC Amp	True Amylose	Fr 2	Fr 3	Fr 3/Fr 2 Ratio
Improved Malagkit Sungsong 2	L	0.0b	3.1a	0.4b	0.1	0.3	24.5	75.1	3.06
Low-AC line	H	7.2b	8.2a	5.0c	0.3	4.7	24.4	70.6	2.88
Kasturi	IH	11.7a	12.3a	8.1b	0.8	7.3	20.4	71.6	3.51
IR2071-137-5	H	12.0a	11.8a	6.6b	0.4	6.2	22.8	70.7	3.10
IR24	L	12.4a	14.3a	8.6b	0.8	7.8	23.0	68.4	2.97
Sinandomeng	L	14.3a	14.3a	12.2b	1.6	10.6	22.7	65.1	2.87
IR64	I	19.2a	19.0a	15.1b	3.6	11.5	22.2	62.7	2.82
PSB Rc14	I	21.0a	20.7a	18.0b	3.5	14.5	21.6	60.4	2.80
PSB Rc12	L	23.3a	22.7a	20.0b	3.8	16.2	21.3	58.7	2.76
NSIC Rc134	L	23.9a	23.2a	19.5b	6.9	12.6	20.3	60.2	2.97
NSIC Rc138	L	24.6a	24.0a	20.2b	4.3	15.9	20.9	58.9	2.82
IR36	I	23.0a	21.8a	19.4b	9.1	10.3	21.0	59.6	2.84
IR8	L	26.1a	24.8a	22.3b	8.0	14.3	22.2	55.6	2.50
IR32	I	26.0a	24.5a	20.2b	5.1	15.1	22.2	57.6	2.59
IR42	L	27.1a	25.9a	19.7b	8.6	11.1	21.7	58.6	2.70
IR36 *ae*	H	32.3a	33.2a	26.4b	4.9	21.5	30.8	42.8	1.39
Mean		19.0	18.9	15.1	3.9	11.2	22.6	62.3	2.79
Correlation coefficient with HPSEC AC[b]				1.00**	0.81**	0.94**	0.048[c]	–0.95**	

[a] In the same row, means for AC followed by the same letter are not significantly different at $P = 5\%$ based on least significant difference.
[b] ** indicates correlation is significant at $P = 1\%$.
[c] $r = –0.69$**, excluding IR36 *amylose extender* (*ae*) mutant ($n = 15$).

minimum AC for high-AC U.S. milled rice varieties from 25 to 23% and for intermediate-AC rice varieties from 20 to 19% (7). The range of AC values for U.S. milled rice varieties is now 6–18% for low AC, 19–23% for intermediate AC, and >23% for high AC (7). The range of AC for Philippine rice varieties is now 10.1–18.0% for low AC, 18.1–25.0% for intermediate AC, and >25.0% for high AC (16). The lower limit for intermediate AC has been reduced from 20.1 to 18.1%.

Milled rice moisture content was 9.2–12.1%, crude protein content was 7.0–11.5% (db), and approximate starch content was 87.0–91.3% (db) (data not shown). When AC values for DSC and ammonium buffer were expressed based on starch and compared with HPSEC values, the latter were lower than those for amylose V complex methods (Table II) despite being expressed on a starch basis; however, the fractionation of starch was done under N_2 to minimize degradation (3). The AC of intermediate- and high-AC starches again overlapped, in contrast to higher values reported for high-AC starches (10–13,21). Fraction 3 decreased with increasing AC ($r = -0.95^{**}$), but fraction 2 did not ($r = 0.048^{ns}$). When the IR36 *ae* value was excluded, *r* for fraction 2 and AC was significant and negative (–0.69**). Waxy rice variety Improved Malagkit Sungsong 2 had trace amounts of true amylose and long-chain amylopectin when determined using HPSEC, in contrast to 0% amylose when using DSC. HPSEC measures apparent amylose (true amylose plus long-chain amylopectin) in starch based on size separation and was lower than values obtained using DSC. Likewise, five ISO milled rice standard samples from INQR with reported HPSEC AC values of 0–23.7% of starch (mean 11.3%) had higher values of 2.3–25.9% (mean 13.7%) when determined by the ammonium buffer method, while AC values of 1.8–29.6% (mean 14.5%) were determined using AACCI Approved Method 61-03.01 (data not shown). AC values determined by HPSEC were consistently lower than those determined by amylose helical V complex formation.

Surprisingly, the high-AC rice samples overlapped in properties with intermediate-AC rice samples. Long-chain amylopectin content in high-AC samples did not exceed 10%, in contrast to previous reports (10–21). Debranched IR36 starch was reported by Hanashiro et al. (10) and Hizukuri et al. (11) to have 9 and 6.8% long-chain amylopectin, respectively, while debranched IR42 starch had 14 and 11.3% long-chain amylopectin, respectively. However, high-AC tropical rice varieties (AC up to 33%) with more than 10% long-chain amylopectin, as determined by HPSEC of debranched starch, and amylopectin are still common (13). With higher levels of AC determined by HPSEC, both true amylose ($r = 0.94^{**}$) and long-chain amylopectin ($r = 0.81^{**}$) contents increased (Table II).

Among intermediate- and high-AC rice varieties, AC values determined by iodine colorimetry were lower than those determined by DSC in rice varieties with high long-chain amylopectin content, such as NSIC Rc134, IR8, and IR42, but not IR36 (Table II). It seems that both true amylose and long-chain amylopectin effectively complexed with lipid and iodine.

The IR36 *ae* mutant had an AC value 3% higher as determined by iodine solution than as determined by DSC, except for the ammonium buffer method (Tables I and II). Its long-chain amylopectin content determined by HPSEC was similar to that of intermediate- and high-AC rice varieties, but it had the highest contents of apparent and true amylose compared with its wild-type parent IR36 (Table II). HPSEC showed that IR36 *ae* had a high fraction 2 content, amylopectin with a medium chain length, and the lowest fraction 3 content, which was consistent with its reported longer mean amylopectin chain length (Table II) (4,22).

Using DSC as a standard for functional amylose based on amylose-lipid V complex formation and positive correlation with cooked rice hardness and amylograph and RVA consistency and setback (12,13,15), the ammonium buffer method offers distinct advantages. It produces a blue color, does not require defatting of samples, potato amylose alone may be used as a standard, moisture content may not have to be determined, and AC values obtained for nonwaxy rice are close to those obtained with the DSC method. Waxy rice had 2–3% higher AC values, although a light reddish brown color was obtained. Evidently, interference from amylopectin and lipid in nonwaxy rice cancelled each other. Addition of ammonium with a pK_a of 9.24 in place of HCl enhanced the stability of the iodine blue color. Neutralizing the solution pH before iodine addition was preferable to simultaneously neutralizing pH and adding iodine in the NH_4I method to minimize iodine decomposition. However, absorbance should be read within 20–60 min of mixing, because the color faded progressively in the alkaline solution after 60 min.

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References

1. AACCI. *Approved Methods of Analysis*, 11th ed. Method 61-03.01, Amylose Content of Milled Rice. Published online. AACC International, St. Paul, MN, 2011. doi: 10.1094/AACCIntMethod-61-03.01
2. Allen, G. C., Flores-Vergara, M. A., Kradsnyanski, S., Kuar, S., and Thomson, W. S. A modified protocol for rapid DNA isolation from plant tissues using cetyltrimethylammonium bromide. New Protocols 1:2320, 2006.
3. Aoki, N., Umemoto, T., Yoshida, S., Ishii, T., Kamijima, O., Matsukura, U., and Inouchi, N. Genetic analysis of long chain synthesis in rice amylopectin. Euphytica 151:225, 2006.
4. Asaoka, M., Okuno, K., Sugimoto, Y., Yano, M., Omura, T., and Fuwa, H. Characterization of endosperm starch from high-amylose mutants of rice (*Oryza sativa* L.). Starch/Stärke 38:114, 1986.
5. Ayres, N. M., McClung, A. M., Larkin, P. D., Bligh, H. F. J., Jones, C. A., and Park, W. D. Microsatellites and a single-nucleotide polymorphism differentiate apparent amylose classes in an expanded pedigree of US rice germ plasm. Theor. Appl. Genet. 94:773, 1997.
6. Bergman, C. J., Delgado, J. T., McClung, A. M., and Fjellstrom, R. G. An improved method for using a satellite in rice *waxy* gene to determine amylose class. Cereal Chem. 78:257, 2001.
7. Chen, M.-H., Bergman, C., Pinson, S., and Fjellstrom, R. *Waxy* gene haplotypes: Association with apparent amylose content and the effect by the environment in an international rice germplasm collection. J. Cereal Sci. 47:536, 2008.
8. Duldulao, J. B. A., and Bergonio, K. B. A true blue amylose test: An alternative reagent improves color and accuracy. Proceedings of the 26th Annual Philippine Chemistry Congress, Cebu City, Philippines, 2011.
9. Fitzgerald, M. A., Bergman, C. J., Resurreccion, A. P., Möller, J., Jimenez, R., et al. Addressing the dilemmas of measuring amylose in rice. Cereal Chem. 86:492, 2009.
10. Hanashiro, I., Matsugasako, J., Egashira, T., and Takeda, Y. Structural characterization of long unit-chains of amylopectin. J. Appl. Glycosci. 52:233, 2005.

11. Hizukuri, S., Takeda, Y., Murata, N., and Juliano, B. O. Molecular structure of rice starch. Carbohydr. Res. 189:227, 1989.
12. Horibata, T., Nakamoto, M., Fuwa, H., and Inouchi, N. Structural and physicochemical characteristics of endosperm starches of rice cultivars recently bred in Japan. J. Appl. Glycosci. 51:303, 2004.
13. Inouchi, N., Hibiu, H., Li, T., Horibata, T., Fuwa, H., and Itani, T. Structure and properties of endosperm starches from cultivated rice of Asia and other countries. J. Appl. Glycosci. 52:239, 2005.
14. Juliano, B. O. A simplified assay for milled rice amylose. Cereal Sci. Today 16:334, 1971.
15. Juliano, B. O. *Rice Chemistry and Quality.* Philippine Rice Research Institute, Muñoz, Nueva Ecija, Philippines, 2007.
16. Juliano, B. O. *Grain Quality of Philippine Rice*. Philippine Rice Research Institute, Muñoz, Nueva Ecija, Philippines, 2010.
17. Juliano, B. O., Perez, C. M., Blakeney, A. B., Castillo, D. T., Kongseree, N., Laignelet, B., Lapis, E. T., Murty, V. V. S., Paule, C. M., and Webb, B. D. International cooperative testing on the amylose content of milled rice. Starch/Stärke 33:157, 1981.
18. Little, R. R., Hilder, G. B., and Dawson, E. H. Differential effect of dilute alkali on 25 varieties of milled white rice. Cereal Chem. 35:111, 1958.
19. Mestres, C., Matencio, F., Pons, B., Yajid, M., and Fliedel, G. A rapid method for the determination of amylose content by using differential scanning calorimetry. Starch/Stärke 48:2, 1996.
20. Resurreccion, A. P., Hara, T., Juliano, B. O., and Yoshida, S. Effect of temperature during ripening on grain quality of rice. Soil Sci. Plant Nutr. 23:109, 1977.
21. Takeda, Y., Hizukuri, S., and Juliano, B. O. Structure of rice amylopectin with low and high affinities for iodine. Carbohydr. Res. 168:79, 1987.
22. Villareal, C. P., Hizukuri, S., and Juliano, B. O. Amylopectin staling of cooked milled rice and properties of amylopectin and amylose. Cereal Chem. 74:163, 1997.
23. Williams, V. R., Wu, W. T., Tsai, H. Y., and Bates, H. G. Varietal differences in amylose content of rice starch. J. Agric. Food Chem. 6:47, 1958.