RICE CRC

FINAL RESEARCH REPORT

ISBN 1 876903 41 4

P3402FR09-05

Title of Project :	Understanding amylose structure, what it controls and what controls it.
Project Reference number :	3402
Research Organisation Name :	Yanco Agricultural Institute, NSW Agriculture, and CSU Wagga Wagga
Principal Investigator Details :	
Name :	Melissa Fitzgerald and Christopher Blanchard
Address :	MF: YAI, PMB, Yanco, NSW 2703 CB: School of Food and Wine Science, CSU, PO Box 588, Wagga Wagga, 2678.
Telephone contact :	69 512 656 (current 63 2 580 5600 exn 5680) 69 332 364

TABLE OF CONTENTS

RICE CRC
Final report – Project 3402

Understanding amylose structure, what it controls and what controls it

1. Summary

Starch accounts for at least 92% (dry weight) of a milled rice grain. Starch is comprised of two fractions, amylose and amylopectin. Amylose content can range from 0% (in waxy rice) to about 30%. Amylose is essentially a linear molecule ranging from about 800 degrees of polymerization (DP) to about 10 000 DP. It carries a few widely spaced chains. Amylose plays a significant role in almost all of the cooking qualities of rice. The process of cooking of rice begins with the softening of the starch granules, which is primarily a function of amylopectin. The next process, swelling, is greatly affected by amylose. As the starch granules swell, amylose leaches from the granules into the solution phase. Behaviour observed in the field of synthetic polymer science suggests that the linear amylose molecules surround the swelling granules and inhibit the swelling. After amylose leaches from granules, it joins the continuous phase and van der Waal forces inside the helices of chains cause double helices to form. The double helices aggregate into a gel; the more double helices, the firmer the gel. The early stages of gel formation would occur in the interval between removing from heat and eating the rice. Long chains of amylose have a higher viscosity than short chains, and this limits the mobility of the long chains. Thus, with long chains, the formation of double helices and aggregations is slower, leading to a softer gel. Therefore, amylose structure could explain why two varieties with the same amylose content differ in cooked texture. In the later stages of gel formation, typically occurring well after cooking the rice, and when the temperature falls below 25 ºC, short chains of amylose will form double helices and crystallites much more readily than long chains of amylose. Therefore, rice that contains short chains of amylose are likely to be hard when cooled after cooking. The knowledge and information that could be provided by developing a method to measure amylose structure will provide a tool allowing greater insights into the effect of amylose structure on different cooking properties, with the ultimate aim of developing the knowledge into a selection tool for rice breeders.

After developing a tool to measure amylose structure, it was applied to understanding a particular nutritional property of rice, namely resistant starch. Literature and early research indicated some link between resistant starch content and amylose content, however, detailed investigations of the structure of resistant starch, hypothesised to reveal more of the secrets of amylose, in actuality, revealed some of the secrets of amylopectin.

2. Background

A rice grain consists only of starch (93 - 95%), protein (5-7%), lipid (0.5 – 1%), and minute amounts of other components like aromatic compounds, yet there is enormous variability in quality. The variability must lie in the processes of grain-filling that lead to structural differences in the grain, which, in turn, lead to perceptible differences in the sensory properties of the rice. The quality of cooked rice includes important traits like texture after cooking, stickiness, increase in hardness when consumed several hours after cooking (retrogradation), and satiety. Amylose accounts for as much as 30% of the starch, and amylose content contributes to most, if not all, of the cooking properties of rice. However, two varieties with the same amylose content do not necessarily have the same cooking properties. Furthermore, when the same variety is grown in different environments, the cooked rice from the different environments will have different properties. Amylose structure has often been cited as a likely explanation for the differences in cooking properties. Techniques were available last century for the sophisticated measurement of amylopectin structure, but not of amylose structure. The knowledge and information that could be provided by developing a method to measure amylose structure, correlating amylose structure with the properties of cooked rice, and understanding the control and heritability of amylose structure will allow the rice breeders to select for a particular structure of amylose. Informed selection for that trait will be a powerful tool that will hasten the development of new varieties with known properties. Increasing our capacity to guarantee quality will improve the reputation of Australian rice, assist in increasing our market-share, and thereby contribute to the economic sustainability of the NSW rice industry.

3. Objectives

The over-arching objectives of the project are:

- Understand some of the factors that control amylose structure at the genetic level;
- Develop a method to measure the structure of amylose;
- Understand how amylose structure contributes to selected cooking properties of rice.

4. Introduction

A single gene, Granule Bound Starch Synthase (GBSS1) (waxy) is known to synthesise amylose. Two alleles have been identified at the waxy locus – Wx^a and Wx^b. The alleles differ by a single base at the 5' end of the splice site of intron 1 and the substitution in the Wx^b allele decreases the splicing efficiency at that site, resulting in less transcript, protein and amylose. Temperate japonica rice predominantly carries the Wx^b allele, and so Australian rice varieties, being predominantly genetically temperate japonica, are expected to carry the Wx^b allele and be of low amylose. Amylose content does not explain cooking properties, thus it is timely to move beyond the gene and to search for allelic variation within the Wx^b allele and associate that with variation in structure and function.

During the course of the project, it was reported that the two waxy alleles in rice could be further divided by a microsatellite in the flanking region of exon 1 which correlated with amylose content. Subsequent collaboration with that group led to the classification of Australian rice breeding program on the basis of that microsatellite.

Little is known about the structure of amylose, and even less is known about the variability in structure across the species. Amylose is a polymer of glucose units, and in rice, can range in size from about degrees of polymerisation (DP) 800 to DP 10 000. Molecules of amylose are described as 'essentially linear'. They consist of a long back-bone which is connected to a small number of distally spaced branches. The branches are thought to range from DP 200 – 1000. It is likely that an isoform of the branching enzyme adds the branches. Branching enzyme 1 mutants show no difference in amylopectin structure in wheat and maize, suggesting either no or a minimal role in amylopectin synthesis or complete compensation by the other isoforms. Moreover, BE1 has shown a preference for inserting branches on long chains, suggesting that it might contribute to amylose synthesis and structure. Figure 1 shows a diagram of the structure of amylose. Linear, or essentially linear, synthetic polymers form gels much more easily than branched polymers, thus the processes of gel formation and entanglement of molecules are likely to be strongly associated with the amylose fraction of the starch rather than the amylopectin fraction. Application of knowledge from synthetic polymer chemistry suggests that different lengths of linear polymers and different branching structures affect the entanglement capacity of molecules, the speed which chains move through gels and thus the properties of the gel. Therefore, if we apply that knowledge to amylose, a biological polymer (essentially a chain), it becomes clear that we must delve deeper than content and investigate amylose structure. If variability exists in the chain length distribution, the variability will certainly contribute to our understanding of cooking properties.

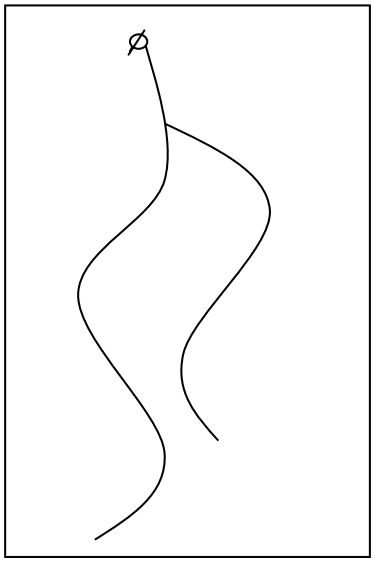


Fig. 1 Schematic Amylose

Amylose content significantly *contributes* to many of the cooking properties of the rice, but amylose content alone is not sufficient to *predict* the cooking properties of rice. Two varieties of the same amylose content can easily differ in cooking properties. For example, Koshihikari, Amaroo and Millin are all varieties of low amylose, but the texture of the cooked rice of each variety differs significantly, and the texture of the cooked and cooled rice (retrograded) also differs significantly. Retrogradation is an important parameter of quality for Australian rice and is likely to be influenced greatly by amylose, thus understanding the association between amylose structure and retrogradation of rice forms part of the second and third objectives of this project.

Quality encompasses physical, sensory and nutritional parameters. As the western world becomes aware of the lower rates of obesity and terminal illnesses in the eastern world where a rice-based diet prevails, understanding the effects of rice on physiological function, well-being, and health is becoming increasingly important. Amylose content is likely to influence retrogradation, which is an important sensory property, and retrogradation is linked to an important nutritional property – resistant starch. Resistant starch is that portion of starch which is not digested in the gut. Retrograded rice is recrystallised and resists digestion. Resistant starch moves into the colon where it is fermented by microflora, producing butyrate and other short-chain fatty acids which have beneficial effects on a number of physiological functions and bowel conditions.

Resistant starch has been classified into four categories: Type 1 is physically inaccessible to digestive enzymes; Type 2 is naturally-occurring granules; Type 3 is retrograded; and Type 4 is chemically modified starch. The third objective of this work is three-fold: to determine the relationship between amylose content, structure, GBSS allele, and resistant starch, to determine the molecule in rice that accounts for naturally occurring resistant starch, and to determine the resistant starch in several 'nationalities' of rice, of cultural and economic importance within the nations, and the effect of cooking and processing on the RS.

5. Methodology

Three varieties of rice were chosen for this study. They were Amaroo, Millin and Koshihikari. All are medium grain and temperate japonica. Amaroo and Millin were developed within the Australian Rice Improvement Program and Koshihikari is a Japanese variety grown in Australia. Amaroo is descended from Californian germplasm, Millin is a cross between a Californian variety and a traditional Japanese variety, and Koshihikari was selected on-farm from a traditional variety. These three rices vary greatly in their cooking properties.

Objective 1: Searching for allelic variation in the GBSS gene

Primers were designed for each exon of the GBSS 1 gene in the three varieties, Amaroo, Millin and Koshihikari. The exons were amplified by PCR and the product was sequenced. Sequences of each exon from each variety were compared to search for variation within the exons that could alter the amino acid sequence and subsequently functionality of the active sites of the enzyme. This work was carried out at Charles Sturt University. Primers were also designed for exons of branching enzyme (BE) 1 and the PCR products of those assays were analysed by gel electrophoresis and any differences in size were sequenced to determine the genetic difference.

For identification of the microsatellite in each variety, DNA was prepared from brown rice, PCR was carried out, polyacrylamide gel electrophoresis was used to separate the fragments and fluorescence was used to visualise the bands. In order to confirm that the three varieties Amaroo, Millin and Koshihikari were temperate japonica in terms of their waxy allele, ie carried the T at the splice site of exon 1, DNA was incubated with the restriction enzyme AccI1 and products were separated on an agarose gel.

Objective 2: A method to measure amylose structure

In order to measure amylose structure, it must be separated from amylopectin. It is well known that amylose leaches from starch granules in hot water (tsai). The method was optimised to maximise the amount of amylose and minimise the amount of amylopectin in the soluble fraction. Different methods of heating, different temperatures and different speeds of centrifugation were tested. The optimised method was used for all further work. The Rapid Visco Analyser was used to solubilise the amylose. Flour (250 mg) was mixed with water (25 ml) and the standard RVA profile was run. After the run, the contents of the canister were centrifuged (10000 g, 10 min). The supernatant contained the hot water soluble (HWS) starch. HWS starch was separated using size exclusion chromatography (SEC). SEC was carried out using a Waters system consisting of an Alliance (2695) and Differential Refractive Index Detector (Waters 2410). Waters software (Empower®) was used to control the pump, and to acquire and process the data. The eluant was ammonium acetate (0.05 M, pH 5.2) flowing at 0.5 ml min^{-1}. Two columns, an Ultrahydrogel 250 (UH 250) and an Ultrahydrogel 500 (UH

500), both from Waters, were tested to determine the range of molecular weights separated. Each column was used independently, and columns were held at 60°C. To determine the range of molecular weights separated by each column, pullulan standards ranging in molecular weight from equivalent to short amylopectin to long amylose chains were injected into each column.

In SEC, molecules are separated either on the basis of their hydrodynamic volume (Vh), which is defined by the shape of each molecule in solution, or (if they are all linear chains) on the basis of the molecular weight distribution (MWD) of the chains.

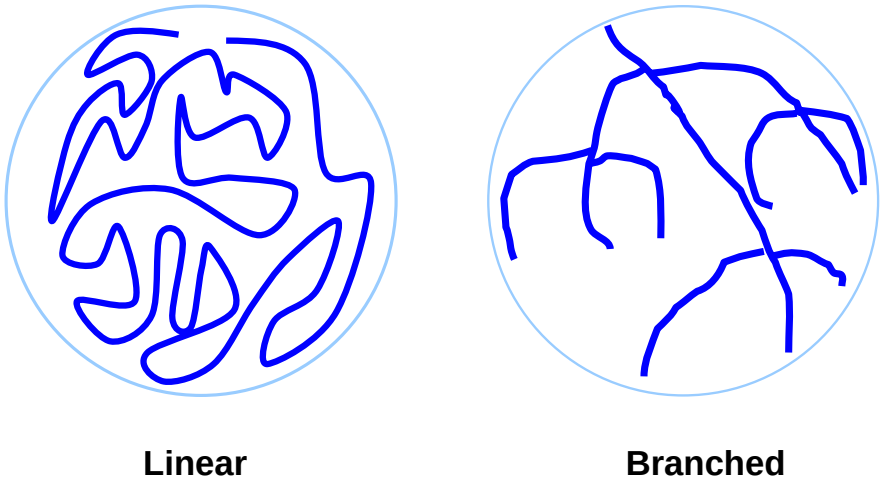


Fig 2: Two molecules of the same volume clearly differ in molecular weight

In SEC the elution time of a molecule, whether it be linear or branched, is a direct function of its size in the eluant, and not its molecular weight. Thus each elution slice of the trace could contain molecules of the same shape, but not of the same MW (Figure 2). In order to understand the relationship between molecules of the same hydrodynamic volume but different structure, HWS starch from each variety was fractionated by eluting with ammonium acetate (0.05 M) from a column (20 x 5 cm) packed with Sepharose 6B. Each fraction was collected for 2 min and the presence of starch in each fraction was determined by addition of a drop of iodine solution to each fraction. Collection was terminated when fractions no longer continued to bind iodine. Fractions were analysed by SEC on the UH 500 column (as described earlier). Linear chains were determined in each fraction by digesting each fraction with iso-amylase (Megazyme), which hydrolyses the α1-6 linkages specifically. This enzyme was used to debranch starch in each fraction. For digestion with isoamylase, aliquots of HWS starch were mixed with sodium acetate buffer (pH 4, 0.5 mM), and then isoamylase was added (7 ul). Samples were incubated (50ºC, 2h). After incubation, samples were boiled and centrifuged as described above. Samples from both enzyme digestions were desalted using mixed bed resin (Biorad) for one hour. Fractions were analysed by SEC (as described above) before and after incubation with each enzyme. After digestion with beta amylase, if no chains remain in a particular fraction, then all molecules in that fraction were linear. If debranching does not increase the elution time of a particular fraction, then all chains in that fraction are similar and of similar length to the starch before debranching. .

SEC with a mass-sensitive detector, like refractive index, does not give the number of chains directly, but the weight average of the chains (w(log M) (Castro et al. 2005) – the bigger the molecule, the more the detector detects it, thus signal does not equate to molecular weight. The number of chains (P) of a particular molecular weight (M) can be determined by detecting each chain once, with ultraviolet or fluorescence detection (Castro et al. 2005). In order to use UV or fluorescence to detect the number of chains, it is necessary to label each chain only once with either a UV absorbing chromophore or a fluorophore. Chains of amylopectin have been successfully labelled and quantified using reductive amination with the fluorophore 8-amino 1, 3, 6, trisulphonic acid (APTS) (O'Shea and Morell), but not chains of amylose. Takeda et al. have attempted to measure amylose with other fluorophores, but problems are cost of the fluorophore, toxicity, co-elution of the label and the starch, and confidence with labelling efficiency being independent of molecular weight. In this project several chromophores and fluorophores were analysed for their capacity to measure the number of chains of amylose of each molecular weight. Attempts were made to separate the label and the starch to overcome the problem of co-elution. Various ion exchange resins and spin columns were used to bind the ions, and the best was incubation for 1h with mixed bed resin (Biorad). SEC conditions were also manipulated to separate the peak of excess label from peaks of interest. Labelling efficiency was tested by utilising the simple relationship of $w(\log M) = M^2P(M)$ by labelling and analysing a range of standards of known molecular weight (M) using inline mass–sensitive detection (w(log M)) and fluorescence/UV detection (P(M)). The signal obtained by the UV/fluorescence detector was then expressed as w(log M) by multiplication of the UV/fluorescence signal by M^2 and the mathematically treated signal compared with the signal from the mass sensitive detection. Fluorophores and chromophores were all sulphonic acids because these can be attached to the reducing end of the starch molecule by reductive amination – the only way to label starch.

Reductive amination was carried out as described by O'Shea and Morell (1998). SEC was carried out as described above, but with the additional detector, either fluorescence or UV, before the RI detector.

Objective 3: Relationship between amylose and resistant starch

** Resistant Starch (RS)*

The objective of this work is three-fold. First, is to determine the relationship between amylose and resistant starch, second is to determine the molecule in rice that accounts for naturally occurring resistant starch, and third to determine the resistant starch in several 'nationalities' of rice, of cultural and economic importance within those nations, and the effect of cooking and processing on RS.

Three sample sets were used for this work. The first was Australian rice, the second was rice from the US, and the third was rice from Asia. The Asian collection included the amylose extender mutant of IR36. The amylose extender mutant is that used commercially in RS applications. Table 2 shows the varieties used and the origin of them. Australian rices were received freshly milled from SunRice, NSW Australia, the US rice was grown in buffer rows at the Beaumont Experiment Station of the USDA in Beaumont, Texas US, and the Asian rice was grown either at the International Rice Research Institute (IRRI) in the Philippines, or at PhilRice, also in the Philippines. Rice grown in Australia, or at IRRI or PhilRice was

dehulled with a Satake dehuller (THU35A 250V 50Hz Test Husker, Satake), and milled for 60 sec (Magill No.2). Rice grown in the US was received as milled.

Resistant Starch (RS) was measured on the different samples of freshly cooked or processed rice using the Resistant Starch Assay (Megazyme) (AACC Method 32-40). Figure 3 shows the steps of the method. Briefly, sample (0.5 g) was incubated with amyloglucosidase (AMG) (4 ml, $xUml^{-1}$) and pancreatic alpha-amylase (10 $mgml^{-1}$) for 16 hours at 37°C to reduce digestible starch to glucose. The reaction was terminated with 4 mL ethanol and the RS pellet was recovered by centrifugation (5000 g, 10 min). The supernatant was decanted and the washing and decanting procedure was repeated with ethanol (50% v/v). The pellet was solubilised in KOH (2 ml, 2 M) in an ice bath, neutralised with sodium acetate (8 ml, 1.2 M) and the RS hydrolyzed to glucose with of AMG (0.1 ml, 3300 Uml^{-1}, 50°C). The glucose oxidase/peroxidase reaction (GOPOD) was used to measure glucose. Absorbance was read (510 nm) (GBC UV/VIS918) after a 20 minute incubation period at 50°C. RS was calculated as a percentage of dry weight of sample. The efficiency of the method was determined by analysing the amount of starch in both supernatants and both pellets.

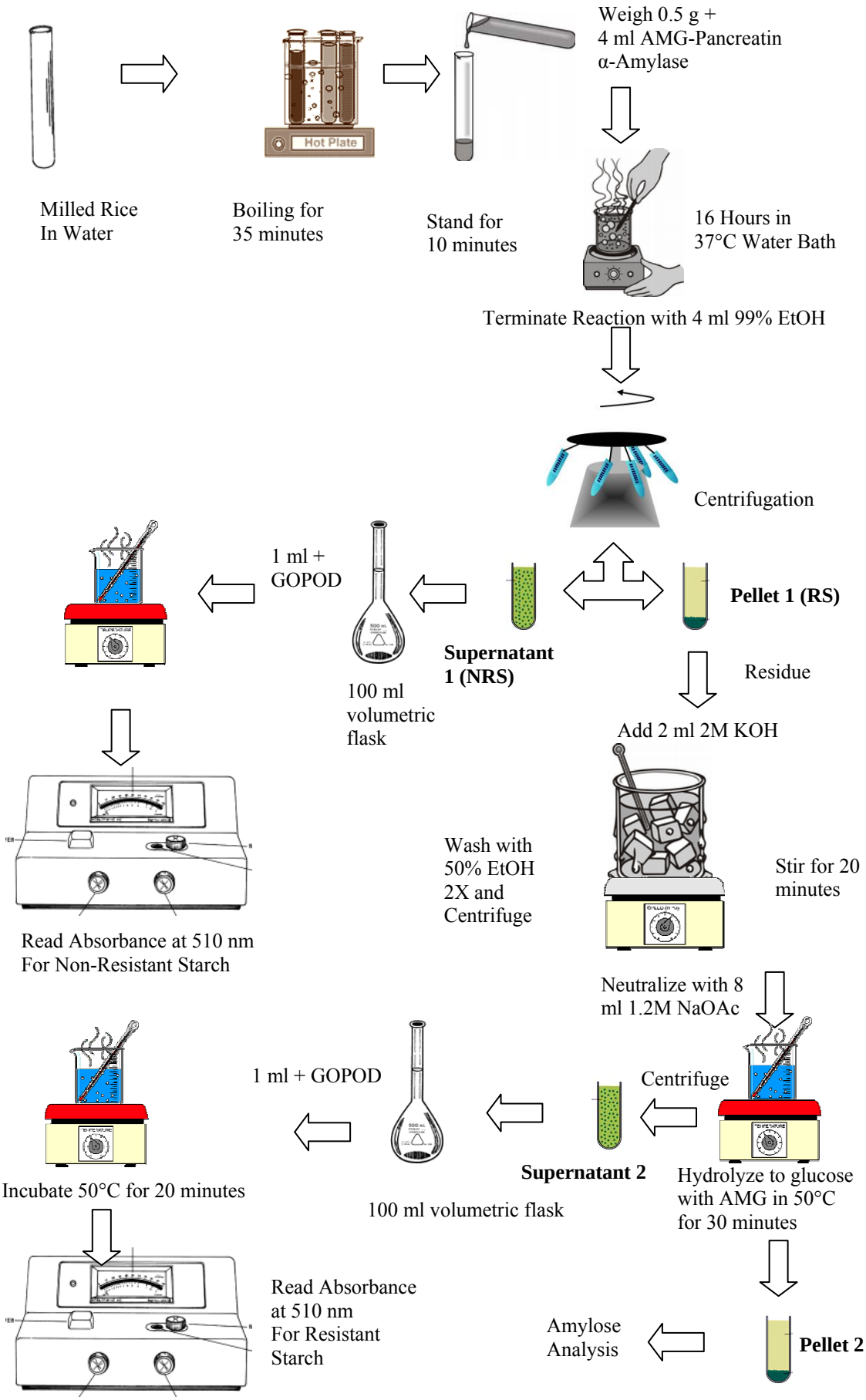
The relationship between resistant starch, amylose content, solubility and amylose allele were also determined. Amylose content was measured by iodine binding (Blakeney et al 1994). Solubility was measured as described earlier, proportion of long chains was determined by debranching gelatinised flour and determining the proportion of chains greater than DP 100 by SEC (described earlier), and genetic variation at the GBSS locus was measured by determining polymorphism in the microsatellite in the flanking region of GBSS using the simplified method described by Bergman et al. (2000). In brief, DNA was extracted in NaOH and the neutralised DNA subjected to PCR using published primers (W484 and W485), and the PCR products were separated with 8% acrylamide gel, and visualised by staining with GelStar and visualising on a Dark Reader.

In order to determine the structure of RS, the RS was solubilised and neutralised (for all varieties) according to the RS assay, but it was not digested to glucose. Instead, it was incubated with mixed bed resin to absorb the acetate ions (Biorad AG 501-X8(D) Resin) and an aliquot of the sample (40 µl) was injected directly into the UH 500 column for SEC analysis (conditions as described earlier). Another aliquot was debranched (as described earlier) and the debranched RS was analysed by SEC. The debranched material was also labelled and the chain length distribution analysed by Capillary Electrophoresis (CE). Labelling was done by reductive amination of the reducing end and attaching a molecule of 8 amino 1,3 6, trisulphonic acid to each reducing end. Labelled chains were separated by CE as described by O'Shea and Morell.

The effect of several processing methods on resistant starch was measured using only the variety Doongara as an example of the way cooking and processing affected RS. Doongara was chosen because of its intermediate amylose, a trait common to the 3 sets of rice. Resistant Starch was measured on freshly cooked brown and white rice, rice cooked by rapid boil or absorption, under- and over-cooked rice, mashed or sieved cooked rice (to simulate chewing a little or chewing a lot), storing (retrogradation) for 1, 2 or 8 days, and freshly cooked and retrograded gels.

The three sets of rice were not analysed simultaneously, and so different data sets were collected for each set, though some data sets are common to all three.

Fig. 3. Resistant-Starch Determination by Megazyme Method



6. Results

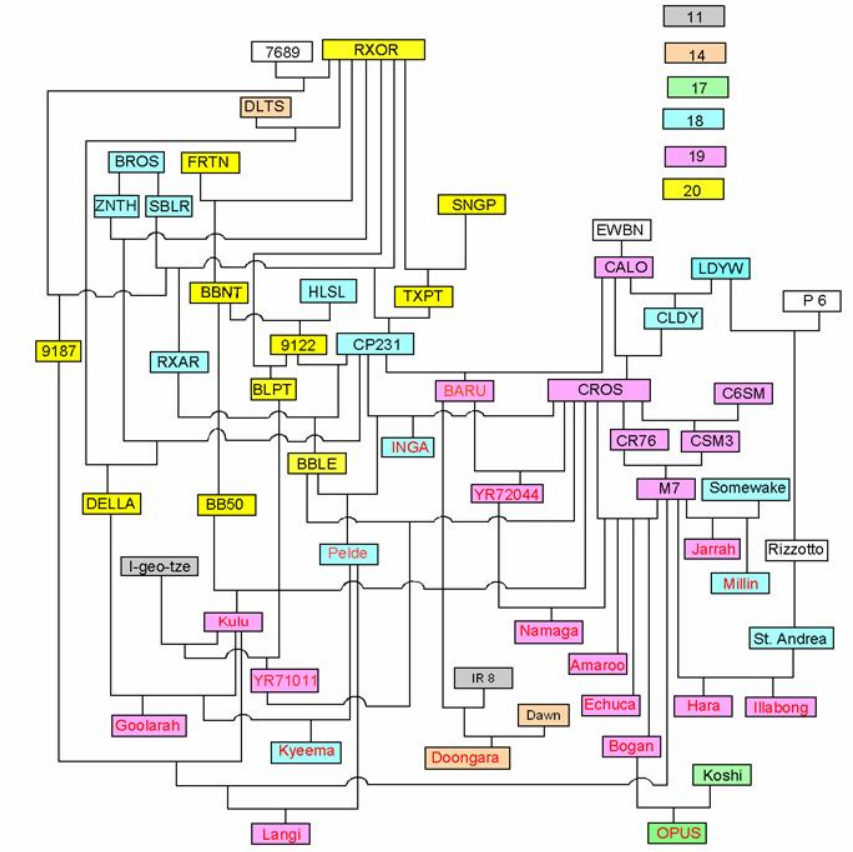


Fig. 4. CT repeat in Aust pedigrees

Objective 1: Searching for allelic variation in the GBSS1 gene

Primers were designed around each of the exons of GBSS1. Exons of GBSS1 were amplified and sequenced from Millin, Koshihikari and Amaroo. No difference in the DNA sequence was found for any of the exons for these three rices. During planning of the next stage, a group in the US, led by Dr Bill Park, published data describing a polymorphic microsatellite in the flanking region of GBSS1 showing how it was inherited throughout the US germplasm and how it segregates with amylose content. Collaboration was developed with the group which led to determining the microsatellite variability in the Australian germplasm. Figure 4 shows polymorphism of the microsatellite throughout the Australian germplasm. Figure 4 shows that the 3 varieties chosen for the difference in their cooking properties, Amaroo, Millin and Koshihikari, all carry a different polymorph-ism of the gene (microsatellite). Amaroo carries the microsatellite that descends from the Californian stock (CT19), Millin from the low-quality, but quite cold-resistant, traditional variety Somewake (CT18), and Koshihikari has CT17, for which the origin is unknown. Efforts examining expression patterns of the genes showed no differences. No difference was found between the three varieties for the sequence in the exons of the Branching Enzyme 1.

Objective 2a: A method to measure amylose structure

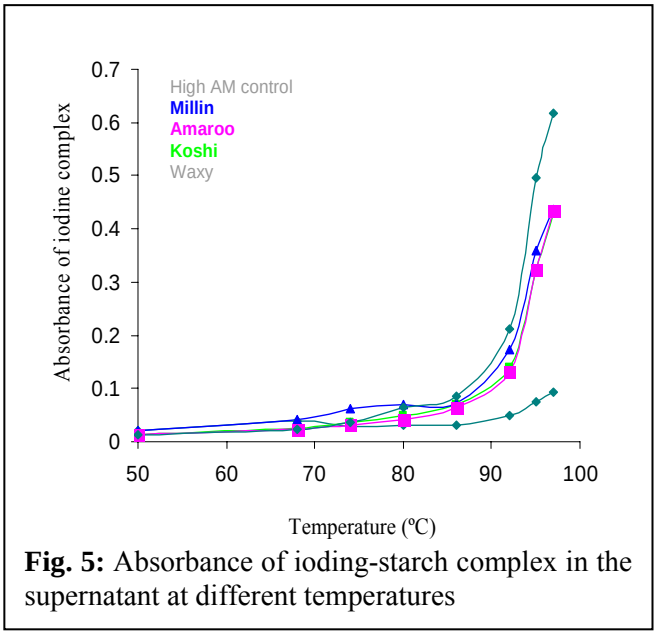


Fig. 5: Absorbance of ioding-starch complex in the supernatant at different temperatures

Separation of amylose from amylopectin was achieved by using a low concentration of flour in water and heating with RVA. The low concentration ensured that the sample was not subjected to any shear. The RVA was stopped at several temperatures between 50 and 97ºC to determine the temperature at which amylose was released from the starch granules. Amylose was determined by iodine binding. Figure 5 shows that amylose was not released until 95ºC. A waxy variety shows that a small amount of amylopectin was released after gelatinisation and again after 95ºC (Figure 5). However, much higher absorbance was noted for the non- waxy varieties, and higher for Doongara (Figure 5), which was included as a positive control due to its higher amylose content.

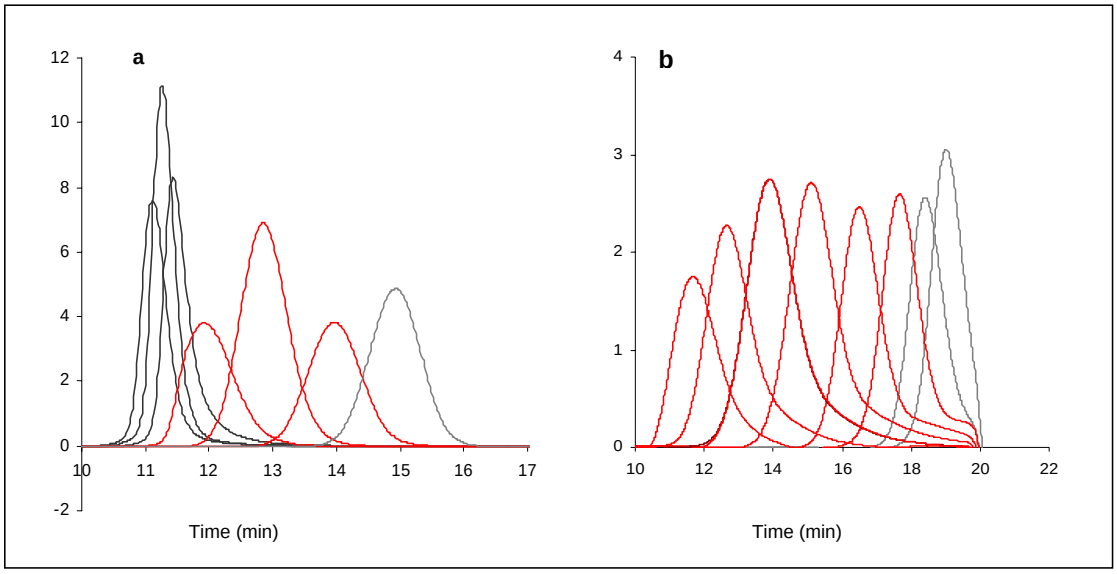


Fig. 6: Separation of pullulan standards on the UH 250 column (a) and the UH500 column (b). Grey curves are either unseparated in the exclusion zone or too small to be within the accepted range of molecular weight for amylose.

Two SEC columns were tested to determine the suitability of use for separating amylose.Both were tested by injection of pullulan standards ranging in molecular weight. A pullulan standard of the same molecular weight as starch will not have exactly the same hydrodynamic volume, so will not elute at the same time as the starch of equivalent MW. However, since pullulan is glucose linked with similar bonding as starch, it was expected that elution times would be close to those of starch of equivalent MW.

Table 1 shows the molecular weight of the pullulan standards, and the equivalent degrees of polymerisation if the standard was starch. The separation of the pullulan standards on two different columns. The first column is of small pore size and the higher molecular weight standards, those corresponding closely to the range of molecular weights found for amylose, are not separated (Figure 6a dark grey). Chains less than DP 1000 elute in the separating phase of the column, and chains of the order of amylopectin chains (the lowest standard) elute towards the end of the separating phase of the column. Figure 6b shows the standards separated on a column with larger pores (UH 500). On this column, the higher molecular weight standards are nicely separated, but the two smallest standards are less well separated. Figure 7 is a calibration curve of elution time vs log M and shows that the relationship is linear, and that the slope alters for the last two, less well separated, standards. Since the UH500 seemed better able to separate chains of the order of amylose (Figure 6b and Table 1), that column was used to determine whether or not amylopectin contributed to the HWS starch and if amylose contributes to the HW insoluble starch.

MW std	DP equivalent of starch
5900	36.4
11800	72.8
22800	140.7
47300	292.0
112000	691.4
212000	1308.6
404000	2493.8
788999	4864.2

Table 1: MW of standards and DP of starch at each MW. The first two are too small to equate to amylose

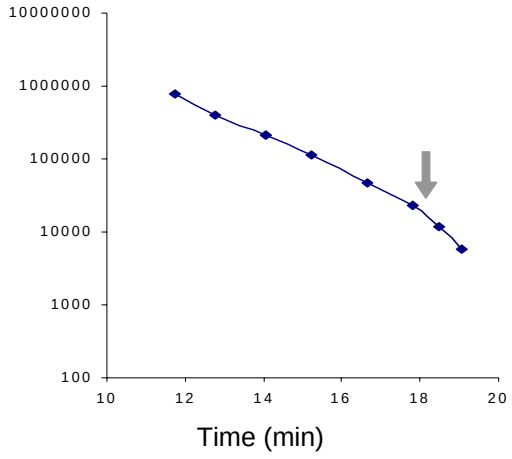


Fig.7: Calibration curve for pullulan standards on the UH500 column showing linearity. Change in slope (arrow) suggests poorer separation

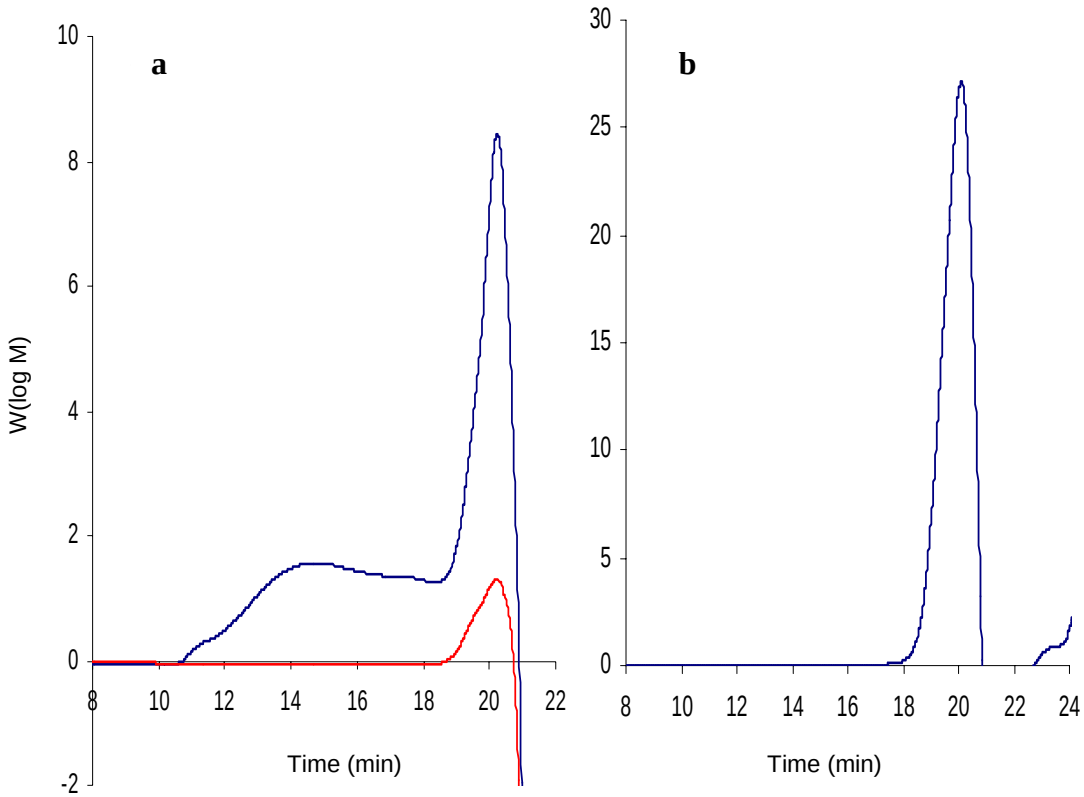


Fig. 8: Debranched HWSS (blue) and pellet (red) of a non-waxy variety (a) and debranched flour of a waxy variety (b) run on the UH500 showing separation of amylose and amylopectin chains, and complete solubilisation of amylose

Figure 8a shows debranched HWS starch and HW insoluble starch of a non-waxy variety and Figure 8b shows debranched gelatinised flour of a waxy variety for reference for elution of amylopectin chains. Figure 8b, the waxy variety, shows that amylopectin chains are not separated on the column and that they elute after 18 minutes, after total permeation of the column. No chains were detected before 18 minutes. Amylopectin chains all eluted in the region where the two smallest pullulan standards eluted (Figure 6b) on the UH500. The debranched HWS starch (Figure 8a) contains many chains that eluted after 18 minutes, as well as chains that eluted between 10.2 and 18 minutes - the entire separation range of the UH 500, and over the same range as the largest six pullulan standards. However, coelution with pullulan standards does not mean that the amylose chains span the same MW range as the pullulan standards. The pullulan standards contain both α1-4 and α1,6 linkages whereas the amylose chains are linked only by α1, 4 bonds. Thus we can imagine that the amylose chains form much tighter coils than the pullulan chains, and so for the same hydrodynamic volume, it is likely that the MW of an amylose chain would be somewhat higher than that of a pullulan chain. The HW insoluble fraction did not contain any chains that eluted before 18 minutes and all chains in that fraction eluted after 18 minutes (Figure 8b).

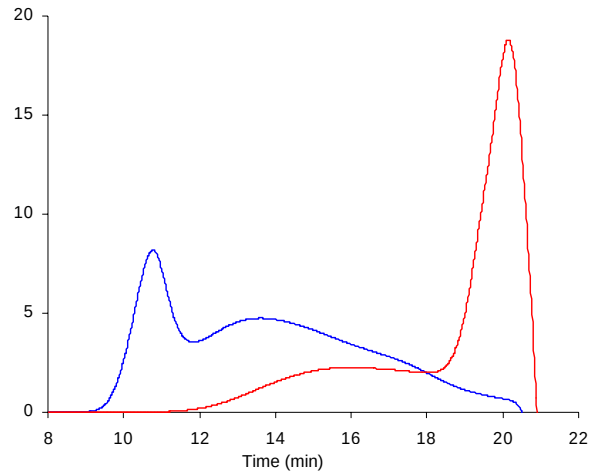


Fig. 9: HWS starch from a non-waxy variety (blue) and debranched HWS starch from the same sample.

Figure 9 shows the HWSS injected into the UH 500 before and after debranching. Very few chains eluted after 18 minutes in the sample that was not debranched, but there was a large peak in the exclusion volume of the column, before 10.5 minutes. The peak in the exclusion volume is material that could not penetrate the pores of the column, so is, by definition, of very high molecular weight. Upon debranching of the HWSS, the peak in the exclusion volume disappeared, and a new peak at the end of the chromatogram appeared. Further, there is a significant difference in the signal between 11 and 18 min between the HWSS and the debranched HWSS; the signal was lower for the debranched HWSS.

Different speeds and different lengths of time of centrifugation were tested to attempt to minimise the contribution of amylopectin to the HWS fraction. Figure 10a shows HWS starch from a waxy variety at three different spinning speeds and Figure 10b shows HWS starch from a non-waxy rice. Speed of spinning or length of time of spinning did not give any difference in the size of the peak in the exclusion volume (Figure 10) or the amount of amylopectin in the soluble fraction of the waxy variety (Figure 10a).

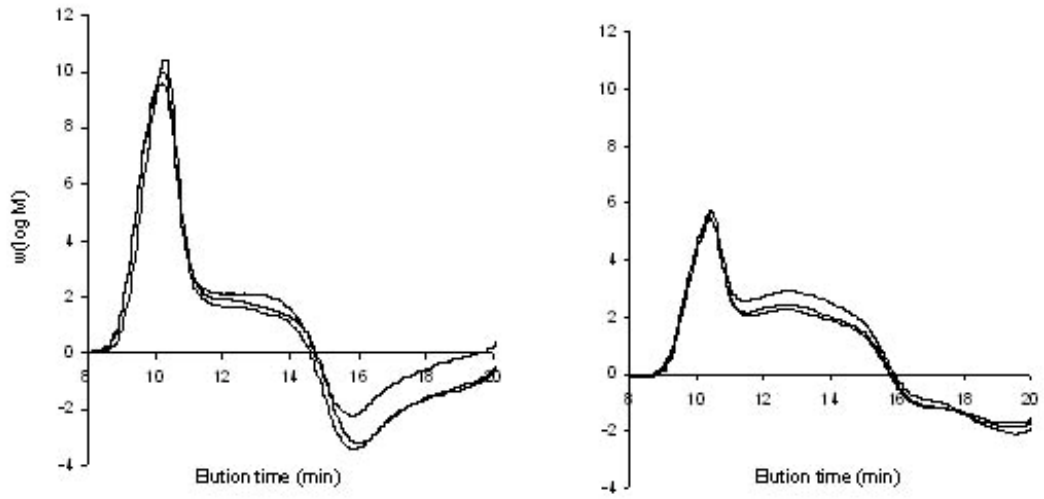


Fig. 10: Three different centrifugation conditions did not remove or change the amount of the HWS amylopectin from either a waxy variety (a) or a non-waxy variety (b).

HWS starch for all further work was collected from flour using 250 mg flour in 25 ml water with heating and mixing in the RVA, followed by centrifugation at 10000 g.

Determining the number of molecules in the amylose of HWSS was attempted using several fluorophores and chromophores. We were unable to reproduce data shown in the literature using the same chromophores (2AP and APTS) for amylose labelling (data not shown). Further, 2AP and APTS are expensive, so very dilute samples were used to try to maximise the utility of the label. Chromatography was poor with these labels since the signal from a large peak of unattached fluorophore swamped the chromatogram. Several methods were used to clean up the sample before chromatography, and all were abandoned because the cost per sample was approaching $15 with label and clean-up. Another sulphonic acid, the chromophore ANDS, was discovered towards the end of the project. ANDS is negatively charged, contains sulphonic acid groups, so is suitable as a label to be attached to the reducing end of amylose chains using the same chemistry as the fluorophores. It required no clean-up and each sample cost only 4c to label. Efficiency of labelling across the MW range was measured by labelling the pullulan standards and analysing them with both RI and UV.

Figure 11 shows the chromatogram of these and shows that the UV trace is not equivalent to the RI trace. By using the simple relationship described earlier, the UV data can be mathematically transformed and plotted as if it was RI data. Figure 12 shows the replotted UV trace as RI data.

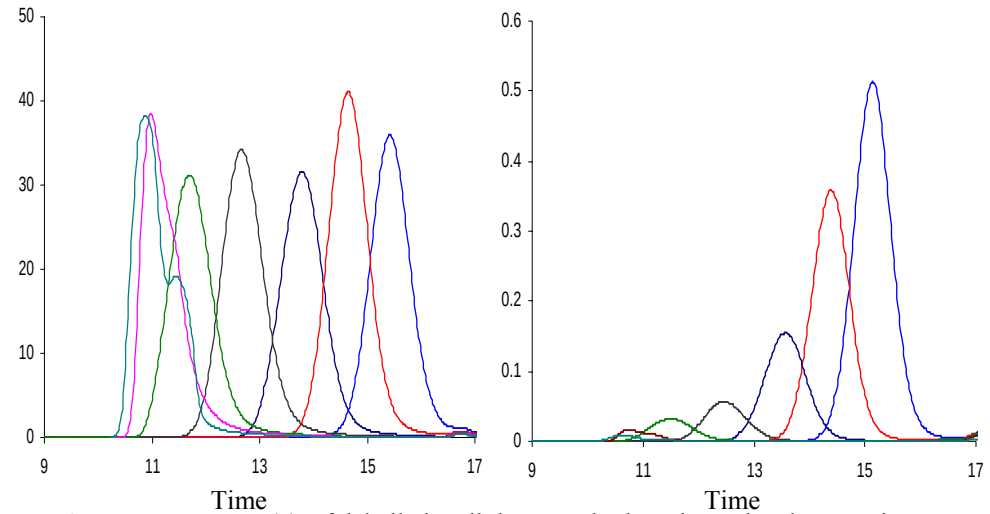


Fig. 11: RI trace (a) of labelled pullulan standards, where the detector is mass-sensitive not label-sensitive, and (b) the UV trace of the same labelled standards where only the label, not mass, is detected.

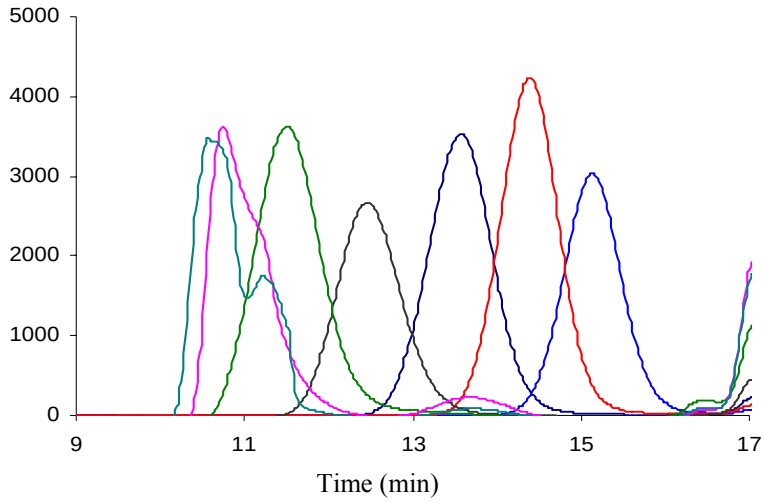


Fig.12: Mathematical transformation of the UV trace in Figure 11b to make it equivalent to the RI trace in Figure 11 a.

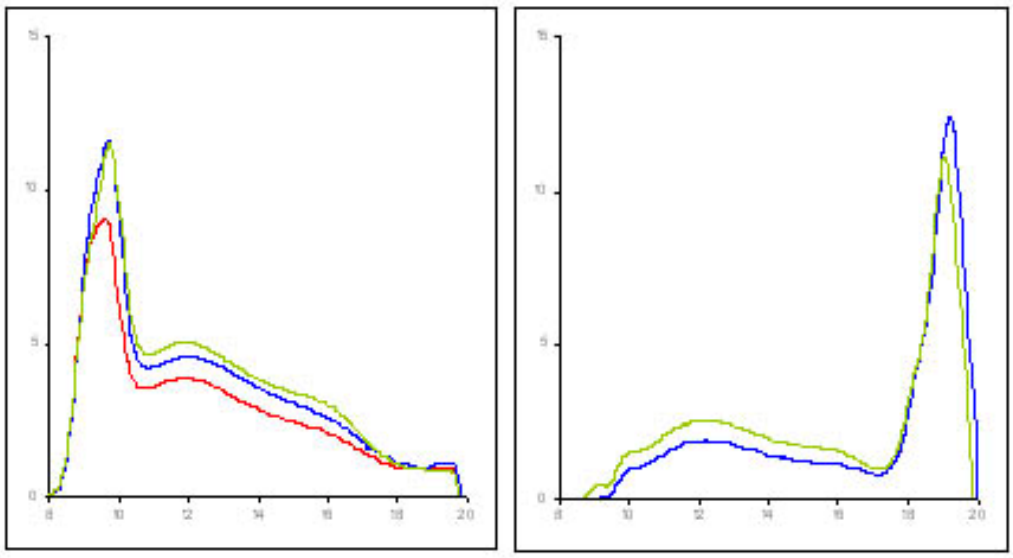


Fig.13: Separation of HWS starch (a) from Amaroo, Millin and Koshihikari, and debranched HWS starch (b) showing slight differences between varieties.

Objective 2b: Amylose structure of three different varieties of the same amylose content

Figure 13 shows the SEC traces for HWS starch and debranched HWS starch (Figure 13b) for Millin, Amaroo and Koshihikari. There are differences in the distribution of the HWS starch and the MWD of the debranched amylose chains in the debranched starch. These differences were further investigated by fractionating the HWS starch. Figure 14 shows the iodine staining of each fraction (Amaroo). The intensity of the blue of the iodine staining shows clearly that fractions 7 – 24 contained amylose, and the green fractions 25 – 30 contained just a small amount of starch. Figure 15 shows the hydrodynamic volume distribution of the fractions of Amaroo. In Figure 15, fractions are grouped on the basis of elution pattern and show a group of early eluting fractions (red) and a group of fractions eluting in the middle of the run (blue), and a group that eluted towards the end of the run (green) molecules. For the

three varieties, each fraction was analysed in terms of its hydrodynamic volume and the proportion of linear chains. Figure 16 shows the hydrodynamic volume distribution of the representative fractions of the three varieties. Figure 17 shows the molecular weight distribution of the debranched chains of each of the fractions for the three varieties.

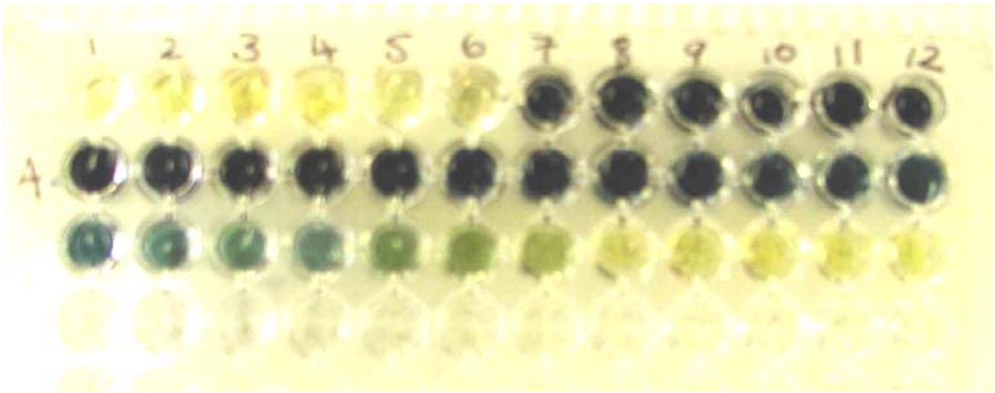


Fig.14: Iodine staining of fractions of HWS starch from Amaroo, each fraction collected for 2 min. Iodine binding, indicating the presence of amylose, commenced by fraction 7 and disappeared by fraction 28.

Figure 16 shows the hydrodynamic volume distribution for the fractions from each variety. For each group of fractions, early, mid and late, each variety shows a different elution profile. Basically there are two peaks: before 10 min (peak 1) and after 10 min (peak 2). The early fractions of Millin show much more starch in Peak 1 than the early fractions of the other two varieties. The early fractions (first column in Figure 16) of Amaroo show a wider distribution in Peak 2 than the other two varieties. The mid fractions (middle column Figure 16) of Koshihikari and Millin show a bimodal distribution for Peak 2, a shoulder at about 15 minutes, whereas the mid-fractions of Amaroo show a normal distribution for Peak 2 with the peak eluting between 10 and 20 min, 2 - 4 minutes longer than for the other two varieties. The late fractions (third column of Figure 16) of Amaroo and Millin show similar distributions, with most starch in the last shoulder of the peak (15 – 20 min), but those for Koshihikari showed least starch in the last shoulder of the peak (Figure 16, column 3).

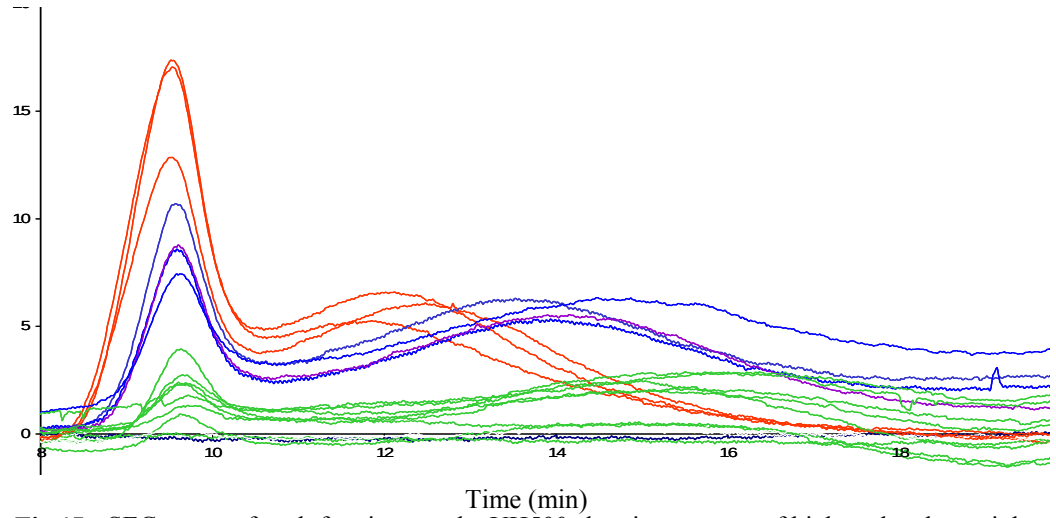


Fig.15: SEC traces of each fraction on the UH500 showing a group of high molecular weight chains (red), intermediate molecular weight chains (blue), and low molecular weight chains (green).

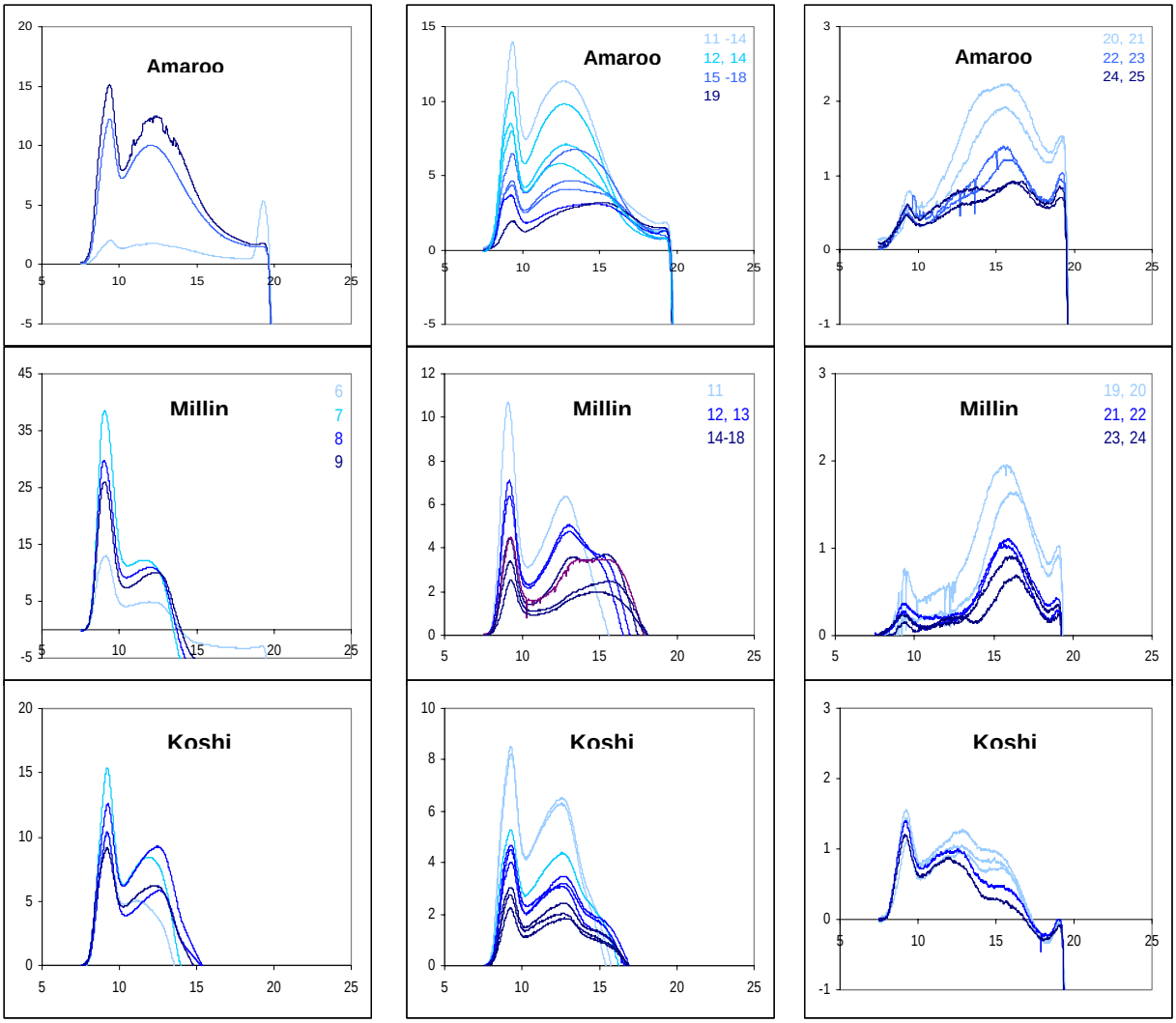


Fig.16 showing the SEC traces of each group of fractions for each variety on the UH500. High molecular weight fractions are column 1, intermediate molecular weight fractions are column 2 and low molecular weight fractions are column 3.

Comparison between Figures 16 (HWS) and 17 (debranched) shows that for every fraction, for each variety, the starch eluting before 10 min, Peak 1, in Fig. 16 was always almost fully eliminated after debranching with isoamylase, and a new, very big peak begins at about 17 min for each (Figure 17). The large peak beginning at 17 min (Figure 17) is not shown within the scale of the figure. Figure 17 shows shows that for the early fractions, peak 2 elutes between 10 and 15 minutes, for the mid fractions, closer to 15 minutes and for the late fractions, after 15 minutes. Figure 17 shows that Peak 2 for Millin is twice as large (compare y axis) as it is for the other two varieties for the early, mid and late fractions.

Figure 18 shows the grains of the three varieties, and shows only small differences between the three, that are probably not distinguishable.

Individual amylose chains were not labelled successfully during the time that this portion of the work was carried out.

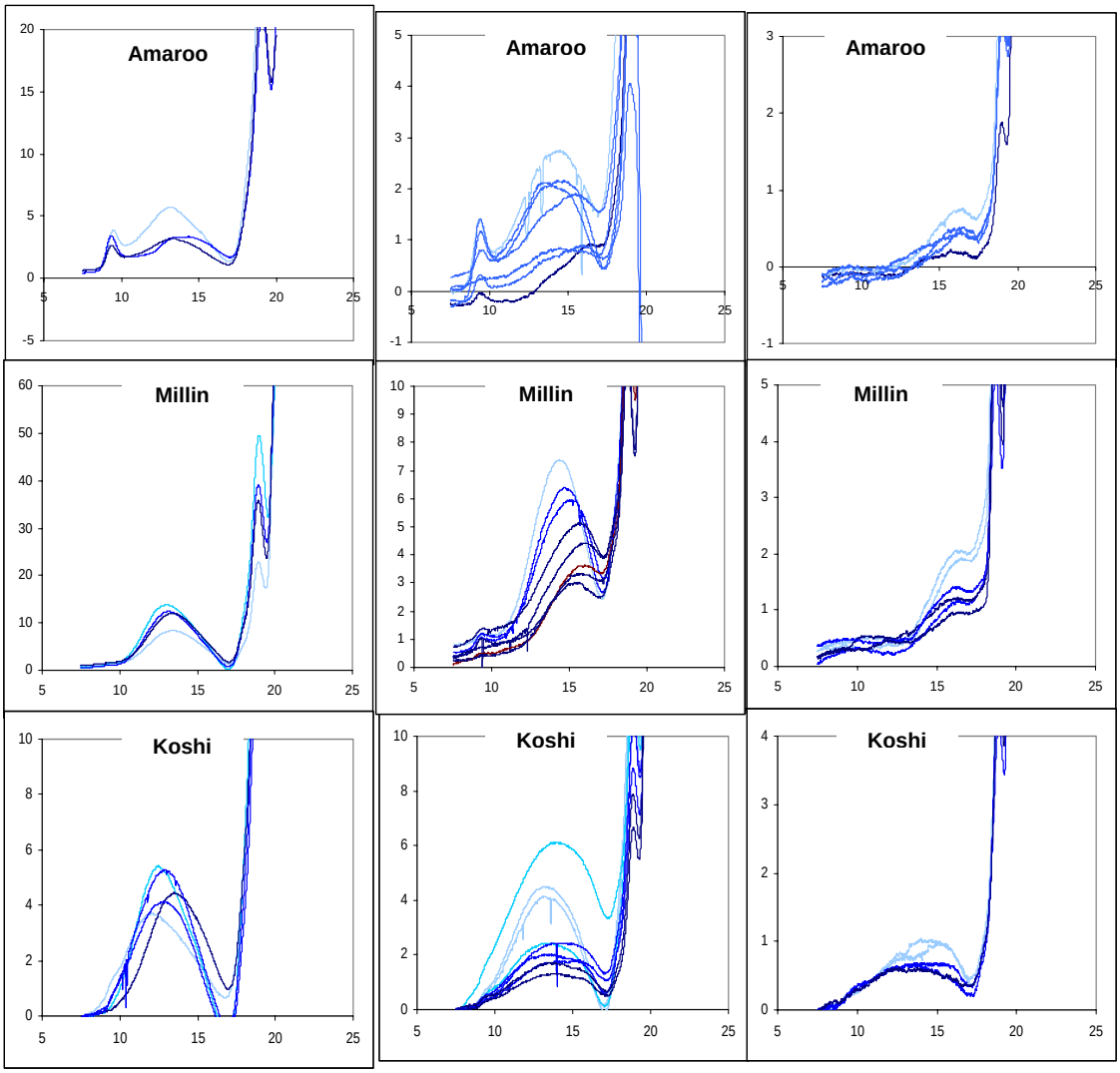


Fig.17 showing the SEC traces of each group of fractions for each variety on the UH500 after debranching. High molecular weight fractions are column 1, intermediate molecular weight fractions are column 2 and low molecular weight fractions are column 3.

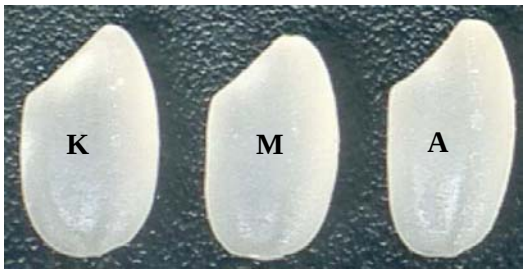


Fig.18: Grains of Koshihikari, Millin and Amaroo showing the similiarity of each.

See Part B – commencing Objective 3: Resistant Starch