

Date project completed:

Research and Development  
**Final Project Report**  
 (Not to be used for LINK projects)

**Section 1 : Identification sheet**

1. (a) MAFF Project Code

(b) Project Title

(c) MAFF Project Officer

(d) Name and address of contractor

(e) Contractor's Project Officer

(f) Project start date  Project end date

(g) Final year costs:

|                             |                      |
|-----------------------------|----------------------|
| <b>approved</b> expenditure | <input type="text"/> |
| <b>actual</b> expenditure   | <input type="text"/> |

(h) Total project costs / total staff input:

|                                     |                      |
|-------------------------------------|----------------------|
| <b>approved</b> project expenditure | <input type="text"/> |
| <b>actual</b> project expenditure   | <input type="text"/> |
| <b>*approved</b> staff input        | <input type="text"/> |
| <b>*actual</b> staff input          | <input type="text"/> |

(i) Date report sent to MAFF

(j) Is there any Intellectual Property arising from this project ?

**\*staff years of direct science effort**

**Section 2 : Scientific objectives / Milestones**

2. Please list the scientific objectives as set out in CSG 7 (ROAME B). If necessary these can be expressed in an abbreviated form. Indicate where amendments have been agreed with the MAFF Project Officer, giving the date of amendment.

- To characterise the granular structure of starches from mutant pea lines at the extremes in terms of their effect, with the aim of establishing a relationship between starch structure and genetic differences for starch content and composition.

- To utilise the information from the pea mutants in the interpretation of the granular structure of wheat starches.

- To determine the functional properties of starches from the most severe pea mutants plus starches from wild type and waxy wheat mutants. The aim is to establish functionality tests relevant to non-food uses, in collaboration with industry, and to begin to relate starch granular structure to function.

- To identify, through discussions with industry, the potential current and future non-food uses for the starches arising from this project and to identify future research needs

3. List the primary milestones for the final year.

**It is the responsibility of the contractor to check fully that ALL primary milestones have been met and to provide a detailed explanation if this has not proved possible**

| Milestones |   | Target date | Milestones met? |         |
|------------|---|-------------|-----------------|---------|
| Number     | Title   |             | in full         | on time |
| 01/01      | Data collected for determining the granular structure of r, rb, rug3 and rug lines  | 01/10/1998  | YES             | YES     |
| 01/02      | Data collected for determining the granular structure of rug5, lam WT and waxy wheats.  | 31/03/1999  | YES             | YES     |
| 02/01      | Data relating relating to functional properties of r, rb, rug3 and rug4 collected and relationship to granular structure determined                     | 01/10/1998  | YES             | YES     |
| 02/02      | Data relating to functional properties of rug5, lam plus WT and waxy wheat starches collected and relationship to structure determined.                 | 31/03/1999  | YES             | YES     |
| 03/01      | Discussions with companies to determine current and possible future non-food uses of starches in project completed and future research needs identified | 31/03/1999  | YES             | YES     |

**If any milestones have not been met in the final year, an explanation should be included in Section 5.**

### Section 3 : Declaration

4. I declare that the information I have given in this report is correct to the best of my knowledge and belief. I understand that the information contained in this form may be held on a computer system.

Signature

Date

29/04/1999

Name

DR C. L. Hedley

Position in Organisation

Project Leader

### Section 4 : Executive summary

Both naturally occurring and chemically induced mutants of pea have been identified that affect the composition and granular structure of the starch accumulated in the seed. The induced mutants were produced relatively recently and, to date, mutations at six genetic loci have been identified that affect starch. Mutations at five of these loci (*r*, *rb*, *rug3*, *rug4* and *rug5*) result in the dry seed being wrinkled in shape. Mutations at the sixth locus (*lam*) result in seeds that are indistinguishable in shape from the round seed of the wild type. A number of alleles, differing in the severity of their effect on starch content and composition, were isolated for each locus during the pea mutagenesis programme. All together the mutants gave starch contents ranging from 1 to 50% of the seed dry weight and starch compositions ranging from 4 to 75% amylose. One aim of the current project was to characterise the granular structure of starches from the mutant pea lines and to establish a relationship between starch structure and the genetic differences for starch content and composition. The structural information gained from studying the pea mutants would be used in the interpretation of starch granular structure of wheat, a species which is genetically less amenable than pea. A second aim of the project was to determine the functional properties of the starches that are relevant to non-food uses and to relate these to the structure and properties of the starch granules. These studies were to be carried out in discussion with industry with the aim of developing a follow-on project with industrial participation. The granular structure of the starches was characterised using solid state NMR, wide-angle X-ray diffraction, DSC and polarised light microscopy. These methods were used to determine the extent of the ordered (proportion of double helices, proportion of crystallinity, type of crystallinity, proportions of A- and B-type crystals and the spacial arrangement of crystals) and disordered (amorphous) material within the starch granules. The influence of moisture on the proportions of ordered/disordered material was studied to determine the correct level to use for characterising and comparing the different starches. A wide range of variation was found between the different pea starches for all of the characteristics describing granular structure. With the exception of mutants with mutations at the *r* locus, all pea starches were found to have both A- and B-type crystals, characterising the starches as C-type. (as against A-type starches, which have only A-type crystals and B-type starches, which have only B-type crystals). Variation was also found when severe and less severe alleles at each locus were compared. In particular, severe mutations at the *r* locus gave rise to starches which contained only B-type crystallites, while a less severe mutation at the *r* locus gave rise to a starch which had about 10% A-type crystals, thus giving it a C-type structure. Following discussions with industry, it was decided early in the project to direct the potential non-food uses of the starches towards their application in thermoplastic films. This decision determined that the functional properties that would be studied would be those relevant to this potential application. Studies were made, therefore, of the gelatinisation characteristics of the starches using differential scanning calorimetry. Different gelatinisation behaviour was found between the starches from the pea and wheat mutants, in particular, peak temperatures ranged from 56-69°C. Starches from two sets of pea mutants (*r* and *rug5*) did not show the narrow gelatinisation peaks demonstrated by all other starches studied. The gelatinisation characteristics of the starches were used to develop a method for producing films based on gelatinisation at either 98°C or 150°C. Thin films were produced by gelatinisation, cooling, evaporation of water and finally equilibration of the resulting films at 57% humidity. The mechanical properties of the films were then determined using an Instron. A wide range of mechanical properties was found between the various starches. The best properties being seen in films produced from the *r* and *rug5* mutants after gelatinisation at 150°C and the worst properties were seen in films produced by lines with very low amylose contents – *lam* mutant of pea and 'waxy' potato and maize starch. The project has illustrated the great potential for utilising pea starches for non-food uses as well a means of understanding the relationships between starch granular structure and functional properties in general, with the potential of applying this information to the development of new starches in less amenable crops such as wheat. The project has formed the basis of a new proposal with industrial collaborators, which will extend the work on thin films to explore the thermoplastic and barrier properties of the different materials.

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## Section 5 : Scientific report

## OBJECTIVES and MILESTONES

The scientific objectives for MAFF project NF0601 were: to characterise the granular structure of starches from mutant pea lines differing in the severity of their effects, with the aim of establishing a relationship between starch structure and genetic differences for starch content and composition; to utilise the information on pea starches in the interpretation of starch granular structure from wild type and waxy wheat; to determine the functional properties of the starches from the most severe mutant pea lines plus the wild type and waxy wheats, the aim being to establish functionality tests relevant to non-food uses and to begin to relate starch granule structure to function; to discuss with industry the potential non-food uses for the starches within the project and to identify future research needs. The primary milestones within the one year project were to collect the data required to determine the granular structure and functional properties of the starches; to relate functional properties important for non-food uses to starch granular structure and to discuss with industry the potential non-food uses of the starches with the aim of identifying future research needs.

## PLANT MATERIAL

The pea material used within the project has been developed over a number of years and consists of a series of mutations at each of six genetic loci, all having a primary affect on the content and composition of starch in the dry mature seed (see Annex 1, Figure 1). The material is unique not only because of the range of genetic variation available but more significantly because all of the mutant lines are essentially in the same genetic background, they are so called near-isogenic lines. A number of different alleles have been identified at each of the six loci (*r*, *rb*, *rug3*, *rug4*, *rug5* and *lam*), which range in the severity of the mutation effect on the resulting starch. Information on the number of alleles available at each locus, on the range of starch contents and compositions within the mutants and on the steps in starch biosynthesis affected by the various mutations is given in Annex 1, Tables 1 and 2 at the end of this report. The wheat starches used in the project were obtained from the Canberra lab of CSIRO in Australia as part of an existing collaboration. The lines, which were in the public domain, consisted of four wild type wheats plus four lines in which one of the three genomes contained a mutation at the waxy locus. In addition to the wheat and pea material, starches from waxy and wild type maize seed and potato tubers were used in the project for comparative purposes and because starches from these species have been used more widely commercially.

## EXTRACTION of STARCH

The maize and potato starches used in the project were obtained from commercial sources and the wheat starches were obtained in a pure form from CSIRO. The pea starches were extracted and prepared as part of the project using the following method, which was developed to produce starch granules in the native, unmodified state necessary for studying granular structure. Pea seeds were ground in a sample pin-mill and the resulting meal was slurried in water at a solid-liquid ratio of 1 to 12, the pH being adjusted to 8.2 with 0.1M NaOH. The mixture was centrifuged at 2500g and the supernatant removed. The precipitate had a very viscous green top layer, which was removed and the rest of the precipitate was resuspended in distilled water. This centrifugation and separation procedure was repeated two more times, until the viscous layer was no longer present. The resulting precipitate was then resuspended in distilled water and screened sequentially through two sieves, 300 $\mu$  and 53 $\mu$ . The resulting starch was washed with distilled water and air-dried.

*Preparation of amorphous starch.* Samples of amorphous starch were obtained by gelatinising a 2% suspension of pea starch for one hour at 98°C for 30 minutes with continuous shaking. The samples were then placed in sealed Pyrex tubes and transferred to a block heater preheated to 100°C. The temperature of the block was then raised to 150°C and maintained at this temperature for 10 minutes. The samples were then cooled to about 100°C for a few minutes, removed from the tubes, rapidly frozen, using a mixture of dry ice and ethanol, and freeze-dried. The freeze-dried samples were then placed under vacuum at 80°C for 30 minutes. The moisture content of the samples was determined by heating to constant weight in a vacuum oven at 80°C.

## METHODS USED

Wide-angle X-ray diffraction measurements were made using a Philips Scientific powder diffractometer operating with CuK $\alpha$ 1 radiation of wavelength 0.154 nm. The diffractometer had a generator that operated at 40kV and 30mA current and a vertical goniometer with an Anton Paar TTK camera. The incident beam of X-rays was collimated using an automatic divergence slit and a 0.3mm receiving slit

with a 1° scatter slit. The samples were scanned in the range 4.95-39.95 °2θ at a speed of 0.004 °2θ/second and a step size of 0.1 °2θ. Data were collected using a proportional detector and then stored and processed on a PC using Microcal Origin Programme. Variation due to the instrument background was determined by scanning the empty holder under the same conditions used for the samples. The Origin software was used to subtract the background variation from the sample scans, to subtract the amorphous starch pattern from the pattern given by the starches and to compute the peak profiles.

*Sample preparation:*- Before X-ray analysis, native starches were equilibrated at a relative humidity of 43% at a temperature of 22°C. The amorphous starches were equilibrated at 57% RH at 22°C and the starch films were cut into pieces and reconditioned in 57% RH at 22°C.

*Calculation of total crystallinity and the proportion of A- and B-type polymorphs:*- The X-ray diffraction pattern of native starch contains a contribution from the amorphous component. In order to determine the proportion of crystalline material in the starches it is necessary, therefore, to subtract the amorphous contribution from the overall pattern. The total proportion of crystalline material in the starch was calculated using the following equation:-

$$P_{\text{Cry}}\% = \frac{S_{\text{ST}} - S_{\text{AM}}}{S_{\text{ST}}} \times 100$$

Where  $S_{\text{ST}}$  represents the X-ray diffraction pattern area for native starch and  $S_{\text{AM}}$  is the X-ray diffraction pattern area for the amorphous starch. The areas were calculated between 4.95 to 27.45 °2θ. The pattern of amorphous starch was fitted to the pattern for native starch (Annex 2, Figure 7). The amorphous starch was produced from potato starch.

A-type starches (mainly cereals), in general, contain only A-type polymorphs and B-type starches (mainly tubers such as potato), in general, contain only B-type polymorphs. Pea starches, however, generally contain both A- and B-type polymorphs and the starch is called C-type. The proportion of A- and B-type polymorphs, therefore, is essential information for characterising pea starches. The method for calculating the proportions of A and B polymorphs from the X-ray diffraction patterns firstly involves subtraction of the amorphous pattern from that of the native starch using Excel. Peaks are then fitted to the resulting total crystalline pattern using an Origin Peak Fitting computer programme. The individual peak positions and peak areas can then be calculated. A large peak at 18 °2θ is characteristic only for A-type polymorphs. The relative area of this peak to the combined calculated total peak areas of peaks at 14, 15, 17, 18, 22, 23, 24 and 26 °2θ gave the proportion of A-type polymorph. The proportion of B-type polymorphs was determined by subtraction of the proportion of A-type from 100%.

Solid state (CP/MAS) NMR spectra were measured using a Bruker MSL-300 spectrophotometer operating at a proton frequency of 300.13 MHz and a <sup>13</sup>C frequency of 75.46 MHz. A standard Bruker double-bearing magic-angle sample-spinning probe was used. The magic angle was accurately set using KBr. 200-300 mg of sample were packed into a 7 mm zirconia rotor with a Kel-F cap and spun at 3.5 kHz. A single contact cross-polarisation sequence was used with a pulse to flip back the remaining <sup>1</sup>H magnetisation after acquisition. The <sup>1</sup>H 90° pulse length was 4.4 μs (equivalent to a spin lock field and <sup>1</sup>H decoupling field of 56.8 kHz), a contact time of 1 ms, spectral width of 16.7 kHz and a repetition time of 5 s were used. The chemical shift was referenced externally to the higher frequency resonance of solid adamantane at 38.56 ppm. The time domain data points were 1 K and the total acquisition time was 31 ms. A total of 1600 scans were accumulated for native starches and 3200 scans were used for amorphous starches.

*Sample preparation:*- Air-dried starch powders were stored at a range of moisture levels; 0%RH (P<sub>2</sub>O<sub>5</sub> in vacuum), 43%RH (K<sub>2</sub>CO<sub>3</sub>) and 97%RH (K<sub>2</sub>SO<sub>4</sub>) for three weeks, by which time they had attained equilibrium. The 97%RH sample was kept at 4°C for this time to prevent bacterial contamination of the samples. The starch films were first ground to a fine powder in liquid nitrogen and then reconditioned to a moisture level of 12-13% in the 43% RH environment for three weeks before determining the NMR spectra.

*Calculation of ordered/disordered components:*- Peak fitting was applied to the CP/MAS NMR spectra using an Origin peak-fitting programme. The C<sub>4</sub> peak at 81 ppm was characteristic only for the amorphous component of the starch and so the proportion of disordered component could be calculated using the following formula:-

$$\text{Disordered\%} = \frac{P_{ST}}{P_{AM}} \times 100$$

Where,  $P_{ST}$  represents the area of the  $C_4$  peak of native starch as a proportion of the total area of the spectrum and  $P_{AM}$  is the area of the  $C_4$  peak of amorphous starch as a proportion of the total area of the spectrum.

Differential scanning calorimetry (DSC) was carried out using two instruments, a DASM-4 M differential adiabatic scanning calorimeter with a Pt cell and a Seteram Micro-DSC. Work with the DASM-4 M was in collaboration with The Institute of Theoretical and Experimental Biophysics, Pushchino, Russia. For both instruments the concentration of starch in suspension was 1.7-2.0%, for those starches giving a large sharp peak of heat capacity change, and 2.0-4.0% for those starches giving a small wide change in heat capacity during gelatinisation. The heating rate was 0.5-1°C/minute. The peak characteristics determined were those related to the functional properties of non-food applications of starch, in particular, to those properties related to the production of films. These characteristics were the peak temperature ( $T^p$ ), the onset temperature of the peak ( $T^o$ ) and the conclusion temperature of the peak ( $T^c$ ); peak width being calculated from the difference between  $T^o$  and  $T^c$ .

Polarised light microscopy was carried out using a Zeiss Axiophot microscope equipped with cross-polarisers with a  $\lambda$  plate and viewed at a 400x magnification.

Film making was carried out by heating starch suspensions (2% w/w) in a water bath to 98°C for 30 minutes at a heating rate of about 0.9°C/minute, with continuous shaking. The gels were then cooled to about 60°C in air, while continuing to shake, and then poured onto polystyrene petri dishes. The gels were dried to films at 22°C at a relative humidity of 10-20%. Films were also made from pastes heated to 150°C. To achieve this the hot pastes from the water bath were transferred to Pyrex tubes and heated in a heating block to 150°C and then cooled to about 60°C to make the film. About 230mg of paste per cm were poured into the petri dish to give films with a final thickness varying from 40-60µm. All the films were reconditioned at 22°C and 58% RH for two weeks before measurement. The moisture content of the film (14-15%) was then determined by heating to constant weight in a vacuum oven at 80°C. During the process of developing the method, films were also made from a higher starch concentration (3 w/w%). The films produced using this concentration of starch were imperfect because the starch pastes were too viscous. The films produced were also very brittle.

Tensile strength and elongation measurements were made on the films using an Instron 1122 machine. The crosshead speed (pulling speed) of the instrument used in the experiments was 1mm/min. The films were cut into 15 x 100mm<sup>2</sup> strips. The film thickness was measured at four positions along the film using a digitised micrometer. The reconditioned film was mounted between two index cards to prevent it from damage during measurement. The film was clamped into the jaws of the machine and pulled until it broke. The tensile strength and elongation prior to breaking were calculated using the following formulae:-

*Tensile strength*      $\sigma = \frac{F}{S}$      where:  $\sigma$  = tensile strength (mega Pascals); F = the load at the break (Newtons); S = the area of cross section (mm<sup>2</sup>).

*Elongation*      $E = \frac{L - L_0}{L_0}$      where: E = elongation at the break (%); L = the length at the break (mm);  
L<sub>0</sub> = original length (mm).

## RESULTS

Starch granular structure was characterised by determining the proportions of ordered/disordered structures, the total crystallinity, the

proportions of A/B polymorphs, the spatial distribution of crystallites in general within the granules and the spatial distribution of A and B type crystallites in the pea starches.

*Proportions of ordered/disordered (O/D) structures* (Annex 2, Figure 6). The proportion of O/D was studied using solid state NMR. It is known that structural water is required for the crystalline structure to exist in starch granules. Since the moisture contents of the starch powders could vary from 0-45% it was necessary, therefore, to study the influence of this moisture variation on the O/D composition of the starches. In order to achieve conditions where all the starch granules in a sample had the same moisture content, the samples were equilibrated at known humidities. Under normal conditions, air-dried starch samples had moisture levels of 10-11%. To reduce this moisture level to 0-3% the samples were equilibrated over P<sub>2</sub>O<sub>5</sub> under vacuum. Higher moisture levels up to 25-30% were achieved by equilibrating the samples in desiccators set to chemically maintain known moisture levels. Moisture contents beyond 25-30% could only be achieved by preparing starch/water suspensions. Using solid state NMR it was shown that the proportion of O/D could be determined in starches with moisture contents of 0-20%. Starches with very low moisture contents (0-3%) had greatly reduced proportions of ordered structures. For starches with intermediate moisture contents (10-20%), however, the O/D was not dependent on the moisture content, demonstrating that under these conditions the O/D could be used as a structural characteristic of starches.

Following an analysis of the pea starches within the project using solid state NMR it was found that the proportion of O/D could be related to the presence of specific mutations. In summary, compared with starch from the wild type pea seeds, starch from the *rb* mutant had a higher proportion of O, starch from the *rug3*, *rug5* and *r* mutants had a lower proportion of O and the proportion of O for starches from the *rug4*, *lam* and *rug5* mutants was similar to the wild type. Comparative studies of the proportion of O/D were also made using starches from alleles at the *r* and *rb* loci differing for the severity of the mutation effect on starch content and composition. With regard to alleles at the *r* locus (*r*, *r-f* and *r-g*), these ranged, according to the severity of effect on the amylose content, such that *r-f* > *r-g*. The starches from these lines were similar with regard to the proportion of O/D and in each case they had a lower proportion of O compared with starch from the wild type. Starches from a severe (*rb*) and a less severe (*rb-f*) mutant at the *rb* locus were also compared for the proportion of O/D. The proportion of ordered structures in starch from the *rb-f* line was similar to that found in starch from the wild type, while the proportion of O from the more severe line was higher.

Comparisons for the proportion of O/D were made between wild type wheats and wheats with a waxy mutation in one of the three genomes and with starches from wild type potato, maize and pea. With regard to the analysis of the starches from wild type wheat, the proportion of ordered structures varied from 20 to 33%. In general, the proportion of O found in these wheat lines was lower than that found for potato and pea, but was similar to the proportion found in wild type maize starch. The range of variation for O found in the waxy wheat lines was slightly greater (18-36%) than that found in the wild type wheats.

*Total crystallinity* (Annex 2, Figures 1, 2, 3 & 6). The total crystallinity of starches is known to be significantly dependent on the moisture content. It was shown for pea starches with moisture contents in the range 13-15%, that the total crystallinity was about 5% higher than for pea starches with moisture contents in the range 11-13%. The crystallinity of starches from wild type wheat and from wheat lines with the waxy mutation was lower than for any of the pea or potato starches studied. The value for wheat was similar to that found for normal maize starch.

*Proportion of A/B polymorphs* (Annex.2, Figures 1, 2, 3 & 8). With the exception of starch from the *r* mutant, all of the pea starches were shown to be of C-type, i.e. they had both A and B polymorphs. It was shown, however, that the proportion of B polymorphs was higher when the starch had increased moisture levels. This result was not unexpected since it is known that B polymorphs are disrupted more easily when the moisture content of starch is reduced. The proportion of B polymorphs in the C-type pea starches varied from 50-70% between the different lines. Since the proportion of B polymorphs changes with the moisture content of the starches, it can only be used as a characteristic of a pea line when the starches are maintained in moisture conditions that stabilise the A/B polymorph proportion.

It is known that when starches are dispersed in excess water the granules swell and that this swelling is reversible and reaches an equilibrium. We have shown in earlier studies that the DSC patterns of C-type pea starch appear as two peak curves when the starch is heated in excess salt solutions. Also, that the first peak is related to the disruption of the B-type crystals and the second peak to the disruption of the A-type crystals. It is apparent that the areas under the first and second peaks are related to the enthalpy of disruption of the B- and A-type crystallinity respectively. Starches from the wild type and mutant pea lines were studied in excess 0.6M KCl solutions using a DASM-4 DSC, in collaboration with colleagues in Puschino, Russia, and using our own micro-Seteram III DSC.

Under these conditions it was shown that starches from the *rb*, *rug3*, *rug4* and *lam* pea mutants had double peak curves (it had been shown previously that these starches had narrow co-operative peaks when heated in excess water using the DSC). The relationship between the areas of the first and second peaks differed for starches from these mutants and in this respect all the mutants differed from the wild type pea. The lines could be ranked according to the proportion of A- and B-type crystallites, the proportion of B-type decreasing and A-type increasing in the order:- *lam-a*>WT>*lam-b*>*rug4*>*rb*>*rug3*. This order corresponded to that obtained using the X-ray diffraction method. The value of the proportions of B/A polymorphs, however, was not always the same, indicating that at a moisture level of 13-15% starch crystallinity had not attained its equilibrium level.

For the starches from the wild type wheat and from wheat lines with the waxy mutation it was shown, using X-ray diffraction, that with moisture levels of 11-40% only A-type crystallites were present.

With regard to studies using the different alleles at the *lam*, *rb*, *rug5* and *r* loci, it was shown that the starches from all of these pea lines were of C-type. The proportion of B polymorphs determined using the DSC differed between the starches produced by the alleles at the *rb* and *lam* loci such that *lam-a*>*lam-b* and *rb*>*rb-f*. Starches from three alleles at the *r* locus were studied, the alleles previously having been ranked *r-f*>*r*>*r-g* in terms of the severity of the mutations on starch content and composition. It was shown that starches from the two most severe alleles (*r* and *r-f*) had only B-type crystallites, while starch from the least severe allele (*r-g*), at 15% moisture, had 10% A-crystallites, making it a C-type starch. Although starch from the three *r* alleles did not show any co-operative peak during gelatinisation, the heat changes in heat capacity during heating were more pronounced for starch from the less severe allele.

*General and specific spatial arrangement of crystallites* (Annex 2, Figure 8). The symmetry for the general arrangement of crystallites in the starch granules was studied using polarised microscopy. With the exception of starches from the *r* and *rug5* pea mutants, all of the starches from pea, wheat, potato and maize gave the typical 'Maltese cross' under polarised light, demonstrating that granules from all of these starches had a concentric symmetrical arrangement of their crystallites. The crystallites in starch granules from the *rug5* pea mutant did not show any concentric symmetry, while the picture for granules from the *r* mutant was complicated because of the 'fractured' nature of the granules.

The specific arrangement of A- and B-type crystallites in the different starches was determined from DSC studies using 0.6M KCl and by observing changes in the crystal structure of single starch granules under polarised light during heating in 0.6M KCl solutions. The DSC study was carried out in collaboration with The Institute of Theoretical and Experimental Biophysics, Puschino, Moscow, Russia, using their DASM4 differential scanning calorimeter. With the exception of starch from the *r* mutant, which has been shown previously to contain only B-type polymorphs, all of the pea starches showed double peak curves in the DSC study. The first peak represents the disruption of the B polymorphs and the second peak the disruption of the A polymorphs. It has been shown previously that all starch granules from wild type peas contain both A- and B-type polymorphs and that the B polymorphs are situated centrally in the granules and are surrounded by the A polymorphs. Likewise, in the present study it has been shown that the polymorph distribution in starch granules from the *rb* and *lam* mutants is similar to that of the wild type.

Functional properties of starches are in general dependent on starch granule structure. The functional properties studied during the project were those related to industrial uses of starches and specifically to use of starch for the production of thermoplastic films. Such films can be made by heating suspensions of starch in excess plasticizer, which in the present project was water. The films were produced, therefore, following gelatinisation of the starches in excess water using the following procedure. The starch suspensions were heated to 98°C or to 150°C and then cooled to 60°C with continuous shaking. The shaking was to produce a homogeneous paste. During heating the starch crystalline structure was destroyed and, at the same time, the granules swelled to a large extent accompanied by the partial solubilisation of amylose. The hot starch paste represents, therefore, the dispersion of the swelled granules in the dilute amylose solution. The viscosity of the hot paste resulted mainly from the degree of swelling, the visco-elastic properties of swelled granules and the interactions between them. It is also, to a lesser extent, dependent on the viscosity of the continuous phase (amylose solution). The hot paste was poured into a dish and allowed to dry at 22°C at 10-20% humidity. While the water was evaporated the structural process developed. We believe that this process is related mainly to the development of a network between the swelled granules plus the development of interactions between the originally solubilised amylose as its concentration grows during drying. At the same time the process of retrogradation develops. Films produced in this way were then equilibrated for 4 weeks at 57% humidity, after which time the retrogradation processes were completed. It is apparent that the nature of the mechanical properties of the films are very complicated and are dependent on every step in the film making process. Film properties are dependent, therefore, on the properties of the paste, these in turn are dependent on the structure of the starch granules

and the plasticizer used. These first experiments with films were carried out using water as a plasticizer and this produced films, which were brittle. In the future we will vary the plasticizers, for example, using mixtures of water and glycerol, which we expect to give films that are less brittle and have improved mechanical properties.

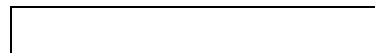
*Gelatinisation behaviour.* This was the first functional property to be measured and was studied using DSC. Two types of gelatinisation behaviour were found with the different starches. With the exception of starches from mutants with the recessive *r* and *rug5* alleles, all other starches produced a gelatinisation peak. Although these starches produced a peak, the peak temperatures differed between the mutants (56 – 69°C) and these differences could be related to changes in the crystalline structure (proportion of O/D and proportion of A/B polymorphs) brought about by the mutations. Starches produced by the *r* and *rug5* mutants did not show a gelatinisation peak but their crystalline structures were broken down during heating to 98°C. The different DSC behaviour of the starches from these two types of mutant is probably related to the different arrangement of crystallites and different properties of the disordered parts of the granules.

*Swelling of granules after gelatinisation.* This functional property was studied using light microscopy. Amongst the pea starches, those showing a peak of gelatinisation had good swelling properties, while those not showing a gelatinisation peak (*r* and *rug5*) had very low swelling properties. It would be expected that these differences in behaviour would be related to viscosity and to film making properties.

*Properties of starch paste.* Changes in the viscosity of starch suspensions during heating up to, and maintenance at, 95°C were studied using a rapid viscosity analyser in collaboration with the British Sugar Technical Centre in Norwich. This procedure is similar to that used widely in the starch processing industry. All of the starches, with the exception of that from the *r* mutant pea, began to increase in viscosity at a temperature which was characteristic for each starch. Starch from the *r* mutant showed very broad changes in heat capacity during gelatinisation with no obvious increase in viscosity. It was evident that the properties of the starch pastes are dependent on the arrangements of A- and B-type crystals in the granules. With regard to starch from the *r* mutant, the structure of the starch granules was broken down during heating even though there was no increase in viscosity. The behaviour of the starch from this mutant correlated with the differences observed using the DSC.

Film making properties (Annex 2, Figure 9) of the different starches were studied after heating the starch suspensions up to two different temperatures, either 98°C or 150°C. The heating was carried out using either a heated water bath (98°C treatment) or a heating block (150°C treatment). In each case the solutions were shaken throughout to ensure uniform conditions to allow the granules to form a uniform network during drying. The effect of two starch concentrations on the film making process was studied, 2% and 3%. The more concentrated solution was found to produce pastes that were too viscous and the films obtained were not flat enough for making strips for studying using the Instron. The more viscous films also contained air bubbles that affected the film properties and reduced film homogeneity. The conclusion, therefore, was that the films should be made using 2% starch pastes. The temperature at which the water was evaporated during film formation was also studied. Two temperatures were compared, 20°C and 60°C. At the higher temperature the starches dried too quickly and the films shrank during drying and so the lower temperature was used. With the exception of starch from the *r* and *rug5* mutants, it was possible to make films following heating of the pastes up to 98°C. Films produced from *rug5* type starch were too brittle and could not be used for making measurements. It was not possible to make film from the *r* type starch after heating to 98°C. When the starches from these two mutants were heated to 150°C, however, they produced excellent films with good mechanical properties. The results of the mechanical tests carried out on the films using the Instron. The mechanical properties differed between the range of starches used. The best properties were found for films made from the *r* and *rug5* type starches heated to 150°C, while the worst properties were found for films produced from the waxy starches, irrespective of the species used. This suggests that the amylose content is important in such types of film, the *r* and *rug5* mutations increasing the amylose content and the starches from waxy mutants are low in amylose.

*Ordered structures in starch films* (Annex 2, Figures 4 and 5). As mentioned earlier, retrogradation processes occur during film production. It was important to determine what structures were developed during this process and how the structuring may affect the properties of the resulting films. The crystalline structure of a number of the films was studied using X-ray diffraction. It was found that all of the films were partially crystalline and partially amorphous. The total crystallinity was calculated for all of the films and found to vary in the range 10-30%. Films produced by starches from wild type peas, the pea mutants and wheat had similar crystallinity.



Comparisons were made with films produced using potato starch. It was found that the total crystallinity of films produced from potato starch was significantly higher than for films produced from pea and wheat starches. The peaks, however, were wider, indicating that the crystallites in the potato films were probably smaller than those produced in pea and wheat films. The increased total crystallinity of films from potato starches correlated with the films having a lower tensile strength. Structuring within the films is mainly due to retrogradation, which we believe to be a competitive process to network formation. This would explain why the higher rate of retrogradation in films from potato starch correlates with lower mechanical properties. A B-type crystalline structure was found in all of the films studied.

It was evident from the current project that more work should be carried out on the influence of plasticizers on the mechanical properties of films. In particular, to determine the role of plasticizers on network formation and on retrogradation.

Discussions with industry on the potential future uses of starches within the project took place over the whole period of the project. The conclusion was to devise a project with industrial input that covered the use of the starches in the generic area of films. In this respect a film was defined as any continuous matrix with a thickness ranging from 0.01mm to 10mm and would cover films for packaging as well as sheets of plastic-type material for moulding. The discussions have led to the development of a LINK proposal for submission into the Competitive Industrial Materials from Non-Food Crops LINK programme. The initial application for support was submitted to the LINK committee and has been accepted with a request to submit a full application. A copy of the initial application is included as Annex 3 in this report.

## MAIN IMPLICATIONS

There are important implications from the project for our fundamental understanding of starch granular structure and how granular structure can be related to starch functional properties and potential uses.

For example, it is evident that the moisture content of starches very much affects the granular structure and hence the properties of the starch. This was a very important observation, which allowed the characterisation of starches produced by mutants containing lesions affecting specific steps in starch biosynthesis. The characterisation of starches from the pea mutants has given us a much better understanding of the genetic basis of starch granular structure in pea and important leads for manipulating starch in other genetically less amenable crops such as wheat.

With regard to the relationship between granular structure and the properties of starch, especially those related to non-food uses, it was evident that particular structural characteristics, such as those found in starches from the *r* and *rug5* mutants, are very useful in the production of starch films with good mechanical properties. Understanding the basis of the relationship between the structure of these particular starches and the resulting films could lead to the development of new types of starches better suited to such non-food applications.

There are also important implications from the project for the future potential uses of starches for non-food applications and for the development of the pea crop as a source of raw materials for industry. Following extraction of the starch, the other raw materials from the pea seed, in particular the protein and fibre fractions, could be useful components of animal feeds, or could be utilised in the human processed food industry. In particular, the protein could replace some of the current applications of soya and, hence, help to overcome consumer concerns with the use of genetically modified soya protein in human foods. In this way, the development of the pea crop would increase the pea seed market within the UK and possibly Europe and provide farmers with an important break-crop to replace those crops that are currently in surplus.

## POSSIBLE FUTURE WORK

The current project was only the first step in the utilisation of the wide range of starches available for industrial uses. Most of the future developments of the project in this area are outlined in the LINK proposal appended to the present report. This new project would give outlets for the accumulated knowledge, from the current project and from many years of research to produce, develop and characterise the material, to be applied in the development of starch films for a wide range of industrial uses. The fundamental information on the starches and the possibility of producing starches with an even wider range of functional properties also opens up the possibility of using this material for food as well as non-food purposes. This will be explored in the near-future by preparing a LINK proposal aimed at the Food Quality and Safety Programme.

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## **ACTION RESULTING FROM THE RESEARCH**

Part of the current Maff project was to establish links with industry relating to the possible non-food uses of starches from the pea mutants and to extend this to non-food uses of wheat starches. Consultations and discussions were carried out with a number of commercial organisations during the course of the project the result of which was the decision to apply for a LINK grant within the Competitive Industrial Materials from Non-Food Crops programme. A copy of the LINK outline application is included as Annex 3. Following acceptance of this outline by the LINK committee, a full proposal is being prepared for submission in July 1999.