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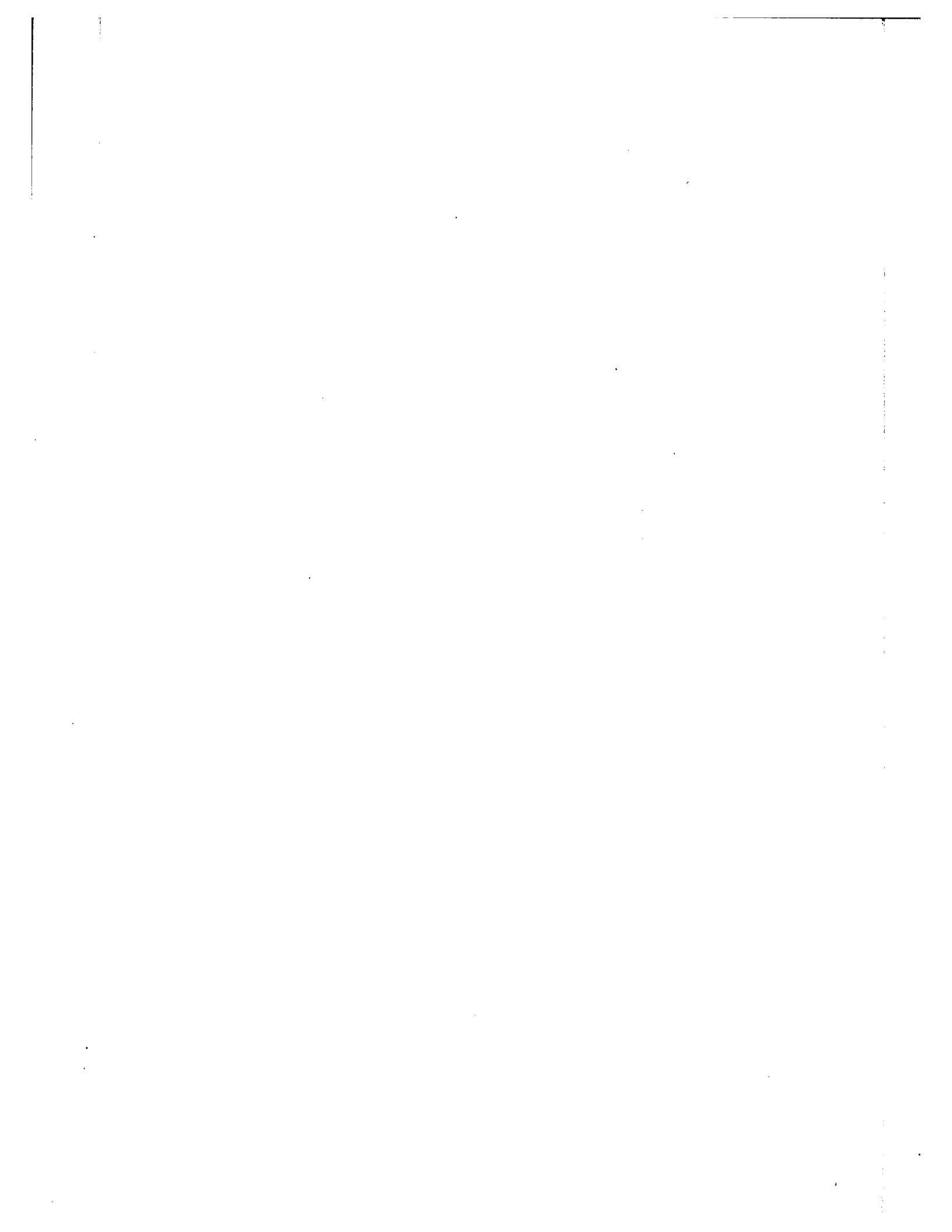
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ARCS-THESIS

Identification, Distribution and Partial Characterization
of Wheat Starch-Associated Proteins

Erin Armstrong

Thesis submitted to
the School of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Biochemistry

University of Ottawa



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Abstract

Wheat starch is composed of two populations of starch granules: large 'A' granules and small 'B' granules. A procedure for the separation of wheat starch into its component populations was developed. Digital image analysis showed that the resulting preparations contained 1% or less of the other granule population and were representative of the corresponding starch fraction in flour. The relative surface areas and volumes of these two types of starch granules were calculated and the results indicated that for an equal volume of starch granules, the small granules have 3.5-4.5 times the surface area of the large granules.

The protein content of each population was determined from the nitrogen content. The small granules were found to contain 0.35% protein as compared to 0.15% protein in the large granules. Microspectrofluorometry was used as a means of indirectly measuring protein content and establishing the distribution of protein within the granules, before and after treatments to extract the protein.

Extracted proteins from the soft wheat variety Fredrick were analyzed by SDS-PAGE to determine their distribution between the large and small starch granules. The large starch granules were found to contain one major protein and the small granules eight major proteins. These nine proteins

were then isolated by electroelution and a partial characterization was carried out by determining amino acid compositions, N-terminal sequences, protease susceptibilities, peptide maps, and carbohydrate content. The results indicated that the nine proteins have several properties in common and they may be composed of similar structural units. In particular, the amino acid compositions, protease susceptibilities and peptide maps varied only slightly. However, there were a few differences among the proteins. Some of the them tested positive for carbohydrate, while others did not. N-terminal sequence analysis indicated that most of the N-termini were blocked, and the two distinct sequences obtained showed no homology to the primary structure of any known protein.

One of the major differences between hard and soft wheats is the degree to which the starch granules are damaged during milling. The extent of damage can be visualized by certain stains, the most common being Congo Red. However, another related dye, Hessian Bordeaux, was found to stain damaged granules much more intensely. The maximum absorbance of starch granules stained with Hessian Bordeaux was more than five times that of starch granules stained with an equivalent concentration of Congo Red. Hessian Bordeaux is therefore a more useful stain than Congo Red for the microscopic detection of damaged starch granules.

I could not have completed this thesis without the support
and encouragement of my friends, local and distant.

This thesis is dedicated to them.

You are not obligated
to complete your work,

but you are not at liberty
to quit.

- The Talmud

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Table of Contents

	Page
Abstract	i
Acknowledgements	v
List of Tables	x
List of Figures	xi
List of Abbreviations	xiv
Chapter One	
Introduction to the Thesis	1
Chapter Two	
Literature Review	6
Chapter Three	
Starch Granule Isolation and Characterization	
Introduction	40
Materials and Methods	
Starch preparation	48
Image analysis	50
Calculation of surface areas and volumes	52
Results	
Starch preparation	54
Image analysis	54
Calculations of surface areas and volumes	60
Discussion	63
	vii

Chapter Four

**The Proteins Associated with Large and Small
Wheat Starch Granules**

Introduction	74
Materials and Methods	
Nitrogen analysis	80
Extraction of starch granule proteins	80
SDS-PAGE	81
Microspectrofluorometry	82
Results	
Nitrogen analysis	84
SDS-PAGE	85
Microspectrofluorometry	88
Discussion	91

Chapter Five

**Partial Characterization of the Proteins
Associated with Large and Small Wheat Starch
Granules**

Introduction	99
Materials and Methods	
Isolation of individual proteins by electroelution	101
Amino acid analysis	102
Peptide mapping	103
Protease digestions	104

	Page
Glycoprotein analysis	105
α -amylase treatment	106
N-terminal sequencing	107
Results	
Amino acid analysis	107
Peptide mapping and protease treatments	109
Glycoprotein analysis	116
α -amylase treatment	119
N-terminal sequencing	119
Discussion	121
Chapter Six	
Concluding Comments	127
Appendix	
Improved Microscopic Detection of Damaged Starch Using Hessian Bordeaux	132
Summary of Scientific Contributions	143
Literature Cited	144

List of Tables

	Page
1. Size distributions of isolated starch fractions	56
2. Size distributions of starch granules in flours milled from Fredrick and Neepawa wheats	59
3. Surface areas and volumes calculated for different starch fractions	62
4. Amino acid compositions of the isolated proteins	108
5. N-terminal sequences	120

List of Figures

	Page
1. Diagram of a wheat kernel	7
2. Starch isolation and separation procedures	51
3. Isolated starch fractions	55
4. Size distributions of starch granules in flour and isolated starch fractions from Fredrick wheat	57
5. Size distributions of starch granules in flour and isolated starch fractions from Neepawa wheat	58
6. Area and equivalent diameter ('Dcircle') distributions in flour from Fredrick and Neepawa wheats	61
7. SDS-PAGE of the proteins extracted from large and small starch granules	86

	Page
8. SDS-PAGE of proteins extracted from large and small starch granules based on equal volumes, equal amounts of protein and equal surface areas	87
9. SDS-PAGE of protein extracts from steps in the isolation of large starch granules	89
10. Distribution of fluorescence intensities in acid fuchsin-stained large and small starch granules	90
11. Maps of fluorescence intensities across individual acid fuchsin-stained starch granules	92
12. Autoradiogram of a peptide map of [¹⁴ C]acetylated proteins digested with pepsin	110
13. Autoradiogram of a peptide map of eluted [¹⁴ C]acetylated proteins digested with pepsin	112

	Page
14. Autoradiogram of a peptide map of the neutral band from HVPE at pH 6.5 of [¹⁴ C]acetylated proteins digested with pepsin and papain	114
15. SDS-PAGE of protease-treated extracts from large and small starch granules	115
16. Autoradiogram of a peptide map of [¹⁴ C]acetylated proteins digested with pepsin and elastase	117
17. Glycoprotein blots	118
18. Starch granules and flour samples stained with Hessian Bordeaux	137
19. Absorbance curves for starch granules stained with Hessian Bordeaux and Congo Red	138

List of Abbreviations

DAF	days after flowering
Dcircle	equivalent circle diameter
DFP	diisopropyl fluorophosphate
Dmax	maximum diameter
ESCA	electron spectroscopy for chemical applications
HVPE	high voltage paper electrophoresis
PITC	phenyl isothiocyanate
PTH	phenylthiohydantoin
PVDF	polyvinylidene fluoride
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SGP	starch granule protein(s)

Chapter One

Introduction to the Thesis

Starch has been one of the most studied of substances, and it is still subject to intense investigation. Sandstedt described how it has been "the subject of an immense amount of microscopic study beginning with the work of Leeuwenhoeck in 1716" (Sandstedt, 1946, p.337). He also cited Badenhuizen as having suggested in 1939 that knowledge pertaining to the structure of starch granules had not progressed in the previous one hundred years. The past fifty years have brought advances in the understanding of starch, but there are still many questions to be answered.

Why study starch? Quite apart from the basic biochemical interest and its importance as a food substance, starch is an extremely important commercial product. It is used in the production of paper, corrugated boxes, textiles, fibre board, coal briquettes, certain adhesives, carpets, 'biodegradable' plastics, cosmetics and toiletries, and a variety of organic chemicals and synthetic polymers. It is also used in oil well drilling, foundry work, and printing processes. New applications are always being sought and become more likely and more effective when the properties to be exploited are understood.

The great diversity of applications and functionalities alone suggests that starch is likely to be a complex substance. Yet starch is described in many texts as simply being composed of amylose, a linear chain of glucose molecules, and amylopectin, a branched chain of the same molecules, packaged in granules of characteristic size and shape, dependent on the source. Unique proteins and lipids are now known to be associated with starch granules, possibly even with the starch molecules, and even the classification of starch molecules as either linear or branched is becoming ambiguous, with references to 'branched amylose' appearing (Lineback and Rasper, 1988, pp.286,290-291). The mechanisms by which the sizes and shapes of granules are established are completely unknown. Even the way in which granule synthesis is initiated is still being actively investigated (e.g. Keeling et al, 1988; Tyson and ap Rees, 1988; Preiss et al, 1991). "Much work remains to be done on the biosynthesis of starch, the formation of the granule, and the organization of the components within the granule" (Lineback and Rasper, 1988, p.298).

Among the various starches, wheat starch is unusual in that it is composed of two distinct types of starch granules, in terms of size, shape, and many other characteristics. Along with the basic questions on starch development in general, wheat starch is studied for the development of these two types of granules and for the effects of the large and small granules on the functionality

of wheat flour in its different applications. Different types of wheat have different applications, and the effect on, or by, the starch from the different types of wheat, particularly in terms of degree of 'hardness', are also extensively studied.

Research often takes investigators in directions they could never have anticipated. This was true of the work which was the inspiration for this thesis project. A group in England, in trying to assess the mechanism by which chlorination of wheat flour leads to improved baking performance in a special type of cake, uncovered a possible explanation for one of the most important functional characteristics of wheat. Six years ago they published a very short but very interesting paper in which they described the analysis of a set of proteins found associated with wheat starch granules, and the connection of one of these proteins with 'softness' of wheat endosperm (Greenwell and Schofield, 1986a). When attempts at the same analysis, at the beginning of this project, verified the published results, an intriguing question was posed. If the wheat starch was separated into its component populations first, would the proteins extracted from each type of granule be the same? The results of this analysis showed that they are not, and these proteins became even more intriguing. This was the real starting point for this thesis.

There are different types of research projects. Some are aimed at developing a method for application to a wide range of questions. Some are aimed at either proving or disproving a particular theory. Some are simply fact-finding missions. Research almost inevitably takes unexpected turns, however, and regardless of the initial goal or direction, it is next to impossible to predict where a project will lead. This project began by asking: what can we find out about these proteins? The focus was not on the specific protein associated with softness as such, but rather on the starch granule proteins as a group and their differential distribution between the large and small wheat starch granules. Are there basic differences among all these proteins or between those associated with the two types of starch granules? Are the properties of any or all of these proteins unique in any way?

The literature review in the next chapter sets up the context of the project. The work which has been published to date on the wheat starch proteins is surveyed, as are papers dealing with some aspects of starch development and the characteristics of wheat, flour, and starch which are relevant to the work reported here. The subsequent discussion of results should be more meaningful with this information as background.

The research on the wheat starch granule proteins for this thesis is described and discussed in three separate

chapters. Each of these chapters has its own, more specific, introduction, followed by a description of the materials and methods used for, and the results obtained from, that set of experiments, a discussion of the results and the conclusions drawn from them. A general discussion follows these three chapters to combine and summarize the various segments of the project. There are two reasons the thesis is structured this way: to simplify the reporting of the work, for the benefit of the writer and the reader; and to emphasize the diversity of ways in which these proteins were studied.

Finally, an appendix is added which describes a novel stain for the microscopic examination of damaged starch granules. This work, while not directly related to the starch granule proteins, is related to the characteristic of wheat hardness, which is relevant to one of the starch proteins.

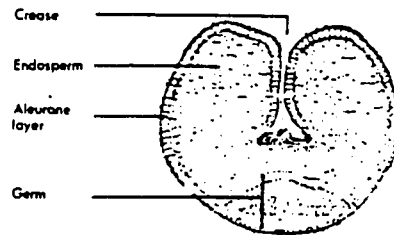
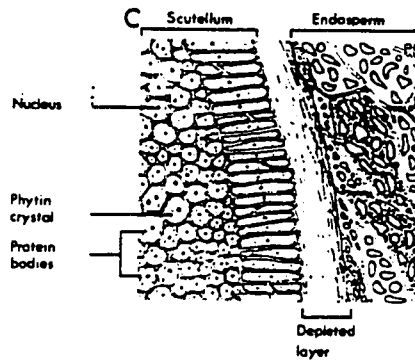
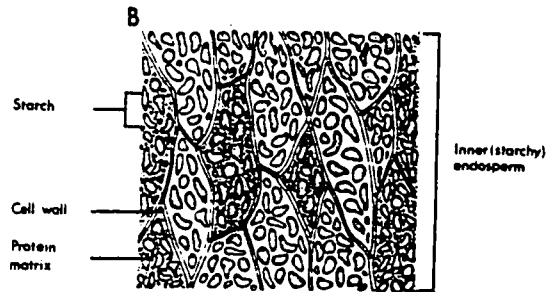
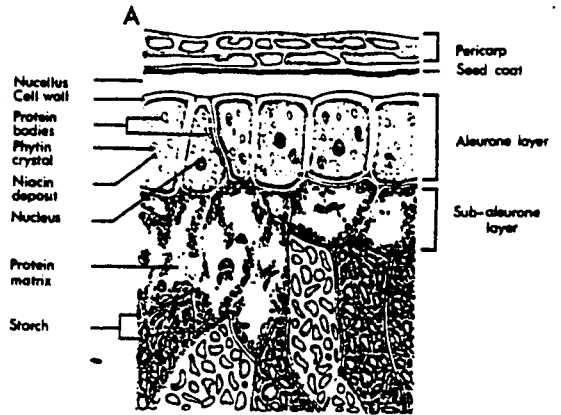
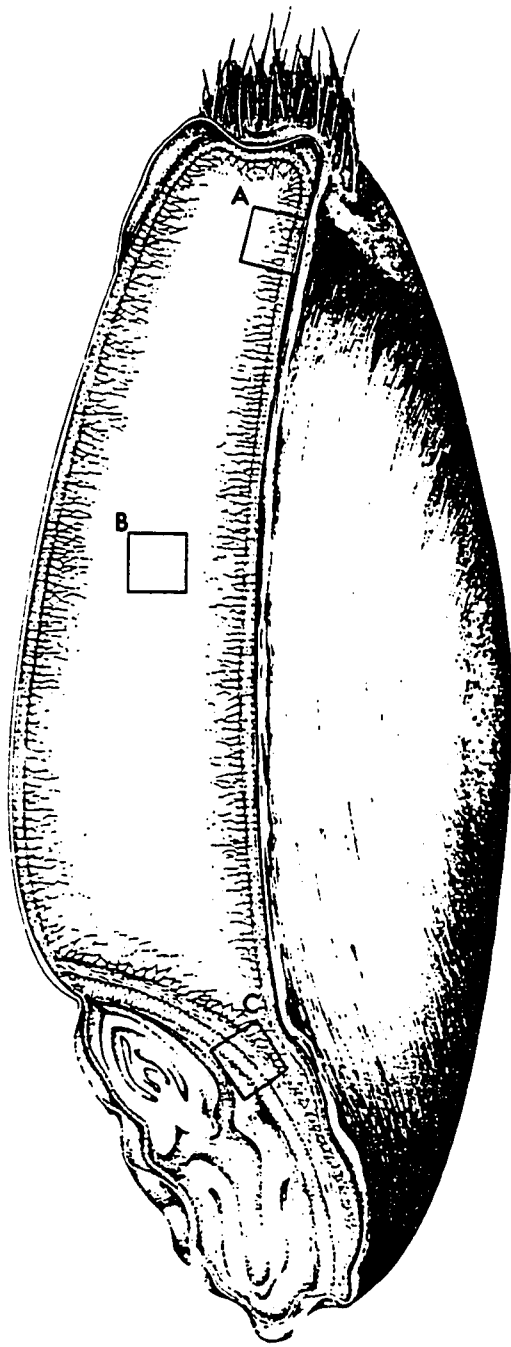
Chapter Two

Literature Review

Wheat has been cultivated as a food crop for approximately 10,000 years and is now the main cereal crop grown internationally (Orth and Shellenberger, 1988, pp.1,5). One of the reasons wheat is such a popular food crop is that the resulting flour can be used to prepare a wide variety of products, including bread which is a primary staple in the Western world's diet. Seventy-five to eighty-five percent of the wheat grain is composed of the starchy endosperm (Figure 1), which makes up the bulk of milled flour, with starch granules occupying 64-74% of this fraction (Pomeranz, 1988, pp.99,137). Starch is a plant's way of storing energy, primarily for the use of the embryo once it germinates. Starch is also the source of more than half of the food calories consumed by the world's population (Whistler, 1984, p.2). Most of the rest of the wheat endosperm consists of proteins collectively referred to as the gluten proteins. The characteristics of wheat starch and gluten proteins, and the way in which these two components interact with each other, determine the functional characteristics of wheat flour in its diverse applications.

In each plant, starch granules have a characteristic size and shape, and in many cases it is possible to identify

Figure 1. Diagram of a wheat kernel.
Morphological and structural features of a typical wheat grain are illustrated. The starchy endosperm (B) occupies the major portion of the kernel. Diagram provided courtesy of R.G. Fulcher and S.I. Wong.



the source of a starch by microscopic examination of the granules. The characteristic size should more accurately be called a size distribution. Wheat starch is unusual in that the size distribution of its starch granules is bimodal. This is also true for the starch granules of other cereals in the Triticeae family: barley, rye and triticale. It is now generally accepted that this characteristic distribution represents two distinct populations of starch granules. This is supported by the events during starch and endosperm development and also by the differences in characteristics and effects on dough functionality of the two types of granules. Each of these topics will be discussed in turn.

In discussing the different starch populations in wheat, large and small granules are commonly referred to as A and B granules respectively. The large granules range in size from 10 to 45 μm diameter and the small ones have diameters of less than 10 μm , unless otherwise defined. 'Prime' starch refers to starch preparations that consist primarily of large granules, but with considerable amounts of small granules present.

Development:

Sandstedt (1946) published a paper in which he presented many photomicrographs and an extensive discussion on the developmental sequence for wheat starch granules. He credited earlier authors for the observation, confirmed in

his study, that cell wall production in the multinucleate cell which is the original endosperm, begins at the periphery of the endosperm, just inside the aleurone layer. He observed that the cellularization occurs within a day, being completed by 4 DAF (days after flowering). This was confirmed by Mares and co-workers (1975) thirty years later. Starch granules were obvious, although extremely small, at this time in the 'inner endosperm cells'. No starch was seen in the peripheral cells. Once starch granules were evident throughout the endosperm, he observed that the granules within any given cell were uniform in size, but that a gradation in size existed from the inner to the outer regions of the endosperm, indicating the different ages of the differently-positioned cells. The starch granules in the subaleurone cells never develop to the same extent as in the older, central, cells. His observation as to the timing of starch granule formation apparently contradicted several earlier authors since he stated that "starch starts to develop much earlier than has been supposed" (Sandstedt, 1946, p.347). Sandstedt also described the shapes the A granules have at different stages of their development before assuming the final 'lenticular' shape characteristic of the large granules. At 12-14 DAF small spherical granules begin to appear among the large granules. These increase in number while the large granules increase in size, and fill the spaces between large granules.

Observations made by electron microscopy on the developing wheat endosperm by Buttrose (1963) agreed with those made earlier on barley. May and Buttrose (1959) reported that barley starch granules initiated up to 15 DAF developed into the large lenticular granules, and that granules initiated after this time developed into small spherical granules. They stated that "it seems that some major change takes place in cell organization at 15 days after anthesis" (May and Buttrose, 1959, p.155). They determined that while 90% of the volume of the starch mass was due to large granules, 90% of the number of granules was accounted for by the small granules. May and Buttrose suggested that heredity may control the number of large granules which are initiated but the number of small granules depends on the final volume and weight of starch mass, with more granules of smaller size being formed as development proceeds to fill in the increasingly smaller spaces left open. Of the granules initiated in the first two weeks post anthesis, those initiated towards the end of this period are formed in the younger, more peripheral cells (Buttrose, 1960). Electron microscopy revealed that there is only one granule per amyloplast during this time. Buttrose (1960) observed tubuli inside the plastid, located around the widest diameter of the enclosed starch granule, where a groove or furrow in the granule is seen. He pointed out that although it had been established that the small granules develop independently of the large, it was not yet known

where they develop. He presented evidence that they develop in amyloplasts which already exist and contain large granules, in evaginations that are formed at 12 DAF. No tubuli were found surrounding the developing small granules. It was not established, however, whether or not these protrusions were eventually separated from the parent amyloplast. In the 1963 paper though, Buttrose stated that while the small wheat starch granules were initiated two weeks after the large granules in the same amyloplasts that already contained a large granule, complete with surrounding tubuli, the small granules were 'extruded' into the cytoplasm.

Evers (1970) also reported that the small granules are initiated 14-16 DAF, and that they fill in the spaces between large granules. In commenting on the reason for the protein concentration gradient which is found across the developed endosperm, he supported the idea of a progression in the development of starch granules from the inner to the outer portion of the endosperm. He found that the amount of protein in any given cell, regardless of location, is essentially the same. However, in the central cells, there is a higher concentration of starch granules, and therefore a greater 'dilution' of protein, producing the wide variation in protein concentration in the differently-located cells previously reported by other authors. Evers also noted that he saw no evidence for continued cell

division past 16 DAF, and that the increase in endosperm size past this stage is due to cell enlargement.

A developmental sequence for the A granules in wheat was presented by Evers (1971). The diagram illustrating this sequence is still referred to today (e.g. Evers and Bechtel, 1988, p.82). His evidence came from examining starch granules from endosperms at different stages of development and from cells in different parts of the endosperm at 14-16 DAF when the full range of stages of developing A granules is present. It is interesting that the sequence he presented is very similar to that described by Sandstedt (1946), who he credited for observations with the light microscope that he confirmed by electron microscopy. The developmental sequence includes: a tiny spherical 'nucleus' to which starch molecules are added, particularly around the equatorial plane; this 'lip-like structure' eventually grows around the nucleus; starch molecules continue to be added all over the granule, with more at the equator than elsewhere, but eventually the nucleus is encased and the bulge no longer shows; the groove, which is originally quite pronounced at the equator, ends up less pronounced but still present. It should be noted that this groove is the one observed by Buttrose (1960) to correspond to the tubuli which are thought to be involved in the deposition of starch molecules on the granules.

Evers and Lindley (1977) measured the size distributions of starch granules in 6-8 wheat varieties by

three different methods and determined the contribution to the total starch population of the small granules by weight and by number. This was an extension of work reported by Evers (1973). Contrary to earlier reports that the small granules represented less than 10% by weight of the starch mass and approximately 90% by number, they found that the small granules compose approximately one third of the total starch mass and 97% of the total number of granules. They concluded that the small granules might be more important in flour functionality than previously thought, given that there are more of them than realized.

Brocklehurst and Evers (1977) showed that small granules contributed at least one third of the weight of the total starch of a given variety of wheat, and the actual proportion was greater for large plump kernels than for smaller or shrivelled kernels. As well as supporting the higher percentage value for small granule mass, this supported the conclusions of May and Buttrose (1959) and Sandstedt (1946) that the small granules continue to increase in number as long as the grain continues to grow.

Briarty and co-workers (1979) published an extensive set of measurements taken from developing wheat endosperms. Their measurements also showed that cell division was complete by 16-20 DAF. As well, their data confirmed that the starch granules in cells of increasing age, i.e. increasingly central, are progressively larger than granules in the younger cells, and that the granules in the youngest

cells never attain the size of the oldest granules. They found that the number of amyloplasts determines the number of large granules and that this number is established by 10 DAF. One slightly confusing distinction they made is that between 'A-type' and 'B-type' amyloplasts. While the A-type are formed by 10 DAF, the B-type are initiated around 16 DAF and the B granules appear only at 22 DAF, approximately one week after the time they are normally observed. Sixty-five percent of the cell volume at maturity is occupied by starch, a value corresponding to that reported for flour (Pomeranz, 1988).

Duffus and Murdoch (1979) confirmed an earlier report that the amylose content of starch in general increases as development proceeds. They also showed that the amylose content of the larger granules does not increase, as a percentage of their total starch content, until later in development, in contrast to the small granules, which show an increase in the proportion of amylose throughout their development. They confirmed earlier reports that the amylose content of the large granules is always greater than that of the small ones.

Dengate and Meredith (1984) studied the granule size distribution in 59 wheat varieties by Coulter Counter, converting the sphere-equivalent diameter measurements into weight values, and found that the proportion by weight of the starch which was composed of A granules was constant, although the exact weight distribution within this

population varied. High weight kernels have A granules which are larger than those in lower weight kernels. This is in agreement with Sandstedt's (1946) conclusion that while the small granules increase in number, the large granules increase in size. Morrison and Gadan (1987) also found that the number of large starch granules is set and the granules continue to grow throughout development, whereas the small granules, once initiated, continue to increase in number. These results also agree with those published by Briarty and co-workers (1979), discussed above.

Parker (1985) presented data which supported the idea that both large and small starch granules are synthesized in the same amyloplasts in wheat endosperm. This was in contradiction to the results of Buttrose (1960). Between these two reports, there had been some debate about where they develop. She claimed that this observation makes sense whether or not there is a difference in the amylose content of large and small granules since differences, when reported, are small, so the same biosynthetic mechanisms could easily be at work in both cases. Again, the time differential in development of the two types of starch granules was shown, with the small granules being initiated approximately two weeks after the large ones. It should be noted that Duffus and Murdoch (1979) observed that the increase in amylose contents occurs at different points in the development of A and B granules, allowing for the

possibility that it starts at the same time, given the difference of two weeks in their developmental sequences.

A third population of starch granules, 'C' granules, was discussed recently by Bechtel and co-workers (1990). They followed the number and size distribution of wheat starch granules during development. Some investigators (e.g. Morrison and Scott, 1986) have discarded the data points at the low end of the measured size distributions as artifact, but Bechtel and co-workers have shown that these measurements are indeed valid and indicate a group of very small granules initiated very late in development. They end up representing almost half the number of granules at maturity, although only 3.4% of the final mass of starch, at least in the variety studied. Dengate and Meredith (1984) also mentioned a third population of starch granules, but this third population seems to be very large A granules as opposed to the very small 'C' granules described by Bechtel and co-workers (1990). Baruch and co-workers (1982) provided evidence from a series of mathematical analyses of Coulter Counter measurements of wheat starch granules which showed that, in the three types of wheat they tested, there were three distinct size classes of starch granules in the mature grain. However, it is quite difficult to determine exactly where the third population may fit into the developmental sequence.

Differences between large and small granules:

D'Appolonia and Gilles (1971) isolated large and small starch granules from a hard red spring flour and compared the two fractions in a variety of tests. They found that the two types of granules differed in their pasting properties, and that the small granules had a higher water-binding capacity and starch damage level, and a very slightly greater density (by 0.002 g/cm^3).

Bathgate and Palmer (1972) analyzed the amylose contents of large and small starch granules from wheat and barley. While they found a significant difference between the barley granules, with the small granules containing considerably more amylose than the large, they found no real difference in the wheat starch granules. Evers and co-workers (1974) published results which also showed that the amylose contents of large and small wheat starch granules were the same. Bathgate and Palmer also found, for both wheat and barley, that the small granules gelatinized at a higher temperature than the large granules.

Kulp (1973) measured a variety of properties for small and 'regular' (by which it is assumed he meant 'prime') starches from different sources. Like the others, he found differences, including: a lower iodine affinity in the small granules, indicating either less amylose or some 'fundamental structural variation'; differences in pasting properties; more lipid in the small granules; a wider range

of gelatinization temperatures for the small granules; a higher susceptibility to digestion by α -amylase in the small granules; a higher water-binding capacity in the small granules; and poorer quality, lower volume loaves of bread made with small granules.

Meredith and co-workers (1978) found that there was a greater amount of lipid in the smaller granules from two varieties of wheat analyzed, and that the amount present was proportional to the surface area in the large granules but proportional to the volume of the smaller granules. Since this lipid is assumed to be present in an amylose-lipid complex, they suggested that a possible explanation for these relationships is that these complexes are not formed until late in granule development, and so are added to the outer layers of the already established large granules, and to the entire structure of the later-formed small granules. This agrees with comments by Duffus and Murdoch (1979) and Parker (1985) on amylose deposition during development. The problem with this idea is that there should then be a corresponding difference in the amounts of amylose found in the two types of granules, but in fact, this is still a debated issue. Where differences have been observed, they have been opposite to that predicted by this hypothesis, that is, several investigators have reported higher amylose contents in large wheat starch granules; for example, Meredith (1981), Soulaka and Morrison (1985) and Morrison and Gadan (1987). Soulaka and Morrison (1985) examined 23

bread wheats and 26 durum wheats for a variety of characteristics. They found more amylose in the A granules of most of the varieties they measured. However, in a few varieties they found the opposite. Following up on the study by Meredith and co-workers (1978), Morrison and Gadan (1987) published results relating amylose and lipid contents in the large and small granules of wheat starch. They also found that the larger granules had more amylose than the smaller granules, and that the amount increased with age, along with an increasing amount of associated lipid.

Meredith, in a paper titled 'Large and Small Starch Granules in Wheat - Are They Really Different?' (1981), presented arguments to support his view that they definitely are. He acknowledged that some of the small granules probably are large granules which never fully developed, but concluded that most of them are distinct from the large granules. He and his co-workers observed a bimodal size distribution and, by documenting the number of granules of given sizes during different stages of development, determined that there are two different points at which granules are initiated, in agreement with reports already discussed. The small granules are assumed to be spherical whereas the large granules are known not to be. By comparing measurements made with a Coulter Counter, which measures volume, with those made by scanning electron microscopy, measuring diameter, he concluded that the small granules are "as asymmetric as large ones" (Meredith, 1981, p.43). This

is not in agreement with most references to lenticular A granules and spherical B granules. Chemical differences between the two types of granules included a different distribution of saturated and unsaturated lipids, a small but consistent difference in the amylose content, with slightly less amylose in the small granules, and a large difference in susceptibility to α -amylase digestion, with the large granules being eight times more susceptible on a surface area basis than the small granules.

Eliasson and Karlsson (1983) used differential scanning calorimetry to measure the gelatinization properties of large and small wheat starch granules. They found that although the small granules begin to gelatinize at a lower temperature, the peak gelatinization temperature is higher. This results in a broader gelatinization interval for the small granules. From their DSC measurements they also determined that the endothermic transition due to amylose-lipid complexes is greatest for the small granules. They related this to the findings of Meredith and co-workers (1978) that the small wheat starch granules contain more lipid than the large granules. Soulaka and Morrison (1985) found no difference in the gelatinization temperatures for the two types of granules, although they did find a wider temperature range for the B granules, in agreement with Eliasson and Karlsson (1983).

Effects of large and small granules on functionality:

Grewe and Bailey (1927) measured the sizes of starch granules isolated from flour milled from 17 varieties of wheat and assigned them to one of three categories: small, $<7.4 \mu\text{m}$; intermediate, or; large, $>14.8 \mu\text{m}$. They found that somewhere between 59 and 92 % of the granules measured were 'small', and that the proportion of small granules was not correlated with the type of wheat from which the flour had been milled, the resulting loaf volume as a measure of 'baking strength', nor diastatic activity. They concluded that any starch characteristics which might affect these properties have nothing to do with the size of the granules.

Stamberg (1939) did an interesting series of experiments adding different starches with different surface areas relative to weight, from rice, corn, wheat and potato, to flour which was then used to bake bread. This was a test of the importance of starch surface area to the baking strength of a flour. His results showed that the minimum absorption occurred at a lower protein percentage when the starch present had a lower surface area/weight ratio, that is, for the larger starch granules. His conclusion was that, with all other factors being equal, this could mean that a flour with a higher proportion of small starch granules would have a poorer baking quality. He stated definitely that more work should be done to determine the relationship

between starch granule size distributions and 'flour strength and baking quality' of different wheats.

Another paper published in 1939, by Sandstedt and co-workers, dealt with work on synthetic doughs of a different type. They took a series of wheat flours and separated them into three fractions: washed gluten; 'pure starch', which is equivalent to prime starch as prepared; and 'amyloextrin', or small granules mixed with damaged large granules, cell wall material and other cellular debris. These components were then reconstituted to see if bread equivalent to that produced from the original flours would result. The results showed that the synthetic doughs did produce loaves similar to ones made from flour. They also showed though, that if the 'amyloextrin' fraction was left out, "the handling properties are completely changed" (Sandstedt *et al*, 1929, p.785). The dough was not sticky like normal dough, and the resulting loaves had a greater volume and greater oven spring. However, the crumb of these loaves was tough and rubbery, as opposed to the tender crumb normally found in bread.

Four decades later, Hoseney and co-workers, in 1971, did a very similar set of experiments, with very similar results. They also substituted several different starches into gluten and water solubles fractions from wheat. They found only the starches from rye and barley, both closely related to wheat, gave similar loaf volumes in baking tests. As for the wheat starch fractions, total wheat starch, a

mixture of previously separated prime starch and 'tailings' (equivalent to Sandstedt and co-workers' (1929) 'amylodextrin' fraction), and prime starch alone, all produced loaves with similar volumes. The tailings fraction alone produced a much smaller loaf. However, if the small granules were separated from the tailings fraction, and then added to a dough, the loaf volume was almost the same as with a control flour. They therefore concluded that granule size has no effect on loaf volume. One other of their findings was in agreement with Sandstedt and co-workers (1939): in bread made without the tailings fraction, although the loaf volume was unaffected, the dough absorption and the 'feel' were very different.

As already discussed, D'Appolonia and Gilles (1971) found differences in certain properties of large and small granules isolated from hard red spring flour. In terms of their effects in doughs, these two fractions differed in farinograph behavior and in the loaf volume which resulted when they were used in a gluten-starch-water-solubles mixture. The resulting loaf volume was essentially the same when gluten and starch alone were mixed. In the case of the difference, it was the small granule formulation that resulted in a lower loaf volume.

Rasper and deMan (1980) performed a range of rheological tests on doughs prepared from mixtures of wheat flour and either starches from a variety of sources or glass beads of different sizes, to test the effect of surface area

on the rheological properties. Water retention of these additives was also measured. They concluded that the surface area alone did not account for the differences in rheology of the doughs, ie. the size of the starch granules did not explain the differences. Some factor, such as water-binding capacity, is probably involved. This means that the different-sized granules, which have been shown to produce doughs and breads with different characteristics, are inherently different in some characteristic(s) separate from size alone.

Lelievre and co-workers (1987) measured breadmaking performance as it is affected by the size of the granules in the starch fraction and the amount of protein in the dough. They first summarized the contradicting results published to date on the effect of granule size on dough performance. Their results indicated that the three different granule sizes they used had no effect on proof time, water absorption or bread volume. But for any given protein level, there is an optimum starch 'type' (size). The interaction of the protein concentration and the starch used determined the 'texture' characteristics of the resulting bread. They also pointed out that many variables, from the method used to isolate a starch fraction to the way components are handled, could affect results, and that effects could also be due, not to the size of the starches used but to different physicochemical properties of the different sized fractions.

This is in line with the concluding comments by Rasper and deMan (1980).

Hardness:

Wheat varieties are classified according to several criteria: whether they are hard or soft, red or white, and when they are planted, in autumn ('winter') or spring. None of these qualities is mutually exclusive and varieties exist with all combinations. Different varieties of wheat have different uses, depending on their classification (see Simmonds, 1974; Pomeranz and Williams, 1990, p.474). For example, hard wheats are used for making bread while soft wheats are used for cookies and cakes. The two types of wheat also require different processing conditions for milling. The starch granules in hard wheat varieties are damaged to a greater extent than those in soft wheat varieties during processing. The topic of starch damage is discussed in the appendix at the end of this thesis.

An excellent review on the topic of wheat hardness was published recently by Pomeranz and Williams (1990). In it they stated that "Kernel texture is the most important single characteristic that affects the functionality of a common wheat" (Pomeranz and Williams, 1990, p.473). Their definition for hardness is the one they found in the Oxford dictionary: "difficult to penetrate or separate into fragments". There are a large number of methods for

measuring hardness and many ways in which it can be described, but the cause has yet to be established.

It is generally accepted that hardness is a characteristic which is genetically controlled by a single major gene (Pomeranz and Williams, 1990). This was first shown by Symes in 1965. The gene responsible has been located to the short arm of chromosome 5D (Law et al, 1978, quoted in: Law and Krattiger, 1987).

Several theories exist to explain the molecular basis for this characteristic, but no definitive proof for any one of them has yet been reported. A theory proposed by Stenvert and Kingswood (1977) suggested that it is the nature of the protein matrix alone which determines the degree of hardness. The more continuous and strong this matrix, the harder the wheat. They seemed to equate vitreosity, which is a glass-like appearance, with hardness. It has been shown, however, that they are independent characteristics (see Simmonds, 1974 and Pomeranz and Williams, 1990, for discussions). This theory is not considered likely to hold true (Hoseney, 1987).

A paper in German by Doekes (1985), as described by Hoseney (1987), proposed that a protein fraction is responsible for hardness, and the net charge of this fraction determines whether a wheat is hard or soft. A wheat will be soft if the net charge is high so that the proteins repel each other. Hoseney commented that while this may be a factor for proteins in solution, it is unlikely in dry

grain, which also exhibits a characteristic degree of hardness.

The prevailing theory (see, for example, Hosney, 1987) for the biochemical cause of hardness is that the interaction between the protein matrix and the embedded starch granules is different between hard and soft wheats. Two papers published in 1973, by Barlow and co-workers and by Simmonds and co-workers, support this idea. In the first paper they reported that micropenetrometer tests of starch and protein fractions from different wheats showed no differences in degree of hardness. In the second paper Simmonds and co-workers (1973) reported no differences in the proteins present in extracts of the matrix proteins of different wheats, and concluded that it is unlikely that hardness is a result of variation in matrix protein composition. In both papers they concluded that the adhesion between the two components is the cause of hardness. They suggested that the water-soluble proteins which surround the starch granules are the 'cementing substance' which probably varies between hard and soft wheat varieties. Hosney and Seib (1973) published a series of electron photomicrographs which also support the idea that it is the protein-starch bonding which determines hardness.

Kulp and Lorenz (1981) made some very interesting observations about the starch granule surface characteristics and the dough properties on mixing. They identified the adhering matter on starches isolated in such

a way as to avoid removing it, as being important to forming doughs without difficulty. If the adhering matter was removed and 'pure' starch added, or if starch from a source other than wheat was added, the starch-gluten interactions formed with difficulty, accounting for poor dough performance. The adhering matter was composed of proteins among other things, and these proteins were water-soluble or gluten-like. The adhering proteins apparently contained thiol groups at a level consistent with that in flour as a whole. They gave no values for protein levels and no distinction was made between large and small wheat starch granules in this respect.

Wheat starch proteins:

Over the past decade a series of papers has come out of the labs at the Lord Rank Research Centre (RHM Research Ltd.) and the Flour Milling and Baking Research Association (FMBRA) in England. These papers have dealt with the proteins found to be associated with wheat starch granules. A very short 'communication to the editor' conveyed the most intriguing result of this work and has subsequently catalyzed work in several other labs around the world, including this thesis project. The report of a particular starch granule protein associated with the surface of soft wheat starch granules (Greenwell and Schofield, 1986a) has raised an enormous amount of interest. As has already been

discussed, although hardness of wheat can be described a number of ways, no one can yet explain it. This report provided another clue to a possible biochemical mechanism for softness. The protein, 'friabilin', is present only in small amounts in hard wheat varieties and absent in durum wheats, which are very hard. Durum wheats lack the D genome, which is known to contain the gene for the factor controlling endosperm texture on the short arm of chromosome 5D (Law and Krattiger, 1987). The idea, first put forward by Greenwell and Schofield (1986a), that the gene coding for endosperm texture and that coding for friabilin might in fact be the same, and therefore friabilin might be the 'cause' of softness, has yet to be proven. It has yet to be disproved either.

In 1981 Lowy and co-workers described a salt-extractable protein associated with large and small wheat starch granules. It is worth noting that they separated the large and small starch granules by repeated sedimentation in water, with microscopic examination of the fractions indicating when they had a clean separation. The size range of the fractions was measured by Coulter Counter. They also reported the amino acid composition of the 30-kDa protein, which is distinct from that of wheat gluten proteins, and its pI, which is greater than 10. One more noteworthy result was a positive reaction on staining for glycoprotein. This protein was declared to be a surface protein based on its extractability at room temperature.

Gough and co-workers (1985) reported the effect of SDS on starch granule properties, especially gelatinization temperature. One important point is that SDS causes a decrease in the temperature at which starch granules begin to swell. An attempt was made to extract only surface components with SDS at 50°C, a temperature below that normal for gelatinization of wheat starch. However, swelling was observed. It is not clear why the extraction was not carried out at room temperature if it was important to avoid swelling. They found that the SDS treatment extracted amylose as well as protein and lysophospholipids.

The effects of various treatments on the way in which starch granules are eroded by amyloglucosidase was investigated by Greenwell and co-workers (1985). The results raised questions about the differences between large and small starch granules which cause them to be eroded differently by starch-degrading enzymes. It was found that starch which had not been treated in any way showed considerable surface pitting due to the action of amyloglucosidase. Starch which had been chlorinated or treated with Pronase, a broad-spectrum bacterial protease preparation, or alkali, while being degraded by the same amount of carbohydrate, showed no surface pitting, indicating that the degradation was via a different mechanism. Toluene, often used to remove surface proteins from starches, showed an intermediate effect. The conclusion drawn from this work was that the surface proteins somehow

protect the granule from direct access by the enzyme. Once this protein coat is removed or altered the enzyme can digest the entire surface, leaving it smooth rather than pitted.

The surface composition of starch granules as measured by ESCA (electron spectroscopy for chemical applications) was used as the basis for the conclusion by Russell and co-workers (1987) that the granule surface is enriched, relative to the whole granule, with a series of elements, including N and P from protein and phospholipids. They proposed "a tentative picture.....of a molecular landscape of glucose polymers, sparsely populated with proteins and (phospho)lipoproteins" (Russell et al, 1987, p.98) and suggested that lipids and proteins are closely associated to form complexes.

The work on the starch granule proteins (SGP), and friabilin in particular, was begun because of an investigation into the mechanism by which chlorination of flour prevents the collapse of high-ratio cakes (Greenwell, 1986). These proteins represent approximately 0.2% of the starch granules and approximately 1% of the total flour proteins. They are distinct from the gluten proteins. Five of the SGP were identified as surface proteins, with molecular weights ranging from 5 kDa to 30 kDa, and five as integral proteins, with molecular weights from 59 kDa to 140 kDa. Antibodies to the 59-kDa protein were used to show its distribution in the endosperm and the results supported

the idea that it is integral to the granules and not found in the surrounding protein matrix. Chlorination was found to cause the degradation of the surface SGP to smaller fragments. The 59-kDa protein was suggested to be an amylose-synthesizing enzyme based on analogous proteins, in terms of molecular weight, in other cereals and its absence in 'waxy' varieties of corn, rice and barley.

A report published by Greenwell and Schofield (1986b), focussed on the 15-kDa protein and its association with soft wheats. A survey of 300 wheat varieties showed a consistently strong presence of the 15-kDa protein in soft wheats and only a very low level in hard wheats. By using chromosome substitution lines and homozygous recombinant lines, the gene which codes for the 15-kDa protein was shown to be located on the same chromosome (5D) as the gene coding for hardness. It was suggested that the 15-kDa protein is a 'non-stick' protein, and wheats would all be hard if it wasn't for the presence in some varieties of this protein, which interferes with the adhesion between the starch granules and the surrounding protein matrix.

These results are extremely interesting in light of the results published by Law and Krattiger (1987) on the genetic control of grain hardness. As was mentioned earlier, Symes (1965) established that a single gene is responsible for this grain characteristic. Law and co-workers (1978, as quoted in Law and Krattiger, 1987) located this gene, Ha, on the short arm of chromosome 5D. Law and Krattiger (1987)

reported several more factors seem to be involved in controlling hardness. Using genetically modified lines, they found that hardness results when Ha is absent, and therefore, Ha is either promoting softness or suppressing hardness, as opposed to promoting hardness as was assumed. Their results also indicated that a second gene on the long arm of chromosome 5A is involved. This gene either suppresses Ha, if Ha promotes softness, or promotes Ha, if Ha suppresses hardness. Morrison and co-workers (1989) reported that at least two genes control the levels of free polar lipids in wheat, which showed a correlation with grain hardness (increased hardness lead to decreased levels of free polar lipids), and that one of these genes is possibly allelic to Ha, and therefore might be involved in softness/hardness. The connection among all these factors is clearly not a simple one.

Skerritt and co-workers (1990) provided immunological confirmation of Schofield and Greenwell's results as to the location of the SGP. High molecular weight SGP are internal and low molecular weight SGP are on the surface of the granule. They also showed that there is some cross-reactivity with antibodies raised to gluten proteins, the extent of which is dependent on the class of gluten proteins the antibodies were raised to. They commented though, that the SGP are not contaminating gluten proteins, and that only one to 10% of the total starch protein is residual gluten.

Malouf and co-workers (1992) did some interesting work on reconstitution studies in an attempt to identify the flour component(s) responsible for hardness. Results from measurement of the tensile strength of different combinations of flour components confirmed the idea that protein on the surface of soft wheat starch granules causes a decreased adhesion between the starch granule and the surrounding protein matrix, in agreement with the currently accepted hypothesis of Barlow and co-workers (1973). Treatment of the starch granule preparations with Pronase removed all associated proteins, with no loss of birefringence, indicating no disruption of the granules. Treatment with SDS left proteins of 59 kDa and higher molecular weight.

A study of the surface proteins associated with wheat starch, in particular the proteins associated with the surface of 'prime' starch from chlorinated flours, has been discussed in several papers by Seguchi. His starch protein work centers around the effect of chlorination on the proteins which adhere to the surface of the starch granules. Greenwell and Schofield (1986a), whose work really catalyzed the study of these proteins, also began focussing on the SGP while they were trying to determine the molecular effect of the improving quality of chlorination of cake flours (Greenwell, 1986).

Seguchi has shown that the effect of chlorination is on the proteins which are associated with the starch granules.

Two papers published in 1984 (Seguchi, 1984a, 1984b) showed that whether starch was isolated from chlorinated flour or chlorinated after isolation from non-chlorinated flour, it exhibited increased lipophilicity. The ability of the chlorinated starch to bind oil was decreased or disappeared after treatment with proteases, α -amylase or dilute acid, indicating that it was a surface protein coat that was being altered by the chlorine treatment. This was confirmed in a paper published the following year (Seguchi, 1985) in which it was shown that glass beads coated with a variety of different proteins and then chlorinated, reacted the same way, i.e. they became lipophilic after chlorination. The specific amino acids being affected by the chlorine were identified as cystine, lysine and tyrosine, all appearing as more hydrophobic derivatives. The one confusing result reported in all these three papers was that treatment of the starch granules with SDS did not have any effect on reducing the increased oil-binding ability like the proteases did, implying it had no effect on the surface proteins. This is surprising given that much of the work reported by others centers around the SDS extraction of the wheat starch proteins. Seguchi's own later work also indicated that SDS does indeed extract the surface proteins from the starch granules (Seguchi and Yamada, 1989; Seguchi, 1990). Why he observed no effect in this early work remains a question.

Seguchi has also shown that heat treatment has the same effect on lipophilicity as chlorination and that the effect

is altered by the same factors, such as proteases (Seguchi, 1984c, 1986a).

Another paper (Seguchi, 1986b) discussed the effect of staining unchlorinated starch granules with a variety of protein-specific dyes. By observing the stained granules with a microscope, he concluded that there is a protein film surrounding the granules which can be flaked off. He also concluded, based on treatment of the starches with a variety of compounds chosen to remove the surface protein, "that protein film is indispensable for the tertiary structure of the granules" (Seguchi, 1986b, p.520) as removal 'caused' gelatinization. This is a questionable conclusion, as it is more likely that the very long treatment times (up to several days) in solution is what caused the swelling he calls gelatinization. Several other workers have reported similar treatments, for much shorter times, with no gelatinization of the granules (e.g. Russell et al, 1987; Malouf et al, 1992).

Seguchi and Yamada (1989) used SDS at room temperature to extract the surface proteins from starch granules, with no swelling of the granules even after 24 hour extractions. Staining the extracted granules as before, they confirmed that the surface protein material had been removed. The extracted proteins were analyzed by SDS-PAGE and then stained for the presence of glycoproteins and lipoproteins. They found no evidence for the presence of either.

The most recent paper by Seguchi on this topic (1990) reported that increased levels of chlorination of a given flour increases the amount of protein which associates with the starch granule surface. The 'general observation' is that chlorination affects 'other' wheat proteins, and somehow causes them to adhere to the starch surface, an interesting possibility in terms of the implications for the proteins which are associated with the starch surface even without chlorination.

Two recent papers by South and Morrison (1990) and Sulaiman and Morrison (1990) addressed the issue of proteins associated with large and small wheat starch granules after isolating starch from individual wheat kernels by a new method. South and Morrison (1990) found that their method of isolating the starch fraction, by passing the crude starch fraction through 80% CsCl and then washing the starch with sodium laurate to remove 'contaminating' surface lipids and proteins, got rid of almost all the protein associated with the surface of the starch granules. The one surface protein left was friabilin, which is important as a marker for kernel softness (Greenwell and Schofield, 1986a). In the second paper, describing the results of the protein analysis, Sulaiman and Morrison (1990) claimed that the proteins extracted from A and B starches were 'qualitatively identical'. It should be noted that the method used to separate A and B granules was a 45 minute sedimentation followed by Coulter Counter analysis to determine the size

distributions of the two fractions. These size distributions were not reported in the paper. They admitted that their method of starch isolation causes almost all of the surface proteins to dissociate from the granules, but the one remaining is the one of great interest to many people as a marker for softness. The single-kernel isolation method does not provide enough starch for protein analysis, but they noted that with a few extra kernels enough starch can be acquired. Sulaiman and Morrison (1990) concluded their paper with an interesting comment on the likelihood that friabilin is the cause of softness by the 'anti-stick' mechanism (Greenwell and Schofield, 1986b; Schofield and Greenwell, 1987). Based on a series of assumptions, they calculated that there would not be enough of a layer of this protein on the surface of the starch granules to have the proposed effect, unless there is actually more present than detected or unless it is present as discrete patches instead of as a uniform layer. The point is an interesting one because no one yet knows whether the presence of friabilin is really the cause of the softness characteristic, or whether it is simply very closely linked to whatever is the cause.

Several points are apparent after reviewing the literature discussed above. The large and small wheat starch granules develop separately and the resulting populations have different properties. These two types of granules affect the functional characteristics of flour differently,

although it is not yet clear what the effect is due to. A unique set of proteins is found associated with wheat starch granules and may be involved in some of the functional characteristics of wheat and flour.

Chapter Three

Starch Granule Isolation and Characterization

INTRODUCTION:

There are almost as many methods published for isolating starch from wheat, and for separating it into its component fractions, as there are investigators working with these materials. Over the years many people have examined a variety of characteristics as they relate to the two granule populations, and as a result, many methods of separating and defining the two types of starch have been developed. The large variety of methods is probably due in part to varying availability of specific equipment, in part to different objectives and in part to personal preference or a conviction that a particular method produces starch granules less subject to artifact.

One approach utilizes dough made from flour and water as a source of starch. In this process, starch is washed from a dough with a stream of water and collected in a beaker after being filtered through cloth to separate any large contaminants. This method was used, for example, by Wolf (1964), D'Appolonia and Gilles (1971) and Kulp (1972).

Many of the methods for the isolation of starch consist of different combinations of an established set of steps.

These steps include steeping grain or separated endosperm in different solutions, grinding or homogenizing, sieving or filtering, centrifuging and sedimenting. Evers (1973), for example, steeped kernels in a solution of Pronase and thiomersal (a fungistat) for five days before using a mortar and pestle to carefully grind the grain. After filtering the suspension through a fine mesh screen, the material which did not pass through was retreated with Pronase to release more starch, re-sieved, and the pooled filtrates were filtered again through a yet finer mesh screen. The material which passed through this mesh (55 μm) was the final starch preparation. Adkins and Greenwood (1966) used a solution of mercuric chloride for steeping grain, which was then minced, squeezed, sieved and centrifuged to produce a prime starch fraction. Meredith and co-workers (1978) steeped the grain briefly in an acid solution to inactivate enzymes, neutralized the solution, added thiomersal and left it to steep overnight. The grain was then 'squeezed' through fluted rollers, to avoid damaging the starch granules by milling or crushing. The resulting suspension was sieved then centrifuged several times, each time scraping off the top, dark, layer, yielding a prime starch preparation.

South and Morrison (1990) developed a method to isolate the starch from single kernels of wheat and barley. It involved steeping cracked, degermed kernels in water overnight, then grinding them, collecting the solids by centrifugation, layering a slurry of the sedimented material

over 80% CsCl in a microcentrifuge tube, and centrifuging. The sediment was washed with water and filtered through cloth.

Once the starch has been isolated, there are also many ways in which the large and small granules can be separated from each other and from contaminating material. D'Appolonia and Gilles (1971) mixed the prime starch fraction with the 'tailings' ('sludge') that had been removed in the preparation of the prime starch, to collect large and small granules. This mixture was resuspended and recentrifuged. The starch remaining after the top, 'sludge' layer was removed was recentrifuged, rescraped, and the bottom layer was considered to be the large granule fraction. The sludge layer removed after the first centrifugation was recentrifuged at a higher speed, and the bottom layer from this treatment was considered to be the small granule fraction. Photomicrographs of the two fractions showed that they were both contaminated with the other fraction to an extent that would tempt one to call the fractions 'enriched' rather than truly fractionated.

Evers (1973) passed his starch preparation through a shaking 'microplate sieve' with 10 μm diameter holes drilled in it, until the different size granules had been separated. Although he said that the separation was not complete, the photomicrographs of his A and B granule preparations showed much better separation than that shown by D'Appolonia and Gilles (1971).

Bathgate and Palmer (1972) used three different methods to separate large and small granules. The first involved sieving a prime starch preparation through nylon mesh (10 μm) using a magnetic stir bar to keep the starch slurry suspended. They found an 'enriched' large granule fraction retained by the mesh, but a small granule fraction contaminated with large granules which managed to pass through the screen. The second method, 'decantation', was used subsequent to the first to remove contaminating large granules from the small granule preparation. This involved allowing the large granules to sediment, then pouring off the still-suspended small granules. The third method was used to remove the small granules remaining with the large granule fraction. To do this they shook an aqueous suspension of starch granules with toluene. The small granules accumulated in the toluene layer.

Meredith (1981), using the method of Meredith and co-workers (1978), separated wheat starch into different size classes by countercurrent sedimentation. He published electron micrographs of the different fractions, which appeared to be cleanly separated. The sedimentations varied from two minutes through to 16 hours.

Duffus and Murdoch (1979) took wheat endosperms and homogenized them in a sucrose solution, filtered the resulting slurry through cloth and centrifuged the filtrate through a discontinuous sucrose gradient. The different size classes were separated from the appropriate interfaces and

collected by centrifugation through diluted sucrose solutions, and finally washed with water.

There are more variations, but those described above are representative of the approaches taken to isolating and separating starch granules from wheat. Most investigators performing such separations monitored their procedure by microscopic examination of the different steps, and of the final products. This remains the simplest, most effective way to determine whether or not a starch preparation is contaminated with other material or with starch granules of a different size. Some investigators, for example Duffus and Murdoch (1979), have also used the microscope to measure the resulting starch granules. Many more use a Coulter Counter to determine the size distribution of their fractions, to the extent that those who do not use this method are in the minority. A paper by Morrison and Scott (1986) dealt specifically with this methodology. Evers and Lindley (1977) used image analysis as well as Coulter Counter analysis to quantify the sizes of isolated starch granules. Recently, Bechtel and co-workers (1990) used image analysis for a detailed study of the size distribution of starch granules during endosperm development.

In measuring the size distribution of starch granule preparations, the assumption is usually made that the granules are all spherical. Particularly when using a Coulter Counter as the measuring device, a spherical shape is assumed because the Coulter Counter is calibrated using

spherical latex beads and actually measures volume based on this calibration, and from this a diameter value is derived. Soulaka and Morrison (1985) calculated granule size distributions from Coulter Counter measurements assuming all granules were spherical: "no allowance was made for larger particles being oblate spheroids" (Soulaka and Morrison, 1985, p.711). Morrison and Scott (1986) referred to the fact that while the small granules are spherical, the large granules are not. Hughes and Briarty (1976) referred to large 'lenticular' A granules, with long axes of 15-50 μm and small spherical B granules with diameters up to 10 μm . Meredith and co-workers also calculated diameters and surface areas from Coulter Counter measurements "ignoring asymmetry of the granules" (Meredith et al, 1978, p.120). Meredith claimed that the small granules are "nearly as asymmetric as large ones" (Meredith, 1981, p.43), based on a comparison of the diameter derived from the volume measurements which result from Coulter Counter analysis assuming sphericity, with the diameters measured from electron micrographs. He suggested that "we see what we expect to see" (Meredith, 1981, p.43). Stamberg (1939) wrote that wheat starch granules, with no distinction made as to the size fraction, are oblate spheroids, but he assumed spherical shape for the calculation of surface area. Eliasson and Karlsson (1983) also assumed sphericity for their calculations.

Density values for starch granules vary widely in the literature. Most investigators stated the value they used for their calculations, but very few gave the source of that value and even fewer made any attempt to measure it. Soulaka and Morrison (1985) assumed a hydrated density of 1.4 g/cm^3 in their calculations of specific surface areas. The density value Meredith and co-workers (1978) assumed for starch at 13.2% moisture content was 1.5 g/cm^3 . Stamberg (1939) also assumed a density of 1.5 g/cm^3 . Buttrose (1960) referred to starch granules as having a high density value of 1.5- 1.6 g/cm^3 . Eliasson and Karlsson (1983) assumed a density of 1.5 g/cm^3 for their calculations. D'Appolonia and Gilles (1971) determined the absolute densities of large and small wheat starch granules, and got values which were virtually identical: 1.488 g/cm^3 for small granules and 1.486 g/cm^3 for large granules. In the introduction to a paper on a new method for determining the density of wheat starch granules, Dengate and co-workers (1978) discussed some of the earlier work on this topic and quoted values ranging from 1.3 to 1.6 g/cm^3 . They also referred to work published in several German journals in the early fifties which found small granules to be denser than large granules, although they quoted a value of 1.50 g/cm^3 without noting which population it applied to. Their own work resulted in values of 1.59 g/cm^3 for a starch fraction with diameters of greater than $20 \text{ }\mu\text{m}$, and 1.60 g/cm^3 for a fraction with diameters of less than $6 \text{ }\mu\text{m}$. This very small difference was not

considered significant. For hydrated density values, they found 1.30 g/cm^3 for large granules and 1.28 g/cm^3 for small granules. They concluded that the lower hydrated density for small granules was a result of higher water content due to a greater degree of starch damage. Their final conclusions were that the dry density of wheat starch granules is 1.6 g/cm^3 , air-equilibrated starch has a density of 1.5 g/cm^3 and the hydrated density is 1.3 g/cm^3 , although this will vary depending on the extent of damage.

The initial step in investigating the proteins associated with large and small wheat starch granules was to separate the starch granules into these fractions. A method was developed which accommodated the requirements of this study and which differed, in the combination of steps used, from previously published methods. The starch, as isolated by this method, was then analyzed by digital image analysis to define which portions of the total starch population were being isolated as 'large' and 'small' granules, and what the sizes of these fractions were. The relative surface areas and volumes of the two types of starch granules were calculated based on these measurements and the assumption that the density of both is the same.

MATERIALS AND METHODS:

Chemicals:

Unless otherwise noted, all chemicals were standard laboratory grade preparations obtained from commercial sources.

Starch preparation:

Wheat flour and sufficient water to develop a dough (e.g. 10 g soft flour + 6.5 ml dH₂O) were mixed either in a home-made farinograph with a 10 g mixing chamber or in a Brabender Farinograph with a 50 g mixing chamber. Once a dough had been developed, the mixing speed was reduced and water was added to wash out the starch. The starch 'cream' was removed by pipetting. This procedure was continued until very little starch was being washed out. The pooled starch slurry was passed through a 355 μ m mesh screen to remove pieces of gluten and large bran fragments. The collected slurry was centrifuged (at approximately 1200 x g) and the supernatant discarded. The starch was resuspended in water and recentrifuged three more times. After the final centrifugation the upper layer of the starch pellet was separated from the lower layer.

The upper layer contained the small starch granules contaminated with cell wall fragments, bran pieces, some

gluten and damaged large granules. These other components were removed from the small granules by sieving successively through 30 μm and 20 μm nylon meshes. The filtering apparatus was a home-made variation of a Millipore-type filter unit. The sintered glass platform normally found in the lower portion of such an apparatus was not incorporated. A piece of metal screen was cut to fit the opening and was used as a support for the nylon mesh screens used to filter the starch preparations. The starch slurry was stirred with a 'rubber policeman', without the application of vacuum, and water added as required until the material retained appeared to be essentially free of starch granules. This was determined by visual inspection - the residue became less white as the starch was washed through - and by microscopic examination. It is worth noting that this would be a good way to isolate wheat endosperm cell walls, which comprised the majority of the retained material. The small starch granules were collected by centrifuging the filtrate and were set out to dry in a small dish at room temperature.

The lower layer contained the large starch granules contaminated with small granules. To remove the small granules, the starch was suspended in water and allowed to sediment several times. The supernatant containing the small granules was either discarded or used as a source of additional small granules. Once the settled starch was free of small granules, as determined by microscopic examination, it was also set out to dry at room temperature. Each starch

fraction was then ground with a mortar and pestle until it passed through a 355 μm screen and was stored at 4°C. The steps in the starch isolation procedure are shown in Figure 2.

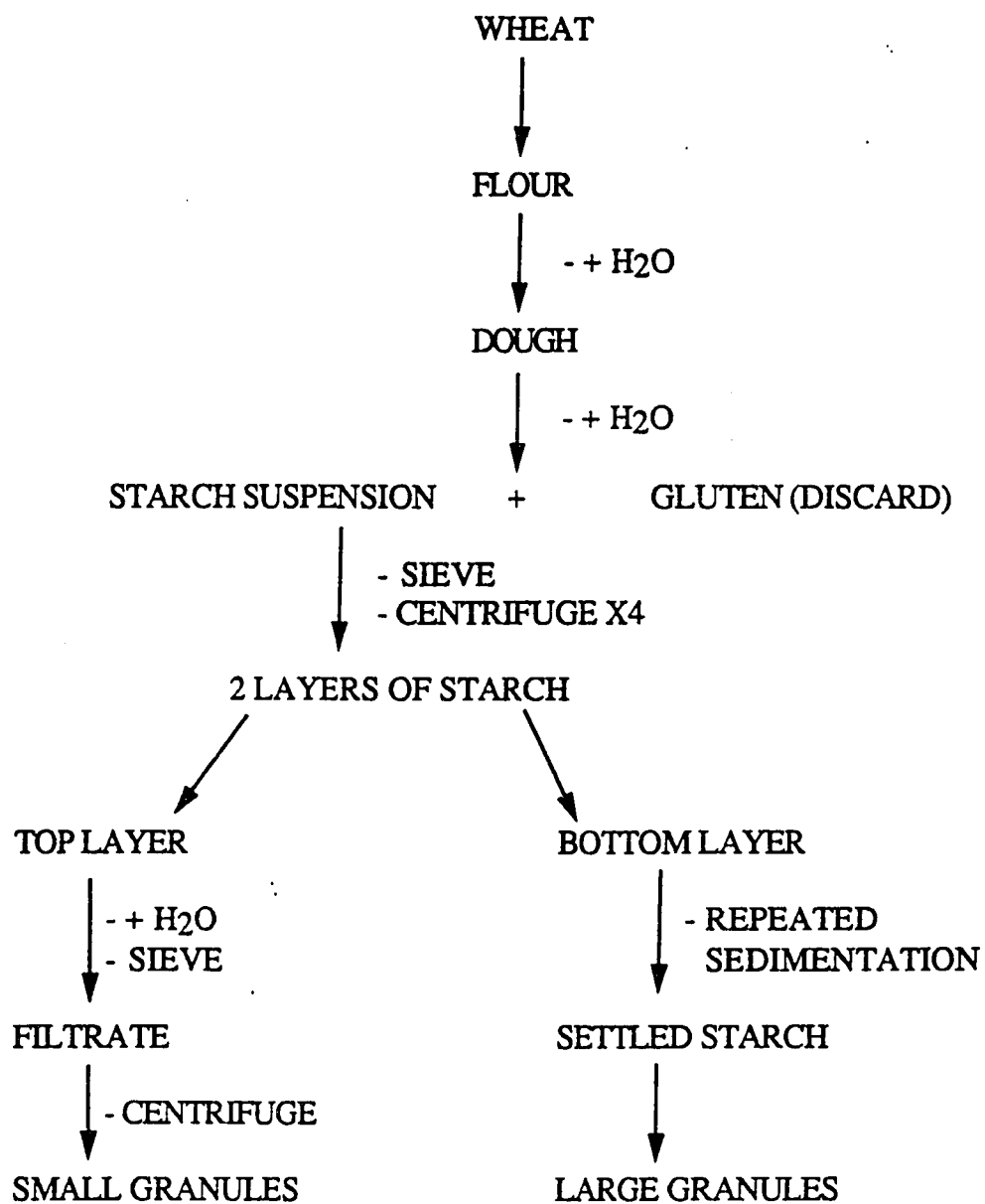
Image analysis:

Image analysis of the starch and flour samples was carried out on a Kontron IBAS system, with images input using a Sony model XC-77 CCD TV camera connected to a Zeiss Universal microscope, with a 40X objective. The system was calibrated by imaging a micrometer and saving the scale data. This scale definition was then used for all sample measurements.

Flour samples from Fredrick and Neepawa wheats, and large and small starch granules isolated from each variety, were analyzed to determine the size distributions of the starch granule fractions. A small amount of each sample was placed on a microscope slide and a drop of IKI solution (1.2% KI, 0.6% I₂, aqueous) was added. Excess stain was removed with absorbant paper after a cover slip was in place. The cover slip was sealed on with clear nail polish to prevent dehydration of the preparation during analysis.

For each of the flour samples approximately 5000 starch granules were measured, and for each of the starch samples approximately 1000 starch granules were measured. Sequential

Figure 2. Starch isolation and separation procedures.



fields were selected until the required number of measurements was reached.

Several parameters were measured, but 'Dmax' (maximum diameter) is the one of interest in this study. It should be noted that a 2-dimensional image is measured by the procedure, and therefore the Dmax values, for the large granules in particular, are from granules which are lying 'flat', given that they are oblate spheroids. All the data were saved and then analyzed with the Kontron Videoplan statistical analysis package.

Calculation of surface areas and volumes of starch granules:

The surface area and volume were calculated for 'typical' large and small starch granules. Three sets of calculations were done using the following parameters: granule diameter values reported in the literature; values for Fredrick granules determined in the present study; and values for Neepawa granules, also determined in the present study. The measured values were those obtained from the image analysis, described above. The small granules were assumed to be spherical and the large granules were assumed to be oblate spheroids. The equations used in each case are shown, along with the resulting values, in Table 3.

The eccentricity value (ϵ) is an indication of the deviation from sphericity. The closer the value is to 1, the more closely the object resembles a sphere. This factor was

required in the calculation of the surface area of the large granules. It was calculated by measuring microscopically the maximum and minimum axes of 100 large starch granules with an eyepiece micrometer and a 40X objective lens. Only granules which were oriented 'sideways' were measured. By preparing a slide with large granules crowded together, sufficient numbers were found in the appropriate orientation to make the measurements. The 100 maximum axis values were averaged, as were the 100 minimum axis values. The resulting averages were then used to calculate ϵ using the following equation: $\epsilon = c/a$, where c is the hypotenuse of a right triangle, side a is the radius of the long axis and side b is the radius of the short axis. The values obtained from the measurements were: $a = 13.8 \mu\text{m}$; $b = 7.2 \mu\text{m}$; and therefore, $c = 11.8 \mu\text{m}$. The result was $\epsilon = 0.85$. The other value obtained through this exercise was the ratio of the short axis to the long one, which equaled 0.52. This value was used to calculate b for the large granules from the Fredrich and Neepawa varieties, for which it was assumed that the values resulting from the image analysis represented maximum diameters.

RESULTS:**Starch preparation:**

Figure 3 shows typical preparations of the two starch populations by the procedure described. The method developed for isolating large and small starch granules resulted in fractions essentially free of the other type of granule and all contaminating cellular debris, such as cell wall fragments, bran and gluten pieces. Using a cutoff value of 10 μm between large and small granules, typical preparations were 98.9 - 100% granules of the appropriate size (Table 1).

Image analysis:

The histograms presented in Figure 4 show the size distribution, as 'Dmax', of the starch granules in Fredrick flour (a) and in the large and small starch granules isolated from this flour (b and c, respectively). In Figure 5, the corresponding histograms for Neepawa flour and starch fractions are shown. Both the large and the small granules from Fredrick, the soft wheat variety, were larger i.e. had a larger Dmax, than the corresponding fractions from the hard wheat variety Neepawa. Table 1 lists the values obtained for all the different samples. Comparison with the size distributions of the starch granules in the flours from which the starches were isolated (Table 2) shows

Figure 3. Isolated starch fractions.

Large (A) and small (B) starch granules as prepared by the procedures described. The scale bars represent 100 μm .

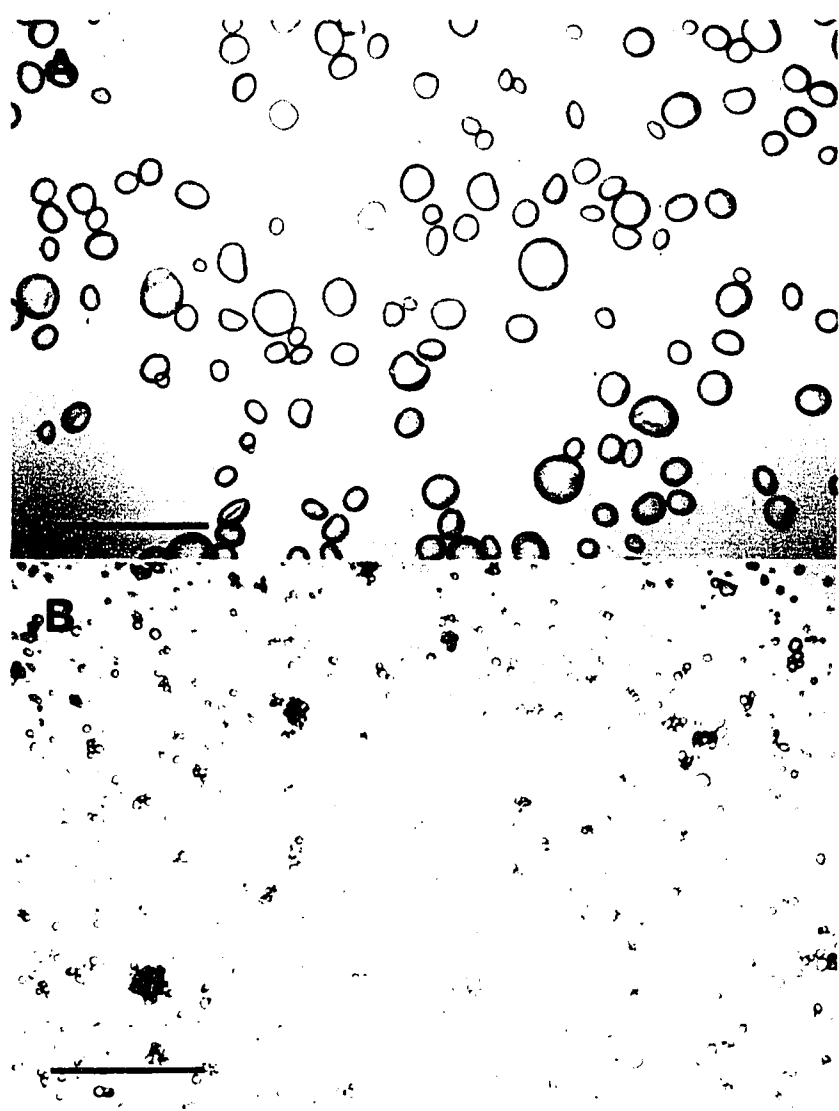
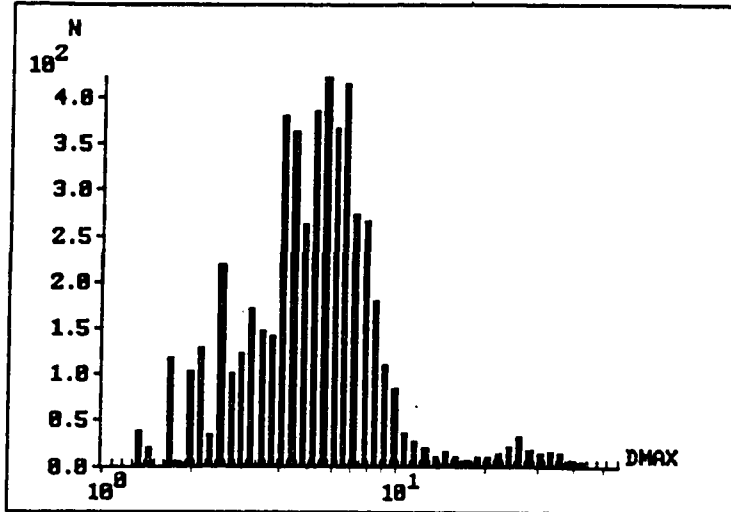


Table 1. Size distributions of isolated starch fractions.
The average diameters of the large and small starch granules isolated from the soft wheat variety Fredrick and the hard wheat variety Neepawa are listed. The values were determined for all the granules in the preparations, and those either above (large granules) or below (small granules) cutoff points of 10 μm and 15 μm . The % values are of the total number of granules measured and indicate the purity of the preparations. The values are derived from the data presented in Figures 4 & 5, parts b & c.

	Fredrick			Neepawa		
	average diameter	# of granules	% of total	average diameter	# of granules	% of total
large:						
all	23.82	1003	100	21.16	1027	100
>10 μ m	23.82	1003	100	21.22	1023	99.6
>15 μ m	24.07	978	97.5	22.08	921	89.7
small:						
all	4.94	1035	100	4.56	1018	100
<10 μ m	4.88	1025	99	4.48	1007	98.9
<15 μ m	4.94	1035	100	4.56	1018	100

Figure 4. Size distributions of starch granules in flour and isolated starch fractions from Fredrick wheat.

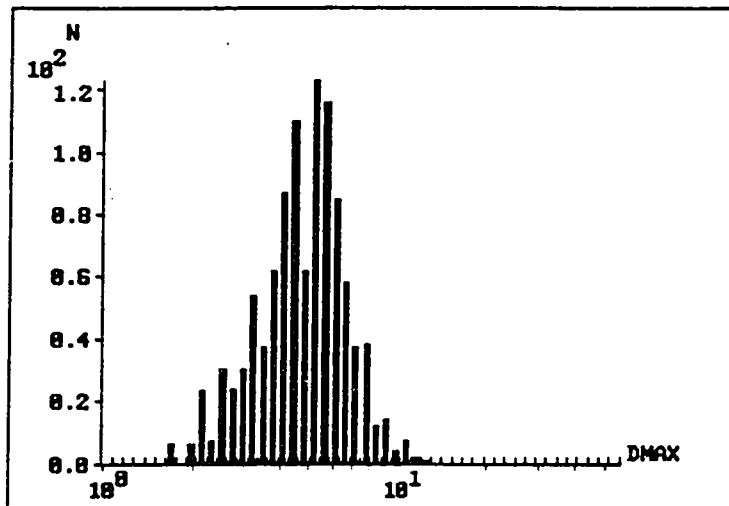
Maximum diameter ('Dmax') as measured by digital image analysis is the parameter shown as an indication of granule size. The distribution in flour milled from Fredrick wheat is shown in A; that of the small granules isolated from the flour is shown in B; and that of the isolated large granules is shown in C. The x axis is in μm and the y axis values are normalized counts. The number of granules measured for each sample is indicated in the report listed beside each histogram.

A

Counts : 5132
 lower bound: 1.00
 upper bound: 56.00
 underflow : 0
 in range : 5132
 overflow : 0
 Classes : 50
 Modul : 1.00

Percentil classes
 25 x : 17
 Median : 21
 75 x : 24
 95 x : 38

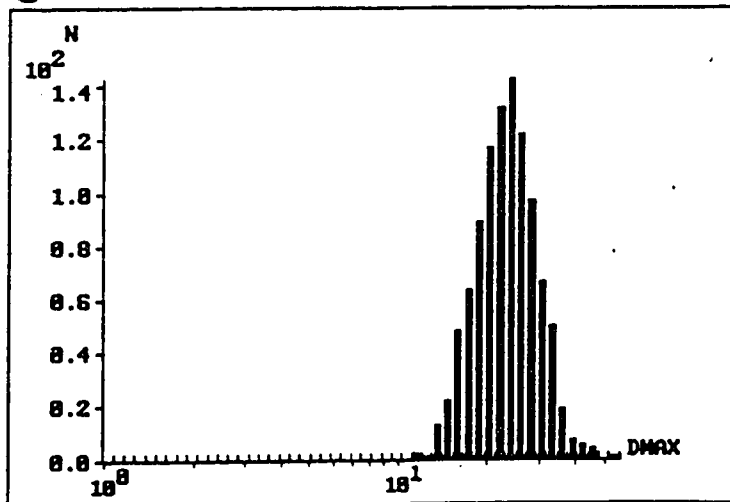
Statistics
 Minimum : 1.33
 Maximum : 43.96
 Mean : 6.00
 Median : 5.63
 Variance : 21.66
 Std.dev. : 4.65

B

Counts : 1835
 lower bound: 1.00
 upper bound: 56.00
 underflow : 0
 in range : 1835
 overflow : 0
 Classes : 50
 Modul : 1.00

Percentil classes
 25 x : 17
 Median : 20
 75 x : 22
 95 x : 26

Statistics
 Minimum : 1.65
 Maximum : 13.84
 Mean : 4.94
 Median : 5.34
 Variance : 2.83
 Std.dev. : 1.68

C

Counts : 1003
 lower bound: 1.00
 upper bound: 56.00
 underflow : 0
 in range : 1003
 overflow : 0
 Classes : 50
 Modul : 1.00

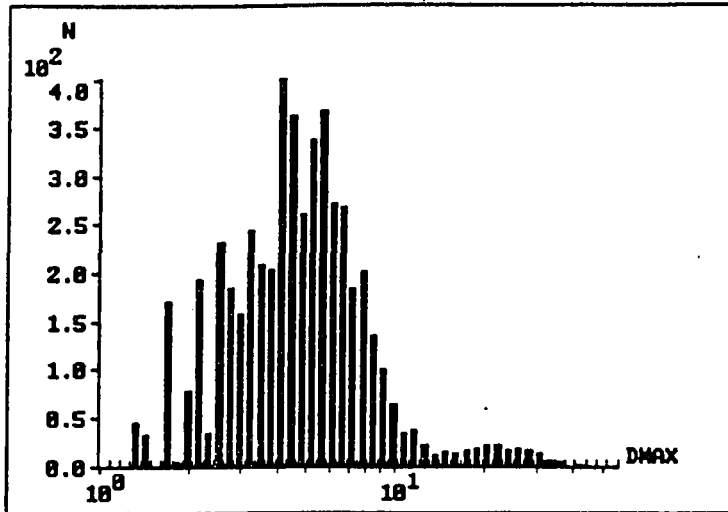
Percentil classes
 25 x : 30
 Median : 40
 75 x : 41
 95 x : 44

Statistics
 Minimum : 11.22
 Maximum : 55.56
 Mean : 23.82
 Median : 23.28
 Variance : 31.69
 Std.dev. : 5.63

Figure 5. Size distributions of starch granules in flour and isolated starch fractions from Neepawa wheat.

Maximum diameter ('Dmax') as measured by digital image analysis is the parameter shown as an indication of granule size. The distribution in flour milled from Neepawa wheat is shown in A; that of the small granules isolated from the flour is shown in B; and that of the isolated large granules is shown in C. The x axes are in μm and the y axis values are normalized counts. The number of granules measured for each sample is indicated in the report listed beside each histogram.

A



Counts : 5038
 lower bound: 1.00
 upper bound: 56.00
 underflow : 0
 in range : 5038
 overflow : 0
 Classes : 50
 Modul : 1.00

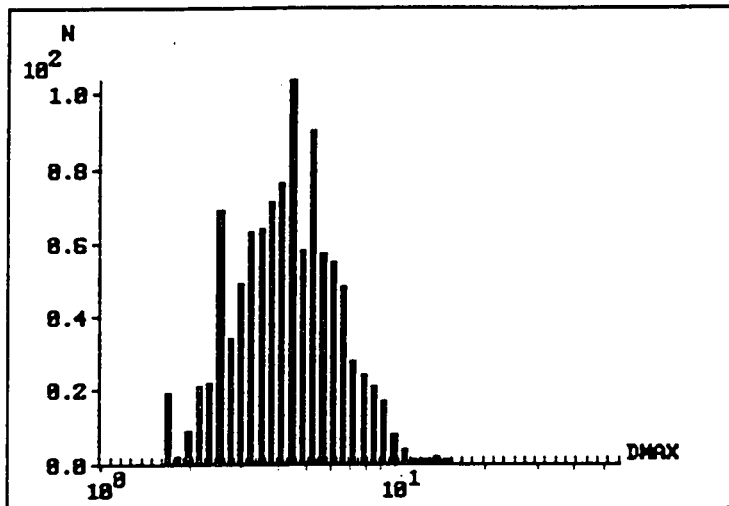
Percentil classes

25 x : 15
 Median : 19
 75 x : 23
 95 x : 38

Statistics

Minimum : 1.33
 Maximum : 41.98
 Mean : 5.47
 Median : 5.24
 Variance : 17.01
 Std.dev. : 4.12

B



Counts : 1818
 lower bound: 1.00
 upper bound: 56.00
 underflow : 0
 in range : 1818
 overflow : 0
 Classes : 50
 Modul : 1.00

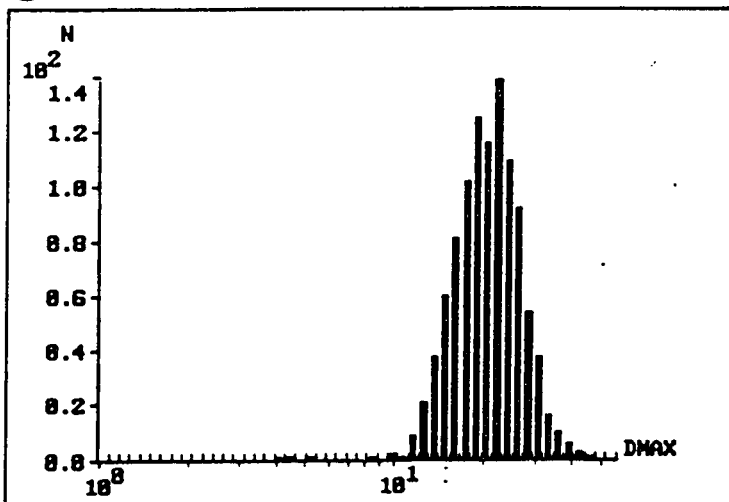
Percentil classes

25 x : 15
 Median : 19
 75 x : 22
 95 x : 27

Statistics

Minimum : 1.65
 Maximum : 14.31
 Mean : 4.56
 Median : 4.36
 Variance : 3.51
 Std.dev. : 1.87

C



Counts : 1827
 lower bound: 1.00
 upper bound: 56.00
 underflow : 0
 in range : 1827
 overflow : 0
 Classes : 50
 Modul : 1.00

Percentil classes

25 x : 36
 Median : 38
 75 x : 48
 95 x : 43

Statistics

Minimum : 3.94
 Maximum : 46.85
 Mean : 21.16
 Median : 28.32
 Variance : 29.29
 Std.dev. : 5.41

Table 2. Size distributions of starch granules in flours milled from Fredrick and Neepawa wheats.

The average diameters were determined for all the granules in each flour and for the large and small granule fractions using cutoff points of 10 μm and 15 μm . The % values are of the total number of granules measured in the flour and indicate the proportions of the total starch populations contributed by each size fraction. The values are derived from the data presented in Figures 4a & 5a.

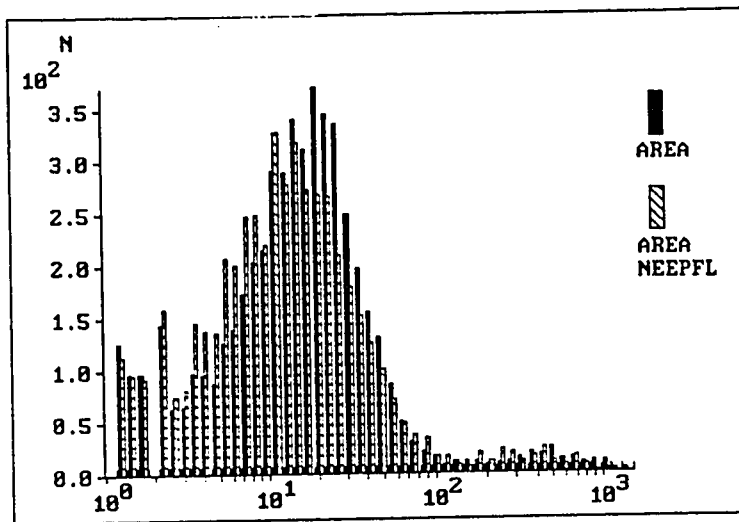
	Fredrick			Neepawa		
	average diameter	# of granules	% of total	average diameter	# of granules	% of total
all:	6.00	5132	100	5.47	5030	100
large:						
>10 μ m	19.99	320	6.2	17.78	320	6.4
>15 μ m	26.33	183	3.6	23.16	172	3.4
small:						
<10 μ m	5.07	4812	93.8	4.63	4710	93.6
<15 μ m	5.14	4949	96.4	4.84	4858	96.6

that the separated starch fractions are representative of those fractions of the whole starch population in the flour. The histograms and derived values also show that the isolated starch fractions are essentially free of contamination from different sized granules. There is also some evidence from the image analysis data from the flour samples, especially in the histograms of the area and 'Dcircle' (equivalent circle diameter) (Figure 6), for the third population of very small starch granules described by Bechtel and co-workers (1990). Apart from this, all the parameters show the classic bimodal distribution.

Calculations:

Table 3 contains the results of the calculations of surface area and volume for the two starch fractions, using a cutoff of 15 μm between large and small starch granules. Results are also listed for the calculations done using values of 5 μm diameter for small granules and 30 μm for large granules. These values were assumed for the calculations, based on the ranges of values published, before the measured values from image analysis were available, and are relevant to some results presented in Chapter Four. The large granules of both varieties of wheat have approximately 16.5X the surface area of the small granules from the same variety, and approximately 60X the volume. For the set of calculations on the estimated values,

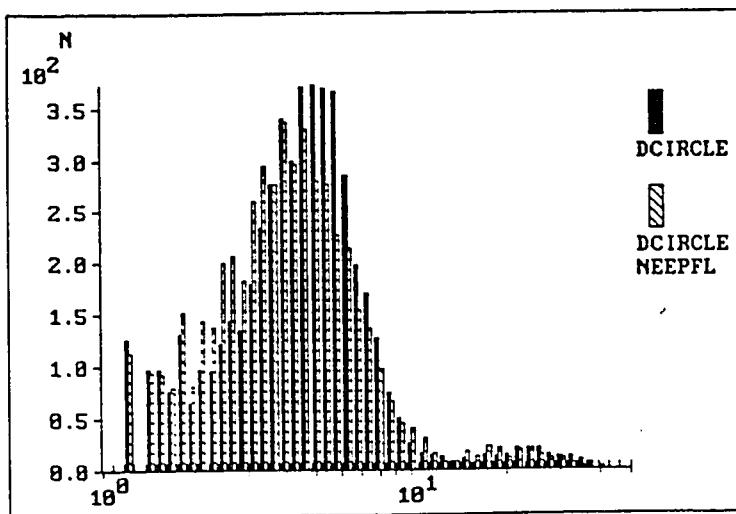
Figure 6. Area and equivalent circle diameter ('Dcircle') distributions in flour from Fredrick and Neepawa wheats. These parameters, measured by digital image analysis, show evidence of the third population of very small starch granules described by Bechtel and co-workers (1990). The x axis in A is μm^2 and in B, μm , and the y axes are normalized counts. The solid bars represent measurements from Fredrick flour and the hatched bars represent measurements from Neepawa flour.

A

Counts : 5132
 lower bound: 1.00
 upper bound: 1500.00
 underflow : 0
 in range : 5132
 overflow : 0
 Classes : 50
 Modul : 1.16

Percentil classes
 25 % : 14
 Median : 19
 75 % : 23
 95 % : 30

Statistics
 Minimum : 1.19
 Maximum : 1426.92
 Mean : 35.56
 Median : 14.81
 Variance : 10424.37
 Std.dev. : 102.10

B

Counts : 5132
 lower bound: 1.00
 upper bound: 50.00
 underflow : 0
 in range : 5132
 overflow : 0
 Classes : 50
 Modul : 1.08

Percentil classes
 25 % : 15
 Median : 20
 75 % : 23
 95 % : 29

Statistics
 Minimum : 1.23
 Maximum : 42.62
 Mean : 5.18
 Median : 4.59
 Variance : 18.47
 Std.dev. : 4.30

Table 3. Surface areas and volumes calculated for different starch fractions.

The average diameter values determined by digital image analysis for the large and small starch granule fractions from Fredrick and Neepawa wheats were used to derive the values for the calculations, and are indicated in the table. Calculations were also performed using the values of $d=5 \mu\text{m}$ for small granules and $d=30 \mu\text{m}$ for large granules based on values commonly cited in the literature. The equations shown at the bottom of the table were used to calculate the values listed.

Reported values:	large (d=30 μm)	small (d=5 μm)	ratio lg/sm	ratio vol./ ratio s.a.
surface area:	1978.6 μm^2	78.5 μm^2	25.2	4.5
volume:	7351.3 μm^3	65.4 μm^3	112.4	
Fredrick wheat:	large (d=24.07 μm)*	small (d=4.94 μm)*	ratio lg/sm	ratio vol./ ratio s.a.
surface area:	1274.7 μm^2	76.7 μm^2	16.6	3.6
volume:	3801.2 μm^3	63.1 μm^3	60.2	
Neepawa wheat:	large (d=22.08 μm)*	small (d=4.56 μm)*	ratio lg/sm	ratio vol./ ratio s.a.
surface area:	1071.7 μm^2	65.3 μm^2	16.4	3.6
volume:	2930.5 μm^3	49.6 μm^3	59.1	

* diameter values (d) for Fredrick and Neepawa assume a cutoff of 15 μm .

equations:

surface area, sphere:

$$=4\pi(r)^2$$

surface area, oblate spheroid:

$$=2\pi a^2 + \pi \frac{b^2}{\epsilon} \log_e \frac{1 + \epsilon}{1 - \epsilon}$$

volume, sphere:

$$=4/3\pi r^3$$

volume, spheroid:

$$=4/3\pi a^2 b$$

the surface area ratio (large/small) is approximately 25 and the volume ratio is approximately 112. Assuming equal densities, samples of an equal weight of both types of granules would have an equal volume, and therefore a small granule sample would have 3.6X the surface area of a large granule sample (4.5X for the estimated values).

DISCUSSION:

The work presented in this thesis was carried out primarily on starch isolated from 'Fredrick', a soft white winter wheat grown in eastern Canada. Some of the work was also done on starch isolated from 'Neepawa', a hard red spring wheat grown in western Canada. Initially, many different wheats, both hard and soft varieties, were analyzed for the starch-associated proteins in prime starch preparations. These varieties were selected from those available from various field trials at the Agriculture Canada Central Experimental Station in Ottawa. Once the focus of the project centered on the difference between large and small wheat starch granules, one soft wheat and one hard wheat variety were selected as the sources of starch granules for the analyses performed. It was established by Greenwell and Schofield (1986b) that, with the exception of minor variations, the proteins associated with starches from hundreds of varieties of wheats were the

same, with the exception of the 15-kDa protein found strongly associated with soft wheat starches but not hard wheat starches. For this reason, it was decided that a soft wheat variety would be used for all analyses carried out in order to include the 15-kDa protein which has raised so much interest, even though this protein was not the focus of this project. For some analyses, starch from a hard wheat variety was also analyzed for comparison.

This work was initiated in the Grain Quality Lab at Agriculture Canada, where many different quality tests are routinely performed on hundreds of new wheat varieties a year. Standard varieties are used for comparison. A soft wheat standard at the time was Fredrick. It was well characterized and readily available, and therefore was chosen as the soft wheat variety to be used. The hard wheat standard in use at the time was a mixture of different varieties. It was decided that a single variety should be used for the extraction of hard wheat starch as well. Hard wheats are not grown to a large extent in eastern Canada, but they are the mainstay of western Canadian wheat farmers. The Winnipeg Station of Agriculture Canada Research Branch provided a well-characterized hard red spring wheat - Neepawa. Should more have been required it could therefore have been obtained with the assumption of consistency.

The method developed to cleanly separate large and small starch granules is yet another combination of the steps frequently used in isolating and separating wheat

starch granules. While similar to several published methods, there are important differences. It was of extreme importance to obtain highly purified large and small granules, but total recovery of the starch was not required, nor was it necessary to avoid damaging the granules during their isolation. Protease treatment of grain (e.g. Evers, 1973) was not an option given that the starch was being isolated as a source of proteins. Damaged granules were not a factor in this work because the extent of water absorption was not an issue (see the appendix for further discussion of starch damage and a method for visualizing damaged granules), and so flour instead of grain was used as the source for starch, which made the procedure easier. A dough was used as the source of the crude starch preparation, as it was by D'Appolonia and Gilles (1971). Also like D'Appolonia and Gilles (1971), the sludge layer removed from above the prime starch layer after centrifugation, was retained instead of being discarded, although these two layers were treated differently (Figure 2). Like Bathgate and Palmer (1972), decantation was used to remove contaminating small granules from the prime starch, which was the source for large granules. The sieving method used by both Bathgate and Palmer (1972) and Evers (1973) was applied to the sludge layer which was the source of the small granules. Instead of simply using a 10 μm mesh screen though, two different-sized meshes were used. The first was a 30 μm mesh screen which removed a large fraction of the

contaminating cell wall and bran material. It also retained quite a bit of the contaminating damaged large granules found in the sludge layer. The filtrate was then passed through a 20 μm mesh screen which removed the rest of the cell wall material and most of the rest of the contaminating large granules. Initially, a 10 μm mesh screen was used as the last step, but it was found that essentially all the contaminants had been removed in the previous steps, and the few remaining large granules were better removed a different way. It was very difficult to get the starch suspension through so small a mesh and even then some large granules, possibly because they tend to be damaged and therefore less rigid, passed through. It was much simpler and quicker to use a final decantation step, saving the supernatant and discarding the settled material, to remove the residual large granules. This method, while tedious, yielded fractions with essentially no contaminating material, as illustrated in Figure 3 and the data in Table 1. It also allowed recovery of the small granules from the layer usually discarded during the preparation of a prime starch fraction. The only solvent to which the starch was exposed was water. Both large and small granule preparations were exposed to large volumes of water, and so any water-soluble proteins which may be associated with the starch granule surfaces were undoubtedly removed. No claim is made to this method being quantitative. There were definite losses of both large and small granules, but the goal was clean

fractions and not total recovery of the starch present in the starting material.

Image analysis of the starch in flour samples was carried out to provide a description of the raw material from which the starch was isolated. Measurement of the size distributions of the isolated starches had two purposes. The first was to see which parts of the total starch population were being isolated and labelled as 'large' and 'small' granules. The second purpose was to check the effectiveness of the isolation procedure as to the purity of the separated fractions, or conversely, the degree of contamination by the other size granule. The histograms in Figures 4 & 5 and the values in Table 1 show that the large and small granule fractions were representative of those fractions in each type of flour. For the two wheat varieties analyzed, it is clear from the size distributions in the flour samples and from the sizes of the separated fractions, that both the large and the small granules from Fredrick, the soft wheat variety, are larger (as measured by Dmax) than the corresponding fractions from Neepawa, the hard wheat variety.

The measurements which resulted from image analysis of the flour and starch samples can be examined in a number of different ways. Convention defines the cutoff between large and small wheat starch granules as a diameter of 10 μm . In the flours measured, an obvious cutoff is approximately 15 μm . The determination of proportion of the total starch

population and the average diameter has been calculated for cutoff points of both 10 μm and 15 μm (Table 2).

The great majority of the granules in the flours are small, as shown by the very low overall D_{max} values and the calculated proportions of granules with D_{max} less than either 10 μm or 15 μm . This is completely consistent with everything else published, as discussed in Chapter Two. The image analysis data also show that the separated large and small granules from both the flours contained 1.1% or less of contaminating granules from the other size fraction. The average D_{max} for the large and the small granules isolated from the hard and the soft wheats were slightly lower than the values derived from the starch granule distributions in the flours. It should also be noted that the measurement of the flours was done at a different time from that of the isolated starches, and it is therefore possible that the conditions on these two occasions were slightly different. The calibration scale was stored and used on both occasions, but the focussing of the sample, for example, could have varied slightly. The isolated fractions therefore do not exactly represent those portions of the total starch populations, but they very nearly do, based on the closeness of the values and the distributions shown in the histograms.

Morrison and Scott (1986) reported mean diameters derived from mean volume results for their Coulter Counter measurements of large and small granules. There was variation of course among the different samples measured,

but the mean diameters of the small granules encompass the values reported here. For the large granules however, the range of mean diameters was considerably lower than that found here or that usually considered typical of large granules. The values ranged from 12.47 through 16.96 μm . This was probably due to the diameters being derived from volume measurements under the assumption that the particles are spherical. If the appropriate calculations were done assuming the large granules are oblate spheroids, as these authors acknowledge, the mean diameters should work out to be higher and closer to expected values.

Based on the average diameter of large and small granules, as determined by the image analysis data, and the assumptions listed in the methods section, the surface area and volume of 'typical' large and small granules were calculated. These values are presented in Table 3. Assuming an equal density for the two types of starch, it can be seen that for an equal weight of starch (and therefore an equal volume of granules) there would be 3.6X the surface area associated with the small granules as with the large ones. It is interesting that, although the values for the granules from the two types of wheat differ, the ratios and the ratio of the ratios (indicating the difference in the weight required for equal surface area) are quite consistent. Again, these calculations are intended only to give some idea of the relationship between surface area and volume for the large and small granules, and not to be absolutely

correct, or statistically significant, values. In this case however, the large granules have not been assumed to be spherical, but rather oblate spheroids, and the appropriate formulas were used. The assumption that the small granules are spherical has been maintained. A more thorough investigation would be required to clarify the assumptions of size, shape, density, size distribution and eccentricity value to determine accurately and precisely such values as have been presented here. Dengate and Meredith (1984) reported that starch granule size distribution is affected primarily by cultivar, and also by location and climatic conditions. Many investigators have examined the effect of surface area on flour functionality, as discussed in Chapter Two, and therefore further, more careful work on these types of measurements is warranted.

The measurements done by Bechtel and co-workers (1990) using digital image analysis which showed evidence of a third, very small starch granule population, are interesting here for several reasons. In the introduction to their paper they mentioned that most studies of starch granule size distribution have used a Coulter Counter as the means of measuring the granules, but that a Coulter Counter doesn't accurately measure granules which are smaller than 3 μm in diameter. These granules are therefore not included in such determinations. This was demonstrated in the paper by Morrison and Scott in which they explained that "False counts were eliminated arbitrarily by setting the lower

limit to be measured..." (Morrison and Scott, 1986, p.14), although they justified this practice by having determined that low channel counts could be generated by background from filtered saline solution and by cell wall debris. The starch preparations measured by Bechtel and co-workers were examined microscopically with crossed polarizers, so it was possible to determine that even the smallest particles were indeed starch granules. When the data from the image analysis measurements done for this project were first examined, those values corresponding to $D_{max} < 2 \mu m$ were thought to be artifact. The paper by Bechtel and co-workers (1990) reminds us that we should not assume that error or artifact account for things we don't expect to see. The authors also raised an intriguing question at the end of their paper. The timing of each new flush of granules was 10 days apart, and they wondered what would happen if the growing season extended past 35 days. The growing season for the wheats examined by Meredith (1981), for example, seemed to be at least 40 days, which would allow time for granules initiated at approximately 34 DAF to become visible and therefore measurable by the method of Béchtel and co-workers (1990). It would be very interesting to know if a fourth population does indeed appear under such conditions.

One final point of interest in this paper has to do with the definitions of the size groups. While the usual cutoff between large and small starch granules in wheat is $10 \mu m$ (Dengate and Meredith, 1984), Bechtel and co-workers

found the cutoff in the starch population they measured to be approximately 16 μm , with the very small 'C' granules being those with a diameter of less than 5.3 μm . As mentioned above, for the Fredrick and Neepawa flours measured, the cutoff appears to be closer to 15 μm than 10 μm . Dengate and Meredith (1984) found that while the proportion of the number of large granules remained constant, the weight proportion varied with the conditions. The cutoff value may also be affected by the hydration level of the granules being measured, the calibration of the equipment used for measurement and optical resolution, if microscopy is part of the procedure. However, it is possible, in fact probable, that the cutoff between the large and small granules is also variable, and might have to be determined for every starch population for truly accurate measurements and interpretation of results.

In summary, a method was developed to separate large and small starch granules from the crude starch preparation extracted from wheat flour by a dough procedure. This method involved centrifugation, sieving and sedimentation, and water was the only solvent to which the starch was exposed. The resulting preparations were shown to be essentially free of contaminating material and the fractions labelled 'large' and 'small' were shown to be representative of those portions of the starch as present in the flour. The size distributions of the A and B granules were consistent with

published values. Calculation of the relative volumes and surface areas provided a basis for considering some of the results to be presented in the following chapter.

Chapter Four

The Proteins Associated with Large and Small Wheat Starch Granules

INTRODUCTION:

Wheat starch granule preparations have consistently been found to contain associated protein which, until recently, was considered to be a contaminant. Greenwell and Schofield (1986b) analyzed the proteins associated with starch granules from a large number of varieties of wheat by gel electrophoresis. They found that the same 10-12 proteins were present in all the preparations. The only exception was one protein with a molecular mass of 15 kDa ('friabilin') which was present in soft wheat varieties and either not present or greatly reduced in hard wheat varieties. The most highly purified preparations contain approximately 0.2% (w/w) protein and it is now generally accepted that these proteins are associated specifically with the starch granules.

The amount of protein found associated with isolated starch preparations has been relatively consistent. Rask and Alsberg (1924) isolated wheat starch in order to compare its characteristics with baking quality. They used the dough procedure for the initial starch isolation, then cleaned it

by washing with a salt solution, centrifuging and removing the top layer, washing with water, several times with alcohol and finally several times with ether, and then dried it. The nitrogen content was determined, and for the 11 samples analyzed, the values ranged from 0.033-0.047%. Using a conversion factor of 5.7 (Tkachuk, 1966), this corresponds to an average protein content of 0.23%, even after such extensive efforts to remove contaminating material.

Sandstedt and co-workers (1939) reported 0.3% protein (N X 5.7) in their isolated prime starch fraction. The prime starch isolated by Sollars (1958) from the starch residue after acid extraction of flour contained 0.13 - 0.18% protein. The starches isolated from 15 flour sources by D'Appolonia and Gilles (1971) contained 0.03 - 0.08% nitrogen, which gives values of 0.17 - 0.46% protein (N X 5.7). It might be interesting to note that the 'poor-quality baking flour' yielded the starch with the lowest nitrogen content (0.03%). They isolated starch by the dough method followed by centrifugation to recover the prime starch fraction. Large and small starch granules were analyzed separately by Bathgate and Palmer (1972). For large wheat starch granules, they reported 0.1% protein, although this value is N X 6.25. Calculated on the same basis as the others (N X 5.7) gives a value of 0.09%. For the small starch granules, their result was 1.6% protein, or 1.5% using N X 5.7. There was a rather large difference in these values for the different starch fractions. Their starch

isolation and separation procedures were rather involved and have been discussed earlier. Evers and co-workers (1974) reported the protein contents of four different starch size fractions. For starch granules $<10 \mu\text{m}$, the protein level was 0.22%; $10 - 15 \mu\text{m}$, 0.27%; $15 - 20 \mu\text{m}$, 0.22%; and $>20 \mu\text{m}$, 0.18%. These levels were considered "essentially protein-free" (Evers *et al*, 1974, p.43). Cauvain and Gough (1975) isolated prime starch, treated a fraction with toluene to remove protein (recovering the separated small granules and adding them back) and aged a fraction of the toluene-treated starch. The protein values for each fraction were: 0.23% for the prime starch; 0.18% for the toluene-treated starch; and 0.16% for the aged, toluene-treated starch. Greenwell (1986) also reported the protein content of prime starch preparations to range from 0.15 - 0.2%.

The protein remaining associated with isolated starch fractions has usually been considered contamination. However, the possibility that protein is an integral component of the granules has been considered. Badenhuizen stated that "It has been known for a long time that proteins are found inside the starch granule, and sometimes they are difficult to remove" (Badenhuizen, 1965, p.90). He referred to Greenwood and Thomson who produced a series of starches "almost completely free from contaminating protein" (Greenwood and Thomson, 1962, p.226) by a 'physical' method which involved filtering and sedimentation followed by repeated toluene extractions of saline suspensions of

starch. The wheat starch preparation they analyzed contained 0.30% protein (recalculating their result of 0.33% using a factor of 5.7 X N instead of 6.25) and appears to have been primarily large granules since they reported an average granule size of 30 μm . They suggested that since some type of chemical treatment is required to remove all the protein from a starch preparation, "the residual protein may well be bound chemically to the granule and might arise from residues of the enzyme-protein responsible for biosynthesis" (Greenwood and Thomson, 1962, p.226). The hypothesis of Greenwell (1986) that the 59-kDa protein might be starch synthase shows that this idea has not yet been discounted.

Schofield and Greenwell were the first to analyze the group of proteins associated with wheat starch granules. Their published work on this topic has been discussed in Chapter Two of this thesis. They have focussed on the proteins extracted from prime starch, defined as "mostly the large, lenticular, A-type granules" (Greenwell, 1986, p.3). 'Mostly', however, doesn't preclude the presence of the small, spherical, B-type granules.

The question of the location of these proteins has also been discussed extensively. Schofield and Greenwell (1987) identified 10 starch granule proteins which they always found associated with wheat starch granules, and located the five smaller proteins (molecular masses: 5, 8, 15, 19, and 30 kDa) on the surface of the granule, and the five larger proteins (molecular masses: 59, 77, 86, 95, and 149 kDa)

inside the granule. The results of immunological analysis by Skerritt and co-workers (1990) on sections of immature grain supported these results. Sulaiman and Morrison (1990) found that the amount of the 15-kDa protein associated with the small granules was greater than that associated with the large granules, but that the difference corresponded to the difference in their surface areas.

The question of contamination of isolated starch granules by proteins from the surrounding protein matrix has always been a concern. Greenwell reported that the starch granule proteins are "a discrete group of wheat proteins that are readily distinguishable" from gluten proteins, as examined by SDS-PAGE (Greenwell, 1986, p.4). Skerritt and Hill (1992) dealt with the contamination of wheat starch by gluten proteins within the context of using wheat starch as a dietary component for those individuals who suffer from gluten intolerance. The samples they analyzed were either industrial or commercial starches, and therefore were probably prime starches. The protein contents (N X 5.7) of the samples varied quite widely, but fell within the range of 0.21 - 0.57%. They analyzed the starches for gluten using a commercial immunoassay kit, developed by them. They claimed it has a sensitivity of detection of 0.0016% gluten in starch and that their antibodies will not cross-react with the starch granule proteins described by Greenwell and Schofield (1986a). If the protein content of the wheat starches was 0.25% or less, the protein tended to be non-

gluten protein. However, above this level, the extra protein tended to be contaminating gluten proteins. They also commented that Kjeldahl analysis of starches is "rather inaccurate and imprecise" (Skerritt and Hill, 1990, p.112), based on large differences in the values reported by different laboratories to which they sent samples for analysis.

The successful development of a procedure for preparing highly purified large and small wheat starch granules, described in the preceding chapter, allowed comparisons to be made between the protein associated with each population. The protein contents were determined and the protein was extracted from both types of granules to determine whether the starch granule proteins identified by Greenwell and Schofield are differentially distributed. The large and small granules were also analyzed as to the location of the associated proteins both by extraction methods and by the use of microspectrofluorometry.

MATERIALS AND METHODS:

Chemicals:

Unless otherwise noted, all chemicals were standard laboratory grade preparations obtained from commercial sources.

Nitrogen analysis:

Nitrogen content was determined using a Kjeltac Auto 1030 Analyzer. One gram of each sample was analyzed, and each analysis was performed in triplicate. The resulting nitrogen values were multiplied by 5.7 to calculate percent protein (Tkachuk, 1966).

Extraction of starch granule proteins:

The protein was extracted from the starch granules by a procedure developed by Greenwell (personal communication). One gram or less of starch was extracted with 10 ml 1% SDS (w/v) ('specially pure', from BDH Chemicals Inc., Toronto, Ont.) in water for 1.5 hours at 50°C in a shaking water bath. When more than 1 g of starch was extracted, a proportionally higher amount of SDS solution was used. The protein was precipitated from the supernatant, after starch extraction, cooling and centrifugation, by addition of 4

volumes of acetone, followed by one hour in a -20°C freezer. This precipitate was then collected by centrifugation and the acetone was removed with a stream of nitrogen gas.

Sufficient protein could be extracted from 0.5 g of large granules and 0.25 g of small granules for detection on a large polyacrylamide gel (e.g. Bio-Rad Protean II system, 1.5 mm gel, 10 or 15 well comb).

SDS-PAGE:

Electrophoresis was carried out on a Bio-Rad Protean II system with the standard Laemmli SDS discontinuous buffer system (Laemmli, 1970), except that the acrylamide stock contained 28.38% acrylamide and 1.62% bisacrylamide instead of 30% acrylamide and 0.8% bisacrylamide as described by Laemmli. Samples were dissolved in sample buffer (approximately 50 μl) and applied to gradient gels of 7.5-17% polyacrylamide, with a 5% stacking gel, then run overnight (approximately 16 hours) at 80 volts. A set of standard proteins for molecular weight estimation (from Bio-Rad Laboratories Ltd., Mississauga, Ont.) was included on all gels and consisted of: lysozyme, 14.4 kDa; trypsin inhibitor, 21.5 kDa; carbonic anhydrase, 31 kDa; ovalbumin, 45 kDa, serum albumin, 66.2 kDa; and phosphorylase B, 97.4 kDa. Before staining, the gels were 'washed' with the destain solution (MeOH-HOAc-H₂O, 3:1:6) for approximately 15 minutes on a shaker to remove excess SDS (which quickly

reduces the efficacy of the stain when it is re-used). They were then stained with Coomassie Blue R250 (0.1% in the destain solution) for several hours, after which the excess dye was removed with several changes of destain solution (the final one usually overnight). The gels were stored in sealable plastic bags at 4°C.

Microspectrofluorometry:

A Zeiss UMSP 80 microspectrofluorometer was used to measure the fluorescence emission of acid fuchsin-stained starch granules. Starch granules from Fredrick wheat were treated three different ways prior to staining. Isolated granules were extracted with SDS, as described above, at room temperature and at 50°C, and an unextracted sample was also measured. Each sample was stained with acid fuchsin (0.01%, aqueous) and the excess stain was washed off with water. The granules were then treated with ethanol, followed by n-propanol, then n-butanol. Finally they were mixed with glycerol. A drop of this preparation was placed on a quartz microscope slide and a quartz cover slip placed on top.

The emission peak of the acid fuchsin was determined using the Lambda Scan software, version 6.5, and was measured as 615 nm. The excitation wavelength was 546 μm and epi-reflectance passed through a dichromatic beam splitter and a barrier filter with transmission of wavelengths greater than 590 μm . Illumination was with a 100 watt

mercury lamp. To measure the fluorescence emission of the stained granules from each of the different treatments, the Photan software, version 5.0, was used, with detection at 615 nm. Two hundred starch granules for each treatment were measured, except for the small, 50°C-extracted granules, in which case only 83 were measured. The photomultiplier tube detector was adjusted against a very bright granule of the appropriate size (i.e. large for the large granule series and small for the small granule series) and all measurements were done for the three treatments with this calibration as the standard. A 10X neofluor objective was used and a pinhole large enough to encompass granules of the size being measured (i.e. one size for the large granule series; one size smaller for the small granule series). The measuring holes used had no mirror on them. Individual granules were positioned within the measuring spot and the intensity measured. All the values were saved and later classified according to intensity classes using the same software.

The Maps software, version 2.2, was used to measure the fluorescence intensities across individual starch granules. This program allows for the measurement of a specific chemical component within a defined physical structure or area. In this case, the distribution of protein, as detected by the fluorescence emission of acid fuchsin, within individual starch granules was determined. The step size in both the x and y directions was 0.25 μm . Again, the photomultiplier tube was calibrated with a brightly stained

unextracted granule and this calibration was used for the extracted granules measured subsequently, with separate calibrations done for the large and the small granules. For these measurements a 40X neofluor objective was used. Surfer software package, version 4 (Golden Software, Inc., Golden, CO) , was used to create the graphs of the data obtained from Maps analysis.

RESULTS:

Nitrogen analysis:

The small starch granules have approximately twice as much protein, by weight, as the large granules based on nitrogen content, as determined by the Kjeldahl procedure. The small granules contained approximately 0.35% protein while the large granules contained approximately 0.15% protein. A factor of 5.7 was used to convert nitrogen to protein. It should be noted that there was no correction for non-protein nitrogen.

Implicit in all following references to percent nitrogen and percent protein is a (w/w) basis.

SDS-PAGE:

The proteins associated with wheat starch granules appear to be differentially distributed between the large and the small starch granules when equal amounts of starch are extracted (Figure 7a). The large granules contain only one major protein with an apparent molecular mass of 59 kDa. In contrast, the small granules contain eight major proteins varying in apparent molecular mass from 14 kDa to 59 kDa. Both populations of granules also contain other proteins as minor components.

If the extraction is carried out on amounts of large and small granules corresponding to equivalent amounts of protein or equivalent surface areas, the asymmetric distribution remains (Figure 8). For equal weight extractions, equal volumes were being extracted, assuming equal densities for both types of granules. For equal protein content extractions, twice as much large granules by weight were extracted, assuming the small granules have twice as much protein as the large granules. For equal surface area extractions, 4.5X as much large granules by weight were extracted, based on the calculated values from Table 3 for the reported values.

Extractions are usually carried out at 50°C, however extraction of the large granules at 25°C did not yield the 59-kDa protein. In contrast, all the major proteins of the small granules are extracted at 25°C (Figure 7b), although

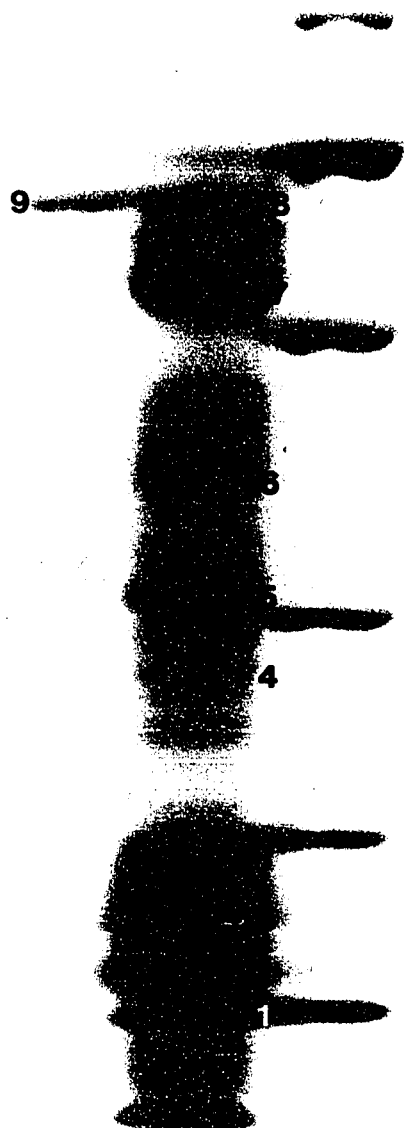
Figure 7. SDS-PAGE of the proteins extracted from large and small starch granules.

a) 50°C extraction: Lane 1, protein extracted from large starch granules; lane 2, protein extracted from small starch granules; and lane 3, molecular weight standards (from the top: 97.4 kDa, 66.2 kDa, 45 kDa, 31 kDa, 21.5 kDa and 14.4 kDa). The numbers 1 through 9 beside the protein bands on the gel indicate the proteins which were isolated and characterized.

b) 25°C extraction: Lane 1, molecular weight standards (from the top: 97.4 kDa, 66.2 kDa, 45 kDa, 31 kDa, 21.5 kDa and 14.4 kDa); lane 2, protein extracted from the small starch granules; and lane 3, protein extracted from the large starch granules.

A gradient of 7.5-17% acrylamide was used for both gels. Starch from the soft wheat Fredrick was used for the extractions.

A

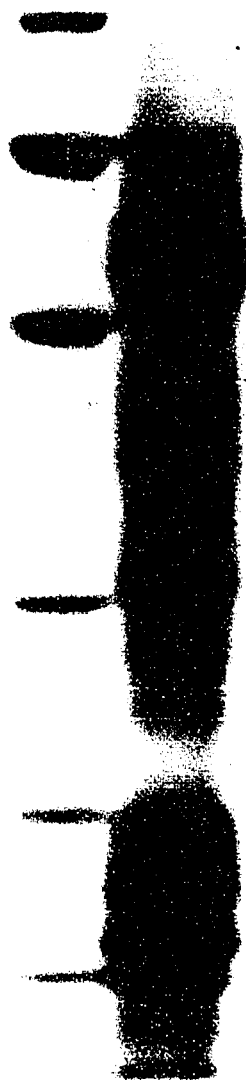


1

2

3

B



1

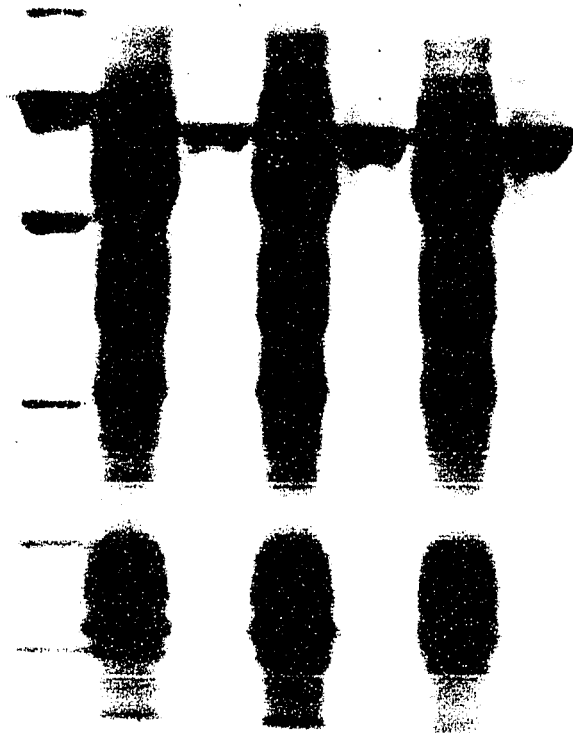
2

3

Figure 8. SDS-PAGE of proteins extracted from large and small starch granules based on equal volumes, equal amounts of protein and equal surface areas.

Lane 1, molecular weight standards (from the top: 97.4 kDa, 66.2 kDa, 45 kDa, 31 kDa, 21.5 kDa and 14.4 kDa); lanes 2, 4 & 6 are small granule extracts; lanes 3, 5 & 7 are large granule extracts. Lanes 2 & 3 are extracts from equal weights of starch granules. Lanes 4 & 5 are extracts from equal amounts of protein, on a weight basis. Lanes 6 & 7 are extracts from equal surface areas, using the values from Table 3 for the reported values.

A gradient of 7.5-17% acrylamide was used for the gel. Starch from the soft wheat Fredrick was used for the extractions.



1 2 3 4 5 6 7

there is a reduced amount of the 59-kDa protein extracted from the small granules at room temperature. The three minor bands with molecular masses greater than 59 kDa are not present in the 25°C extracts from either the large or the small granules.

Figure 9 shows the proteins extracted at 50°C from a prime starch preparation and from subsequent steps during removal of the contaminating small granules by discarding the supernatants after repeated sedimentation of the large granules (as described in Chapter Three). The protein profile changes from that characteristic of small starch granules to that characteristic of large granules, indicating that the proteins typical of the prime starch fraction are predominantly contributed by the small granule portion of that fraction.

Microspectrofluorometry:

Figure 10 shows the difference in protein content in unextracted granules and granules extracted at room temperature and at 50°C, as measured by the fluorescent emission of acid fuchsin, a dye which is known to stain endosperm proteins (Fulcher and Wong, 1980). Both large (Figure 10a) and small (Figure 10b) granules exhibit the same trend, with extraction under either condition removing a large proportion of the protein. The exception of an apparent increase in protein in the large granules extracted

Figure 9. SDS-PAGE of protein extracts from steps in the isolation of large starch granules.
Lane 1 shows the proteins extracted from the bottom starch layer after centrifugation. Lanes 2 through 5 show the extracts from the starch fractions resulting from progressive removal of the contaminating small granules, with lane 5 showing the extract from isolated large granules. Lane 6, molecular weight standards (from the top: 97.4 kDa, 66.2 kDa, 45 kDa, 31 kDa, 21.5 kDa and 14.4 kDa).

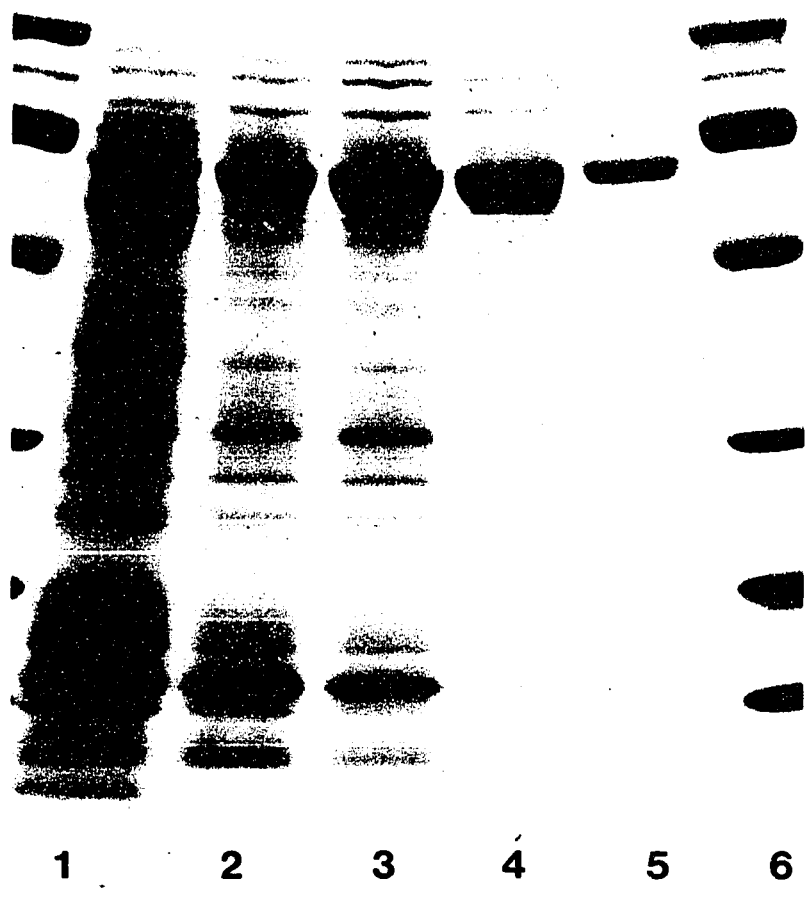
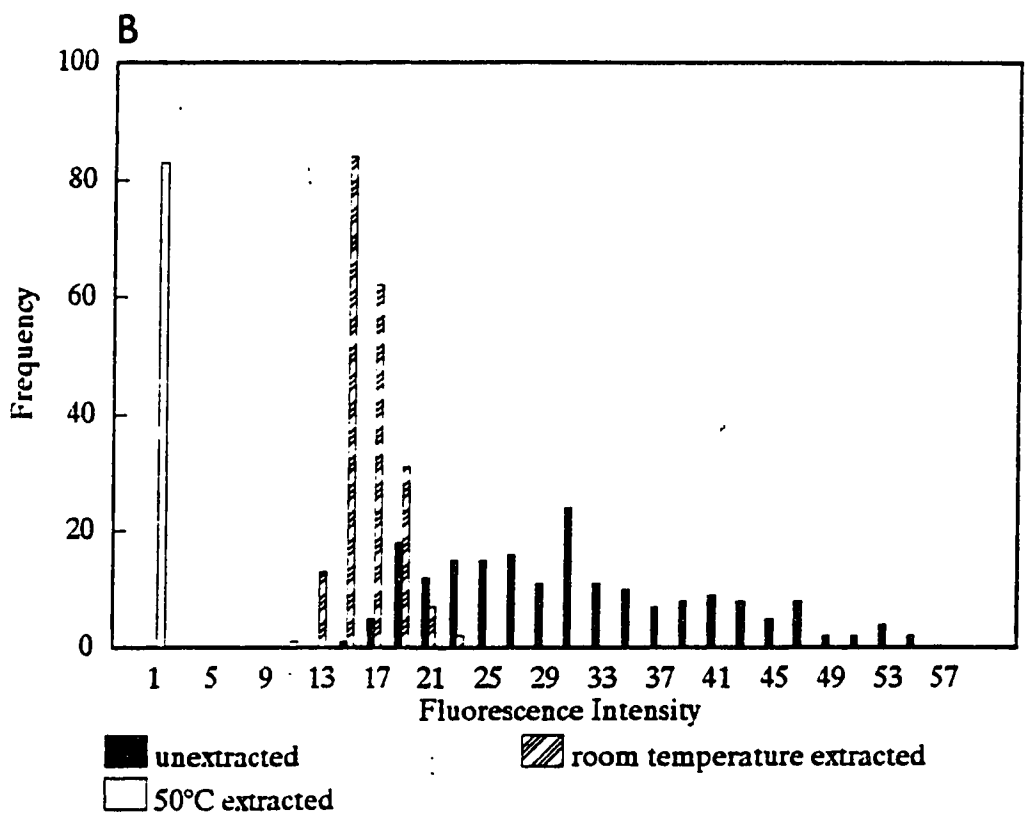
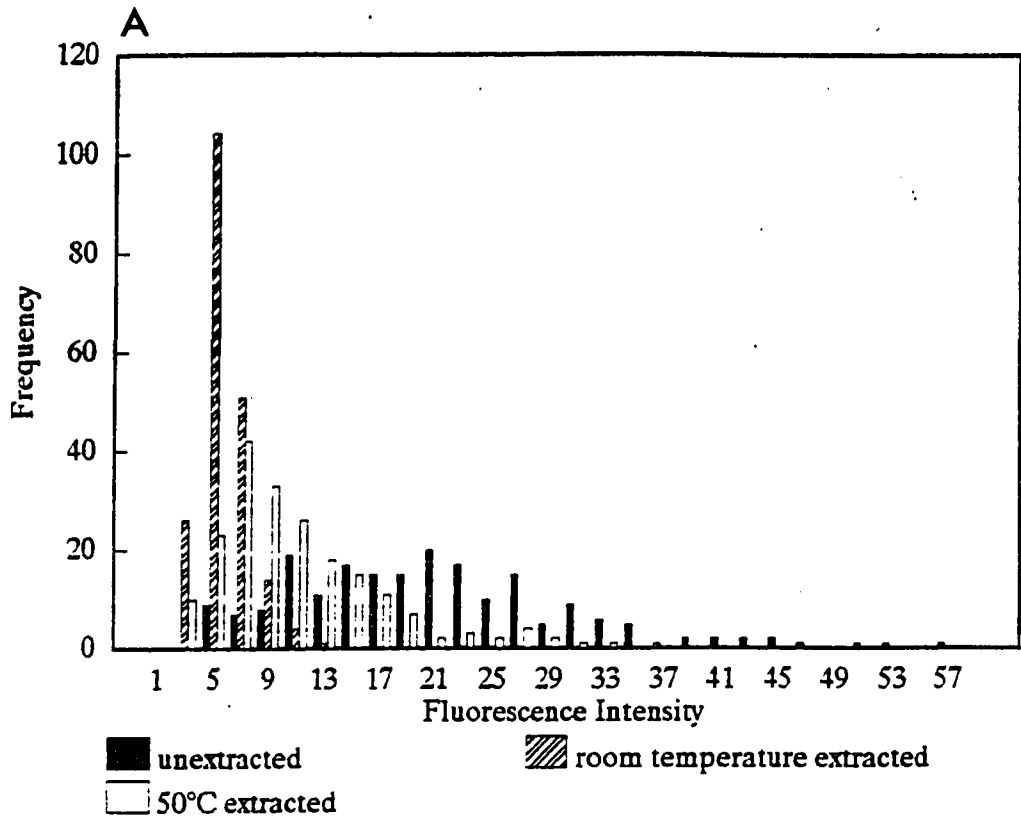
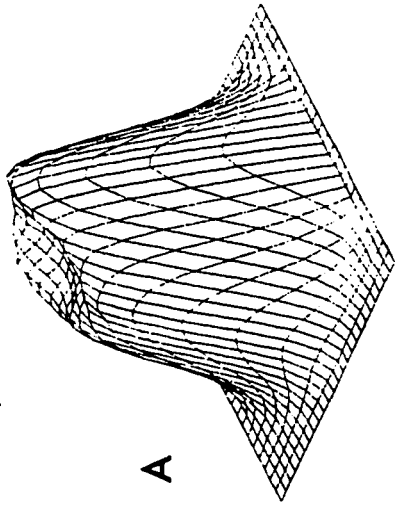


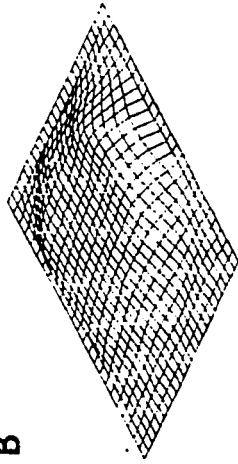
Figure 10. Distribution of fluorescence intensities in acid fuchsin-stained large and small starch granules.

- a) Large starch granules.
- b) Small starch granules.

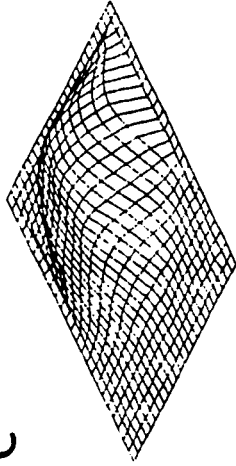




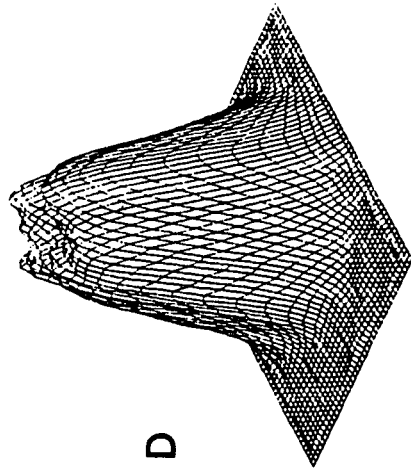
A



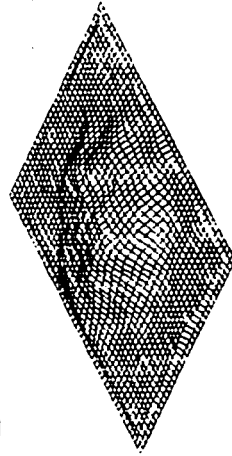
B



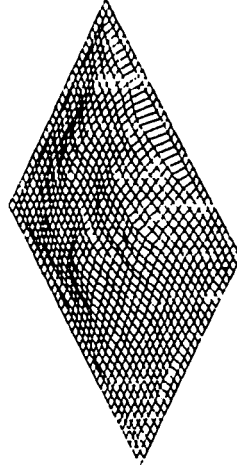
C



D



E



F

at 50°C, given the increase in fluorescence after this treatment, is believed to be an artifact of sample preparation. Kjeldahl results on extracted starches show there is no protein left.

The protein distribution across individual starch granules was also measured by fluorescent dye binding. Figure 11 shows the 'maps' of protein in the same series of samples, i.e. large and small granules, unextracted and extracted under two different conditions. These results graphically demonstrate the reduction in protein content after treatment with SDS.

DISCUSSION:

The protein contents of the two starch fractions are very similar to the values previously reported and listed in the introduction to this chapter. The values are surprisingly consistent, especially for prime starches. This history of 'protein-free' wheat starch containing approximately 0.2% protein argues against the conclusion by Skerritt and Hill (1992) that Kjeldahl analysis of starch is 'inaccurate and imprecise'. The small starch granules were found to have a higher protein content, 0.35%, and the large granules a lower protein content, 0.15%, than a typical prime starch preparation. Since prime starch consists of mostly large granules with residual small granules, a

Figure 11. Maps of fluorescence intensities across individual acid fuchsin-stained starch granules.

- a) Large, unextracted starch granule.
- b) Large, 25°C extracted starch granule.
- c) Large, 50°C extracted starch granule.
- d) Small, unextracted starch granule.
- e) Small, 25°C extracted starch granule.
- f) Small, 50°C extracted starch granule.

For the large granule maps, each line in both the x and y directions represents a 1 μm step (every fourth scan line measured), and for the small granule maps, each line in both the x and y directions represents a 0.25 μm step. Relative fluorescence intensities are shown on the z axes on the same scale.

protein content of 0.2% for such a mixture is consistent with the results presented here for the separate populations.

It has been established that a portion of the nitrogen content of the starch granules is due to the presence of lysophospholipids. The ratio of N:P in wheat starch lysophospholipids is 1:2 (South and Morrison, 1990). The non-protein nitrogen is corrected for by measuring the phosphorus content and deriving the non-protein nitrogen value, which can then be subtracted from the total nitrogen level determined. In the results reported by South and Morrison (1990), the phosphorus content of a bulk wheat starch sample was less than 0.07%. Since phosphorus was not determined in the present study, this non-protein nitrogen has not been accounted for. The use of the factor 5.7 for converting percent nitrogen values to percent protein values does not take into account non-protein nitrogen either (Tkachuk, 1969). The phospholipid contents of different size fractions of several varieties of wheat were published by Meredith and co-workers (1978) and Morrison and Gadan (1987). The fractions of smaller granules did contain more phospholipid than the large granule fractions. The non-protein nitrogen content of the small granules is approximately 0.02% and that of the large granules is approximately 0.03% as determined by calculating the percent phosphorus from the phospholipid values published, using the factors given in the papers, and then using the above ratio

to determine levels of non-protein nitrogen. Even considering this difference, it is clear that the protein content of the small granules, as isolated by the method described here, is greater than that of the large ones.

The large and small wheat starch granules have distinctly different associated proteins. There are approximately 25 protein bands in the small granule extract (Figure 7a). In contrast, there are many fewer bands from the large granules and only one of these, the 59-kDa band, is present in a significant amount. This protein appears to be common to both the large and small granules based on what appears to be the same mobility on gradient gels. The difference in protein profiles supports the conclusion that the difference in protein content as measured by Kjeldahl analysis is real and not due to non-specific contamination of the small granule preparations. It should be stressed again that the only solvent to which the starch was exposed was water, and copious amounts were used during the separation steps for both types of granules. Bathgate and Palmer (1972), also reported a higher protein content for small wheat starch granules. They reported that the small granules contained not twice, but 17 times as much protein as the large granules. The results of Evers and co-workers (1974) showed relative amounts that were much closer to the results presented here, although their size classes are not completely analagous.

The granule proteins have been classified as either internal or external based on their extractability in SDS at 50°C or room temperature (Greenwell, 1986). At 50°C the granules swell in the presence of SDS (Gough *et al*, 1985), and the internal proteins can be extracted, whereas at 25°C only the surface proteins are extracted. The results indicate that the 59-kDa protein from the large granule is internal, in agreement with results reported by Schofield and Greenwell (1987). In the case of the small granules, all the proteins, except for the three minor bands with molecular masses greater than 59 kDa, appear to be surface proteins, in agreement with Malouf (1992), although there is a reduction in the amount of 59-kDa protein in the room temperature extract when compared with the 50°C extract. It is possible that 'integral' proteins are more accessible to extraction from the small granules than from the large granules due to the greater surface area-to-volume ratio, even without swelling of the granules.

Sulaiman and Morrison (1990) reported that there was no difference in the distribution of friabilin between the large and small granules purified on cesium chloride gradients. They found that the difference in amounts of friabilin observed on protein gels when equal amounts of starch are used, is accounted for by the difference in surface area. However, in the present study it was found that the difference in surface area does not account for the apparent asymmetric distribution of, not only friabilin, but

also several other proteins. The proteins in the large granule extracts which appear to be present in trace amounts are still present in lower amounts than in the small granule extract after correction for surface area (Figure 8). As these proteins appear to correspond to major proteins from the small granules, it is not clear whether they are simply present in lower quantities in the large granules or whether they are contaminants. It is also possible that, even though both types of granules are exposed to the same solvent conditions during the isolation and separation procedures, the majority of large granule proteins are removed during these processes. If this is the case, it would require a different strength of association of proteins with each type of granule and would still imply that, in addition to size, there are differences between the two populations of granules. Seguchi's proposal (1990) that chlorination of flour causes increased adherence of protein to starch granules, and the finding of Skerritt and Hill (1992) that protein contents of more than 0.25% represent contaminating gluten proteins, are especially intriguing in this light. If the higher protein content of the small starch granules is persistent contamination, then the question arises as to what property of the small granules causes or promotes this strong adherence. Under such circumstances, is 'contamination' even an appropriate term for this protein?

The histograms in Figure 10 indicate the reduced protein content of the starch granules after extracting the

protein from the starch with SDS. The one anomalous result, that of an apparent increase in the protein in the large granules extracted at 50°C, as measured by an increase in fluorescent emission, is probably explainable by the swelling which takes place at this temperature in the presence of SDS. This swelling is obvious by microscopic examination of granules so treated. The stain probably penetrated the swollen granules and got trapped. In the small granules, given the much higher surface area-to-volume ratio, excess dye could more easily be washed out of the interior of the granules. The solvents used to remove excess dye apparently did not remove it from inside the large granules. It should also be noted that there is always a range of intensity of staining, with any of the samples. Trying to find granules to measure in the sample of 50°C-extracted small granules was extremely difficult. This is the explanation for the much lower number of granules examined in this sample.

The 'maps' of fluorescent emission of acid fuchsin (Figure 11), used to stain for protein, are a graphic representation of the results shown in the histograms (Figure 10). Fulcher and Miller (1992) used this method to show the distribution of β -glucans in oat endosperm cell walls. It allows for the visualization of specific chemical components within a given physical structure, in this case the protein distribution within individual starch granules. While the polyacrylamide gels show the proteins which were

extracted from the starch, the maps illustrate what remained - very little protein as compared to unextracted granules. This is in agreement with the Kjeldahl results which show no protein in extracted starch samples.

In conjunction with the calculated surface areas and volumes presented in Chapter Three, it appears that surface area alone does not account for the difference in protein contents of the two starch populations. As discussed, for an equal volume (and therefore weight, assuming equal densities), a small granule sample would have almost four times the surface area of the large granule preparation, whereas the protein content of the small granules is only double that in the large granules. Because the calculated values can not be relied on to be more than a general indication of relationships, caution should be exercised concerning the implication that there is only half as much protein on a surface area basis as would be expected if it were the factor governing protein contents, as suggested by Morrison and Sulaiman (1990). However, along with the finding that no surface proteins appear to be associated with the large granules (Figure 7b) and almost all the protein associated with the small granules appears to be on or near the surface, it seems reasonable to consider the possibility that some other factor is responsible for the difference in protein contents between the large and small granules.

Chapter Five

Partial Characterization of the Proteins Associated with Large and Small Wheat Starch Granules

INTRODUCTION:

Schofield and Greenwell stated that "very little is yet known about (the) biochemical properties" of the starch granule proteins (Schofield and Greenwell, 1987, p.413). The information that is available has been reported primarily by these investigators. In addition to the molecular masses (see Chapter Four), they determined that the isoelectric points of the integral proteins are slightly acidic, with pI values of approximately 5.7. The 30-kDa surface protein is very basic, with a pI of greater than 10.0. The amino acid compositions of two of the proteins, the 30-kDa and the 59-kDa proteins, were determined and reported to be distinct from those of gluten proteins (Schofield and Greenwell, 1987).

The starch granule proteins were referred to as "still largely uncharacterized" by Skerritt and co-workers (1990, p.124). Their work addressed the question of possible structural similarities between the starch granule proteins and gluten proteins, using an immunological approach, as discussed in Chapter Four.

The results of the present study show that the proteins associated with the large and small starch granules in wheat are differentially distributed between the two starch granule populations. A question that arose was whether these proteins are distinctly different from each other or related in some way. An additional point of interest was whether or not the one protein (59-kDa) which appears to be common to the two types of granules, based on molecular mass as determined by gradient SDS-PAGE, is indeed the same protein. A preliminary characterization was undertaken with a view to answering these questions and providing data on the structural properties of the starch-associated proteins.

MATERIALS AND METHODS:

Chemicals:

Unless otherwise noted, all chemicals were standard laboratory grade preparations obtained from commercial sources. For those noted, the suppliers were as follows: Sigma Chemical Company, St. Louis, MO; BDH Chemicals, Inc., Toronto, Ont.; NEN Research Products from DuPont Canada, Inc., Mississauga, Ont.; and Millipore Corp., Bedford, MA.

Isolation of individual proteins by electroelution:

Preparative polyacrylamide gels were run as described in Chapter Four. A sample, extracted from 5 - 10 g of starch, was applied to a sample well which spanned the width of the gel. After electrophoresis was completed, one plate of the gel sandwich was carefully removed and the edge strips, extending approximately 0.5 cm into the gel below the sample well, were sliced off with a razor blade. These strips were stained and destained as usual and the rest of the gel was carefully wrapped in plastic and stored at 4°C. Once destaining was completed, the strips were rehydrated so that their length matched that of the stored gel. They were lined up with the gel and the strips containing the separated proteins were sliced out. These strips were then cut into small pieces and placed on the bridge in the four sample cups of an ISCO Electrophoretic Concentrator model 1750. The membrane on both sides of the sample cups had a molecular weight cut-off of 3500 kDa. The buffer used for the electroelution was the same as the running buffer used for SDS-PAGE except that it contained no glycine, and was the same throughout the electrophoresis tank. The power was set at 3 W and electroelution continued for approximately four hours, until the current dropped to approximately 16 mA from an initial level of 22 mA. The buffer was collected from the entire anode side of the cup, which was also washed several times with buffer in order to collect as much of the

eluted protein as possible. The pooled sample was dialyzed against water then freeze-dried. The gel pieces were always collected after electroelution and stained to be certain that the protein had actually been eluted. The homogeneity of the isolated protein was checked by SDS-PAGE of the freeze-dried samples on a 10 or 12% acrylamide mini gel.

It should be noted that care was taken in selecting the source of SDS for use during electrophoresis prior to and during electroelution. According to Lacks and co-workers (1979), SDS preparations containing longer-chain-length alkyl sulfates are more difficult to remove from proteins. Their analysis of a variety of commercial sources indicated that SDS from BDH contains no contamination by different-sized alkyl sulfates (Lacks et al, 1979).

Amino acid analysis:

Samples were prepared for amino acid analysis by hydrolysis at 110°C for 24 h with 6 N ultrapure HCl (BDH, 'Aristar'). Analysis was carried out at the National Research Council in Ottawa, on an Applied Biosystems analyzer, consisting of a 420A derivatizer (based on PITC derivatization), 130A separation system (with a C18 column) and a 920A data analysis module. Cysteine content was determined as cysteic acid by performic acid oxidation (Hirs, 1956) of the samples prior to acid hydrolysis.

Peptide mapping:

Individual proteins were trace-labelled with [¹⁴C]acetic anhydride (NEN Research Products, specific radioactivity: 9.2 mCi/mmol) according to the method of Kaplan and co-workers (1982). Samples of protein were dissolved in 1 ml of 8 M urea, which had been adjusted to pH 3 to remove traces of cyanate and then adjusted to pH 9. An aliquot (50 μ l) of [¹⁴C]acetic anhydride (125 μ Ci) in acetonitrile, was added with rapid stirring. The amounts of each protein labelled were: #1, 1.1 mg; #2, 1.0 mg; #3, 1.1 mg; #4, 0.7 mg; #5, 0.8 mg; #6, 0.2 mg; #7, 1.0 mg; #8, 0.7 mg; and #9, 0.3 mg (the numbers correspond to the proteins as identified in Figure 7). After 5 min, excess unlabelled acetic anhydride was added as 2 X 10 μ l aliquots and the pH was maintained at 9 by addition of 1 M NaOH with a pH stat. A sample (1 mg) of albumin (Sigma, No. A-4503) which had been oxidized with performic acid (by the method of Hirs, 1956) was then added as a carrier protein. The samples were dialyzed against distilled water and freeze-dried. The labelled proteins were then digested in 10% formic acid with 50 μ g pepsin (Sigma, No. P-6887) at 37°C for 3 h, dialyzed, and freeze-dried. The digested samples were dissolved in pyridine(10%)-acetate(0.3%) buffer at pH 6.5 and approximately equivalent amounts of radioactivity from each sample were applied to Whatman 3MM paper. High voltage paper electrophoresis (HVPE) was carried out for

approximately 45 minutes at 60 V/cm at pH 6.5. The material from the strips of the electrophoretogram on the anode side of the origin was eluted with the pH 6.5 buffer and rerun at pH 6.5. Samples were also run in formic(8%)-acetic(0.2%) buffer at pH 2.1, and the neutral band from the pH 6.5 electrophoretogram was re-run at pH 2.1. A portion of the samples which had been digested with pepsin were subsequently digested with 10 μ g papain (Sigma, No. P-3125) at pH 6.5 and the peptides were separated using the same electrophoretic conditions. After HVPE, X-ray film was exposed to the dried paper electrophoretograms until bands could be seen. The radioactive material which had not moved from the origin of a pH 6.5 electrophoretogram of pepsin-digested samples was cut out, eluted with pH 6.5 buffer and digested with 10 μ g elastase (Sigma, No. E-0127) at pH 8 overnight. The samples digested with both pepsin and elastase were then subjected to HVPE as described above.

Protease digestions:

Protein extracts from the large and the small starch granules were subjected to digestion by a series of proteases. For each enzyme, digestion took place overnight, approximately 16 h, at 37°C. The digested samples were dialyzed against distilled water and freeze-dried. The enzymes used were: pepsin, from porcine stomach mucosa (Sigma, No. P-6887); papain, from papaya latex (Sigma,

No. P-3125); elastase, from porcine pancreas, type III (Sigma, No. E-0127); protease, from Streptomyces griseus, type XIV ('Pronase', Sigma, No. P-5147) and protease from Tritirachium album, type XI ('proteinase K', Sigma, No. P-0390). Stock solutions were made for each enzyme at a concentration of 1 mg/ml. Pepsin was dissolved in 10% formic acid, papain in pyridine(10%)-acetate(0.3%) buffer at pH 6.5 with 1 μ l β -mercaptoethanol/2 ml, and the other three in 1% ammonium bicarbonate, pH 7.9. Typically, 10 μ l of stock solution would be added to protein extracted from either 0.25 g of small starch granules or 0.5 g of large granules in 1 ml of the appropriate buffer, for digestion as described above. An enzyme blank was always run, with conditions as described except no starch protein was present. The extent of digestion was determined by gradient SDS-PAGE as described in Chapter Four.

Glycoprotein analysis:

Three methods were used to stain the proteins which had been separated electrophoretically on gels. These were: Alcian Blue staining as described by Wardi and Michos (1972); thymol-sulfuric acid staining (Racusen, 1979); and by the periodic acid-Schiff technique (Zacharius et al, 1969). One other method involved detection of carbohydrate using concanavalin A as a lectin. In this procedure, individual proteins were used instead of whole extracts, as

above. They were run on 12 or 15% mini gels and electroblotted onto polyvinylidene fluoride (PVDF) membrane (Millipore) using a BRL Mini-V 8.10 Vertical Gel Electrophoresis Apparatus for the electroblotting. The staining was done by following the procedures described by Bartles and Hubbard (1984) and Kijimoto-Ochial and co-workers (1985). A positive control, deoxyribonuclease (Sigma, DN-25), and a negative control, albumin, were also included.

α -amylase treatment:

The whole protein extracts from both starch populations were treated with α -amylase, from Aspergillus oryzae, type X-A (Sigma, No. A-0273) then run under normal SDS-PAGE conditions (see above). The enzyme was dissolved in 0.02 M Na_2HPO_4 , 0.006 M NaCl, pH 6.9, at a concentration of 1 mg/ml, and 10 μl of diisopropyl fluorophosphate (DFP) was added to inhibit any serine proteases. An aliquot (10 μl) of the enzyme solution was added to the sample dispersed in 1 ml of the same buffer. Digestions were carried out for 1 minute, 15 minutes and 1 hour. The stacking gel was removed from the separating gel after electrophoresis but before staining. The separating gel was stained normally with Coomassie Blue and the stacking gel was stained with a solution of IKI (1.2% KI, 0.6% I_2 in dH_2O) for the presence of starch.

N-terminal sequencing:

N-terminal sequencing of the nine purified proteins was carried out at the Biotechnology Research Institute in Montreal, on an Applied Biosystems model 470A gas phase sequencer, equipped with an on-line model 120A phenylthiohydantoin (PTH) analyzer, employing the method of Hewick and co-workers (1981). The samples were prepared for analysis by electroelution, as described above, followed by electroblotting of an SDS-PAGE gel with the individual proteins, as described for the glycoprotein analysis, but using a Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell for the electroblotting. The PVDF membrane with the electroblotted proteins, stained briefly with Coomassie Blue, was submitted for analysis.

RESULTS:

Amino acid analysis:

The amino acid compositions of the eight major proteins from the small granules, the single major protein from the large granules and the 5-kDa band resulting from protease treatment are summarized in Table 4. Glutamate, glycine and alanine are the most abundant amino acids while methionine is the least abundant. Cysteine is also present in

Table 4. Amino acid compositions of the isolated proteins.
The values are in mole %. The numbers refer to the proteins indicated in Figure 7a, and '5K' is the fragment which appears to be generated by protease digestion of the samples.

A.A.	Protein #									
	1	2	3	4	5	6	7	8	9	5K
Asp	6.2	7.6	6.4	7.1	7.6	8.1	8.1	9.0	10.0	6.8
Glu	15.6	16.0	16.0	14.0	13.6	14.5	13.0	14.0	11.4	15.5
Ser	7.8	6.4	6.3	7.2	6.4	7.8	7.4	12.4	5.6	6.3
Gly	13.4	13.2	10.4	11.8	10.2	11.2	11.8	14.3	11.8	11.4
His	2.8	1.6	2.2	2.4	2.4	2.4	2.6	2.0	1.6	3.0
Arg	7.6	7.9	8.2	6.0	5.7	6.8	7.8	4.6	4.6	7.8
Thr	4.6	4.0	5.0	4.7	5.9	4.5	4.0	4.6	4.2	3.6
Ala	8.0	7.8	9.6	9.8	10.4	10.0	10.5	9.6	9.6	6.2
Pro	6.0	7.3	6.6	6.6	7.2	6.8	6.0	5.2	6.2	7.2
Tyr	3.0	2.1	2.3	2.9	2.4	2.2	2.8	2.9	3.9	1.4
Val	5.1	5.2	5.4	6.0	6.2	5.8	6.4	5.4	7.4	5.8
Met	1.0	0.6	0.4	1.0	0.9	0.3	0.5	0.0	0.8	1.2
Ile	3.2	3.0	3.4	3.5	3.0	3.1	3.0	2.8	3.4	3.5
Leu	6.9	7.5	7.4	7.7	8.5	7.4	7.6	6.4	8.7	8.5
Phe	3.0	3.4	3.2	3.8	3.6	3.6	3.8	3.0	4.0	3.4
Lys	5.6	6.2	7.2	5.3	6.1	5.2	4.6	3.9	6.3	6.5
Cys	1.9	4.9	1.3	1.8	1.6	0.5	1.0	1.0	0.7	2.6

relatively low amounts except in the 15-kDa protein where it is relatively abundant. There is no amino acid which is present in an unusually high or low amount. Tryptophan was not determined due to its destruction under the conditions of acid hydrolysis.

Peptide mapping and protease treatments:

Peptide mapping was used to define further the similarities or differences among the proteins. Because only small quantities of each protein could be isolated, the amino groups were labelled with [^{14}C]acetic anhydride in order to increase the sensitivity of detection of the peptides generated by enzymatic digestion. In this approach, the peptide maps should be greatly simplified since only lysine-containing peptides and amino-terminal peptides will appear. Figure 12 is an autoradiogram of a pepsin digest of each of the ^{14}C -acetylated proteins after high voltage paper electrophoresis (HPVE) at pH 6.5. All the proteins gave very similar peptide maps. The most notable feature of the autoradiogram is that for every protein most of the digest did not migrate in the electric field but remained fixed at the origin, indicating the presence of peptides which were too large to move through the matrix of the paper. Of the material which did migrate, the neutral band (the radioactive peptides which moved slightly toward the cathode) was the strongest. The basic peptides generated

Figure 12. Autoradiogram of a peptide map of [¹⁴C]acetylated proteins digested with pepsin.
HVPE was carried out at pH 6.5. The numbers identify the protein, as indicated in Figure 7a, in that lane. The position of the origin is indicated by 'O' and the position of the dansyl sulphonic acid marker by 'D'.

+

← D

← O

1 2 3 4 5 6 7 8 9

-

(those which migrated ahead of the neutral band towards the cathode) appear to be very similar for all the proteins. Of the acidic peptides (those which migrated towards the anode), the slower migrating peptides gave a smear while the faster moving peptides gave distinct spots. Only these faster moving acidic peptides appear to differ among the proteins. In this region there are some peptides which are common to all the proteins and several which differ. The lower molecular mass proteins (lanes 1-5) and the higher molecular mass proteins (lanes 6-8) from the small granules gave two different peptide patterns. The single high molecular mass protein (lane 9) from the large granules gave an identical pattern to that of the high molecular mass proteins from the small granules.

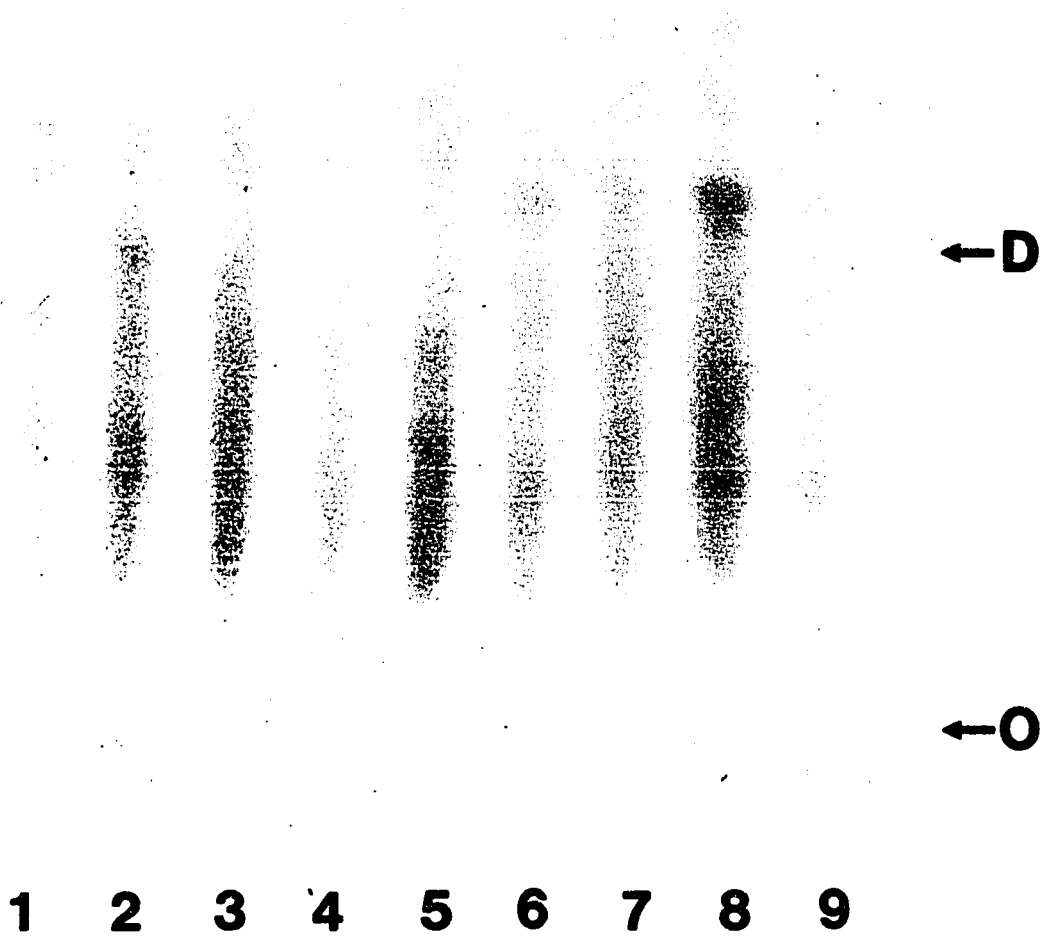
In order to determine whether the material at the origin was the cause of the smearing associated with the peptides which did migrate, each lane of the pH 6.5 electrophoretogram was cut out on the anode side of the origin and the material eluted with the pH 6.5 buffer. When these samples were then run again at pH 6.5, the smearing was still very evident (Figure 13), implying that it was a function of the material which was migrating and not the material at the origin through which it migrated.

Treatment of the pepsin digests with papain still left the bulk of the radiolabelled material at the origin after HVPE at pH 6.5 and, while a few more peptides were generated, did not qualitatively alter the results obtained

Figure 13. Autoradiogram of a peptide map of [¹⁴C]acetylated proteins digested with pepsin.

HVPE was carried out at pH 6.5, the material from the strips of the electrophoretogram on the anode side of the origin was eluted and rerun at pH 6.5. The numbers identify the protein, as indicated in Figure 7a, in that lane. The position of the origin is indicated by 'O' and the position of the dansyl sulphonic acid marker by 'D'.

+



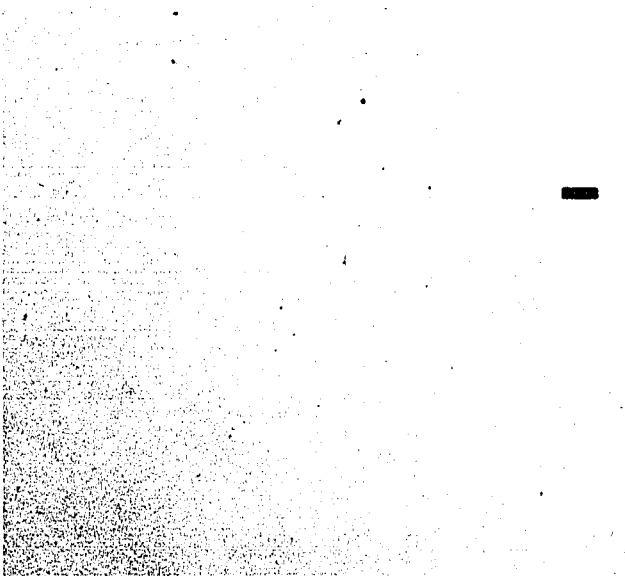
with pepsin alone. The neutral peptide band, i.e. the band containing peptides with no net charge at pH 6.5, was excised and run at pH 2.1 (Figure 14). Again, there was considerable background smearing but a few distinct peptides were discernible. As in the case of the acidic peptides, some peptides were common to all nine proteins and the others fell into the same two groupings noted above, i.e. the lower molecular mass proteins and the higher molecular mass proteins.

The results from high voltage paper electrophoresis indicated that large polypeptides remained after pepsin and pepsin-papain digestion of the starch proteins. SDS-PAGE was used to examine digests of the total protein from the small and from the large granules. The results shown in Figure 15a,b,c confirm that the starch proteins are not extensively digested by either pepsin or papain, although papain does digest the higher molecular weight proteins to a greater extent than pepsin does. It is notable that, as in the case of the peptide autoradiograms (Figures 12,13,14), there is considerable background material. In contrast, digestion with elastase, Pronase or proteinase K resulted in a single band with an apparent molecular mass of 5 kDa (Figure 15e,f) and little background.

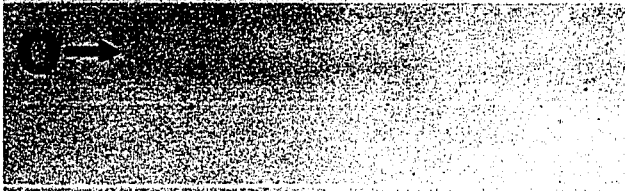
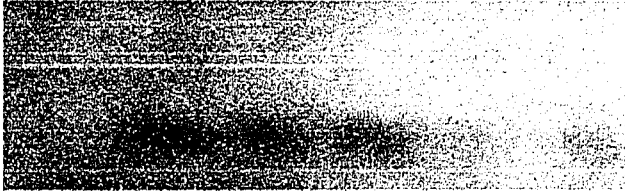
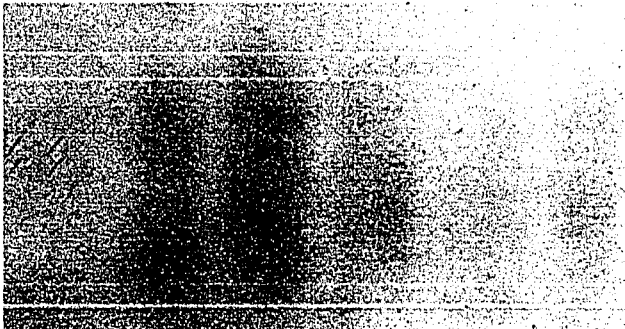
Based on the observation that the proteins are much more susceptible to proteolysis by elastase than by pepsin, the material from the pepsin digest which remained at the origin (Figure 12) was excised, eluted and digested with

Figure 14. Autoradiogram of a peptide map of the neutral band from HVPE at pH 6.5 of [^{14}C]acetylated proteins digested with pepsin and papain.

HVPE was carried out at pH 2.1. The numbers identify the protein, as indicated in Figure 7a, in that lane. The position of the origin is indicated by '0'.



-



0 →

1

2

3

4

5

6

7

8

9

+

Figure 15. SDS-PAGE of protease-treated extracts from large and small starch granules.

a) Pepsin digestion: lane 1, molecular weight standards (from the top: 97.4 kDa, 66.2 kDa, 45 kDa, 31 kDa, 21.5 kDa and 14.4 kDa); lanes 2-5, extracts from large granules from Fredrick wheat, small granules from Fredrick wheat, large granules from Neepawa wheat and small granules from Neepawa wheat, respectively; lanes 6-9, the same samples, but treated with pepsin; lane 10, pepsin.

b) Papain digestion: as for the pepsin digestion; lane 10, papain.

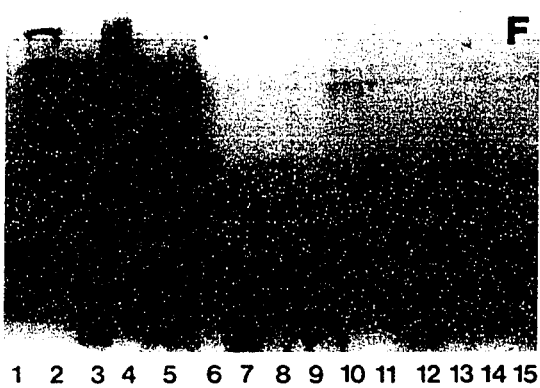
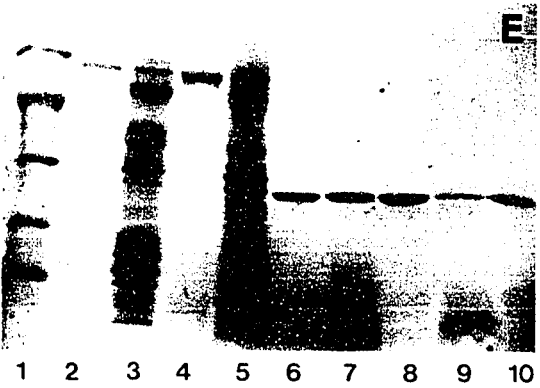
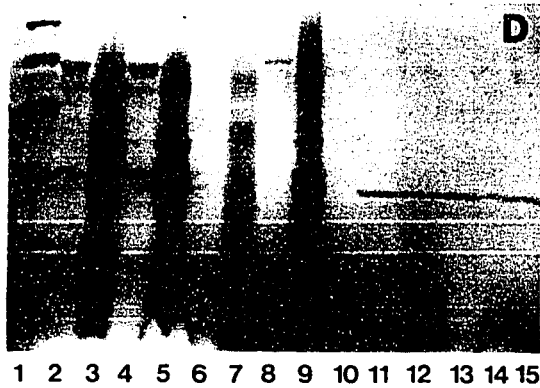
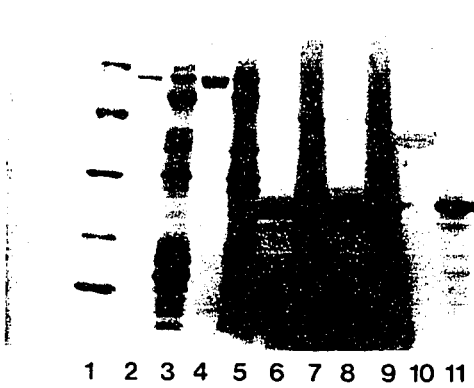
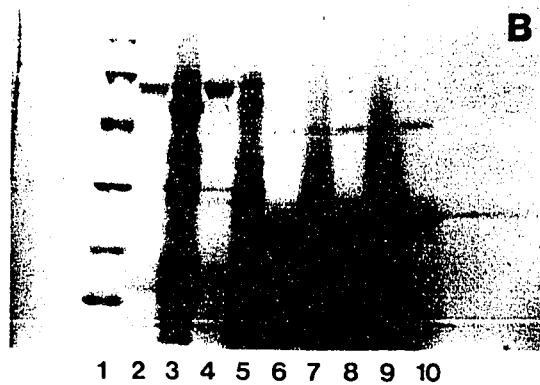
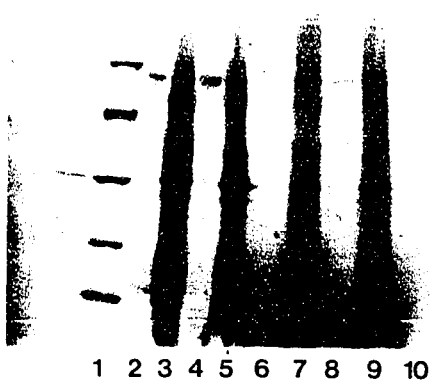
c) Pepsin and papain digestion: as for a) & b); lane 10, pepsin; lane 11, papain.

d) Pepsin and pepsin-and-elastase digestions: lanes 1-10 as for pepsin digestion; lanes 11-14, pepsin and elastase-digested samples; lane 15, elastase.

e) Elastase digestion: lanes 1-5, as above; lanes 6-9, elastase-digested samples; lane 10, elastase.

f) Pronase and proteinase K digestions: lanes 1-5, as above; lanes 6-9, Pronase digestions; lane 10, Pronase; lanes 11-14, proteinase K digestions; lane 15, proteinase K.

All the above samples were run on 12% acrylamide mini-gels.



elastase. This digest was subjected to HVPE at pH 6.5 and subsequently, the neutral band was rerun at pH 2.1. There was too little material on the pH 2.1 electrophoretogram to draw any conclusions, however, at pH 6.5 distinct bands were obvious. As expected, peptides were generated from the pepsin-resistant material by elastase (Figure 16). As observed from the pepsin digest alone, some peptides are common to all nine proteins while others are common either to the five lower molecular mass proteins or to the four higher molecular mass proteins. The only exception was a very distinct basic peptide present in protein 1 which did not appear in any other protein.

Glycoprotein analysis:

The Alcian Blue, thymol/sulphuric acid and periodic acid-Schiff procedures gave no indication of glycoprotein. However, the lectin blot procedure was performed on the individual proteins and gave a positive result for five of the nine proteins (Figure 17). Proteins 7 and 8 stained quite strongly, proteins 2, 5 and 6 stained less strongly and proteins 1, 3 and 9 did not stain at all. Protein 4 appears to have broken down, and so the result is inconclusive. As the amount of the individual proteins on the gel varied, the differences in the intensity of staining observed may arise from different amounts of protein.

Figure 16. Autoradiogram of a peptide map of [¹⁴C]acetylated proteins digested with pepsin and elastase. HVPE was carried out at pH 6.5. The numbers identify the protein, as indicated in Figure 7a, in that lane. The position of the origin is indicated by 'O' and the position of the dansyl sulphonic acid marker by 'D'.

1

2

3

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+

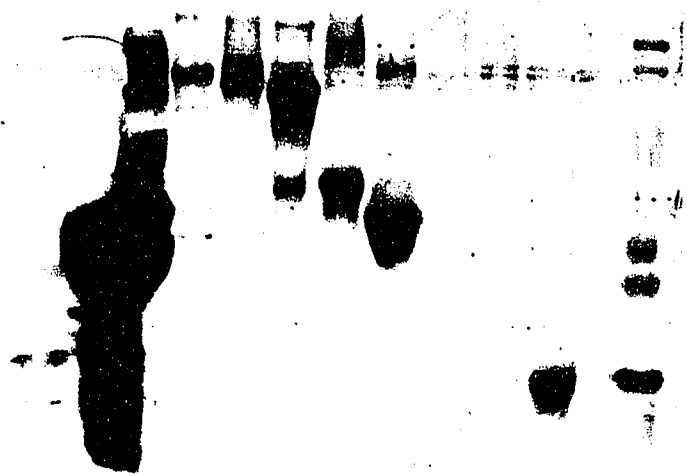
←D

Figure 17. Glycoprotein blots.

a) Samples blotted onto PVDF membrane from a 15% acrylamide mini-gel and stained with concanavalin A as described. Lanes 'S', pre-stained molecular weight standards (from the top: 106 kDa, 80 kDa, 49.5 kDa, 32.5 kDa, 27.5 kDa and 18.5 kDa); 'A', albumin (negative standard); lane 'D', deoxyribonuclease (positive standard, vastly overloaded); lanes 9-1, the proteins, as indicated in Figure 7a.

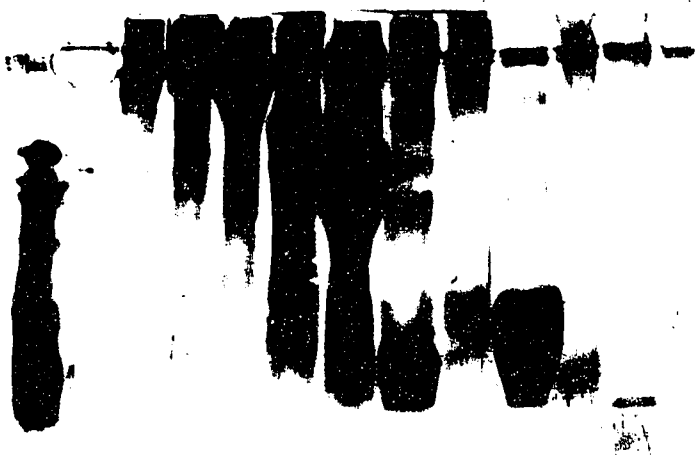
b) Samples blotted onto PVDF membrane from a 12% acrylamide mini-gel and stained with concanavalin A as described. The lanes are as described for a, and lane 5K is the fragment which appears to be generated by protease digestion of the samples.

A



S A D 9 8 7 6 5 4 3 2 1 S

B



D A 9 8 7 6 5 4 3 2 1 5K S

α -amylase treatment:

Treatment of the protein extracted from the granules with α -amylase had no effect on the mobility of the proteins on SDS-PAGE. Below the wells which had contained untreated starch protein samples, there was intense black staining when the stacking gel was immersed in an IKI solution. There was none below those wells which had been loaded with α -amylase treated samples.

N-terminal sequencing:

Very short sequences were obtained for four of the nine proteins. Of these, two were likely contaminated, since more than one amino acid was found at most positions. The sequences for the 15-kDa protein and the 30-kDa protein, were distinct. They are listed in Table 5. These sequences were compared with those in the NBRF-PIR database and the Swiss Prot database of 'Laser Gene' (by DNA Star, Madison, Wis.), but were not found to be homologous to any listed. The other five proteins gave no sequences, implying that the N-terminals are blocked.

Table 5. N-terminal sequences.

Protein #:	Sequence:
2	mixed-Val-Ala-Gly-Pro-Ser-Gly-Ala-Gln-Gln
3	mixed-Pro-Gln-Gln-Glu-Ala-Tyr-Leu-(Pro/Glx- Ser-Ala-Pro-Ile-Leu-Leu)

note: residues in parentheses are less certain.

DISCUSSION:

The selection of eight of the proteins from the small granules for further study was based on the intensity of staining with Coomassie Blue after SDS-PAGE as being indicative of those present in the greatest amounts, although given the varying intensities, this judgement was somewhat subjective. The eight proteins chosen are indicated in Figure 7a, with the numbers by which they are referred to labelled.

In the large granule extract, only the 59-kDa protein stained strongly with Coomassie Blue. This protein appears to be common to both the large and small granules based on their mobilities on the gradient gels. Although they have similar peptide maps, susceptibility to proteases and amino acid composition, only the 59-kDa protein from the small granules stained positively for carbohydrate and, in homogeneous gels, a slight difference in their mobilities was occasionally observed. Therefore, it is not clear at present whether these bands are indeed the same protein.

Electroelution was used as the method for isolating the nine proteins to be characterized because of its relative simplicity and the ability to directly isolate the proteins of interest. Relatively large amounts of the proteins could be obtained by using preparative SDS-PAGE. The main disadvantage was that the proteins could not be considered SDS-free. Also, the purity of the isolated proteins depended

on the degree of separation during electrophoresis and the accuracy with which the bands were excised from the gel. The proteins were always checked after isolation for the presence of contaminating protein, and on those occasions when contamination was apparent, the procedure was repeated. Due to the types of analyses performed on the isolated proteins, electroelution was considered an acceptable method in terms of the amounts of protein which could be isolated and the purity of those proteins, and avoided the necessity of developing other isolation procedures for all nine selected proteins.

Given the large distribution in the apparent molecular masses of the starch-associated proteins, it was surprising to find such striking similarities among them. Other than the relatively higher cysteine content in friabilin, there is very little to distinguish among these proteins in terms of amino acid composition. The amino acid composition of the 5-kDa fragment is very similar to that of the rest of the SGP. The compositions are not very different from those reported by Schofield and Greenwell (1987) for the two proteins they analyzed. Comparison with wheat proteins as a whole or with gliadins (alcohol-soluble gluten proteins) or glutenins (alcohol-insoluble gluten proteins) however, demonstrates that these proteins are indeed quite different, as a group, from the wheat storage proteins in terms of their amino acid composition. Wrigley and Bietz (1988) published several tables of amino acid compositions of

different groups of the wheat proteins. There are some notable differences between the SGP amino acid compositions and those of the wheat proteins. The glutamic acid content (which includes glutamine) of wheat and flour proteins is approximately double that of the SGP, whereas the SGP contains more than three times as much glycine. The proline content of the SGP is lower, as is phenylalanine. Alanine and lysine are several times higher in the SGP. The gliadins as a group have an even higher percentage of glutamic acid (glu + gln), and therefore the proportion in the SGP is even lower in comparison. As well, they contain much less glycine and almost no lysine, whereas the SGP have approximately 5% lysine. The glutenins also contain much more glutamic acid (glu + gln) and very little lysine in comparison with the SGP. The 15-kDa protein is the only SGP to have a cysteine content greater than that of the wheat proteins.

The resistance of the nine proteins to proteolysis by papain and pepsin and their susceptibility to elastase, proteinase K and Pronase are remarkably similar. Only minor differences were observed in the peptide maps and are associated with two distinct groupings of these proteins based on molecular mass.

The treatment of the wheat starch granule proteins with Pronase has been used by several investigators to remove the surface proteins. The finding here that the isolated proteins are susceptible to this enzyme is consistent with published results. Seguchi (1984a,b, 1985), Greenwell and

co-workers (1985), and Russell and co-workers (1987) all used Pronase to digest the proteins on the granule surface during studies on the effect of chlorination on starch. All found that the Pronase treatment effectively removed the surface proteins. Pronase was used by Malouf and co-workers (1992) in investigating the surface proteins and wheat hardness. They treated starch with the enzyme, then extracted the starch for any residual protein, but found none. This was considered an indication that all the starch proteins are surface-associated.

Glycoproteins have been identified in wheat, and are thought to be important functionally in breadmaking (Wrigley and Bietz, 1988). Five of the nine proteins appear to be glycoproteins which are recognized by binding of concanavalin A. This result is in contradiction to that of Seguchi and Yamada (1989), who found that the proteins they extracted from starch granules did not stain positively as glycoproteins. It should be noted though, that they used the periodic acid-Schiff method, and when that method was applied in this study, negative results also were obtained. The two other methods used which involve staining proteins in the gel, also yielded negative results, and it was only on using the more sensitive method of staining blotted proteins with a lectin that positive results were obtained. Whether or not this has to do with the type or amount of carbohydrate attached to the proteins remains unanswered. Lowy and co-workers obtained a positive result for the

'30-kDa protein' (protein #5 in this study), when they used the thymol/sulfuric acid method. Although this method (Racusen, 1979) gave negative results in this study, their result is in agreement with the result of the lectin staining procedure, in that protein #5 was one of the proteins which stained positively.

Lectins are proteins which bind sugars with characteristic specificities. Concanavalin A is specific for α -linked mannose, α -linked glucose and their derivatives (Hughes, 1983, p.60). The staining of six of the nine proteins after treatment with concanavalin A indicates that these proteins contain some amount of these types of sugar residues. It gives no indication of the exact sugars present nor the way in which they are attached to the protein. Those proteins which did not stain at all are not necessarily devoid of carbohydrate moieties, but only of detectable amounts of glucose or mannose. Further analysis using other lectins with different specificities would be required to establish if other types of sugars are present on any or all of these proteins. Additional work will be required to characterize the carbohydrate portion of the glycoproteins. None of the proteins were affected by treatment with α -amylase. This indicates that the carbohydrate is not attached starch or starch-like chains.

The observation that there is a 5-kDa fragment that is resistant to proteolysis suggests that there may be a common structural component to all or some of these proteins. This

fragment is present in untreated samples and appears to increase in intensity in protease-treated samples. Digestion of the isolated proteins was inconclusive on this point as some appeared to generate a faint 5-kDa band whereas others did not. It is not clear whether this variation was due to the small amounts of protein used. Wheat storage proteins are known to contain large numbers of repeating units (Mifflin et al, 1984, Wrigley and Bietz, 1988, Tatham et al, 1990). In their review of wheat proteins, Wrigley and Bietz stated that "Repeating structures appear characteristic of all wheat proteins" (Wrigley and Bietz, 1988, p.246). It has also been shown that there is a large degree of cross-reactivity with antibodies raised against individual storage proteins (Wrigley and Bietz, 1988), and Skerritt and co-workers (1990) have shown that there is even some degree of cross-reactivity with the starch granule proteins. It is therefore possible that, like the storage proteins, the starch-associated proteins consist of different amounts of some common unit, yielding different-sized proteins. The existence of common structural units would account for the similarities in amino acid composition, peptide maps and susceptibility to protease digestions. All this suggests that, while the starch-associated proteins are distinct from the gluten proteins (Greenwell, 1986), they may be distantly related, and their structure may be similarly patterned.

Chapter Six

Concluding Comments

Unexpected results were encountered at almost every step of this project. Far more questions remain at the end than were ever considered at the beginning. This has been both the driving force and the source of much frustration. Having established that there is a difference in the type, amount and location of the proteins associated with the two types of wheat starch granules, the aim was to investigate some of the basic biochemical characteristics of a selection of these proteins.

As frequently happens, an assumption was recognized only once it was shown to be incorrect. That assumption was that nine completely different and independent proteins were being chosen. The first major surprise, after that of finding distinctly different ranges of proteins, according to size, associated with the large and the small granules, was the similarity of the amino acid analyses. When the peptide maps and the protease-susceptibilities were also found to differ only slightly, it became clear that these proteins are much more closely related than was ever considered. Equally intriguing though, are the results showing differences between some of the proteins. The peptide maps, although very similar, split the nine proteins

into two groups clearly divided between higher and lower molecular weight proteins. The reports in the literature of the lower molecular weight proteins being surface and the higher molecular weight proteins being 'integral', make this an even more interesting observation, especially when the extraction studies performed for this project found all but the large granule-protein to be surface-associated.

Another possibly-mistaken assumption was that the 59-kDa proteins isolated from both the large and the small granules are the same protein. The very slight difference in mobility that was observed on a few occasions when they were run on homogeneous polyacrylamide gels to check the purity of the isolated proteins, initially raised the possibility that they are not the same. The amino acid results show them to be about as different from each other as are any of the proteins, given the overall similarity. Finally, the results of the glycoprotein analysis again showed a difference between the two 59-kDa proteins: that from the small granules gave a positive result and that from the large granules, a negative result.

As has often been noted by many researchers, in attempting to answer one question, several others arise. This project was no exception. Just how closely related are these proteins? Is the resistant 5-kDa peptide a common building block? If it is, how is it incorporated into each of the proteins? Are the 59-kDa proteins the same or different? Do the proteins which did not react with

concanavalin A contain carbohydrate of a different sort? Of those proteins which did react with concanavalin A, exactly how much of which sugars are attached, and where? Are the N-terminals of the proteins for which sequences could not be obtained really blocked, and if so, in what way? If sequences could be obtained, would any homology with sequenced gluten proteins become apparent?

The predominant question is why are different proteins found associated with the two types of granules? It was suggested many times that this result was an artifact of the isolation procedure. It is always a possibility, however this conclusion is considered unlikely. Both the large and the small granules were exposed to the same conditions during their isolation. It was only part way through the procedure that they were treated differently, and then, that difference was one of physical handling, not chemical treatment. Both were exposed to large amounts of water, and nothing else. The large granules were not washed more extensively than the small, removing the protein in the process. If anything, the small granules may have been exposed to a greater amount of water.

As for the argument that these proteins are contaminating storage proteins, again, this does not seem likely. The consistency of the protein profile, regardless of the variety of wheat examined, strongly suggests these proteins are starch-associated. Out of the large number of possible storage proteins present in wheat, that the same

10-15 would happen to contaminate starch preparations consistently would appear to be more than coincidental.

It is possible that there are specific gluten proteins which preferentially associate with the small granules, or that they associate with the small granules in a different manner than with the large granules. It is also possible that the association with the small granules is less susceptible to disruption by the isolation procedure. Two points are important here. First, there would still be something inherently different about the chemistry of the small granules which would allow or cause this different association. Second, the proteins are very similar based on a variety of analyses, which does not preclude their being gluten proteins, but the amino acid compositions are quite different from those of the gluten proteins, supporting the idea of these proteins as distinctly starch-associated. The way in which these proteins are associated with the starch granules is an unanswered question.

It is possible that these proteins are involved in the different functional characteristics documented between large and small wheat starch granules. The surfaces of these two types of granules appear to be different, whether due to the proteins themselves or whatever is responsible for the specific association of the proteins. Mixing characteristics could easily be affected by the presence or absence of starch surface proteins for the gluten proteins to interact with. It would be particularly interesting to study the

sulfhydryl groups of the surface proteins - especially the 15-kDa protein with the relatively high cysteine content - to determine whether these groups are involved in disulfide bridges with the proteins forming the gluten network. There is a much greater surface area associated with the small granule component of a flour than with the large granule component, and so any interactions specifically occurring at this interface will be very important.

The work which has been reported in this thesis lays the groundwork for future examination of the proteins associated with wheat starch granules. Many questions have been raised, as listed above, and finding an answer to any one of these questions would be a serious undertaking. The information which would be generated, though, could help explain some of the most basic questions about starch functionality, and possibly about wheat starch development as well. Why are there two types of wheat starch granules and what causes them to be different? Are the different proteins a possible cause of the different types of granules or are they a result of the difference in development between them? The information gathered by this preliminary characterization indicates that these would be very interesting proteins to study in greater detail whether one is interested in their chemical, biological or functional role.

Appendix

Improved Microscopic Detection of Damaged Starch Using Hessian Bordeaux

ABSTRACT:

A dye which stains damaged starch granules more intensely than Congo Red is described. This dye, Hessian Bordeaux, stains damaged starch a very bright pink/fuschia color, in comparison to a fainter orange-pink with Congo Red staining. Using equivalent concentrations of both dyes, the absorbance of the stains in starch granules was spectrophotometrically measured between 380-700nm. The maximum absorbance of wheat starch granules stained with Hessian Bordeaux was 1.6 ($\lambda_{\max}=530$), whereas that of starch granules stained with Congo Red was 0.3 ($\lambda_{\max}=504$). Hessian Bordeaux is therefore a more useful stain than Congo Red for the microscopic detection of damaged starch due to its more intense staining.

INTRODUCTION:

Congo Red (3,3'-[[biphenyl]-4,4'-diylbis(azo)]-bis[4-amino-1-naphthalenesulfonic acid] disodium salt (C.I. 22120), is a direct dye and has been used as a stain for histological work for more than a century (Clark and Kasten, 1983). Huss, in 1922, described the uptake of Congo Red by mechanically damaged starch granules (as cited by Alsberg and Griffing, 1925). Later work by Alsberg and Griffing (1925), Pulkki (1938), and especially Jones (1940) further demonstrated the susceptibility of damaged starch to staining with Congo Red.

Another direct dye - Hessian Bordeaux (4,4'-bis(4-amino-1-naphthylazo)-2,2'-stilbenedisulfonic acid (C.I. 24860) - was patented as a dye in 1886 (1975). It has been found to stain damaged starch with greater intensity than Congo Red, since it has a higher extinction coefficient.

Jones (1940) described Congo Red as staining starch "ghosts" orange-pink; gluten, brown; and cell walls pink. Hessian Bordeaux stains damaged starch granules a very bright pink/fuschia; protein, red; and cell walls purple. These effects can be seen when a flour sample is used for staining instead of isolated starch.

Starch damage is an indicator of the conditions to which grain has been exposed during milling, and exerts a strong influence on how the resulting flour will perform in

processes such as bread production. A comprehensive review of starch damage was published recently by Evers and Stevens (1985). They pointed out that no concrete, simple definition of starch damage exists. The term refers instead to a variety of changes in starch granule structure and estimation depends on the context of its use and the method of analysis.

Despite this, microscopic detection of damaged starch granules has been accepted on the basis of staining with Congo Red as described above. The staining of damaged starch granules by Hessian Bordeaux has now been characterized and compared quantitatively to staining by Congo Red.

MATERIALS AND METHODS:

Starch was isolated from a hard wheat flour (var. Neepawa, a hard red spring wheat) which had been developed into a dough, by a simple washing procedure. The final starch sample was essentially free of protein and cell wall pieces. Flour samples (Fredrick, a soft white winter wheat, and Neepawa) were also used for observations, although all measurements were done on the isolated starch samples.

Congo Red was obtained from Aldrich Chemical Company, Inc., Milwaukee, Wis. (81% purity) and Hessian Bordeaux from Eastman Kodak Company, Rochester, New York (min. 99% purity). Both stains were used at a concentration of 0.1%

(w/v) (corrected for stated purities) in carbonate buffer ($\text{NaHCO}_3/\text{Na}_2\text{CO}_3$), pH 10.5. The Congo Red was also used in distilled water (0.1% w/v), with a final pH of 6.1.

Starch was placed on a microscope slide, a drop of stain added, and a coverslip sealed on with nail polish. Absorbance was measured on a Zeiss UMSP 80 microspectrophotometer. Illumination was by a halogen lamp. The objective lens was a Zeiss neofluar, 40X. Stained starch granules were located and positioned under the measuring spot and scanned from 380 to 700 nm in 2 nm increments, with a slit width of 20 nm. The measuring spot was the same size for all granules scanned, and did not overlap the edge of any of the granules. Twenty-five large granules and 25 small granules were measured for each dye. To standardize each measurement a blank spot next to each granule was measured. The data from the individual scans were then meaned. Unstained granules were also measured, following the same procedure.

Photographs were taken on a Zeiss Universal microscope using a halogen light source and Kodak Ektachrome ASA 400 film.

RESULTS AND DISCUSSION:

The main advantage of the use of Hessian Bordeaux for the observation of damaged starch granules is the much greater intensity of staining which results, as compared with Congo Red, due to its higher extinction coefficient, making it much easier to detect the damaged granules. There is a range in the extent of dye uptake, in terms of both the proportion of a granule stained and the intensity of the stain in the granule. This variation is also more easily detected with Hessian Bordeaux than with Congo Red.

The difference in intensity between the two dyes can be seen in the photographs (Figure 18a,b). Figure 19 shows the large difference in absorbance between Hessian Bordeaux- and Congo Red-stained starch granules. Starch stained with Hessian Bordeaux showed a maximum absorbance of 1.6 at 530 nm compared with 0.3 at 504 nm for starch stained with Congo Red. These absorbance curves were compared to that for unstained starch granules.

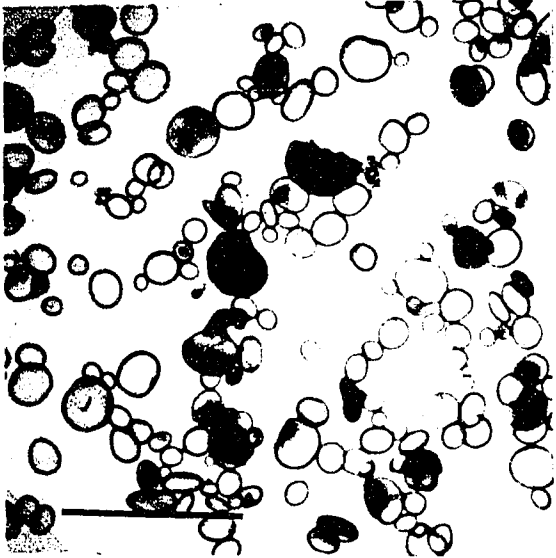
Hard wheats are damaged to a greater extent than soft wheats during milling. This is visually obvious when flour samples are stained with Hessian Bordeaux and compared. More starch granules will appear stained in a hard wheat flour sample than in a soft wheat one (Figure 18c,d).

A pH-related color transition occurs for both dyes. Hessian Bordeaux changes from blue, at pH 8.0, to red at pH 9.0, while Congo Red undergoes a similar color transition

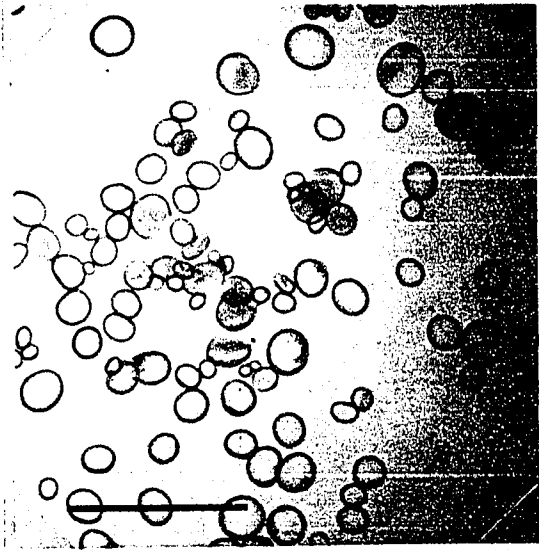
Figure 18. Starch granules and flour samples stained with Hessian Bordeaux.

Starch granules were stained with 0.1% Hessian Bordeaux (a) and Congo Red (b). A hard wheat flour (c) and a soft wheat flour (d) were also stained with 0.1% Hessian Bordeaux. A damaged starch granule is labelled 'D', an unstained starch granule is labelled 'S', protein is labelled 'P' and a cell wall is labelled 'CW'. The scale bars represent 100 μm .

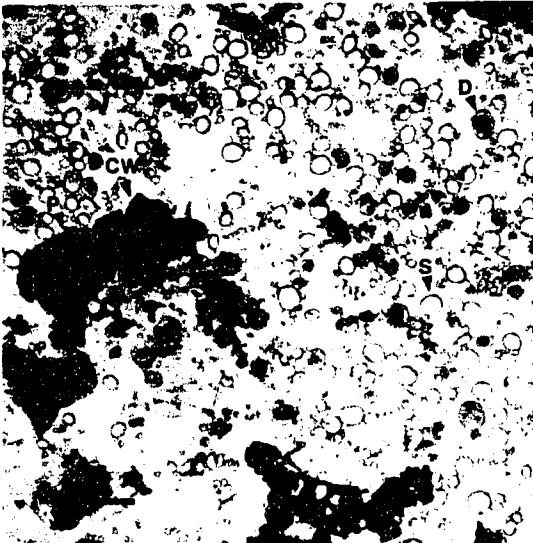
A



B



C



D

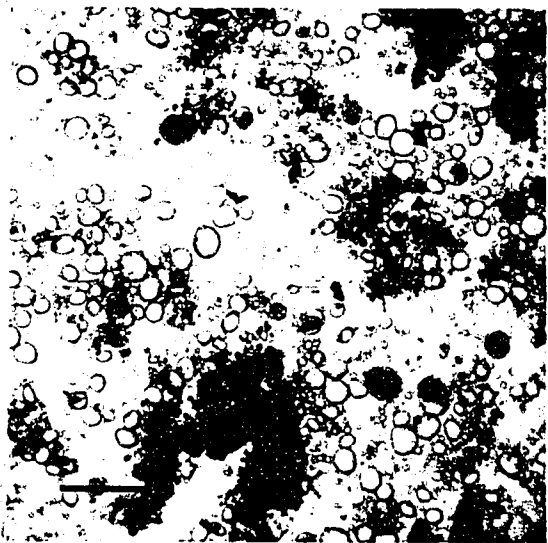
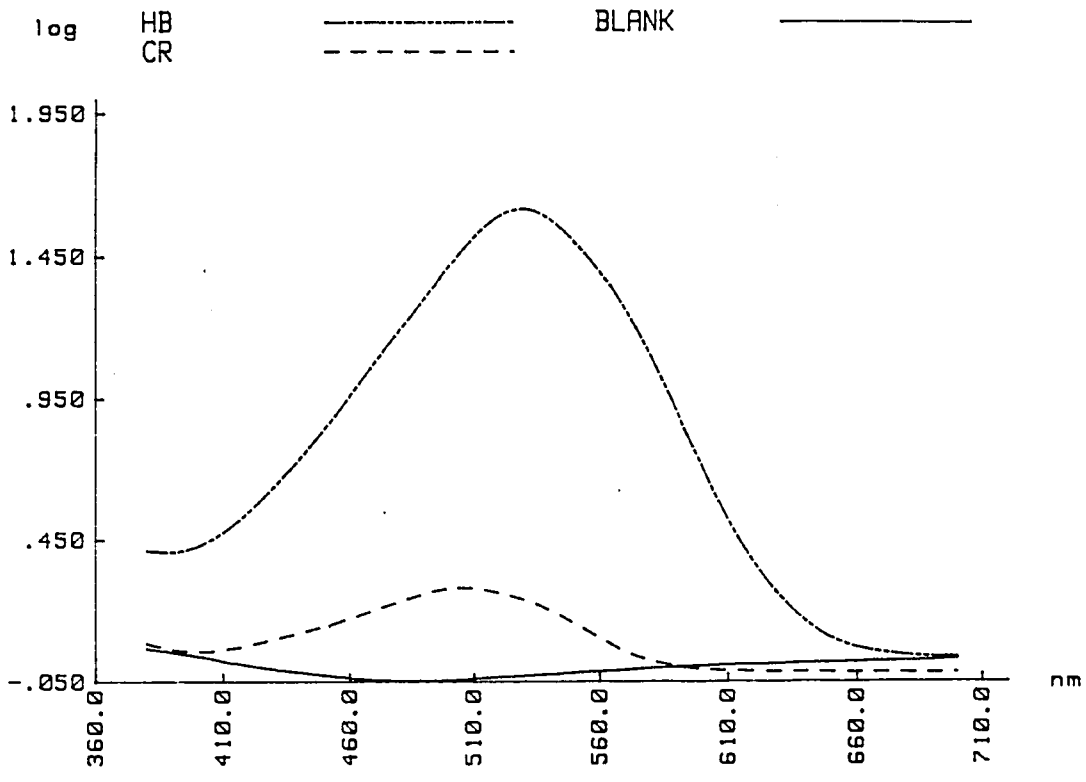


Figure 19. Absorbance curves for starch granules stained with Hessian Bordeaux and Congo Red.

Starch granules stained with each of the dyes were measured for absorbance between 350-700 nm. Unstained granules were measured as a blank. 'HB' is Hessian Bordeaux and 'CR' is Congo Red.



from pH 3.0 to 5.0. At pH 10.5 both dyes are red in color. For the Hessian Bordeaux the pH of the stain was important. At a pH close to 9.0 precipitation was a problem, while increasing the pH above 10.5 led to a gradual decrease in the intensity of the staining. An increase in pH to 13 caused a loss in the staining intensity of starch granules with Hessian Bordeaux. In comparison, Congo Red at a range of pH from 5.5 to 11.5 showed no visible differences in staining intensity. However, the absorbance of granules stained with Congo red in the buffer at pH 10.5 was almost double that of the granules stained with Congo Red in distilled water, although still low compared to the absorbance of Hessian Bordeaux-stained granules. The measurements reported were therefore done at the same pH, so that any pH or buffer effects would be the same for both dyes.

References to the preparation of Congo Red for use as a starch stain frequently state nothing other than 'Congo Red', or 'aqueous Congo Red'. Jones (1940) referred to 0.1-0.35% aqueous, and Pulkki (1938), 1% aqueous. Wolf (1964) also stated 0.1%, although he did not say what the solvent was. His reference (MacMasters, 1964) said 'saturated in ethanol solution or in phosphate buffer, pH 8'. Because of the paucity of information regarding any sort of standard preparation of Congo Red, 0.1% (w/v) was used and the pH adjusted as described above. Hessian Bordeaux is equally

effective at concentrations lower than 0.1% (w/v), but it was used at the same concentration as the Congo Red.

To check that the Hessian Bordeaux was staining with the same specificity as Congo Red, starch granules were stained with Congo Red and the preparation was then irrigated with Hessian Bordeaux by placing a drop of stain at one side of the coverslip and drawing it through the sample with a piece of absorbent paper placed on the opposite side of the coverslip. Observation during this process showed that Hessian Bordeaux was taken up by the Congo Red-stained starch granules. It was also noted that the granules which took up either stain showed no birefringence in the stained areas, another indication of damage.

Congo Red is thought to interact with polysaccharide chains through hydrogen bonding between the amide groups of the dye molecule and the hydroxyl groups of the polysaccharide (Pearse, 1985, p.579). Since Hessian Bordeaux is so similar structurally to Congo Red and also contains two amide groups per molecule, it is reasonable to assume the staining interaction is the same. As to why these dyes only stain the damaged portion of a starch granule when the rest of the granule also contains hydroxyl groups, it is possible that the hydroxyl groups in the undamaged portion of the granule are not available due to prior hydrogen bonding interactions. The damage might allow access both physically and chemically that was not possible before.

If starch was left in Hessian Bordeaux overnight, all granules eventually took up the dye to some extent. Although this was not observed with Congo Red at the concentration used, this result with Congo Red was mentioned in the review by Evers and Stevens (1985). It may be that the more lightly Congo Red-stained granules were not detectable. With time, the dye molecules probably diffuse throughout the granule and replace more and more of the molecules previously hydrogen bonded to the starch molecules.

In addition to staining damaged granules more intensely, the Hessian Bordeaux appeared to stain a larger number of granules. This may be the result of granules which were so faintly stained with Congo Red as to go undetected, while with Hessian Bordeaux the more intense staining allowed detection because even the more faintly stained granules could be seen.

The interaction of Hessian Bordeaux with the other flour constituents, protein and cell walls primarily, caused them also to appear more intensely stained than with Congo Red. The cell walls in particular were stained a vivid purple, and it is possible that different shades of purple corresponded to different types of cell walls.

It was evident when starch was stained with Hessian Bordeaux that there are different types of starch damage. Granules which were cracked or cleaved or otherwise physically damaged did not necessarily take up the dye, and if they did, it was not always in the vicinity of the

physical damage, although staining could coincide with these areas. Conversely, those granules which were partially or totally stained with Hessian Bordeaux often showed no signs at all of physical damage. This observation is not new, having been discussed quite extensively by Jones in 1940 and more recently by Evers and Stevens in their review of starch damage.

CONCLUSIONS:

Hessian Bordeaux is a better stain than Congo Red for damaged starch. It is a similar chemical compound but stains damaged granules much more intensely. This makes them much easier to detect and observe. Hessian Bordeaux is also very effective at concentrations a fraction of that used in the work described here, while Congo Red is much less effective at lower concentrations.

Summary of Scientific Contributions

1. Developed an improved procedure for the separation of large and small wheat starch granules.
2. Characterized the size distribution of the starch granules in Neepawa and Fredrick wheats and in starch fractions isolated from these wheats, by digital image analysis.
3. Measured the distribution of protein within individual starch granules using microspectrofluorometry.
4. Established that there is an asymmetric distribution of the starch granule proteins between the large and the small wheat starch granules.
5. Obtained evidence for the structural similarity of the starch-associated proteins.
6. Established that Hessian Bordeaux is better stain for damaged starch granules than Congo Red.

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