Part B: Final Report Project 3402

Objective 3: Resistant Starch (RS)

This is the first report of the use of the Megazyme method for the analysis of resistant starch (RS) in rice, so investigations were carried out to validate the method before it was used for routine determinations of RS. Figure 3 shows the method. The first incubation produces supernatant 1 which contains the digested and non-resistant starch (NRS), and pellet 1 which contains the RS. The second step produces supernatant 2 which contains the resistant starch and pellet 2, which is discarded. Table 3 shows the amount of NRS (supernatant 1 (discarded), RS (supernatant 2) and the amount of amylose in pellet 2 of the Australian rices and of one from the Philippines (PSBRc 18). When protein and moisture is accounted for, the total of the starches in the different fractions almost accounts for the starch in the milled rice.

Variety	RS	NRS	AM P2	TOTAL
Amaroo	1.26	67.8	1.50	70.6
Basmati	2.67	69.5	1.64	73.8
Doongara	2.15	70.6	1.36	74.1
Koshihikari	1.30	66.9	2.66	70.9
Koshi Japan	0.97	71.8	1.91	74.7
Kyeema	0.89	62.2	1.35	64.4
Langi	1.24	66.0	1.93	69.17
Opus	1.17	73.1	1.17	75.44
PSB Rc10	1.88	69.2	1.83	72.91

Table 3. Resistant starch (RS) non-resistant starch (NRS), amylose in pellet 2, and total starch. Allowing for moisture and protein, the Megazyme method accounts for almost all of the starch in a milled grain.

RS from freshly cooked rice

Table 4 shows the varieties, their origin, their amylose content, the amylose allele for each and the RS content of freshly cooked rice of each. Figure 19 shows the relationship between RS in freshly cooked rice and amylose content (Figure 19a), amylose allele (Figure 19b), and proportion of long chains (Figure 19c). The figures show some relationship to amylose content with a correlation coefficient of about 0.7, and to long chains, but little relationship with the amylose allele.

Figure 20 shows the HWS starch of a selection of varieties where RS in freshly cooked rice is low (red), intermediate (blue) or high (green). There is no clear relationship between the amount of HWS starch and the amount of RS in freshly cooked rice. Figure 21 shows chromatograms of the same varieties after debranching and again shows very little difference in MWD of the chains and RS content. These results were unexpected so the structure of the molecule that accounts for the RS reading was investigated.

Figure 22 shows HWS starch from Doongara and the molecule that accounts for the reading of RS. Clearly, the molecule that accounts for the RS reading is very low in hydrodynamic volume, and there is only a very small amount relative to the HWS starch.

Variety	Origin	Amylose (%)	CTn of GBSS	RS (%)
Amaroo	Sunrice, Australia	19	19	1.26
Basmati	Sunrice, Australia	24	17	2.67
Doongara	Sunrice, Australia	25	14	2.15
Koshihikari	Sunrice, Australia	19	17	1.30
Koshihikari-	Sunrice, Australia	18	17	0.97
Kyeema	Sunrice, Australia	20	18	0.89
Langi	Sunrice, Australia	19	19	1.24
Opus	Sunrice, Australia	19	17	1.17
IR60	IRRI	26	10	2.99
IR8	IRRI	26	11	3.40
IR5	IRRI	27	17	3.38
IR64	IRRI	23	17	2.54
IR24	IRRI	18	18	0.83
AE	IRRI	34	11	6.24
PSB Rc10	PhilRice,Philippines	25	11	1.88
PSB Rc98	PhilRice,Philippines	30	11	3.09
PSB Rc12	PhilRice,Philippines	24	20	2.47
PSB Rc16	PhilRice,Philippines	28	20	2.57
PSB Rc18	PhilRice,Philippines	23	20	1.84
Dawn	YAI	22	14	1.60
Newbonnet	USDA	22	14	1.80
Tebonnet	USDA	22	14	2.00
Kaybonnet	USDA	23	14	2.00
Jodon	USDA	26	20	2.40
Rexmont	USDA	24	11	2.70
Dixiebelle	USDA	27	11	2.70
Bolivar	USDA	25	11	2.80
Cocoderie	USDA	26	20	2.90
L205	USDA	25	11	3.00
TX3042	USDA	27	11	3.20
Te Qing	USDA	26	11	3.30
TX 4175	USDA	27	11	3.60
Sierra	USDA	25	11	3.70

Table 4: Varieties used, their origin , amylose content, CT number of amylose allele, and RS content of freshly cooked rice.

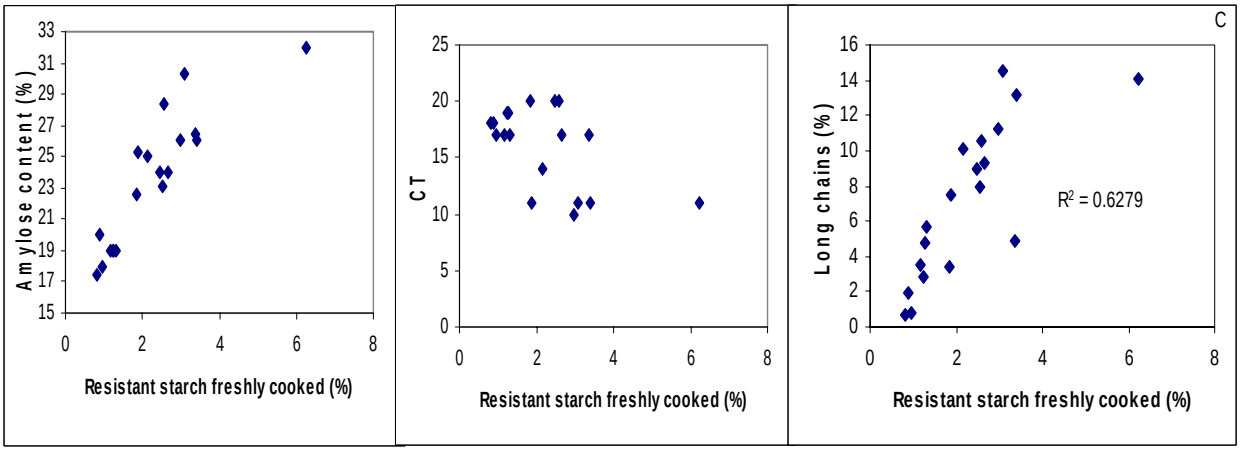

Fig.19: RS in freshly cooked rice correlates reasonably with amylose content, poorly with CT and reasonably with % long chains (ie chains > DP100).

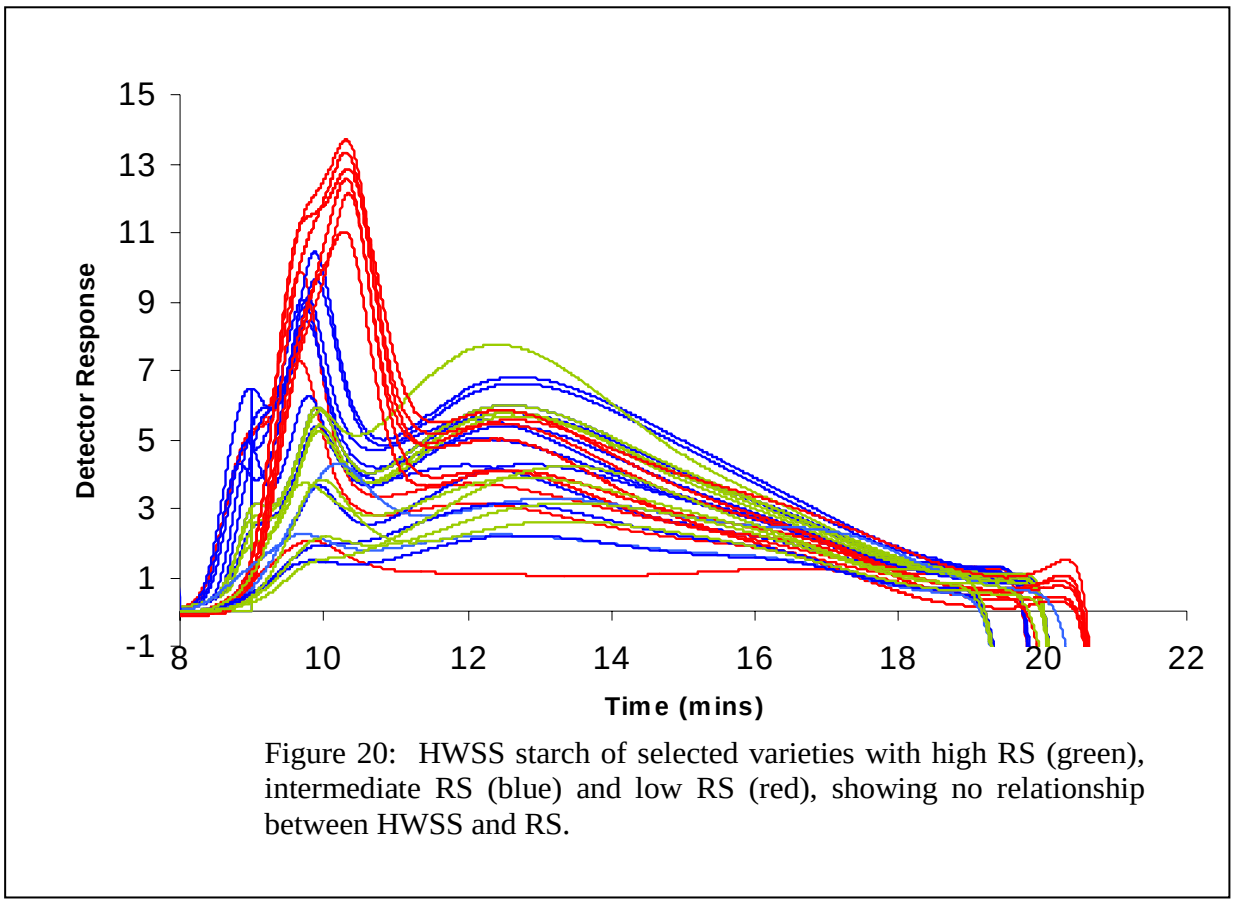

Figure 20: HWSS starch of selected varieties with high RS (green), intermediate RS (blue) and low RS (red), showing no relationship between HWSS and RS.

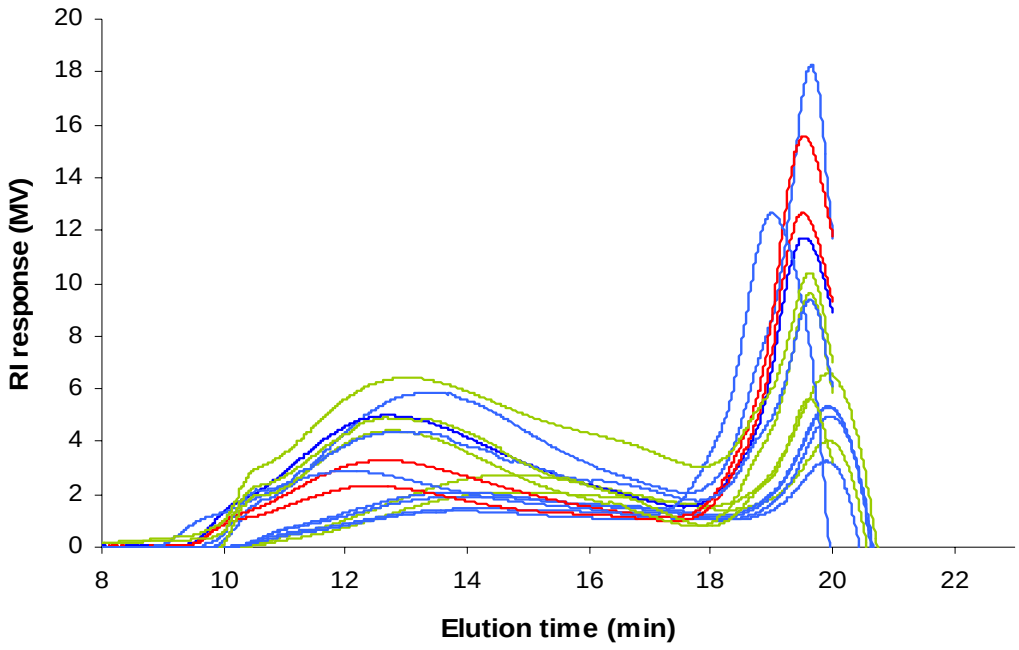

Figure 21: Debranched HWSS starch of selected varieties with high RS (green), intermediate RS (blue) and low RS (red), showing no relationship between MWD of long chains and RS.

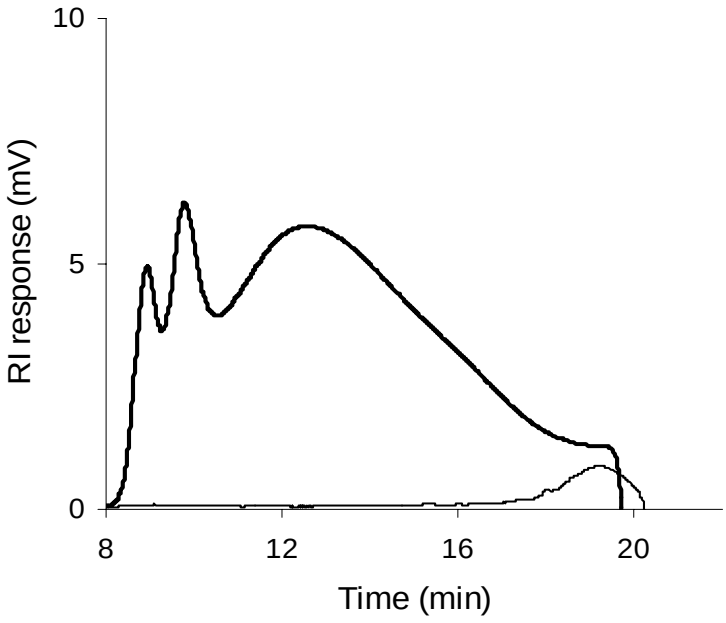

Figure 22: Amount and elution profile of HWSS of Doongara and the of molecule that accounts for the RS reading from Doongara

The data in the previous figures suggests that the molecule that accounts for the reading of RS is much smaller than amylose molecules. This was not expected. A selection of varieties was made from the set of Australian and Asian rices, spanning the range of RS, to explore the structure of RS further. Figure 22 suggests that RS molecules are small and so they were re-analysed using the UH 250 column, which separates smaller molecules better than the UH 500. Figure 23 shows the chromatograms of whole RS molecules on the UH 250 column for the sub-set of low (red), intermediate (blue) and high (green).

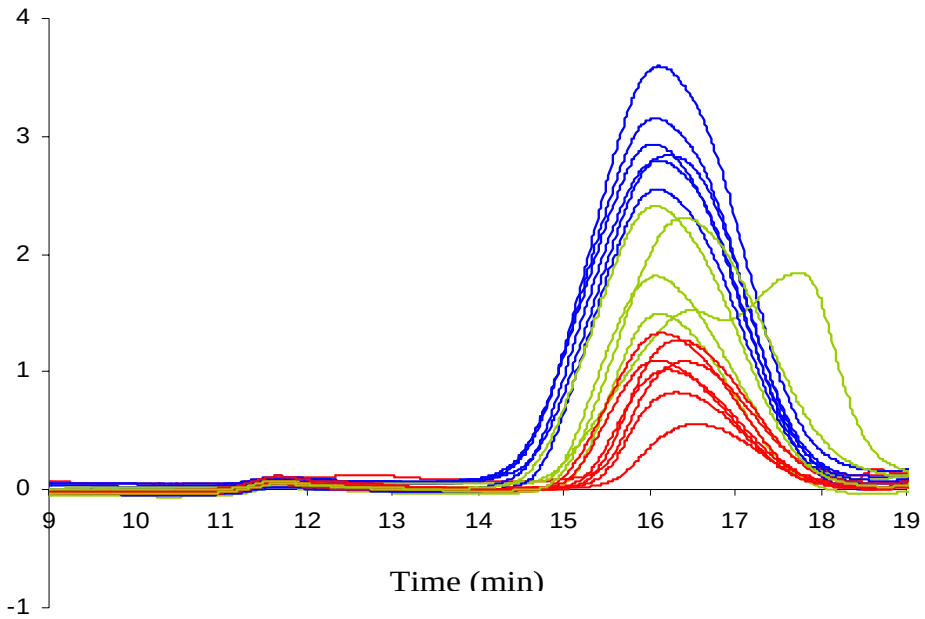

Figure 23: Whole molecules of RS of Australian and Asian varieties showing good grouping of high (blue), intermediate (green) and low (red) RS measured by the Megazyme method. Separation is SEC on the UH250.

Figure 23 shows that the high, intermediate and low RS values measured by the Megazyme assay relate well to the relative amounts of RS measured as detector response on the SEC. Figure 24 is a typical chromatogram of full flour showing the separation of amylose and amylopectin chains, for reference when comparing the elution time of RS molecule relative to the amylose and amylopectin chains. Note though, the RS molecule is a complete molecule, so its elution pattern relates to its hydrodynamic volume (size in the eluant) and not to its molecular weight, whereas the amylose and amylopectin chains in Figure 24 can be interpreted in terms of the MWD. Amylose chains elute between 11 and 12 minutes, and amylopectin chains elute between 15 and 19 minutes. Figure 23 shows that RS molecules elute between 14 and 19 minutes, so the size of the molecule in the eluent is the same as the size of the linear chains of amylopectin that elute between 14 and 19 min. There is a small amount of material that elutes at the same time as the chains of amylose elute (by reference to Figure 24).

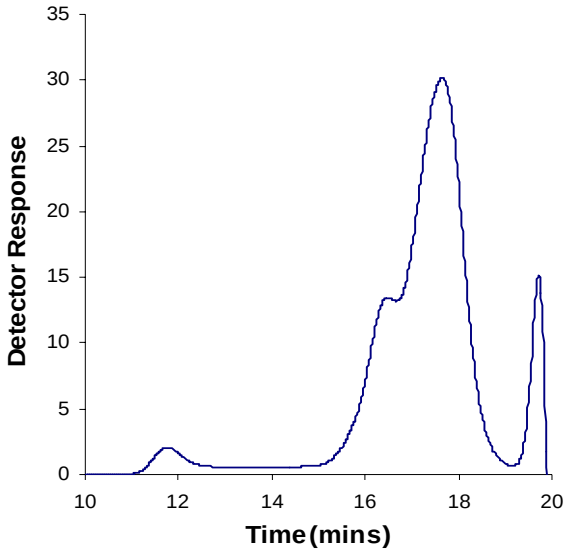

Figure 24: Typical chromatogram of debranched flour on the UH250 showing amylose chains eluting between 9 and 14 min and amylopectin chains eluting between 15 and 18 min.

The UH250 column has recently been calibrated for the analysis of the absolute MWD of linear starch chains. In order to determine the nature of the RS molecule, its MW was analysed using a light scattering detector (DAWN) and compared with the expected MW for that elution time, for linear chains. Figure 25 shows the expected MW and the actual MW of the RS at each elution time. Figure 25 shows that the MW of linear chains is proportional to elution time, and shows that at the elution time of RS, the molecular weight of a linear chain would be of the order of 1000 - 10000, whereas all the samples presented show that the average molecular weight of the molecules at each elution time for all the samples of RS is higher in MW than a linear chain at the corresponding elution time, and furthermore, there is some variation in the molecular weight of the RS molecules.

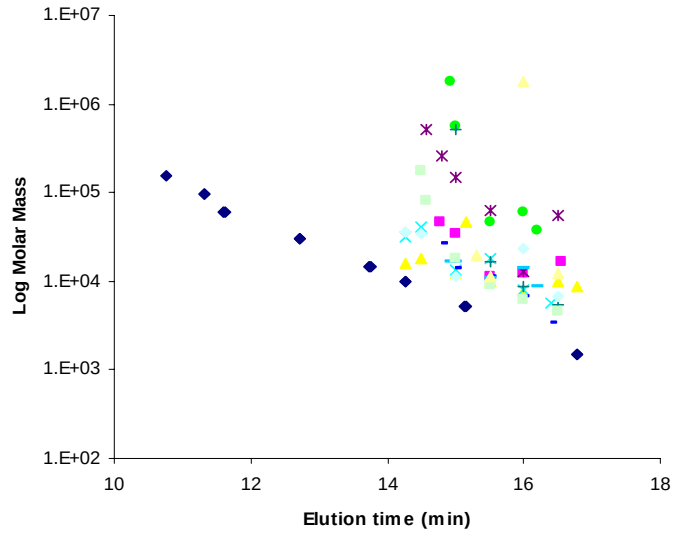

Figure 25: Expected MW vs Elution time for linear chains fo starch (Castro et al. 2005) (blue diamonds) and actual MW of molecules of RS from different varieties (colours).

In order to test whether or not the structure of RS resembled digested amylose or digested amylopectin, the RS molecules were debranched with isoamylase and the debranched RS analysed on the UH250 (Figure 26).

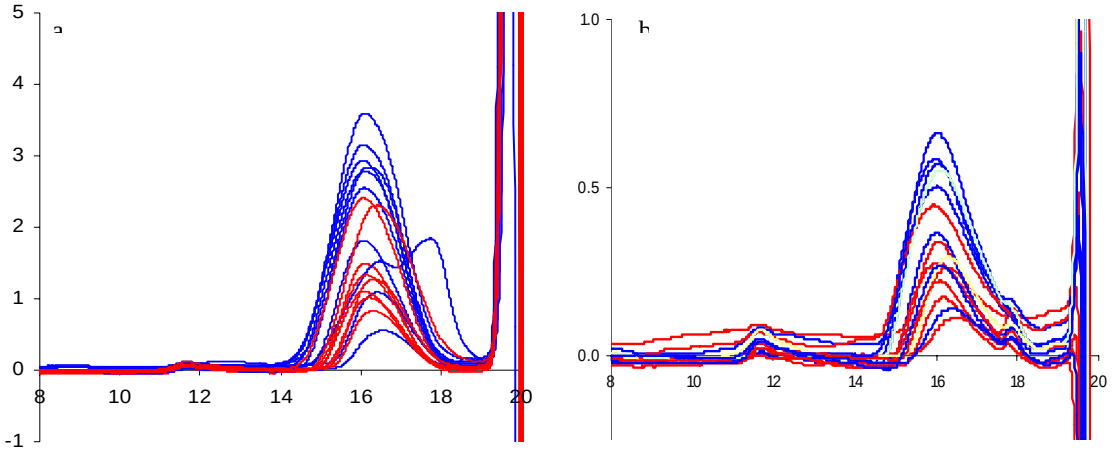

Fig. 26: Whole molecules of RS (a) from freshly cooked rice of Australian (red) and Asian (blue) origin, and (b) debranched RS from the same varieties. Note the difference in the scales.

Figure 26 shows chromatograms of the whole (Figure 26a) and debranched (Figure 26b) RS for the Asian and Australian varieties. Figure 26b shows that the debranched molecules are of similar hydrodynamic volume to the whole RS molecules, and for all, there is a significant increase in the proportion of very short chains in the debranched RS. Further, there is a very small peak eluting in the area of amylose. The short chains of the RS molecules (debranched) were analysed further with CE and the amylose-like molecules by SEC.

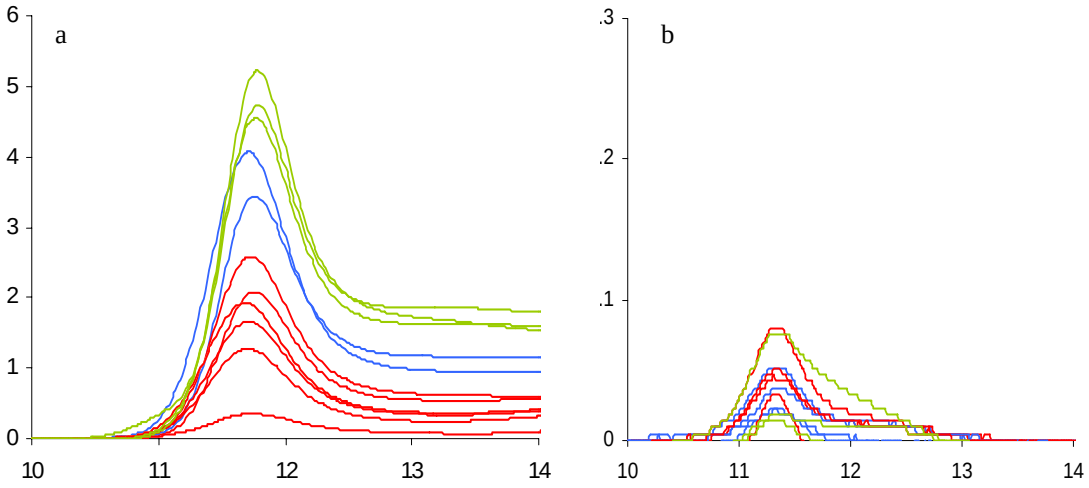

Figure 27 showing (a) amylose chains from flour of 3 high, 2 intermediate and 6 low amylose varieties and (b) showing the amylose in the RS from the same varieties. Note, the scale of b is 1/20th of a..

Figures 27a and b show the amylose chains in debranched starch and in RS for the varieties. Concentration is taken into account and the amount of chains in the debranched RS and full flour can be directly compared. Note the differences in the scale of the y axis. For all the varieties, very little amylose remains in the RS relative to that in the full flour and the amount of amylose remaining in the RS is independent of the amount in the full flour. The remaining amylose is between 1 and 2% of the original. Figure 28 shows a CE trace of the chain length distribution (debranched) of amylopectin of Doongara for comparison with the chain length distribution of the debranched RS from the Australian varieties (Figure 29) (this project is still current so data is unavailable for the Asian rices).

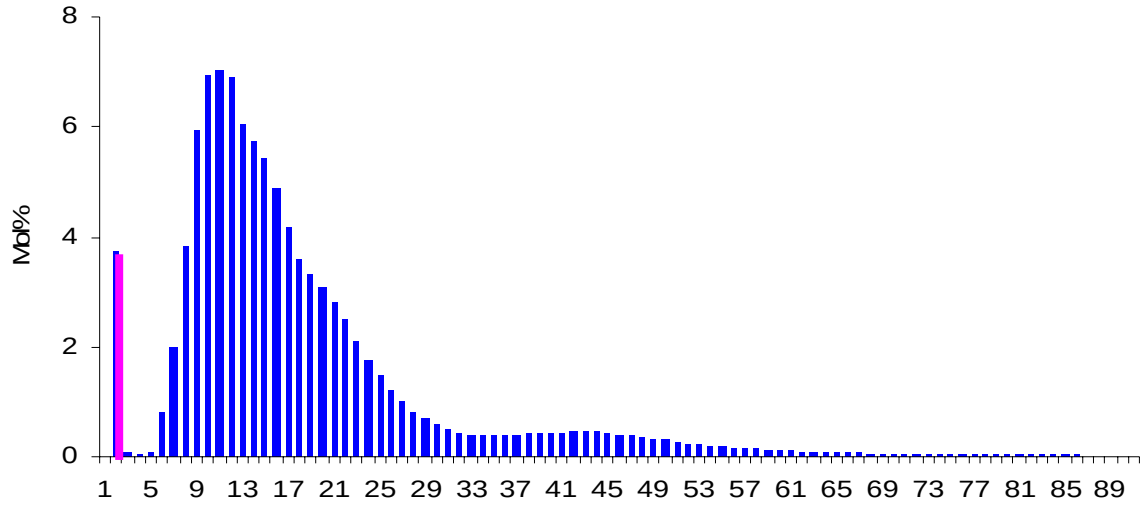

Fig. 28: CE trace of debranched flour showing baseline separation for each chain length (DP). The internal standard is maltose (DP 2) and is shown in pink.

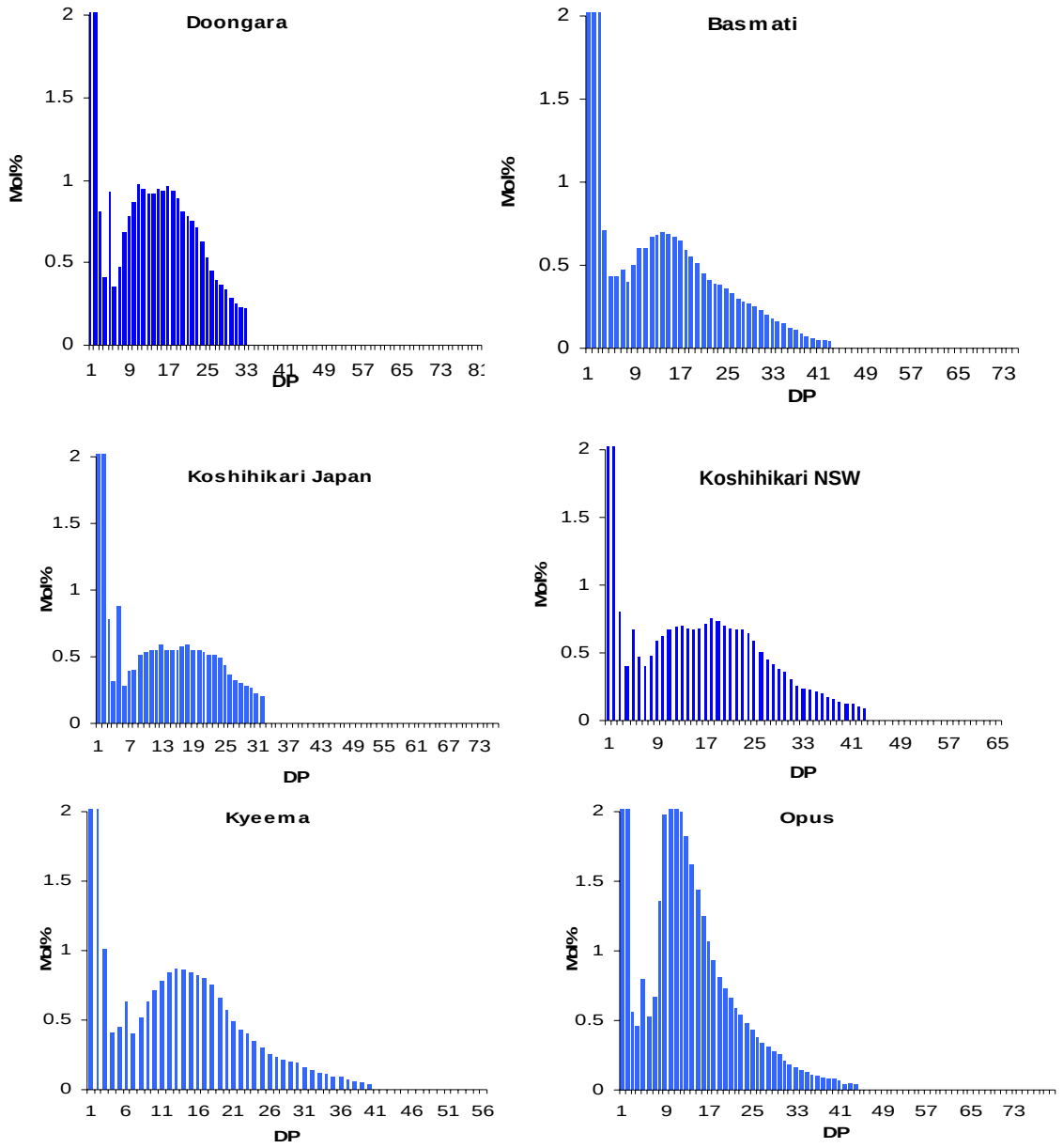

Figure 29: Chain length distribution of molecules of RS from Australian rices.

Figure 28 shows two populations of chains in amylopectin of full flour, one population between DP 6 and DP 33 and the other between DP 38 and DP 61. For each variety, there are clear differences between the chain length distribution of amylopectin and that of RS. Figure 29 shows that there are far fewer chains in the RS than in the amylopectin (amounts can be directly compared), and for all, there are far fewer chains above about DP 25, and there are no chains beyond about DP 40. Further, the chain length distribution of all the RS samples shows a much higher proportion of chains of DP 2 - 6 than the chain length distribution of amylopectin shows and the RS samples contain large peaks of glucose and maltose (DP 1 and 2), which the amylopectin does not show . Figure 29 also shows that there is not a consistent chain length distribution for RS from the different varieties.

Resistant starch in processed rice

Analysis of the effect of different cooking and processing techniques was carried out using the Australian variety Doongara. The structure of RS in retrograded rice was analysed using a range of Australian and Asian rices.

Resistant starch was measured on freshly cooked samples of both brown and milled rice of Doongara, and was 3.7% in freshly cooked brown rice and 2.1% in freshly cooked milled rice.

The effect of different cooking times and methods was compared using milled rice of Doongara. Different cooking times showed little difference in resistant starch content. When rice was under-cooked, RS was 1.9%, when it was over-cooked, RS was 2.2%. Rice cooked by the absorption method retained more resistant starch than rice cooked by the rapid-boil method (2.1% versus 1.3% respectively). The cooking water from the rapid boil method was retained and the water evaporated. The RS content of the dried slurry from the rapid boil methods was 5.3%. Cooking rice and then mashing or sieving it did not alter the RS content relative to freshly cooked milled rice.

The white rice was ground to a flour and gels prepared (8% flour). RS was determined on fresh gels and on gels that were stored for a week at 4 ºC. The RS content of fresh gels was 0.1% and of stored gels was 0.5%.

The effects of retrogradation were observed when cooked rice was chilled for 24 hours. The RS value increased significantly after 24 hours at 4°C (from 2.2% to 3.6%), but the value did not change with storage beyond 7 days.

The structure of RS in retrograded rice

RS in retrograded rice was explored further using the set of Australian and Asian rices. Table 5 shows the RS content of retrograded rice for the set of Australian and Asian rices.

Variety	Origin	Amylose (%)	CTn of GBSS	RS (%)
Amaroo	Sunrice, Australia	19	19	1.67
Basmati	Sunrice, Australia	24	17	3.70
Doongara	Sunrice, Australia	25	14	3.17
Koshihikari	Sunrice, Australia	19	17	1.73
Koshi Japan	Sunrice, Australia	18	17	0.92
Kyeema	Sunrice, Australia	20	18	1.60
Langi	Sunrice, Australia	19	19	1.50
Opus	Sunrice, Australia	19	17	1.41
IR60	IRRI	26	10	4.50
IR8	IRRI	26	11	3.59
IR5	IRRI	27	17	4.85
IR64	IRRI	23	17	2.57
IR24	IRRI	18	18	1.20
AE	IRRI	34	11	11.97
PSB Rc10	PhilRice,Philippines	25	11	4.02
PSB Rc98	PhilRice,Philippines	30	11	5.90
PSB Rc12	PhilRice,Philippines	24	20	2.63
PSB Rc16	PhilRice,Philippines	28	20	4.07
PSB Rc18	PhilRice,Philippines	23	20	3.92

Table 5: Varieties used, their origin, amylose content, CT number of amylose allele, and RS content of retrograded rice.

Figure 30 shows the relationship between RS in retrograded rice and amylose content (Figure 30a), RS in retrograded rice and long chains of starch (Figure 30b), RS in retrograded rice and amylose allele (Figure 30c), and RS in retrograded and freshly cooked rice (Figure 29d). Amylose content correlates better with RS in retrograded rice (Figure 30a) than it does with RS in freshly cooked rice (Figure 19a), however, in a contradictory sense, the proportion of long chains shows no correlation with RS in retrograded rice (Figure 30b) or with amylose allele (Figure 30d). However, the amount of RS in freshly cooked rice correlates nicely with the amount of RS in retrograded rice (Figure 30c).

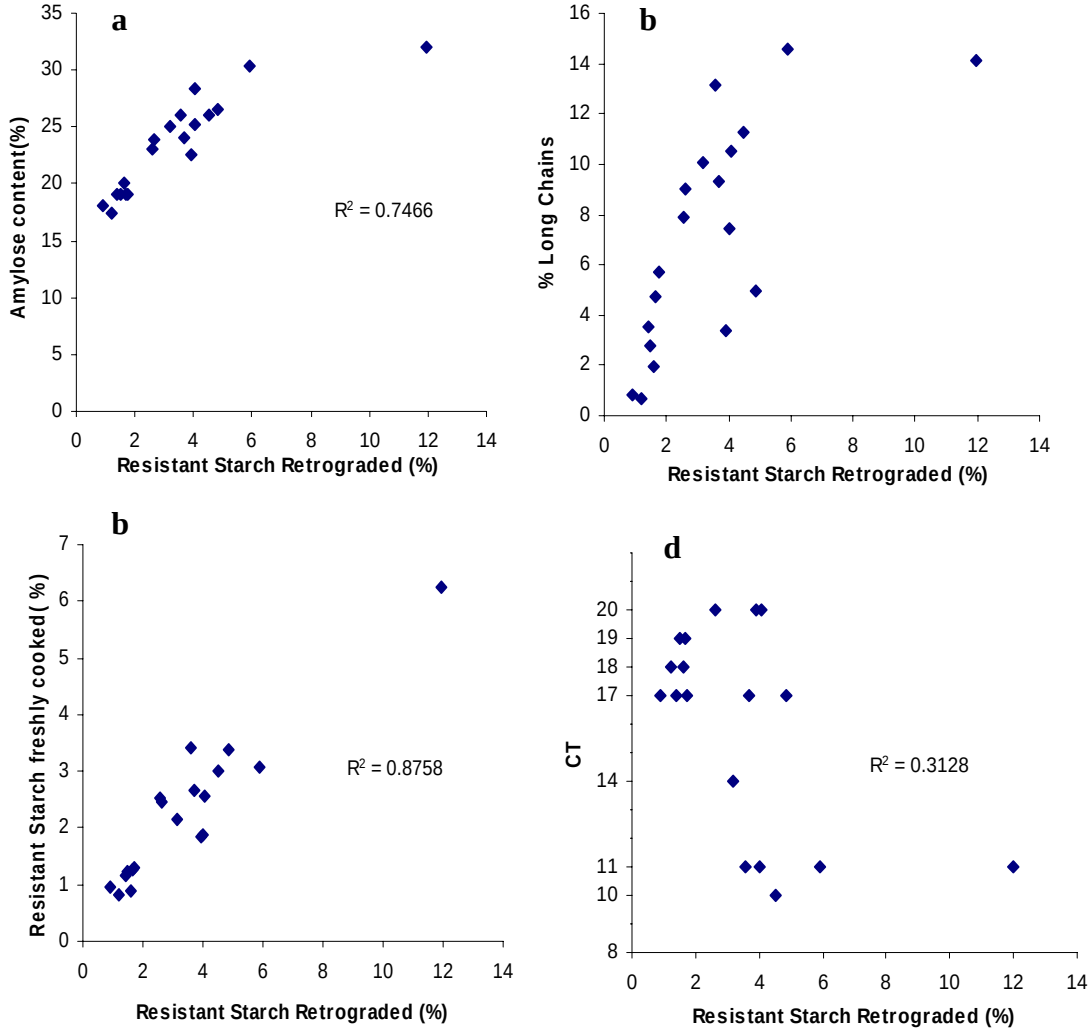

Fig.30: Correlation between RS from retrograded rice and amylose content (a), % long chains (b), resistant starch from freshly cooked rice (c) and CT number of GBSS1 (d).

The structure of RS in retrograded rice was determined by SEC of the RS molecules and of the debranched molecule. Figure 31a shows the chromatograms of the whole RS molecules from retrograded rice, and Figure 31b shows the chromatograms of the debranched RS from retrograded rice. Figure 31a shows that no chains elute where amylose chains elute, but the molecules elute in the region of amylopectin chains. Debranched RS from retrograded rice

shows that the molecules consist of chains of the order of amylopectin. The ae variety shows more RS when retrograded and its chains differ in distribution from the wild-type rices and Koshihikari grown in Australia shows much more RS when retrograded than does Koshihikari grown in Japan.

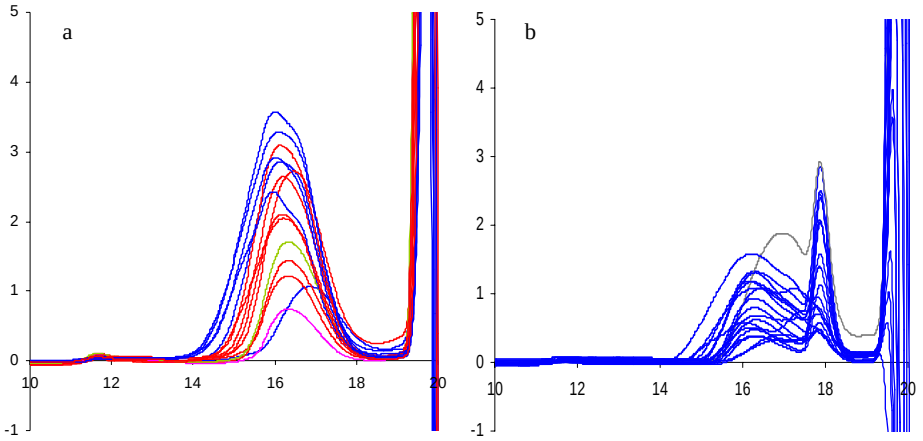

Figure 31: showing the SEC traces of RS from retrograded rice from Australian (red) and Asian rices (blue). Australian Koshi is green and Japanese Koshi is pink, and (b) debranched RS from retrograded rice. The trace from the ae is in grey in (b) and the highest in (a). Amylose elutes around 12 min

Figure 32 amalgamates the data from Figure 31b and from Figure 26b and shows the debranched RS molecules from freshly cooked rice and from retrograded rice. Figure 32 shows that there is more RS in retrograded rice, the RS molecules from retrograded rice elute earlier than those from freshly cooked rice, there is no difference between the amount of amylose chains (eluting at 12 min) in RS from freshly cooked and from retrograded, but there is a peak representing short chains (eluting at 17.5 – 18 min) in the RS molecules from all the retrograded samples but there is not a corresponding peak in the RS molecules from freshly cooked rice. Studies are ongoing investigating the chain length distribution of RS in retrograded rice using CE.

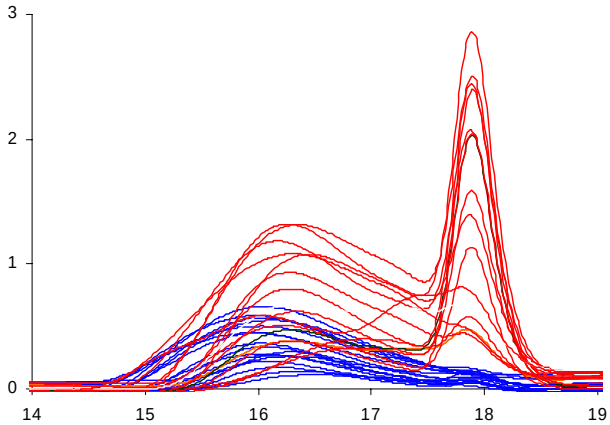

Figure 32: Debranched RS from Australian and Asian rices from freshly cooked (blue) and retrograded (red), showing a different distribution of chains that comprise each type of RS.

See Part C – Discussion etc.