

Ratio of A Chains to B Chains in Rice Amylopectins

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ABSTRACT

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The ratio of A chains to B chains of rice amylopectins from two pairs of near isogenic lines differing in the waxy gene was determined by the action of pullulanase and of β -amylase plus pullulanase on the β -limit dextrins followed by the determination of maltotriose in the digests by quantitative gel filtration chromatography. Maltotriose is derived from A chains by the

action of pullulanase on the β -limit dextrin and from A and B chains by the combined action of β -amylase and pullulanase. Waxy and nonwaxy (low-amylose) rice amylopectin were shown to have similar ratios of A to B chains of 1.1-1.5, despite differences in $[\eta]$ and freeze-thaw stability.

A number of revisions have been proposed for the fine structure of amylopectin (Enevoldsen 1985, Manners 1985). In most of these, an important parameter is the ratio of A chains to B chains (Marshall and Whelan 1974). The A chains are unsubstituted chains linked to the rest of the amylopectin molecule through an α -D-(1-6)-glucosidic linkage; B chains are linked through an α -D-(1-6)-glucosidic linkage but also carry one or more substituent chains joined to primary (C-6) hydroxyl groups.

Amylopectins from waxy varieties of cereals are frequently used for structural analysis. This is a somewhat risky shortcut when the results are extrapolated to encompass nonwaxy amylopectin without reservation. For example, amylopectin from waxy sorghum and waxy corn has an A chain-B chain ratio of 2.6 as compared to a ratio of 1.7 for nonwaxy corn amylopectin (Marshall and Whelan 1974), suggesting that waxy amylopectin may have a different branching pattern to nonwaxy amylopectin. An A chain-B chain ratio of 1.5 is reported for nonwaxy rice amylopectin (Marshall and Whelan 1974) and a ratio of 1.3-1.5 was obtained for waxy rice starch β -limit dextrin (Umeki and Yamamoto 1977, Asaoka et al 1985).

The measurement of A chain-B chain ratio reported by Marshall and Whelan (1974) is based on the comparison of reducing power generated when amylopectin β -limit dextrin is digested with isoamylase or a mixture of isoamylase and pullulanase. When digested with isoamylase, maltotriosyl (derived from odd-numbered glucose unit A chains) should be released but not maltosyl stubs (from even-numbered glucose unit A chains), whereas the mixture of enzymes should debranch all of the A chains. The difference in reducing power should be equivalent to one-half of the A chains. Although the method was novel, small errors in measuring reducing sugar led to large errors in the final A chain-B chain ratio (Altwell et al 1980). Moreover, the A chain-B chain ratio appears to be affected by isoamylase concentration (Manners and Matheson 1981). Alternately, the maltose and maltotriose released may be estimated after separation by chromatographic methods (Manners 1985).

Amylopectins of three pairs of isogenic lines from brown rices differing in the waxy gene have been isolated and compared (Vidal and Juliano 1967). In the present study, the A chain-B chain ratios of two of the three pairs of rice amylopectin were determined from the amount of maltotriose released by subjecting their β -limit dextrins to the action of pullulanase and to the combined action of pullulanase plus β -amylase. Quantitative gel filtration chromatography was used to measure maltotriose in the digests because of its built-in controls (Enevoldsen 1978). A preliminary communication on this study has been presented (Enevoldsen 1980, Juliano 1985).

MATERIALS AND METHODS

Grains of two pairs of near isogenic lines, Caloro and its near isogenic waxy line Cal 5563A1 and Century Patna 231 and Waxy Century Patna 231, were obtained from the multiplication plots of the International Rice Research Institute farm, Los Baños, Philippines, during the 1965 drying season (Vidal and Juliano 1967). Rough rice was dehulled with a McGill sheller, and contaminant translucent grains were removed from the waxy brown rices. Starch was prepared from brown rice by soaking in 1.2% sodium dodecylbenzene sulfonate/0.12% sodium sulfite solution, homogenizing, and passing through a 160-mesh sieve followed by several protein extractions with the same detergent solution (Reyes et al 1965). The purified starch was washed repeatedly with water, air-dried at 35°C, ground to a fine powder with mortar and pestle, and defatted with refluxing 95% ethanol for 24 hr in a Soxhlet apparatus. Purified starches (25 g) were fractionated by autoclaving for 2 hr at 125°C in 1,250 ml of water and 125 ml of a 1:1 (v/v) mixture of Pentasol 27 (mixed amyl alcohols, Pennsalt) and 1-butanol; the dispersion was filtered through glass wool, reheated to boiling, and 100 ml more Pentasol/butanol mixture was added (Reyes et al 1965). The mixture was cooled to room temperature in Dewar flasks for one to two days and for another day at 4°C. Amylose-alcohol complex was removed by centrifuging at 1,000 × g at 0°C for 20-30 min. The amylopectin supernatant was carefully decanted and precipitated by pouring into 5 vol of 95% ethanol with stirring and allowing the precipitate to settle for at least two days, triturated into a powder, washed with 95% ethanol, and dried.

Intrinsic viscosity of amylopectins was measured at 30°C in 1.0N KOH using no. 50 Cannon-Fenske viscometers (Reyes et al 1965). Mean chain length was estimated by periodate oxidation (Reyes et al 1965). Freeze-thaw stability of 5% rice starch gels stored at -20°C and thawed alternately at room temperature was adapted from Schoch (1967). Starch gel was prepared by heating duplicate 5% starch dispersions in water in plastic test tubes for 10 min and cooling. The gels were initially placed at -20°C for 15 hr, then thawed by immersing in tap water for 1.5 hr. The tube was then tested for water separation or syneresis by centrifuging at 760 × g for 10 min and watching for free water. Stable gels were refrozen for at least 5 hr at -20°C and then rethawed and tested until syneresis was observed. The number of freeze-thaw cycles required for syneresis was recorded.

At Copenhagen, β -limit dextrin was prepared from 200 mg of rice amylopectin and waxy rice starch boiled in 20 ml 0.1N NaOH for several minutes, cooled, and neutralized with 2 ml of 1.0N H₂SO₄ to pH 5.0-6.5, and 2 ml of 0.2M acetate buffer (pH 5.07) was added. Then, 0.4 ml of the sweet potato β -amylase (Boehringer) solution (500 units per milliliter, 1 U/mg of amylopectin) and 0.5 ml toluene were added to the polysaccharide dispersion, and the mixture was incubated 24 hr at 30°C. The digests (about 25 ml) were boiled for 5-10 min to inactivate the β -amylase. An aliquot of 2.0 ml was taken for total carbohydrate by phenol-H₂SO₄ (Dubois et al 1956), and for reducing sugar (Somogyi 1952) and quantitative gel filtration chromatography. The main digest (about 20 ml) was dialyzed for several days against

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deionized water and freeze-dried. The yield was 65–73 mg.

β -Limit dextrin (20 mg) was dispersed in 0.90 ml of water and 0.10 ml of 0.2M acetate buffer pH 5, and mixed with 20 μ l of *Aerobacter aerogenes* pullulanase (Boehringer) (28 U/ml, 0.56 U). A 100- μ l aliquot was diluted with 400 μ l of water, and 1 μ l of β -amylase (0.5 U) was added. Toluene was added to the remaining 920 μ l of β -limit dextrin plus pullulanase and to the aliquot with added β -amylase and incubated at 30°C for 24 hr. Aliquots were also analyzed for total carbohydrate by phenol-H₂SO₄ (Dubois et al 1956) and for reducing sugars (Somogyi 1952).

Quantitative gel filtration chromatography used Bio-Gel P-2 (Bio-Rad) lot no. 11324 as described by Enevoldsen (1978). Sample size injected was 10- μ l digests for amylopectin + β -amylase, 16 μ l for β -limit dextrin + pullulanase, and 20 μ l for β -limit dextrin + β -amylase + pullulanase. Carbohydrate concentration was estimated at 420 nm by orcinol-sulfuric acid reagent. One of every 10 runs was a calibration mixture of dextran, maltose, and glucose. Quantification was based on the area of the orcinol-sulfuric acid curve for 40 μ g of glucose. The void volume peak eluted in about 3 hr and elution time for total volume was about 8 hr. Results were expressed as weight percent of total carbohydrates (as glucose) in the digest. The coefficient of variation of the method was 1–2%.

RESULTS AND DISCUSSION

β -Amylolysis limits, which were calculated from maltose estimated by quantitative gel filtration chromatography, overlapped for waxy rice starch and amylopectin and nonwaxy rice amylopectin for the two pairs of isogenic lines (Table I). The other major fraction was the β -limit dextrin plus trace amount of glucose (Fig. 1).

Maltose (M₂) and maltotriose (M₃) recoveries from the action of pullulanase on amylopectin β -limit dextrin to hydrolyze A chains showed the expected 1:1 molar ratio (Table I). Higher maltooligosaccharides recovered were 0.9–1.8% M₄, 2.3–3.8% M₅, 2.9–3.6% M₆, 2.2–2.6% M₇, 2.4–3.2% M₈, 2.8–3.3% M₉, and 64.8–67.9% \geq M₁₀. The highest value for M₄ (1.85%) coincided with the lowest degree of β -amylolysis (53.5%), and thus perhaps reflects that some even-numbered A chains were not completely degraded to maltose stubs, which in turn could explain the comparatively low molar ratio of M₂/M₃ (0.94) in this case (Cal 5563A1 amylopectin).

Combined action of β -amylase and pullulanase on β -limit dextrin yielded mainly M₂ and M₃ (Table I) and some glucose (0 to trace), except for 0.91% glucose for Century Patna 231 waxy starch, and 1.00–3.06% high molecular weight fraction in all except

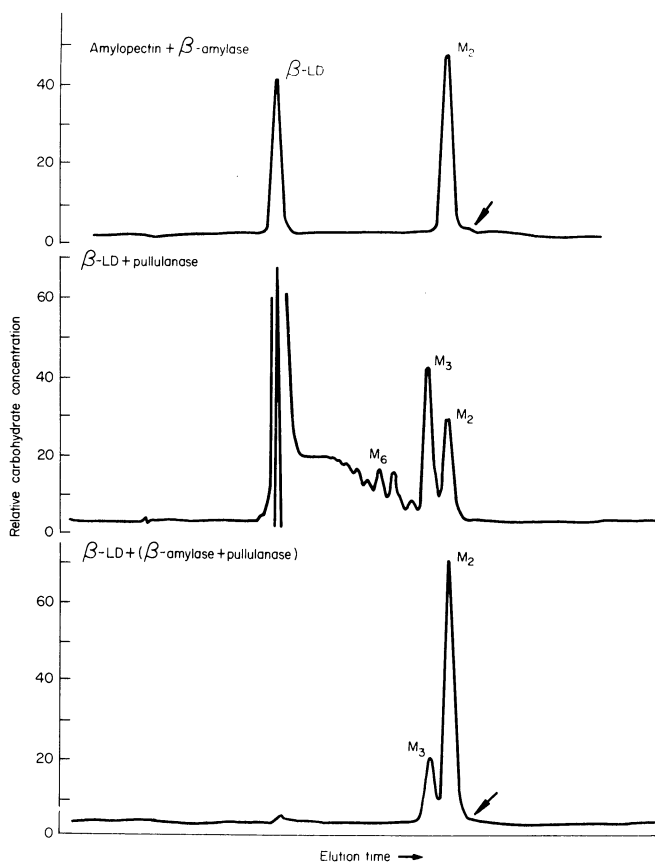


Fig. 1. Typical Bio-Gel P-2 gel filtration elution profile of the products of the action of β -amylase on amylopectin, of pullulanase on β -limit dextrin (β -LD), and of β -amylase and pullulanase on β -LD. Sample: Waxy Century Patna amylopectin. Arrow refers to glucose. M₂–M₆ refer to maltose to maltohexaose.

TABLE I
Properties of Waxy Starch and of Amylopectins Obtained from Two Pairs of Near-Isogenic Rice Starches Differing in the Waxy Gene (Vidal and Juliano 1967)

Amylopectin Property	Cal 5563A1 Waxy		Caloro Nonwaxy Amylopectin	Century Patna 231 Waxy		Century Patna 231 Nonwaxy Amylopectin
	Starch	Amylopectin		Starch	Amylopectin	
β -Amylolysis limit, %	56.1	53.5	55.6	59.4	56.7	58.7
β -Limit dextrin + pullulanase						
Maltose (%)	7.26	6.49	7.66	6.82	6.96	7.13
Maltotriose (%)	10.6	10.3	11.4	10.2	10.5	10.3
M ₂ /M ₃ molar ratio	1.03	0.94	1.01	1.01	1.00	1.04
β -Limit dextrin + pullulanase + β -amylase						
Maltose (%)	79.1	80.1	79.6	77.6	80.1	81.1
Maltotriose (%)	17.8	18.9	19.0	19.2	18.8	18.9
$\overline{CL}_{\beta-LD}$ ^a	8.16	7.85	7.78	7.56	7.88	7.95
\overline{CL} ^b	18.6	16.9	17.5	18.6	18.2	19.2
A chain/B chain ^c	1.45	1.20	1.49	1.12	1.25	1.20
Exterior \overline{CL} ^d	12.5	11.1	11.8	13.1	12.4	13.3
Interior \overline{CL} ^e	5.1	4.8	4.7	4.5	4.8	4.9
Intrinsic viscosity (ml/g)	...	146	177	...	124	160
Freeze-thaw stability (no. of cycles)	...	10	4	...	9	5

^a $\overline{CL}_{\beta-LD} = 1.5 (M_2/M_3 + 1)$.

^b $\overline{CL} = \overline{CL}_{\beta-LD} / (1 - \% \beta)$.

^cA/B = M₃ from pullulanase / (M₃ from β -amylase + pullulanase - M₃ from pullulanase).

^dExterior $\overline{CL} = \overline{CL} \times \% \beta + f(A/B) \times 2.00$.

^eInterior $\overline{CL} = \overline{CL} - \text{Exterior } \overline{CL} - 1.00$.

Century Patna 231 nonwaxy amylopectin, which had only M₂ and M₃.

Chain length of β -limit dextrin was calculated as 1.5 (M₂/M₃ + 1):

$$\overline{CL}_{\beta-LD} = (M_2 + M_3)/^{2/3}M_3 = 1.5 (M_2/M_3 + 1)$$

For conversion to glucose, M₃ had to be divided by 3 and multiplied by 2 to take care of the 50% even-numbered chains. Values were similar between waxy and nonwaxy amylopectins for both pairs. Mean chain lengths (\overline{CL}) of amylopectin were 16.9–18.6 glucose units for Caloro and 18.2–19.2 glucose units for Century Patna 231. These values were lower than those obtained by periodate oxidation of 23–25 glucose units (Vidal and Juliano 1967).

The ratio of A chains to B chains was based on M₃ from the action of pullulanase on β -limit dextrin representing 50% of A chains (with odd-numbered glucose units) and the additional M₃ from the combined action of β -amylase and pullulanase on β -limit dextrin as 50% of B chains (with odd-numbered glucose units). Similar ratios were obtained for waxy and nonwaxy amylopectins for both pairs of lines (Table I).

Exterior chain length was calculated for $\overline{CL} \times \% \beta + f(A/B) \times 2.00$. The use of $f(A/B)$ is based on the difference in mean residual A chain of 2.5 glucose units and mean residual B chain of 1.5 glucose units. Mean residual chain length is 2.00 at A/B = 1.0, 2.10 at A/B = 1.5, and 2.17 at A/B = 2.0. Exterior \overline{CL} values were similar for waxy and nonwaxy amylopectins for both pairs of samples (Table I). Interior \overline{CL} also was similar for waxy and nonwaxy amylopectins in both pairs of lines.

The weight of A chains in β -limit dextrin corresponded to that of M₂ and M₃ released by pullulanase (Table II). The weight of B chains represents the remainder of the digests. The A chain-B chain ratio was also calculated from the A chain-B chain ratio per 100 glucose units, where the weight of A chains/2.50 represents the number of A chains, and the weight of B chains/ \overline{CL}_B represents the number of B chains. Values obtained for A chain-B chain ratio were similar for waxy and nonwaxy amylopectins in both pairs of lines (Table II). The values were similar to those obtained based on M₃ content of digests of β -limit dextrin (Table I).

Mean \overline{CL} of B chains can also be calculated from the additional M₂ and M₃ released by β -amylase and pullulanase on β -limit dextrin compared with pullulanase action on β -limit dextrin. Using the weight of A chains from pullulanase action β -limit dextrin, the A chain-B chain ratios obtained using the \overline{CL}_B values above were slightly higher than those obtained from data for pullulanase action on β -limit dextrin alone (Table II). However, waxy and nonwaxy amylopectins in both pairs of lines had similar values using all three methods.

The A chain-B ratio of Caloro nonwaxy amylopectin (from 17.2%-amylose starch) was higher than that of its near isogenic line Cal 5563A1 waxy amylopectin but similar to that of Cal 5563A1 waxy starch (Tables I and II). With the Century Patna 231 pair, the waxy and nonwaxy amylopectins (from 14.4%-amylose starch) had similar A chain-B chain ratios, but that of waxy starch was lower. The results obtained by quantitative M₃ analysis showed that isogenic waxy rice amylopectin and nonwaxy low-amylose (10–20%) amylopectin have similar A chain-B chain ratios of 1.1–1.5. Reported values were 1.3 (Umeki and Yamamoto 1977) and 1.4–1.5 (Asaoka et al 1985) for waxy rice amylopectin, and 1.5 for nonwaxy rice amylopectin (Marshall and Whelan 1974). Thus, waxy rice amylopectin may be used as a model system for amylopectin of low-amylose nonwaxy rice starch. Waxy rice starch may be used in place of waxy rice amylopectin, provided the sample is not contaminated by nonwaxy grains.

The method described for determining the ratio of A chains to B chains developed by Enevoldsen (1980) is not new but exploits the benefits of quantitative gel filtration chromatography in determining the hydrolysis products of the action of pullulanase and of β -amylase and pullulanase on amylopectin β -limit dextrin. The method has a few built-in controls. Contaminant α -amylase or glucosidase in β -amylase will show up in the complete elution profile of amylopectin β -limit dextrin. In the pullulanase reaction, M₂ and M₃ released can be verified if in the expected 1:1 molar ratio. If the reaction with β -amylase was not complete, then more M₄, M₅, etc., will show up in the pullulanase reaction. From the combined action of β -amylase and pullulanase on β -limit dextrin, the reaction products can be verified to be maltose and maltotriose only. Chain lengths of β -limit dextrin and its B chains can be calculated. The method is about twice as sensitive as that of Marshall and Whelan (1974) based on reducing sugar measurement.

The ratio of A chains to B chains must have an implication on the fine structure of amylopectin. At A/B = 2 instead of 1, exterior $\overline{CL} = \overline{CL} \times \% \beta + 2.17$ instead of $\overline{CL} \times \% \beta + 2.00$. The degree of multiple branching is defined as the average number of substituent chains that each B chain in the amylopectin molecule carries, and is equal to A/B + 1 (Enevoldsen 1985).

Intrinsic viscosity of nonwaxy amylopectin was higher than that of the corresponding waxy amylopectin in both pairs (Vidal and Juliano 1967) (Table I). The freeze-thaw stability of their 5% aqueous gels was better for the waxy amylopectins because they require more freeze-thaw cycles for syneresis. Regardless of differences in intrinsic viscosity or freeze-thaw stability, the amylopectin in each pair had similar A chain-B chain ratios.

Recent study of amylopectins of high-amylose rices showed that they have higher iodine affinity and more long-chain linear fraction than amylopectins from low-amylose rices (Takeda et al

TABLE II
Alternative Methods of Calculating Ratio of A Chains to B Chains from the Action of Pullulanase and Pullulanase Plus β -Amylase on β -Limit Dextrins of Waxy and Nonwaxy Amylopectins and Waxy Starch of Near-Isogenic Rice Lines

Amylopectin Property	Cal 5563A1 Waxy		Caloro Nonwaxy Amylopectin	Century Patna 231 Waxy		Century Patna 231 Nonwaxy Amylopectin
	Starch	Amylopectin		Starch	Amylopectin	
β -Limit dextrin + pullulanase ^a						
A chains in β -LD (wt %)	17.8	16.8	19.0	17.0	17.4	17.4
\overline{CL}_A	2.50	2.50	2.50	2.50	2.50	2.50
B chains in β -limit ^b (%)	82.2	83.2	81.0	83.0	82.6	82.6
A chain/B chain ^c	1.42	1.16	1.47	1.08	1.23	1.22
β -Limit dextrin + pullulanase + β -amylase						
ΔM_2 (%)	71.8	73.6	72.0	70.8	73.1	74.0
ΔM_3 (%)	7.27	8.60	7.63	9.06	8.36	8.56
\overline{CL}_B ^d	16.3	14.3	15.6	13.2	14.6	14.5
B chains in β -LD (% $\Delta M_2 + \Delta M_3$)	79.1	82.2	79.6	79.9	81.5	82.6
A chain/B chain ^c	1.47	1.17	1.50	1.13	1.25	1.22

^aA chains in β -limit = $\% (M_2 + M_3)$.

^bWeight of B chains in β -limit = $100 - \% (M_2 + M_3)$.

^cA/B = (Wt of A chains $\times \overline{CL}_B$ in β -limit) - (Wt of B chains $\times 2.50$).

^d $\overline{CL}_B = 1.5 (\Delta M_2 / \Delta M_3 + 1)$.

1987). The ratio of gel filtration fraction 3 (DP 16–17) to fraction 2 (DP 41–44) for three isoamylase-debranched high-amylose amylopectins was 3.2–3.5, compared with 3.9–4.3 for three low-amylose amylopectins. The ratio of gel filtration fraction 3 (iodine $\lambda_{\text{max}} < 525$ nm) to fraction 2 (iodine $\lambda_{\text{max}} 525$ –600 nm) for isoamylase-debranched starch of waxy and nonwaxy Taichung 65 was 2.1–2.7 for waxy amylopectin and 2.2–2.8 for nonwaxy amylopectin (Asaoka et al 1985). Correspondingly, a high-amylose (29%) starch mutant of Kinmaze had an isoamylase-debranched starch fraction 3-to-fraction 2 ratio of 1.4 as compared to 2.7 for debranched Kinmaze starch (22% amylose) (Asaoka et al 1986). Thus, high-amylose rice amylopectin may have a lower A chain-B chain ratio than waxy and low-amylose nonwaxy rice amylopectins.

CONCLUSION

Amylopectins from two pairs of near isogenic lines differing in the waxy gene had similar ratios of A chains to B chains in waxy and nonwaxy (low-amylose) rice starches. Ratios were determined by maltotriose assay by Bio-Gel P-2 gel filtration of digests of the action of pullulanase and of β -amylase plus pullulanase on amylopectin β -limit dextrans.

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