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Studies on the Amylolytic Breakdown of Damaged Starch in Cereal and Non-Cereal Flours

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BSc. Applied Sciences

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Thesis submitted for the award of a
Masters in Philosophy

To be awarded by the
Dublin Institute of Technology

Under the Supervision of Dr. Paul Mathias

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Dublin 8.

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Signature Karen Bailey Date 16/10/98

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Glossary

HPLC – High Performance Liquid Chromatography

HPLC HPAEPAD – HPLC system with an anion exchange column and pulsed amperometric detection.

HPLC RI – HPLC system with an amino bonded reverse phase column and a refractive index detector.

SEM – Scanning Electron Microscope

AACC – American Association of Cereal Chemists

ACS – American Chemical Society

CCFRA – Campden and Chorleywood Food Research Association

RS – resistant starch

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ABSTRACT

The term starch damage refers to a number of changes to the starch granule structure, which are detectable by different analytical techniques. Damaged starch is the substrate for amylases, which in turn provides carbohydrates that are used in the production of certain foods and food ingredients; as a result, therefore, determination of levels in flours is a procedure carried out routinely by many manufacturers of starch based products. Enzymatic assay methods emulate the susceptibility of damaged starch granules to enzymatic attack.

The first aim of this study was to evaluate enzymatic methods[1,2] to determine damaged starch in wheat flours and non-wheat flours. The most reliable and simplest method tested was the AACC 76-31 method[2] (used in the form of a kit); this could be replaced with other enzymes and reagents, which was just as effective and less costly.

The effect of different types of enzymes (fungal alpha amylase and pancreatic alpha amylase) and the effect of their incubation times on a variety of flour samples was also examined. The products of enzymatic digestion of damaged starch were identified and quantified using two HPLC systems[3]. It was found that the two enzymes tested produced different combinations of reducing sugars (mainly glucose, maltose and maltotriose) in the different flours tested and that the rate of breakdown of these flours over a 60 minute time period also varied. "Percent maltose values" correlated well with percentage damaged starch results obtained by an enzymatic colorimetric method (r^2= 0.98).

These effects on the starch granule were observed using a Scanning Electron Microscope. No difference was observed in the breakdown patterns of the two enzymes but differences in the way in which the A and B type crystalline structures digest was seen. The general progress of breakdown of the starch granules could be seen but it was not clearly distinguishable at ten minute intervals. Finally, starch granules were examined with regard to starch crystallinity using x-ray powder diffraction. No reduction in crystallinity of the starch in the flours tested was found following digestion for up to a period of sixty minutes.

CHAPTER 1.

INTRODUCTION

1:1 Starch

Starch is one of the main components of human diets all over the world. Potatoes were once the staple food of this country and apart from water are almost entirely made up of starch (85% dry weight approx.). Further afield starch containing foods can be identified as the main dietary components of other countries for example pasta in Italy, bread in France and rice in China and other Asian countries. These foods provide people with carbohydrate, which is the main source of energy. After cellulose, starch is probably the most widely commercially utilised of all the polysaccharides [4].

Starch is obtained from a variety of botanical sources. It is produced photosynthetically and stored in plants as their own source of carbohydrate in roots, seeds, fruits, stems, leaves and pollen. One of the most well known cereals used, as a source of starch, is wheat, which is used to produce flour for bread making. Barley another cereal containing starch, is used in the production of beer, a second major food product made in this country which requires starch as a raw material.

While starch is very clearly used to produce the foods mentioned above it has other uses in the food industry, which are not as clearly recognisable. Starch can be physically or chemically modified to produce food additives, which can, for example, stabilise, emulsify and thicken other food products. Glucose syrups used in a wide variety of food products for many different purposes, are made from starch. Starch may also be used in non-food products particularly in paper and textile manufacture.

Why and how starch is used in so many different ways is related to its wide availability and to its structure and composition. Certain characteristics of starch structure are common to all types of starch, and other characteristics vary depending on:

a. Botanical source
b. Any physical or chemical treatment, which the starch may have undergone.

1:1:1 Amylose and Amylopectin

All types of starch are composed of polymers of glucose molecules with an alpha -D-glucopyranose structure, which are attached to one another in this polymeric form through hydrogen bonds. These chains of glucose molecules may be either linear or branched to varying degrees and are known as amylose (mainly linear with alpha 1-4 bonds and a minor amount of branching formed by alpha 1-6 branch points) or amylopectin (mainly branched). These structures are shown in Figure 1. Amylose has an average chain length of 500 AGU (anhydro glucose units) and amylopectin an average chain length of 20 AGU.

Alpha 1-6 bonds make up 5% of the total number of bonds in amylopectin [5] and the branching rate increases as a function of molecular weight. If amylopectin is digested using starch de-branching enzymes such as pullulanase or isoamylase three or more populations of chains are obtained whose length can be determined using another starch degrading enzyme beta amylase [5]. These include short chains [average DP (degree of polymerisation) 12-20], long chains (average DP 40-45) and chains with average DP greater than 60. Evidence for the branched structure of amylopectin may be derived from the following facts:

1. The molecular weight of amylopectin is great but it is made up of only short chain lengths.

2. Isolated 2,3 di-o-me-D-glucose is formed due to methlyation and hydrolysis of the amylopectin molecule.

3. In partial hydrolysis there was no further evidence for the structure giving an isomaltose 1-6 link[5].

Depending on the source of the starch the ratio of these two types of polymer will differ. Amylose generally makes up 25 to 27% of the total mass of the polymers. Some starches e.g. waxy maize, consist almost entirely of amylopectin. The amylose and amylopectin content of different starches is shown in Table 1. Starch also contains a quantitatively minor amount relative to amylose and amylopectin of another unknown substance.

1:1:2 Starch Granules

Starch is a reserve polysaccharide occurring in granular form in higher plants. It is most abundant in seeds, roots and tubers. Starch granules vary in their susceptibility to enzymatic digestion. This is partly due to their morphology and crystalline structure. The size of the granules varies from 2-100um in length. When the morphology of starch is examined under a microscope it is composed of round or angular, rough or smooth granular structures found in plant cells which vary in size depending on their source. The size and also the shape are prescribed by the biosynthetic system of the host plant and by the physical constraints imposed by the tissue environment. Therefore, the shapes and sizes of granules can be used in the genotyping of a particular starch or flour. All starch granules show a cleft called a hilum, which is the nucleation point of growth and development. This has been observed through experiments involving electron microscopy techniques on starch granules.

Granules may be simple or compound (polydelphous) i.e. rounded but comprising of many sub-granulli which are flat or concave, subsequently producing an angular multifaceted appearance to the starch granule. Rice and oats consist of compound granules, whereas wheat, barley and rye contain both large and small simple lenticular granules. In wheat the large granules account for between 50% and 70% of the total starch mass [8]. There are exceptions whereby other cereal starches may exist as compound granules. These are small and polyhedral e.g. maize. Variation in size and shape quantifies least among maize starch granules.

Figure 1
Schemes of the linear, helicoidal amylose macromolecule (A1 and A2) and of the grape-like clustered amylopectin macromolecule (B1 and B2). Amylose in A1 shows one reducing (open circle) and only one non-reducing (closed circle) end-chain group. Amylopectin in B1 shows one reducing (open circle) but several thousands to several millions of non-reducing closed circles) end-chain groups [6].

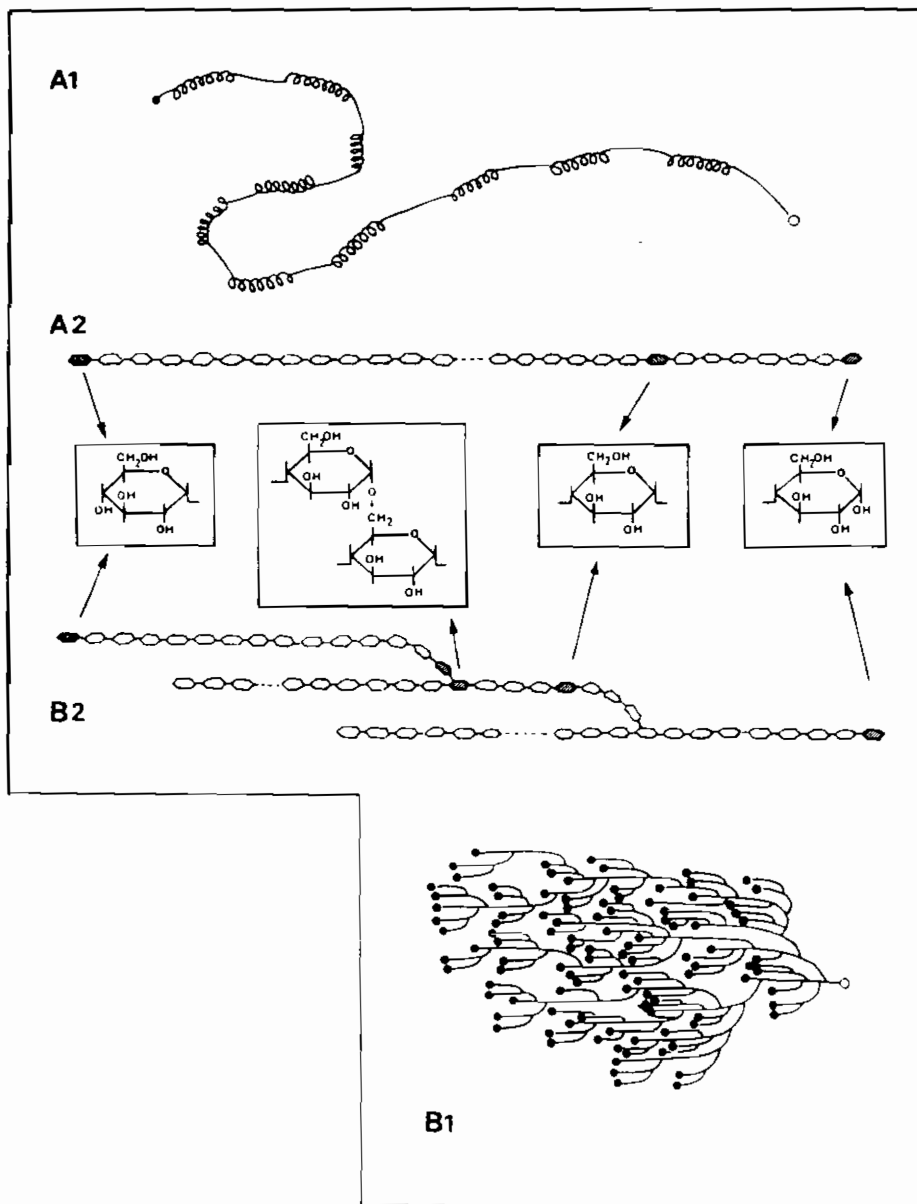


Table 1
Amylose and amylopectin content of starch sources [7].

	Amylose (%)	Amylopectin (%)
Standard maize	24	76
Waxy maize	0·8	99·2
High-amylose maize	70	30
Potato	20	80
Rice	18·5	81·5
Tapioca	16·7	83·3
Wheat	25	75

All granules are of one simple shape, spherical, but are sometimes distorted by impact of other granules due to space limitations. Cereals are generally polyhedral in shape [5]. Amylomaize is an exception to this general rule because parts of the granules are filamentous [6].

Potato and tuber starch granules are for the most part larger than granules in cereal starches and have an ellipsoid as well as spherical shape. There are some exceptions to this including cassava, sweet potato, coleus, taro and yam, which possess smaller and sometimes, polygonal starch granules [9]. Legume starch granules are kidney shaped or ovoid with a well-defined shell centred along an elongated hilum. An exception to this is the wrinkled pea, which contains sphero-pyramidal units [5].

1:1:3 Hydrolysis of Starch

Starch may be hydrolysed by starch degrading enzymes. The most commonly found starch-degrading enzyme is amylase.

Enzymatic degradation of carbohydrates is of major significance in the industrial processing of cereals and fruits. In the production of beer, barley is germinated under well-defined conditions (malting) to induce maximum enzyme synthesis with maximum respiration of reserve carbohydrates. In the production of baked products alpha and beta amylases degrade starch to fermentable sugars so as to sustain yeast growth and gas production.

Alpha amylases are often used to modify starches. They rapidly catalyse the hydrolysis of starch into low molecular weight dextrins attacking alpha 1-4 linkages and bypassing alpha 1-6 linkages.

The two types of amylase, alpha and beta break down the starch polymers in different ways. Amylase breaks down the alpha 1-4 bonds of starch polymers. Alpha amylase may digest the alpha 1-4 bonds between the branch points, and not hydrolyse the alpha 1-6 bonds at the branch points. Beta amylase may digest the alpha 1-4 bonds until it reaches the alpha 1-6 bonds but cannot digest beyond the branch points. The sugars produced by breakdown with amylases include mainly maltose but also glucose and maltotriose and to a lesser extent longer chains of glucose molecules. Alpha amylases are endo-enzymes and can produce glucose, maltose and dextrins. The products of alpha amylase digestion of amylose and amylopectin are shown in Figure 2. Beta amylase and glucoamylase are exo-enzymes. Beta amylase breaks down starch to mainly maltose. Glucoamylase can hydrolyse both the alpha 1-4 and the alpha 1-6 bonds.

Beta amylase by itself attacks approximately one third of amylopectin producing beta limit dextrins. In the presence of minute amounts of alpha amylase beta amylase can resume its cleavage of alpha 1-4 linkages from the non-reducing ends of the chains released by the alpha amylase. Therefore, both enzymes are often used together.

Amyloglucosidase finds widespread application in the conversion of starch and maltosaccharides to glucose. Its rate of hydrolysis has been measured by reducing sugar methods and by the measurement of released glucose using glucose-oxidase-peroxidase reagent.

The two types of amylase, alpha and beta, can be derived from different sources. These sources include fungal (*Aspergillus oryzae*), pancreatic (porcine) and bacterial (*Bacillus licheniformis*) amylases. As well as starch source, the amylase source also effects the susceptibility of starch granules to degradation [11,12]. Fungal alpha amylases were found to be less active on starch granules than pancreatic, salivary, malt and bacterial alpha amylases [12]. It has been shown microscopically that the alpha amylase of *Aspergillus oryzae* produced larger pores on the surface of maize starch than other amylases [13]. The pH and temperature at which the enzymes are active also varies depending on their source.

Starch granules contain both hard and soft material. During amylolysis soft material is more likely to be degraded than hard crystalline material. Hardness of a wheat flour may be due to increased protein content producing a harder texture and a more complex matrix.

Electron microscopy shows that hydrolysis of starch occurs by penetration of pitting and fissures formed initially on the surface of a starch granule during processing. Starches whose surfaces are readily attacked by amylases are quickly digested with the formation of canals into the starch granule e.g. wheat, corn and waxy maize. According to Gallant et al [5] attack by amylases results in the formation of concentric patterns and radial canals, orientating from the periphery of the granule. Wheat starch granules treated with alpha amylase also show collapses, cracks and erosions similar to potato starches [15].

It has been observed that there was "no evidence of potato starch granule erosion even after 56% hydrolysis"[14]. This means that undamaged potato starch is very resistant to digestion. However, another experiment by Gallant et al [5] found that although the extent of damaged grains in the starting sample was less than 0.1% there were at least five times more starch grains damaged after 24 hours of hydrolysis. It was found that when digested with bacterial alpha amylase the area around the hilum is largely attacked, the external part of the granule being more resistant [5].

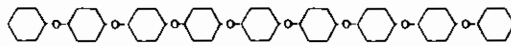
Hydrolysis of the starch is related to the type and arrangement of the crystalline structures, the granular structure, amylose amylopectin ratio, the type of amylodextrin, the average molecular weights of the components and existence of other material (lipid and protein) in association with the starch [16].

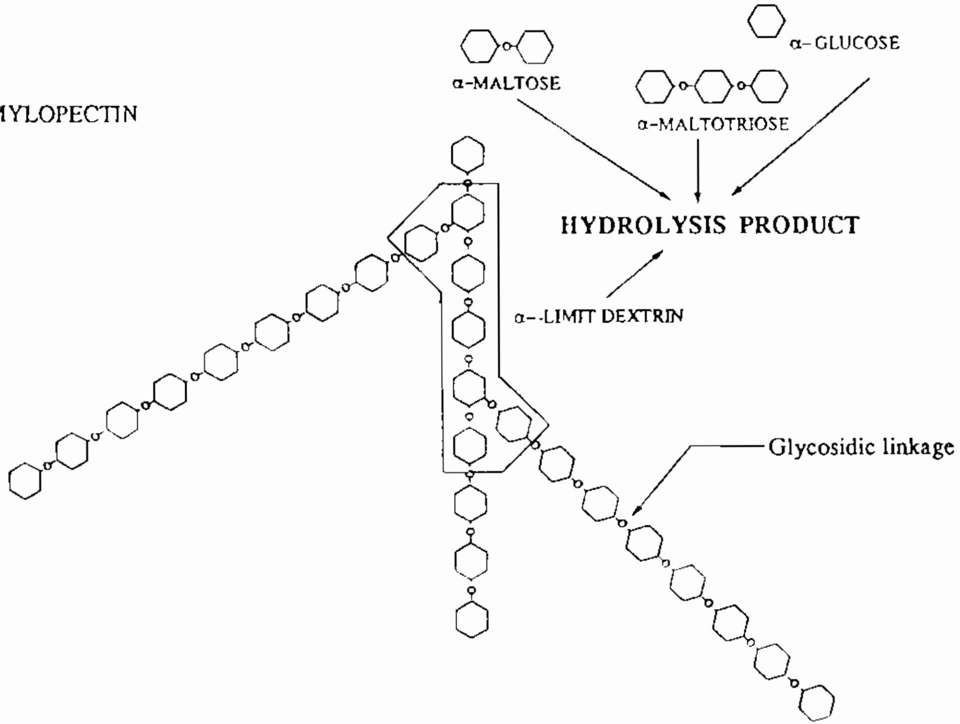
1:1:4 Resistant Starch, (RS)

It had been assumed that all food starch, which forms the majority of available dietary carbohydrate, was completely digested in the human small intestine. However, in 1982 [17], the presence of a starch polysaccharide was reported, which was termed "Resistant Starch", that was resistant to digestion by enzymes in the human small intestine, but degraded by the action of large bowel bacteria. Thus, Resistant Starch (RS) was seen to have a similar biological fate to that of Dietary Fibre.

Figure 2
End products of alpha amylase action on amylose and amylopectin [10].

AMYLOSE





Dietary Fibre is a heterogeneous group of non-digestible non-starch polysaccharides (cellulose, hemicelluloses, pectins and gums) which are located in the cell walls of plants. Fibre is now accepted as important in the diet, with numerous beneficial effects to human health, such as the relief of constipation and possible protection against heart disease and colonic cancer. Some of these beneficial effects relate to the resulting degradation of fibre by bacteria in the large bowel. Thus as resistant starch shares the same fate as fibre in the gut, it has the same potential for promoting beneficial health effects. It is not surprising, therefore, that there is much interest and research on resistant starch including a European Collaborative project (EURESTA) [18].

There are three different ways in which starch escapes digestion in the small intestine, each giving rise to different types of resistant starch called RS1, RS2 and RS3. RS1 is starch, which is resistant to digestion in its natural form. RS2 is starch, which is resistant to digestion due to its crystalline structure. RS3 is starch, which is resistant to digestion due to the fact that it has recrystallised or retrograded following gelatinisation. These three types of resistant starch will be further explained below. The amount of each of them present in the particular food depends on its botanical origin and on the processing conditions, which the food has undergone. Most starch containing food will undergo some form of processing treatment before human consumption.

1:1:5 Starch Damage

For the most part, starch in its natural form is indigestible or not readily digestible. This may be classed as RS1. Beta amylase is unable to attack intact starch granules at all [11] but alpha amylase will, over time, carry out some endocorrosion on intact starch granules. Very few starch containing foods are eaten in their natural form, one of the only types being fruit. Bananas, in particular, contain a large amount of starch and starch is also present to a greater extent in fruits, which are in an un-ripened state. The term starch damage represents a number of changes to the starch detectable by different techniques. Starch granules may be damaged or disrupted from their original structure or configuration by mechanical means, for example by grinding and milling which, releases natural resistant starch for digestion. This generally accepted view was extended [19] to include changes brought about by other means e.g. cooking. Mechanical damage physically disrupts the surface of the starch granule producing cracks and fissures. This physical damage increases surface area and decreases particle size.

Due to damage the granule is made more accessible to the enzyme, increasing its susceptibility to attack. Starch granules can lose crystallinity partially, or completely, as a result of damage. Damaged starch is the substrate for amylases, which in turn provide fermentable carbohydrates that are used by yeast in fermentation e.g. during the bread manufacturing process. Damaged starch also has an increased ability to absorb water as well as dissolved dyes. In an intact granule water enters only via amorphous zones. On disruption of crystalline regions access to the whole granule becomes possible, allowing hydrogen bonding between water and exposed hydroxyl groups on starch molecules hence giving rise to increased absorption due to starch damage.

In order to understand the effect damage has on a starch granule one must understand its structure and the way in which enzymes break down damaged starch. Most of the starch we consume is processed. During processing of starch in the production of different foods the structure of the starch is altered. This can be done in different ways and to varying degrees. Therefore, the physical form of the food itself may affect its resistant starch content. Electron microscopy of starch shows that hydrolysis occurs by penetration of pitting and fissures formed initially on the surface of the starch granule during processing. It is in this damaged condition that wheat, corn and waxy maize starches are readily attacked by enzymes. The surface of potato starch granules is more resistant until the enzyme penetrates the surface and at this point hydrolysis is far more rapid than for the cereal starches.

Some of the properties of starch damage are not manifested until contact with water has been made as in gelatinisation. Gelatinisation destroys the ability of starch to exhibit x-ray diffraction patterns and it has also been shown that the same applies to starch damaged by mechanical means [20]. By the method of mechanical damage amylopectin fragments are produced [21, 22, 20] whereas in the case of gelatinisation amylose is released out into the surrounding solution [23]. Damage to starch granules is controlled to cause changes in physical structure which exhibit important advantages in technology concerning the production of starch based products.

a. Production and Control of Mechanically Damaged Starch

Damaged starch is required for example in the bread making industry. In the production of flour, starch in cereal grains is separated from the bran and germ. The starch is physically damaged mechanically by milling of wheat to produce flour. The starch is physically crushed between rollers. The extent to which the granules are crushed can be changed depending on how much damaged starch is required by altering the amount of physical pressure required to damage different types of wheat or other cereals. Mechanical damage will break up the granules in different ways. Granule surfaces may become rough and cracked [24] or may be broken into pieces or sliced in two. Cereals are more susceptible to hydrolysis when finely ground [25]. Also during mechanical damage glycosidic bonds may be broken [26, 24]. Dry milling was found to increase the susceptibility of starchy materials to digestion when tested with enzymes[27 28].

Starch in cereal grains is damaged during the reduction stages of processing. The pressures of shear, attrition, compression or impact disrupt the granule structure during grinding or milling procedures.

Two factors influence starch damage levels by this method of processing:

1. Type of starch used

2. The method and severity of grinding

Related to this are the following sub-factors which have an effect on the level of starch damage in a flour [29, 30]:

1. Wheat type: the harder the endosperm the higher the level of damage measured. In a standard milling process the amount of starch damaged will increase with the hardness of the wheat [31].

2. Feed rate: decreasing the feed rate releases a higher flow of flour thereby increasing the level of damage produced.

3. Roll speed: increasing the roll speed increases the rate of flow and consequently the rate of damage.

4. Roll differential: increasing this ratio increases starch damage especially for coarse feed material.

5. Roll pressure: increasing roll pressure increases starch damage level especially for fine feed.

6. Roll surface: matt surfaces produce more starch damage than smooth surfaces.

It has been found that there exists a considerable range in the content of mechanically damaged starch between flours milled from different wheat varieties[32].

The importance of damaged starch in flour used for bread making is two fold. By the milling process a certain amount of starch in the form of flour must be available for digestion (fermentation) by yeast. Damaged granules are susceptible to amylolysis thereby producing maltose as the substrate for yeast in the late stages of fermentation when supplies of readily available fermentable sugars in flour are exhausted. Yeast fermentation produces carbon dioxide, which gives bread the aerated structure and its general texture and volume. Starch damage affects rate of fermentation, crumb strength, loaf volume and gassing power. Inadequate gassing is as a result of either insufficient damaged starch or lack of alpha amylase. The amylase activity must not be allowed to become too high or too low in order to produce the correct amount of gassing. A consequence of lack of substrate on which yeast can ferment is poor gas retention. The result is production of bread with low volume and heavy texture. Protein content and degree of starch damage contribute to its water absorption. Increase in starch damage will increase water absorption. Allowing the starch to absorb a certain amount of water is also necessary in bread production.

Too much starch damage causes excessive water absorption and swelling, particularly in the presence of an abundance of water. On increased enzymatic activity, much of this water is released or exuded causing slackening of the dough. Excess water gives rise to sticky crumb texture. This occurs particularly during longer traditional bread making processes. Consequently starch damage levels must be kept to a low level during these processes. However, with adoption of modern processes such as the Chorleywood bread process in which fermentation time is much reduced the limits to levels of starch damage may be expanded.

Following production of flour for bread manufacture, approximately 10 % of starch [33] is damaged. In the production of beer, damaged starch is also fermented this time to produce alcohol. Therefore, both of these major industries require damaged starch as their main raw material.

The production of satisfactory bread requires a balance between the abundance of water in dough making, the protein content of the flour, its damaged starch content and the level of alpha amylase activity. The latter can be controlled by the use of thermo-unstable enzymes e.g. fungal alpha amylase where the enzyme activity is inactivated during cooking or heating.

Following milling, amylopectin from barley is preferentially soluble in cold water. This may be because the larger size of amylopectin leads to greater susceptibility to rupture of linkages between groups of short chains. Molecules of lower molecular weight are more soluble, and therefore, more easily degraded by enzymes.

Rice is one of the few cereals usually consumed in its granular structure. It has been found that rice and lentils gave a higher blood glucose content after they had been ground compared to when they were consumed whole [34, 35]. The higher blood glucose content showed that more starch had been made available, by grinding, for digestion in the small intestine with the release of glucose. Whole rice contains 3.1% resistant starch (RS) but the same rice when ground contains only 0.7% RS [36]. Starch in raw rolled cereals (i.e. mildly crushed) is hydrolysed more slowly than starch in corresponding cereal flours (i.e. milled) [25]. The rolled cereal has a more tightly packed physical form with less surface area relative to milled flours. Even on cooking the rolled cereal starch is less readily digestible than the starch in the milled flour. When different types of breads were compared, it was found that the bread which contained whole-grain wheat, rather than milled wheat, (white or wholemeal), contained the highest levels of RS [37]. During the milling process dietary fibre may be removed which would have limited the access of the enzymes to the starch [38].

Structural resistance is not always related to the original form of the food. In tests pasta gave lower blood glucose levels than bread. This relates to the fact that the pasta has a firmer physical structure [39] and this protects some of the starch from digestion. The association of starch with other food components may also inhibit digestibility. Protein, for example, may physically encapsulate the starch and, therefore, make it unavailable for digestion [40, 41]. Likewise, fibre may also form a physical barrier limiting the access of enzymes to the starch. This was shown when whole brown rice was found to be hydrolysed more slowly than whole white rice [25]. However, when whole brown rice and whole white rice were ground hydrolysis was the same.

Thus, it can be seen that the extent of physical, mechanical damage which some starch-containing foods are subjected to, during processing, will have a considerable effect on how much starch is available for digestion, and how much will pass through to the colon and behave in the same way as dietary fibre.

b. Gelatinisation of Starch

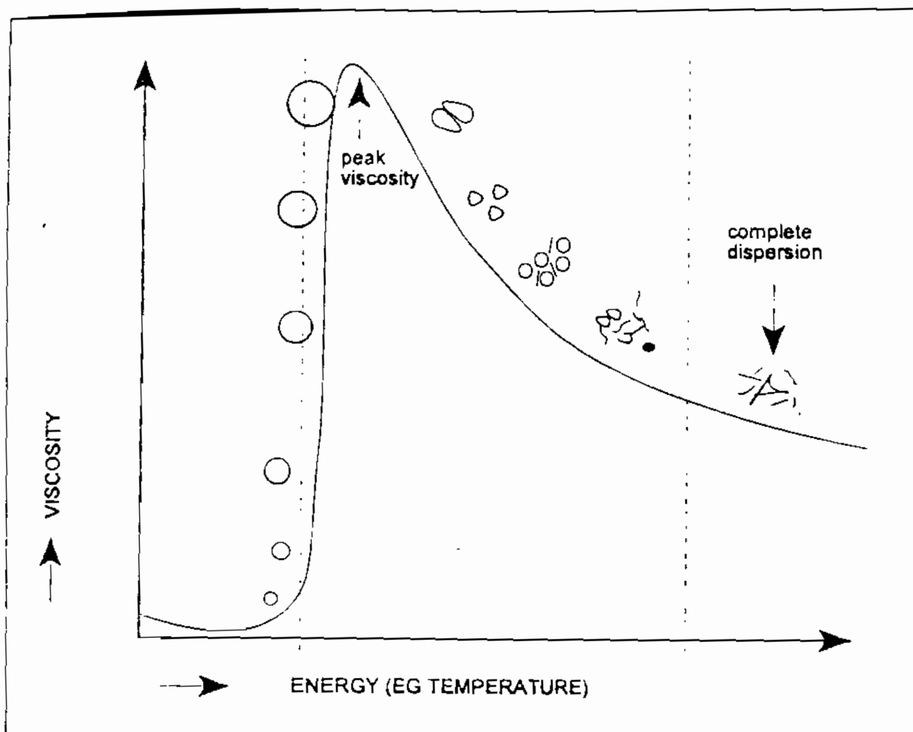
As has been said, already starch reversibly absorbs water in its natural state. Granules can reversibly imbibe water resulting in slight swelling until the total water content is approximately 35% of the total mass. This is the case however, only up to a temperature of approximately 60°C. At this temperature and above it starch in excess water solution becomes damaged. Starch molecules on absorption of thermal energy vibrate. Intermolecular hydrogen bonds are broken which allows more water imbibing to take place. The granule swells. Randomness increases as crystallinity decreases. The starch granules irreversibly absorb water and swell to many times their original size which eventually disrupts the granule, leaching the amylose component out into solution and leaving behind an amylopectin skeleton which becomes progressively disordered. The rupture of granules at this stage may be caused mechanically even as a result of just gentle stirring. The stages of gelatinisation of starch are shown in Figure 3.That gelatinisation has actually occurred can be evidenced from the fact that birefrigence can no longer be seen, and crystallinity, which can be observed by x-ray powder diffraction is lost. The hydrogen bonds between the linear segments of the crystallites and to a lesser extent between molecules in the amorphous regions are broken [10]. Native starch has average water absorption of 0.33g per gram of starch whereas samples of 100% starch damage by milling absorb 1g of water per gram [10].

Sixty degrees Celsius is an average gelatinisation temperature for all types of starch. The temperature at which each individual type of starch gelatinises depends on its botanical source but the range for most starches is from 50 to 70°C. Eventually crystallinity is completely lost, shown by disappearance of birefringence in x-ray diffraction patterns. At 100°C complete conversion to the amorphous state will occur. This process occurs during the preparation of many starch-containing foods, for example, during the production of bread or in cooking potatoes. Certain modified starches are prepared by pre-gelatinising the starch, followed by drying the starch on a roll or drum dryer. This will give a starch, which will can be readily dispersed in cold water and easily digested. Also the extent of gelatinisation will vary depending on starch source, temperature, gelatinisation time and other factors (amount of amylose present and degree of crystallinity of the granule).

The water content of the starch solution for gelatinisation is generally more than 300% added on a dry matter basis. However, often the water content of food, which is being cooked is generally below 50%. In low moisture conditions melting of crystallites occurs at much higher temperatures 100 to 150°C than gelatinisation temperatures [42, 43]. Crystallinity is lost but the granule shape may remain. According to the Flory equation as the water content decreases the melting temperature increases up to 150°C [10].

Therefore, when the amount of water is limited e.g. biscuits or bread or the structure does not allow the granules to swell, no disruption and limited solubilisation of granules occur [10]. Complete gelatinisation may be prevented by other food components such as mono or oligosaccharides or the presence of salt causing the melting temperature to increase [10]. Partial gelatinisation and melting renders starch granules more accessible to enzymes, but it also increases their water binding capacity, which creates better accessibility for enzymes. Therefore, when samples are completely gelatinised hydrolysis occurs at a greater speed.

Figure 3
Schematic diagram of a starch granule undergoing gelatinisation.[44]



Although cooking increases starch susceptibility to hydrolysis, a wide variation in the level of starch modification still remains, depending on the processing intensity and storage conditions [45]. Using a method involving a centrifuge step, a linear relationship can be found between starch digestibility and water retention [46]. However, higher water absorption capabilities are obtained on starch gelatinised in low shear conditions [47]. Starch can exhibit extensive swelling and still maintain a high degree of crystallinity as shown by x-ray diffractometry [48]. Therefore, processing effects may be explained in terms of structural modifications interpreted by physicochemical mechanisms. This approach enables understanding of structural modifications and therefore, allows a better design of starchy foods with special structural features [49].

Different levels of damage can result from variation in commercial heating processes. Comparative tests on whole grain wheat and white wheat flour were performed. It was found that steam flaked and dry autoclaved wheat samples at the lower limit of conditions normally used commercially were incompletely gelatinised[50]. Unless strictly controlled the damage to starch samples under some of the main types of processing conditions can vary considerably.

c. Chemically Modified Starches.

Starch may be damaged chemically: for example by solubilisation or hydrolysis with acid. Chemical modification may also involve combining the starch with other substances e.g. phosphate groups in order to give the starch other functional properties.

1:1:6 Granule Crystallinity

Starch may be resistant to digestion due to its crystalline structure. This form of resistant starch requires gelatinisation to make it available for digestion. Different starches have different susceptibilities to gelatinisation. For example, high amylose starches are more resistant than potato starch due to granule structure and composition. Damaging destroys the crystalline organisation of starch granules.

The granular structures are composed of the amylose and amylopectin mentioned above. However, exactly how the amylose and amylopectin are arranged in different types of starch is as yet uncertain. It is believed that the amylopectin, which usually comprises about 70% of the starch granule, gives the granule an ordered crystalline structure and the amylose is arranged in some way around this, dispersed between its branched chains. The linear structures in amylose and amylopectin are not flat but form a natural twist which is coiled into a helical structure (about 6AGU per turn) [51, 52]. Two of these helical structures align together side by side to form a long double helix and are joined by hydrogen bonding. This helical structure is shown in Figure 4, which is a micrograph of a starch double helix. The linear part of the amylose and amylopectin is composed of these double helices. It is the double helices within the amylopectin, which in ordered form within the granule gives it its crystallinity and the interjacent linear segments form the crystalline lamellar domains [53].

Figure 5 shows a schematic model for the arrangement of amylopectin in potato starch. It is believed that the amylopectin is primarily responsible for the crystallinity because waxy starch granules still exhibit crystallinity [54]. The amylose and the branch points of the amylopectin are disordered or amorphous and are arranged around the ordered crystalline structure. About 70% of the starch granule is amorphous [55]. The botanical source of the starch determines the degree and type of crystallinity within it.

The semi-crystalline structure of potato starch granules has been studied using electron optical tomography in combination with cryo electron diffraction [55]. It was concluded, following examination of the starch, (which had been partially hydrolysed with acid to allow the crystalline structure to be made more visible) that the crystalline domains in the amylopectin form a continuous network of left handed helices which appear as a well ordered skeleton for the starch granule. It was found from a three-dimensional reconstruction that the crystalline lamellae are tilted relative to their axis and that they form short helical stretches. Closer inspection showed that the complete semi crystalline structure is built up from more or less continuous left handed helical segments. A model was proposed from the information gathered. In the model the helices form a continuous regular crystalline network which appears as a skeleton around which the rest of the starch granule is built. The linear segments which are approximately 5nm long form double helices which are crystallised into 5nm thick lamellae alternating with amorphous layers in which the alpha 1-4 and alpha 1-6 branch points are located. Neighbouring helices interpenetrate each other and the crystalline lamellae form a continuous super helical structure. The central cavity of the helices is approximately 8nm in diameter [55].

When starch granules are viewed under polarised light they display a cross pattern in the shape of a Maltese cross indicating a radial orientation of the crystal structure around a principle axis [9]. This pattern formation is known as birefringence. It remains unchanged in both polar and equatorial sections of elongated starch granules. This is only possible if it is considered that the crystallites are extremely small and show multiple orientations which interfere during observation [9]. The birefringent properties have been attributed to the presence of spherulites (spherical aggregations of needle shaped crystals) which have no definite surface and are made up of small crystalline regions held together by secondary valence forces[54].

It was suggested, that either the grouping of a number of branches of the highly ramified amylopectin molecules form the spherulites into radially oriented crystalline bundles, or by the alignment of the outside branches of several molecules to form a fringe micelle. Some branches of one amylopectin molecule may align themselves to form part of a micelle and the rest including the branch points may be in the amorphous region where secondary valence forces act tangentially to hold the granule together [54]. This structure is consistent with x-ray data available [4].

The inside of the helical structure is hydrophobic and the outside contains hydrophilic hydroxyl groups. Therefore, the hydrophobic cavity allows complexation with hydrophobic molecules such as iodine. Figure 6 shows the structure of an amylose-fatty acid complex. About 20mg of iodine complexes with 100mg of amylose and 1mg of iodine complexes with 100mg of amylopectin [5].

Figure 4
Central section, showing the packing of the helices. The central holes in the helices are black. The roughly tetragonal packing is indicated by dashed lines[55].

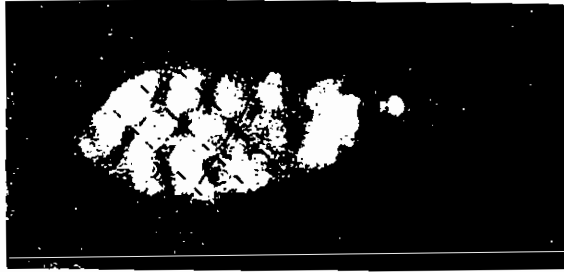
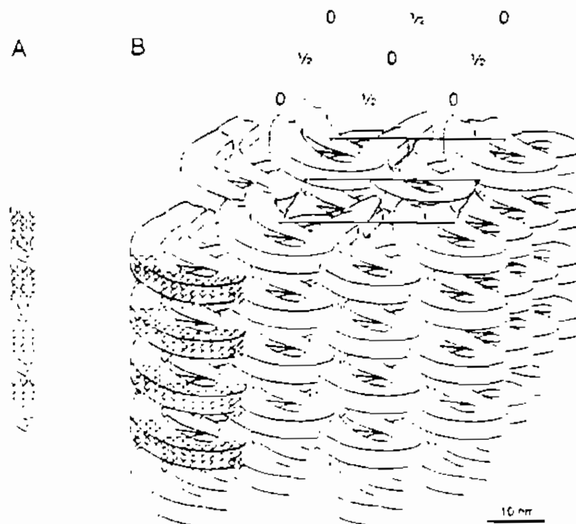


Figure 5
Schematic model for the arrangement of amylopectin in potato starch. (A) Model for the amylopectin molecule showing the clustering of the alpha (1-4) alpha (1-6) branch points and the double helical linear alpha (1-4) glucan chains. The layers containing the branch points are amorphous whereas the linear segments are crystallised to form 5nm thick crystalline lamellae. (B) The crystalline layers containing the double helical linear segments in the amylopectin molecules from a continuous network consisting of left handed helices packed in a tetragonal array. Four amylopectin molecules are projected into one of the helices[55].



The type of crystallinity which different starches possess, has been examined by x-ray diffraction and has been categorised into three different types A, B and C. This does not follow the morphological classification of the starch granules. The structures depend partly on the chain length making up the amylopectin lattice, the density of the packing within the molecule and the presence of water [57, 58, 59].

A-type starches are found mainly in cereals. They are generally the easiest types of starch to digest in the human small intestine and have a low degree of crystallinity. They may be digested without cooking. However, gelatinisation of these starches increases the availability of the starch. Cooked oats have 0.028g/g dry weight resistant starch compared to a value of 0.161g/g dry weight for uncooked oats [60]. This would mean, for example, that muesli prepared with rolled oats would contain more resistant starch than porridge made by cooking rolled oats. This type of starch consists of chain lengths of 23-29 glucose molecules with further crystalline molecules interspersed.

B-type starches are found in bananas and tubers such as potato and are the most resistant type of starch to digest unless damaged (cooked). One study showed that, in using porcine pancreatic alpha amylase to digest starches, rice or wheat starch is degraded over six times faster than banana starch, and over 20 times faster than potato starch [61]. The fact that potato starches are less readily hydrolysed than cereal starches is often attributed to the higher content of crystalline structure in potato starch [62],due to the fact that alpha amylase is believed to hydrolyse the amorphous parts of the granule first [63]. Another reason why potato starch is less readily hydrolysed is attributed to the granule size [64]. This category also contains amylose rich starches such as amylomaize, some waxy starches, barley and wrinkled pea starches [51, 52].

Up to 75% of banana starch may be resistant and as banana is usually consumed uncooked this is a very high source of resistant starch. The amount of banana starch (in green tipped bananas) not hydrolysed in the human small intestine and passing into the large intestine may be up to eight times more than the non starch polysaccharides (dietary fibre) in this food [65]. Even though B-type starches are more difficult to digest according to diffractometric patterns the A pattern appears to be more dense than the B pattern [5] which consists of chain lengths of 30-34 glucose molecules with water interspersed.

C-type starches are considered by some to have a distinct crystallographic structure or are considered to be made up of either mixed populations of starch granules with the A or B pattern respectively, or of populations in which all the starch granules have the two A and B structural patterns [5]. This type of starch can be found in legumes such as soybeans, peas and beans. This type of starch is intermediary between A and B - type starches in its ability to be digested. Raw legume starch contains 21 - 44 % resistant starch, but on cooking this is reduced to 3-15%. After extrusion cooking, resistant starch is reduced even more to 1 - 7% [66]. These starches consist of chain lengths of 26-29 glucose molecules.

The rate of hydrolysis of non-solution starch depends on accessible surface area [67]. A-type spherilites are uneven whereas B-type are smooth [16]. Studies using the SEM have found that resistant shells in starch granules are composed of spherical blocklet like structures which are larger in size in the B and C type crystalline structures than in the A type starch granules. These blocklets are shown in Figure 8.

A V-type pattern, which has a different type of crystalline structure may be formed when starch complexes with hydrophobic molecules such as fatty acids.

A, B and V type crystalline patterns determined by diffraction are shown in Figure 8.

Figure 9 shows diffraction patterns of starch at various levels of hydration. This shows that very different patterns can be obtained depending on the moisture content of the sample being tested.

Studies have been carried out on the resistant starch content of breads, due to the fact that they make up a large part of the starch content of our diet. Commercial breads are found to be hydrolysed more rapidly than home-made breads. This may be due to greater exposure to heat during the commercial process. As mentioned previously there is no significant difference, however, between wholemeal and white breads as regards hydrolysis. A clear distinction should be made between wholemeal and whole grain breads. Whole grain wheat bread contains, as would be expected, the highest levels of resistant starch. Generally the processing which many starch-containing foods undergo seems to be severe enough to leave the starch readily available for digestion.

Therefore, as has been seen, some starches must be cooked before consumption to allow the starch to be digested. The time and temperature of heating will release different amounts of starch for digestion. The producer and consumer should, therefore, be aware of the type and extent of heat treatment the food has undergone, and is likely to be subjected to following purchase. This in turn will affect the amount of starch that may be digested and how much starch will be resistant and potentially behave in the same way as dietary fibre.

1:1:7 Starch Re-crystallisation

The process of gelatinisation produces an unstable solution of starch. It was explained earlier that the linear components of amylopectin give the starch granule its crystallinity, however, it was also discussed that the amylose contains more linear, less branched structures than amylopectin. Once these helical linear structures are leached out into solution following gelatinisation and separated from the ordered structure they are unstable and tend to re-associate and bond together alongside each other forming hydrogen bonds. This occurs in the case of both amylose and amylopectin but at different rates.

Figure 6
Structure of amylose fatty acid complex [56].

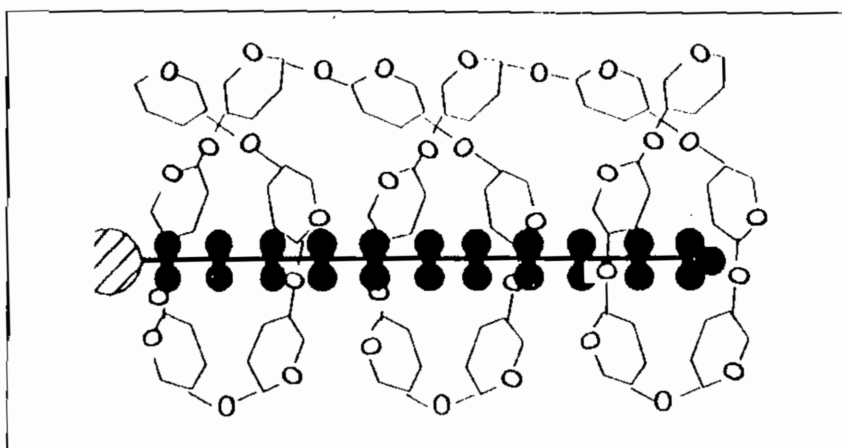


Figure 7
Scanning electron micrographs of starch granules after alpha amylolysis. Potato (a), wheat (b), maize (c), manihot (d) smooth pea (e), wrinkled pea (f), and the hybrid of (e) and (f), (g). SEM shows that resistant shells are composed of spherical blocklet-like structures. These blocklets are larger in size in the B and C type starch granules than the A type starch granules [9].

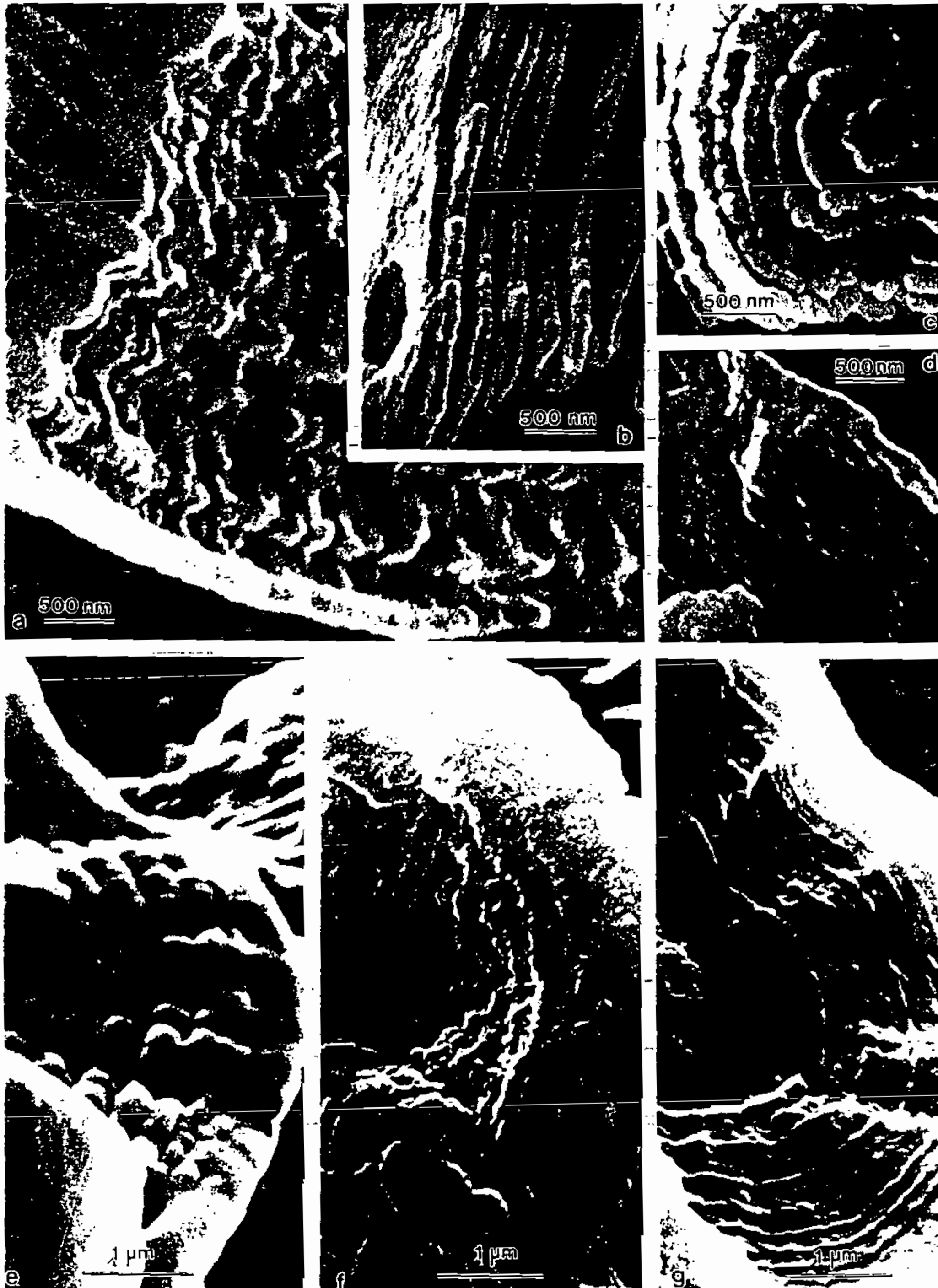
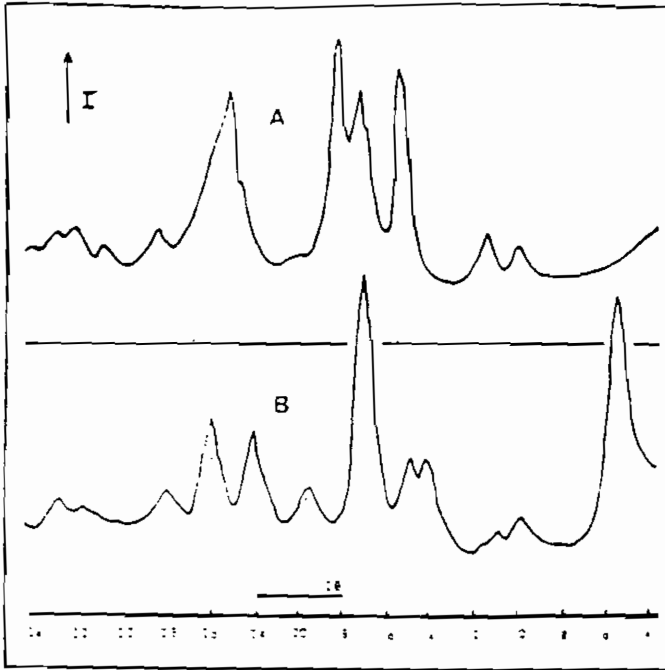


Figure 8
Diffractometry Spectra with A, B and V Crystalline Patterns of Starch. The C Spectrum is marked with Stars in the A and B Spectra[68].



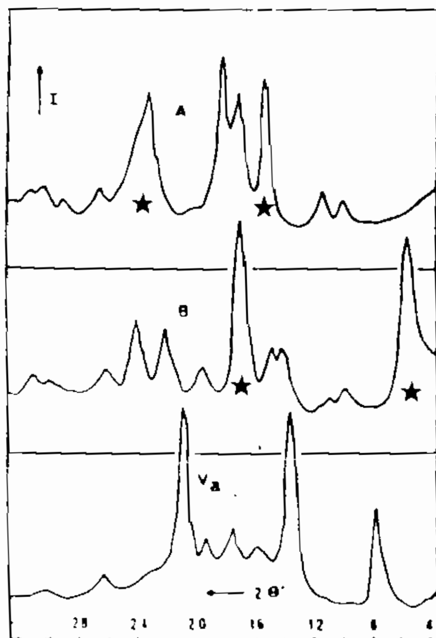
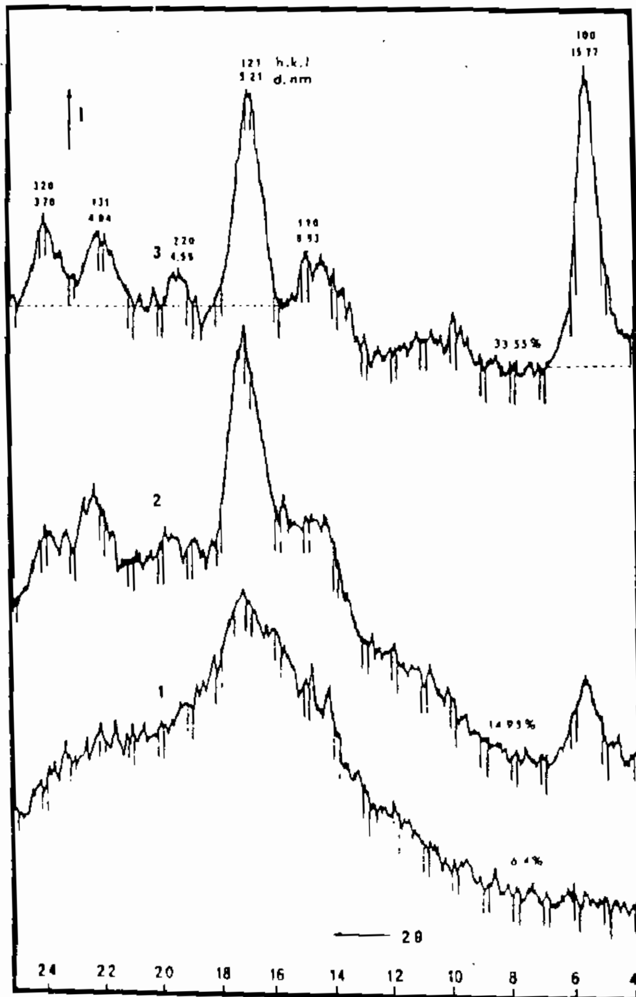
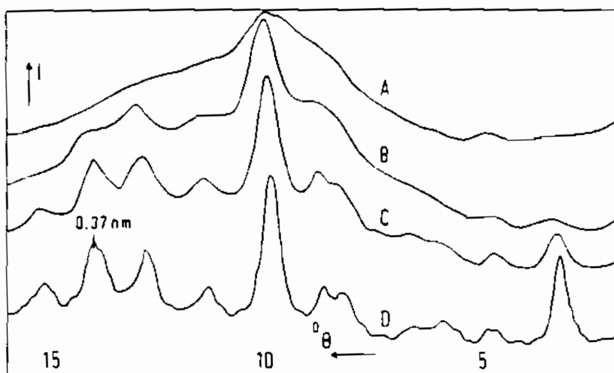


Figure 9
X-ray Diffraction Patterns at Various Levels of Hydration[72]





A - near to 0%

B - 8%

C - 11 %

D - 40%

This re-association or re-crystallisation may occur in different ways. A viscous paste may form or in higher concentrations >5% a gel may be formed[10]. This is as a result of a ternary phase separation occurring in the water-amylose-amylopectin system yielding a coarse network of a polymer rich phase [69, 49]. This phase separation is followed by a nucleation phase, leading to the development of a thin three-dimensional network. Viscosity rises as hydrogen bonding occurs. This is also caused by impingement of the swollen granules causing extrusion of amylose molecules. Water is also lost. The aggregation of starch to form a gel is shown in Figure 10. Elementary junction zones are established between macromolecules, which are thought to adopt a double helical conformation. These junction zones aggregate on ageing and develop a B-type crystallinity [10] forming what is known as resistant starch. The processes of gelatinisation and retrogradation and gelation of starch are shown in Figure 11. Figure 12 shows x-ray diffraction patterns of native, gelatinised and retrograded starches.

Amylose gels are formed on cooling semi-dilute solutions of amylose (>/= 15mg/ml) and aggregates are formed in dilute systems (<15mg/ml) [70]. Amylose gels are poorly crystalline composed largely of an amorphous fraction [10]. Amylopectin gels have higher polymer concentrations (>100mg/ml). This is due to the branched structure of the molecule and to the lower degree of polymerisation of the segments involved in the crystallites. (DP is approximately 15 as opposed to DP 40 to 60 in amylose crystallites) [10]. On cooling of starch solutions the amylose and amylopectin form mixed gels of two phases one continuous and one discontinuous. Pure amylose gels have better rigidity and thermostability and longer crystallites than pure amylopectin gels [10]. These characteristics are responsible for increased cohesiveness, cooking stability and hydrolytic resistance in amylose rich mixed gels such as pasta products from Mungo starch and rice flour [71].

Starch retrogradation occurs during ageing of cooked foods especially baked goods such as bread. Retrogradation of amylose is a rapid process taking only a few hours in bread whereas amylopectin retrogradation may take several days leading to hardening of bread. Redistribution of water from the crumb to the crust also occurs during this change accelerating retrogradation [10]. Resistant starch is insoluble in water, cannot be re-gelatinised and cannot be digested by the human small intestine. This resistant starch may be used as a replacement for fibre in foods as evidence shows that it behaves in much the same way in the human diet. V-type crystallinity may be formed attributable to the helical complexation of fatty acids and monoglycerides by the amylose molecules. Complexation is more rapid (<1h) than formation of B-type crystallinity.

Retrogradation of amylopectin is limited by its branched structure. However, when amylopectin is de-branched (with de-branching enzymes such as pullulanase) it has been shown that the linear components, which remain, increase the amount of resistant starch formed. Amylopectin was found to form 0.2 - 4.2% resistant starch, but following de-branching and heat treatment gave 32 - 46% resistant starch [73]. The resistant starch formation due to retrogradation of amylopection takes place over several days and is reversible by heating (e.g. re-heating softens stale bread).

Figure 10
Conformational changes occurring during amylose gelation[10].

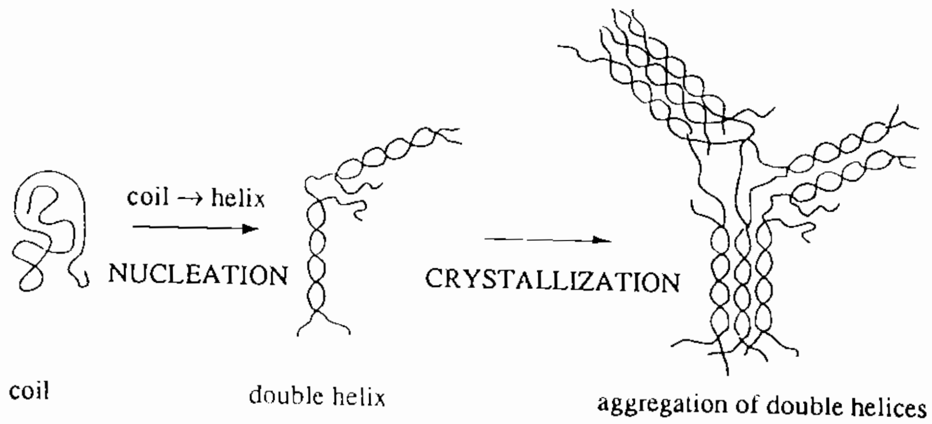


Figure 11
Thermal transitions of starch[11].

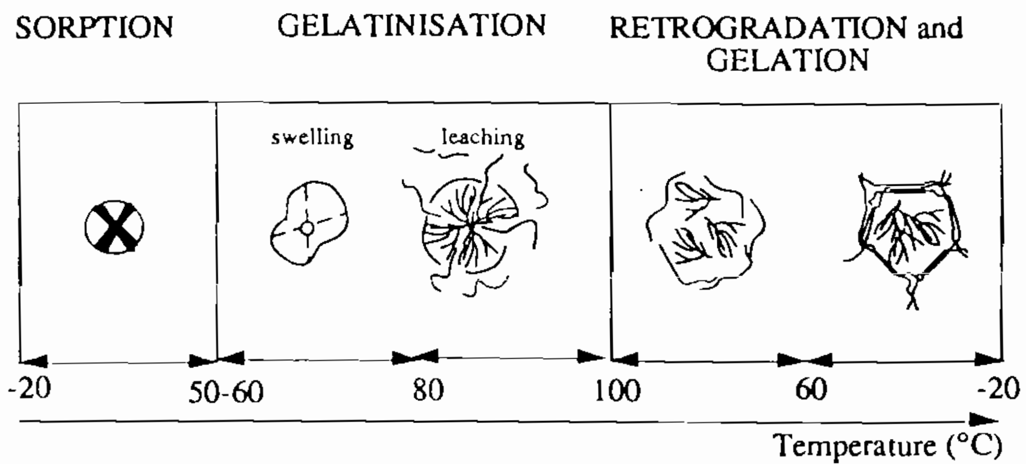
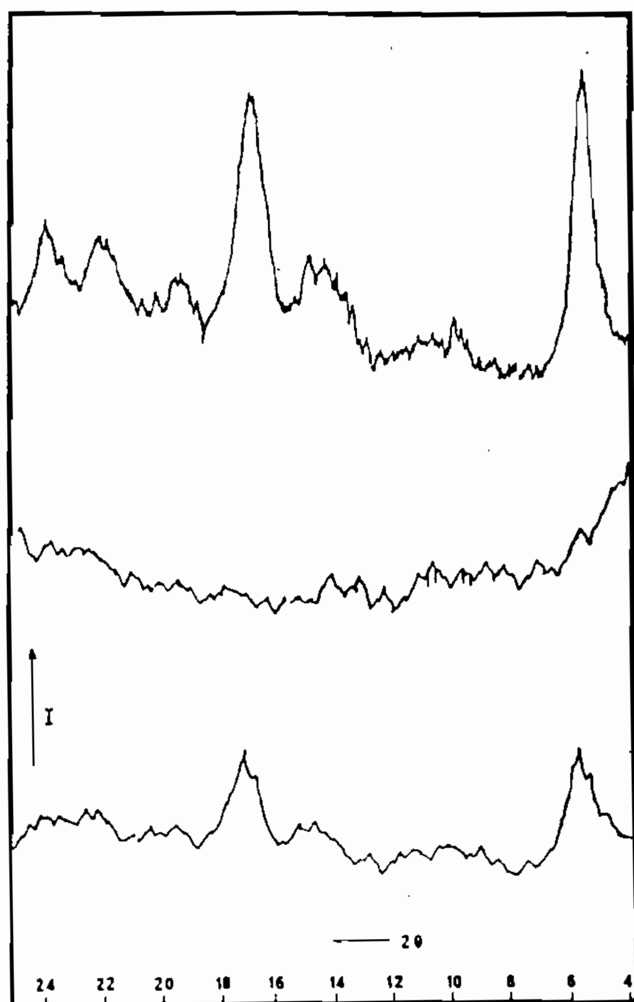


Figure 12
X-ray Diffraction patterns of Native (upper), Gelatinised (middle) and Retrograded (lower) Starches[10].



According to the conditions discussed above, in biscuit production starch is un-gelatinised and in puddings it is completely retrograded [10]. Browning and Maillard reactions produce un-digestible starch [74]. Resistant starch is present in small quantities in different breads but much less is present in the flours from which they were made. This would implicate a type of resistant starch known as retrograded amylose. Highest resistant starch yields are obtained from flour with the greatest amylose content.

Fibre, in the form of resistant starch, thus produced, is of particular interest to food manufacturers who could potentially influence the dietary fibre content of their foods. This form of resistant starch, once manufactured, could also be added to other foods.

Water and heat must be present for the production of resistant starch. The amount of water and the temperature at which the starch containing food is cooked will have an effect on the amount of resistant starch formed. It has been found that cooling a cooked potato overnight at 4°C resulted in a 2.8 fold increase in resistant starch [60]. The resistant starch, as a percentage of total starch, recovered from ileostomy patients was 3% resistant starch for a freshly cooked potato, 12% resistant starch for a cooked and cooled potato, and 7.4% for a cooled and reheated potato. This reduction, from 12% to 7.4%, on re-heating was probably due to a reduction in retrograded amylopectin however, the resistant starch produced was not RS1 as the potato was presented for consumption in different physical forms (in pieces, sieved etc.) or RS2 as the sample was well gelatinised [75]. These results are significant because potato is one of our main sources of carbohydrate. Potato may also be added to many ready to eat foods and used to produce various potato products, which may be sold frozen or dried. The processes which the potato has undergone may make an important difference to how much resistant starch is being produced and, therefore, how much potato is actually being digested. Technological advances include reduced retrogradation and improved freeze thaw stability of pastes as compared with those made from pregelatinised starches[20].

Autoclaving a wheat starch suspension at 100°C, for a given time followed by cooling, produced 2.5% resistant starch. Autoclaving for the same time at 134°C, followed by cooling, produced 9% resistant starch. This indicates that the production of resistant starch requires considerable damage to the native starch granule [73]. Amylose may need to dissociate from various components such as lipids, amylopectin or indeed from other amylose molecules before it can re-associate with itself to form resistant starch. Further cycles of heating and cooling produced an increase in resistant starch [73]. Chemically modified starches may also contain resistant starch and be less easily hydrolysed.

Therefore, low temperature, high starch concentration and long chains of amylose and amylopectin [10] enhance retrogradation of starch.

This non-digestibility of resistant starch has meant that a number of methods for fibre estimation in food include any resistant starch present in the result for total dietary fibre [76, 77]. An exception was found to this, which corrects for any resistant starch present in the food[17]. Hence reported figures for dietary fibre in the same foods can vary, in some cases quite appreciably, depending on the method of analysis. This can be seen in the UK Food Composition Tables [78], which give two values for dietary fibre for each food based on the two different methods used [17, 77]. This has the potential for giving rise to confusion for nutrition labelling, as well as the recommended intake for fibre. In the future high starch foods may be manipulated to maximise their resistant starch content in order to provide our diets with a substance, which behaves like dietary fibre. This will involve either less processing as in the case of the use of whole cereal grains, or more processing as in the case of retrograded amylose. As well as providing a dietary fibre-like component to our food resistant starch may well also be used in the future in diabetic diets, as it releases less glucose, or in low energy foods for the same reason. If it is included, then the dietary fibre value may vary depending on how the food is prepared or cooked before consumption. Either way resistant starch, particularly retrograded amylose RS3, may well become an important consideration in the future for both the manufacturer and the consumer, who wishes to obtain a more nutritionally beneficial diet.

In fact some food companies in the USA and Europe are taking advantage of this particular property of some starches and 'creating' high fibre foods by altering processing techniques e.g. by heating and cooling potato flour many times to produce a product with a high resistant starch / "fibre" content.

1:2 DAMAGED STARCH ANALYSIS

1:2:1 Damaged Starch Determination

Determination of damaged starch levels in flours is a procedure carried out routinely by many manufacturers of starch based products. Its relevance lies in the effect, however advantageous or adverse, it may have on the rheological properties of the end product. To ensure quality of the highest standard this type of experimental procedure is necessary. Assays emulate the susceptibility of damaged starch granules to enzymatic attack.

A very wide variety of procedures and equipment have been used for the starch structure to be elucidated and the changes in the structure due to processing determined.

The most well known characteristics of damaged starch include:

a. Increased enzyme susceptibility or digestibility
b. Increased cold water extractability
c. Increased ability to absorb water
d. Increased ability to complex with dye.

Two main categories of measurement have evolved from these specific characteristics. They are based on:

1. The susceptibility of the starch to digestion by amylolytic enzymes
2. The extractability of amylose from starch in cold water [79].

Methods used to measure starch damage are broadly grouped into four classes:

1. Enzyme digestion procedures
2. Dye staining procedures
3. NIR procedures
4. Extraction procedures

A frequently used criterion, to evaluate starch damage level is whether the procedure usefully predicts a flours water-absorbing capacity, due to starch. Many protocols have been established for the measurement of starch damage levels, based on either extractability or digestibility parameters, and comparisons made on the basis of reproducibility, speed and potential for automation. There exists no definitive method for indicating starch damage levels. Therefore, evaluation of results is often necessary and correlation factors are required between different methods. All such methods [80] are empirical, since the complete differentiation between damaged and undamaged starch granules is virtually impossible. Determination of damaged starch by enzymatic methods has no definite end point. Therefore, we cannot be sure exactly where digestion of damaged starch ends, and endocorrosion of undamaged starch begins.

Most methods rely either on the use of amylases to hydrolyse damaged starch or the iodometric measurement of soluble polysaccharide exuded from the damaged granules.

The expected level of percentage starch damage in wheat flours is 10% [33], which contributes to optimal baking properties in the production of bread. Therefore, much study has been done to find the easiest, simplest and most reliable way of determining starch damage in flour. Standard enzymatic damaged starch determination tests which are internationally used include the Farrand Method [1] and the AACC 76-30A Method [33].

1:2:2 Enzymatic Methods

Much of the work done on starch analysis has been in the area of determining the availability or digestibility of different starch fractions. This means that in each starch containing food which has undergone damage part of the starch will be readily digestible and part of it will be less damaged and therefore, less easy to digest. Starch degrading enzymes can be used to determine the extent of digestibility of the starch. What is in fact being determined by the enzymatic methods is digestible rather than damaged starch. The digestible starch is then taken as apparent damaged starch.

Enzymatic methods all involve the addition of an excess concentration of alpha amylase to a buffered solution containing the starch sample to be tested and digestion of the damaged portion of the starch granules. The reducing sugars produced enzymatically are quantitatively evaluated to determine the digestible or apparent damaged starch value. As well as amylases another amylolytic enzyme amyloglucosidase can be used to digest the reducing sugars produced by amylases to glucose.

Digestibility and damage are, as was mentioned above, not necessarily the same thing. Also, within individual starch granules damaged sites exist, which differ in their susceptibility to amylolytic hydrolysis [81, 2]. While starch in its natural form is for the most part not readily digestible, some starch which is not damaged may be digested by endocorrosion. Therefore, the amount of damaged starch present which is initially extracted is responsible for determining the level at which sound starch commences to be digested, and an estimation of the amount of damaged starch present may be derived from the final amount of maltose produced [82].

The Farrand [1] and the AACC 76-30A[33] methods are enzymatic methods of determining percentage damaged starch in wheat flours. The Farrand method involves the measurement of reducing sugars produced under controlled conditions and expresses starch damage in arbitrary units (Farrand units). This method is designed to be used to determine damaged starch in wheat flours, which have a total starch content in the range 68 to 72%. The AACC 76-30A method is similar except that a different enzyme is used over a shorter incubation time. The AACC 76-30A method involves the use of alpha amylase from *Aspergillus oryzae* and the Farrand method uses amylases derived from malted barley. The factors affecting damaged starch determination depend mainly on the enzyme used, the length of time for which digestion is carried out, and the type of starch being digested.

A recent study was carried out to develop another enzymatic method, the AACC 76-31 Method [2, 83]. The main modification to previous enzymatic methods is that 100mg samples are used instead of 1g or 5g samples, which are used in the AACC 76-30A method and the Farrand Method respectively, and instead of determining reducing sugars produced the reducing sugars are converted to glucose using amyloglucosidase and the glucose is determined using a glucose-oxidase-peroxidase chromogen reagent. It was found that this method is simple and reliable and enables up to 20 samples to be measured in duplicate in 2 hours. The greater size of the samples in the traditional methods mean that fewer samples can be analysed in the same time than could be determined using the modified method. It has, however, been found that variations in enzyme activity and stability may cause difficulties with consistency over time with the AACC 76-31 method [84]. However, another study previous to this showed that enzyme instability was not detected [85]. Standard errors for the AACC 76-21 method are approximately 5%.

Another study has been carried out which was similar to the AACC 76-31 [79] method. It involves the use of 50mg samples [85] and was compared with the AACC 76-30A method and the Farrand method. Unlike the AACC 76-31 method described earlier, in this modified method the amylase reaction is not terminated using acid but instead the samples are centrifuged without delay and the sugar solution then removed and the sugars converted to glucose using amyloglucosidase. When comparing the traditional methods with the second modified method above, it was found that the AACC 76-30A method overestimates damaged and gelatinised starch by about 30% compared to the modified method, and the Farrand method gives corresponding values about three times greater than these methods when carried out on mixtures containing known proportions of native and milled starches [85].

As was said above the AACC 76-30A and Farrand methods quantify damaged starch as maltose. Maltose, however, makes up only 64 to 70 % of the starch digested by alpha amylase. Therefore, these methods must then use conversion factors to find the total damaged starch value. Farrand uses a scale in which zero represents the damage in the least damaged commercial flour available and 100 represents the level in a flour with starch that absorbed its own weight of water. With the Farrand method it was decided that 3.5g is the average amount of starch in 5g of wheat flour (70%). The Farrand method can consequently give negative values for damage [1] and can also give values above 100% [85] in some flours. In the AACC 76-30A method it is assumed that 100g of damaged or gelatinised starch gives 61g maltose. The AACC 76-31 method, however, does not assume that only maltose is produced and quantifies all reducing sugars (in the form of glucose) as being part of the digestible and hence apparent damaged starch fraction.

It has been found that some amylose may be complexed with lipids and this is not determined or accounted for by enzymatic damaged starch determination methods. A test was carried out to examine the effect of the addition of lysophosphatidyl choline to potato starch during gelatinisation at 100°C in acetate buffer. It was found that the yield of maltose was lowered, showing that it had complexed with the amylose and could not be degraded by alpha amylase in the time scale of 15 minutes [85]. This, therefore, shows that when lipids are complexed with starch, this starch is not degraded and is not included in the final damaged starch value. Consequently the presence of lipid in the flours affects the damaged starch value.

To summarise, the main basic steps in enzymatic damaged starch determination include:

1. Incubation of the flour sample with alpha amylase to produce reducing sugars from the damaged starch present in the flour.

2. Incubation of the filtrate from above with amyloglucosidase to produce glucose from the reducing sugars.

3. Determination of the glucose present in the filtrate using the glucose oxidase peroxidase reagent and conversion of the value for glucose to a value for anhydro-glucose or starch.

1:2:3 High Performance Liquid Chromatography Analysis of Damaged Starch

Reducing sugars may also be quantitatively or qualitatively determined using HPLC (High Performance Liquid Chromatography). With HPLC the sugars produced by the amylolytic enzymes are separated and internal standards allow determination of the type and quantity of each sugar produced.

Reverse phase, amino bonded high performance liquid chromatography was found to provide a rapid and accurate quantitative analysis of sugars produced by incubation of flours with added alpha amylase [86]. Instead of providing a value for either total reducing sugars or glucose this method allows observation of the individual reducing sugars produced by alpha amylase. It can then be shown whether maltose is the main sugar produced or whether the amylase is producing other types of sugar in the particular starch being analysed. It can also be determined, whether different amylases, produce different reducing sugars and whether the ratio and quantity of sugars produced change with increased incubation time. The columns are made from polar bonded phase materials prepared from 5µm and 10 µm silica. Amino bonded columns give good resolution, a stable base line and have a long lifetime. The mobile phase used is acetonitrile:water usually used at a ratio of 75:25. The acetonitrile content may be increased to 85% in order to allow determination of longer chain sugars. The mobile phase composition allows a choice between resolution and molecular weight range eluted. Refractive index detection allows detection by differential refractometry. Very little work has been done to determine both quantitatively and qualitatively the actual reducing sugars produced in damaged starch in different types of flours by different starch degrading enzymes.

High performance anion exchange chromatography using pulsed amperometric detection (HPAEPAD) allows quantification of "non-derivatised" carbohydrates. Carbohydrates are weakly acidic and by this method are separated at a high pH using a strongly basic hydroxide form anion exchange stationary phase. This is due to the fact that at high pH they are at least partially ionised and thus can be separated by anion exchange mechanisms. The columns permit the separation of mono, oligo and polysaccharides. They are packed with polymeric nonporous resins, which exhibit rapid mass transfer, high pH stability (pH 0-14) and excellent mechanical stability that permits back pressures of more than 28Mpa. The carbohydrates are detected by measuring the electrical current generated by their oxidation at a gold electrode. The products of the oxidation reaction poison the surface of the electrode, which means that it has to be cleaned between measurements. Cleaning of the electrode is carried out by first raising the potential to a level sufficient to oxidise the gold surface. This causes desorption of the carbohydrate oxidation products. The electrode potential is then lowered to reduce the electrode surface back to gold. The detector measures the current integrated over time. Current integrated over time is charge so the detector response is measured in coulombs. Detection limits are as low as 30ppb for sugar alcohols and monosaccharides and about 100 ppb for oligosaccharides [87]. HPAE-PAD is extremely selective and specific for carbohydrates because pulsed amperometry detects only those compounds that are oxidisable at the detection voltage employed and neutral or cationic sample components in the matrix elute in or close to the void volume of the column. Therefore, even if such species are oxidisable they do not usually interfere with analysis of the carbohydrate components of interest [3].

This HPLC method is more expensive than the above method using amino bonded columns but allows measurement of a wider range of reducing sugars under the same conditions. Also, this method does not require column heating unlike the anion exchange column used by the above method [3]. The Dionex system also involves minimal sample preparation and clean up. This method has not been previously used to determine damaged starch in flours.

1:2:4 Iodometric Methods

Assays used to measure reducing sugar levels include : dinitrosalicylic acid (DNSA), Nelson-Somogyi and ferricyanide procedures [88, 89]. However, with cereal and fruit products these procedures cannot be used because of the very high levels of reducing sugars present. To overcome these problems, assays which exploit a specific reaction characteristic, or a solubility property, of a particular polysaccharide have been developed. An example of the latter is the reaction of starch with iodine (this colour decreases in its intensity as starch is de-polymerised by alpha amylase). Damage may also be evident by staining with Congo red dye. The degree of damage is directly proportional to the swelling and intensity of staining with the dye. This is observed using a microscope.

Limited damage was identified microscopically in granules in one particular test by the fact that areas had become swollen and stained with Congo red dye, which indicates that they took up water[31,90]. They also lost birefringence, and were digested by amylases [90], which are other indications of damage. Early work showed a positive correlation between the amount of cold water extract and the proportion of granules stainable with aqueous Congo red dye[91].

Iodine stains damaged starch by the formation of an inclusion complex. Starch complexes with hydrophobic molecules e.g. lipids. This reaction is most commonly associated with amylose component of dissolved starch. On reaction with potassium iodide solution, iodine ions are entrapped within the helices along the axis of the amylose. The resulting colour intensity may be measured spectrophotometrically or the reaction may be measured amperometrically [92] as well as by using a microscope. Complexation is important with regard to enzymatic digestion. As was mentioned above starch complexed with lipids is more resistant to enzymatic digestion. During bread production anti-staling agents are added. These complex with amylopectin preventing retrogradation of the polymeric material, which prevents staling and increases the shelf life of the product. Starch damage may therefore, be qualitatively determined by iodine absorption [82, 93].

It has been found that microscopic methods following dye staining do not lend themselves to quantitative estimation of starch damage in flour, being laborious and subject to large sampling errors. Starch granules are also damaged to varying degrees during their passage through roll mills [82], also resulting in error and difficulties in measurement. Measuring damaged starch using iodine [82,93] does not readily correlate with methods based on enzymatic digestibility [79], especially at high levels of starch damage [85].

A method has been developed based on an amperometric procedure which measures the differential rate of absorption of iodine by flours of different damaged starch content [94]. The AACC method 76-30A [33] and the AACC method 76-31 [2, 83] were compared with this method known as the Chopin Rapid Flour Test method [84]. The Chopin Rapid Flour Test (RFT) system depends upon the fact that under acidic conditions a standard iodine solution reacts with potassium iodide to form free iodine which is absorbed by the starch and particularly the damaged starch granules. The unit measures the current flowing between two electrodes attached to a platinum sensor. The system converts the electrical current to units of starch damage. High degrees of correlation were found between the Chopin electric current and the reducing sugars determined by the AACC 76-30A (r=0.94) or the spectrophotometric 76-31 method (r=0.95). It was concluded that the Chopin electric current value could be used as an appropriate indicator of flour starch damage.

1:2:5 Extraction Methods

When a granule becomes damaged, in a way that can be recognised by dye absorption and loss of birefringence, its ability to absorb water corresponds to three times the weight of the damaged material. If the granules are ground further they will not increase their water holding capacity. However, the amount of extractable amylose can increase. But the greatest level of extractability that can be achieved by grinding is only about one third of that obtained by gelatinisation. Different ways of creating starch damage therefore, lead to different relationships between extractability and digestibility.

Cornstarch properties were examined after various periods of ball milling. The rate of dissolution of the starch in DMSO (an alkaline substance, which breaks down starch) was found to increase to near maximum after 17 minutes of grinding at which time discernible x-ray diffraction patterns were absent. Water binding behaviour was similar in that it reached a maximum after 30 minutes of grinding [79]. Reducing power, limiting viscosity, and iodine binding capacity all progressively declined as a result of prolonged ball milling to 120 minutes [20], indicating the molecular size of the polymers was reduced by the treatment. The more rapidly changing properties were considered to reflect a reduction in the hydrogen bonding in the granules. However, to achieve complete cold water extraction of amylose, fragmentation of covalent bonds seems to be necessary. It was also found that on removal of branches of amylopectin, extraction becomes easier [95]. This indicates loss of crystallinity. Mechanically damaged starch granules were also more rapidly extracted by saturated ammonium sulphate solution than sound starch granules. The results of this test were recorded as absorbance and compare well with damaged starch estimations made on a series of flours by conventional enzymatic procedures.

A study was carried out to compare results of extractability and digestibility methods [96]. An extraction method using saturated ammonium sulphate solution was compared with the AACC 76-30A and the AACC 76-31 digestibility methods[97]. The disproportionately high values from the extractability method in terms of its greater accessibility to damaged granules were explained. This was assumed to result from the effects of constituents of the extraction solution on permeability of cell walls in endosperm particles [79]. Therefore, methods of this kind should give the most similar results with digestibility measurements when finely ground flours are tested.

Consistent relationships were found between water absorption and digestibility for starches and flours ground by various methods. The enzymatic methods were found to be more reliable in determining water absorption [79] than extractability methods. The relationship between extractability and water absorption depended, therefore, on the grinding method [98].

It has been found [96] that the relationship between extractability and digestibility of flour milling stocks varied with three factors:

1. Granularity of the stock examined
2. The milling system employed
3. The millstream selected

The methods of measurement of water absorption in flours, which are commonly used by millers are by means of the Brabender Farinograph [99] or the extrusion method of Halton [100].

1:2:6 Microscopic Analysis of Damaged Starch

Starch granules can be observed using a light or an electron microscope. The shape and size of the granule can be observed using a light microscope and the surface, crystalline structure, and degradation by various processes, can be observed using the electron microscope. The microscope has been used to distinguish between different botanical sources of starch and the degradation of starch granules by enzymes. Starch granules in most plants can be easily isolated [101]. Protein can be removed by shaking a saline suspension of the granules with an immiscible liquid such as butanol, and boiling. 80% aqueous methanol will extract fats [4], by extraction in a blender, in the presence of mercuric chloride to inhibit enzymatic activity. The proteins will readily sediment out from the extract. More detailed studies on potato starch have also been done using an electron microscope to determine the actual crystalline microstructure of the starch granule. The Transmission Electron Microscope has been used to observe the crystalline lamellar section and the interjacent clustered branch points of amylopectin [53, 102]. However, the use of traditional microscopy in quantitation has problems associated with it as to the interpretation of images and, therefore, results are difficult to measure.

1:2:7 NIR (Near Infra Red) Analysis of Damaged Starch

NIR analysis may also be used for determination of damaged starch [103]. Using this method free and bonded H and OH bonds are detected. The wavelengths at which reflectance measurements are made correspond to overtones and combinations of vibration frequencies due to free and hydrogen bonded -OH bonds in starch. NIR can be used to predict water absorption by simple rapid dry methods.

1:2:8 Analysis of Damaged Starch by X-ray Diffraction

X-ray powder diffraction has been used to categorise the crystallinity of different starch sources [68]. Starches may be treated by mild acid hydrolysis to remove some of the amorphous material and give a better pattern of the remaining crystalline material. X-ray fibre diffraction is another method, which can be used to display the A and B diffraction patterns. Samples of high DP amylose are cast from solutions as films. They are dried and stretched, pulling the crystallites' axis into alignment. These long amylose molecules give the same patterns as a short segment of amylopectin.

X-ray crystallography is used to determine the spacings between atoms or crystals and these spacings are used to determine crystalline structures. X-ray diffraction can also be used for qualitative or quantitative analysis. One of the instruments, which can be used for x-ray crystallography is the x-ray powder camera.

If x-rays of known wavelength impinge on a crystal, whose lattice planes are separated by the distance d, the radiation will be strongly reflected at specific diffraction angles q. A prerequisite for the occurrence of a reflection is that the Bragg condition l = 2d sin q is fulfilled. If the wavelength is known and the 2 q angles of the reflections measured, the corresponding values of d (lattice plane spacings) can be calculated.

A plane sample is rotated at a constant angular speed q in such a manner that the angle of incidence of the primary beam changes, whilst a detector is moved at twice this speed around the sample, at the same time. Whenever the Bragg condition is fulfilled the primary radiation is reflected at the sample and reaches the detector, which converts the x-ray quanta into electrical pulses. These may be counted digitally or recorded. In this way pulse counts or diffraction patterns are obtained. In the normal case the sample surface lies at the bisecting line of the angle between the incident and reflected beam (1:2 condition). For special purposes the sample can also be rotated out of this symmetrical position. Slits between the x-ray tube and the sample as well as between the sample and the detector limit the beam.

1:3 Aims of the Present Study

Ongoing work is constantly being done to further elucidate the microstructure of starch granules, which will, therefore, affect the behaviour of starch under certain conditions, particularly those related to cooking, processing and chemical modification. Generally speaking, starch damage tests are designed for determination of damage in wheat flours, for use in the bread making industry. Therefore, in order to examine the methods of analysis of starch, and the suitability of these tests for damaged starch determination, and to determine which is the most versatile and simplest to use, a range of starch types should be looked at. One of the most common ways in which starch is used in the food industry is in the form of flour and a wide range of starch types can be obtained in this form. It is also important that damaged starch values are tested in starches from each of the three crystalline types A, B and C.

The first aim of these studies is to examine and compare enzymatic starch damage determination methods. Therefore, two methods for the determination of damaged starch are to be compared, one standard method, the Farrand Method [1], which is used to determine damaged starch in wheat flours with different degrees of damage and one more recently developed method, the AACC 76-31 method [2]. The AACC 76-31 method will be carried out in its standard form and will also be carried out by replacing the kit with reagents and enzymes from other sources. These methods will be used to test a variety of starch types in the form of flour.

A simple dye test will be performed using iodine. The aim of this method is to qualitatively determine starch damage. The presence of damage, and general intensity of damage, may be estimated and compared with the percentage, damaged starch determined using the enzymatic methods.

The second aim of this study is to examine some of the factors, which influence damaged starch determination. These include:

a. The type of enzyme used.
b. The type of starch being measured, starch from different flours.
c. The sample size used.
d. The incubation time with the enzyme.
e. The simplicity of method and time taken to carry out the method.

As well as use of a variety of cereal and non-cereal flours the main factors influencing starch damage determination which are to be analysed are the determination of what happens when damaged starch is left incubating with an added enzyme for increased periods of time. Two different types of enzyme will be tested. The first enzyme to be tested is porcine pancreatic alpha amylase and the second enzyme is pure fungal alpha amylase, which is the enzyme normally used for determination of damaged starch.

As little work has been done comparing the use of the HPLC to the use of enzymatic colorimetric methods for damaged starch determination, further work will be carried out to determine the different reducing sugars produced by two different types of enzyme while incubating for increased periods of time. Two HPLC systems will be used, the first has a system which includes a reverse phase amino bonded column using acetonitrile/water mobile phase and a refractive index detector (HPLC RI) and this will be compared with a cation exchange system using pulsed amperometric detection (HPLC HPAEPAD). The reverse phase HPLC system has been used before to some extent to determine damaged starch content of samples but previous methods have relied on maltose only as an indicator of percentage damaged starch. The HPAEPAD system has not been previously used for damaged starch determination in flour samples. This study will assess its suitability for damaged starch determination because of its greater sensitivity and ability to separate an increased number of reducing sugars under the same conditions compared to use of the other HPLC system. Using HPLC systems, sugars other than maltose can be detected and quantified.

The appearance of the damaged starch in flour samples will be examined using the SEM. This procedure will be carried out on two types of starch in the form of flour, an A-type, maize flour and a B-type, potato flour. These samples will again be examined following digestion with the above amylases at increased incubation times. These tests will be carried out to see whether it is possible to visually determine the rate of digestion of the starch granules as well as quantifying the breakdown products using the HPLC. Finally a test will be carried out using x-ray diffraction to see whether enzymatic digestion under the aforementioned conditions has any effect on the crystallinity of the starch granules.

Percentage damaged starch determined using the modified method above, the HPLC and the SEM will be compared.

Some of this work has been carried out before but much of it is an initial insight into the effects of enzyme type and incubation time on the damaged starch granules. Added to this other non-cereal and cereal flours will be tested here that had not been tested in previous studies.

Further tests will also be carried to examine some more of the factors affecting damaged starch determination. These include:

1. Method of termination of enzymatic activity

2. pH at which different enzymes can be incubated

3. Incubation of flour with more than one enzyme at the same time.

Finally a test will be carried out to examine the effect of heat on percentage damaged starch.

Chapter 2

MATERIALS and METHODS

2:1 Materials for All Methods

Wheat flour samples, including the CCFRA (Campden Chorleywood Food Research Association) [104] damaged starch standard sample, various bread-making (high protein wheat, Quality wheat, Constancy wheat and Superbaker wheat) and one biscuit making wheat flour samples were obtained from Odlum Mills Ltd., Dublin, Ireland. The damaged starch standard sample has a damaged starch composition of 36 F.U. (Farrand Units). Non wheat flour samples were obtained from Fitzpatrick's Fruit and Vegetable Store, 40A Camden Street, Dublin 2, Ireland. These included maize, potato, barley and soy flour. The chickpea or gram flour was obtained from Natures Way, Hebron Road, Kilkenny, Ireland.

Pregelatinised Wheat

The pre-gelatinised wheat flour sample was produced by steam injection for twenty minutes. The pre-gelatinised wheat flour was obtained from E. Flahavan and Sons Ltd., Kilmacthomas, Co. Waterford, Ireland.

Potato Flour

Potato flour was sourced to Grain Process Entreprises Ltd., Ontario, Canada. It is made from Idaho Russett potatoes that are peeled, trimmed, cooked and then flaked on a single drum drier and ground in a flourmill. The product is made from pure dehydrated potatoes with no additives. It contains 85-86% carbohydrates.

Maize Flour

The maize flour used in these methods was finely milled. Milling maize involves de-germing of moisture-conditioned corn and de-hulling by friction followed by partial drying

Barley Flour

Abrasive de-hulling (resulting in pearled barley) and then grinding produce barley flour.

Soya Flour

Soya flour is produced by steam cooking, followed by drying and milling. Soya beans must also be treated prior to consumption generally for 10 minutes at 100°C to inactivate trypsin inhibitors.

Chickpea Flour

Chickpeas must be detoxified. This involves cooking in excess water, draining, soaking overnight in cold water and steeping the de-husked seed in hot water. The chickpeas are dried on a sieve in air and milled.

Resistant Starch Standard:

This is an extruded retrograded high-amylose cornstarch (Euresta product[18], supplied by Cerestar). It is prepared from Hylon 7 (high-amylose cornstarch) by extrusion, milling, subsequent storage at 40°C for 48 hours, drying and renewed milling.
Resistant starch content includes physically inaccessible starch, resistant starch granules and retrograded starch. It contains 29.5% resistant starch.

Following purchase these flours were stored in airtight plastic containers prior to analysis.

The flours used in each test may vary due to their availability while a particular test was being carried out, however, in order to compare methods, some of the flours were used in all methods.

Enzymes

Fungal alpha amylase (A0273, 40 U /mg solid)
Porcine Pancreatic alpha amylase (A3176, 29U alpha amylase /mg solid, 3.6U beta amylase/ mg solid)
Amyloglucosidase (A 7255, 20800U/g solid)
All were obtained from the Sigma Chemical Company, The Old Brick Yard, New Road, Gillingham, Dorset FP8 4XT, U.K.

Other Materials

The sugars used to make up standard solutions for calibration purposes were also obtained from Sigma Chemical Company. Chemicals used were of analytical grade. Glucose oxidase peroxidase chromogen glucose determination method and reagent were obtained from Boehringer Mannheim U.K., Diagnostics and Biochemicals Ltd., Bell Lane, Lewes, East Sussex BN7 1LG, U.K.

2:2 Specific Materials and Methods

2:2:1 Damaged Starch Determination by a Dye Test using Iodine Solution

Materials

Microscope
Microscope slides and coverslips
Dilute iodine solution 0.0625M

Method

A few milligrams of flour sample are placed on a microscope slide and covered with a coverslip. One drop of dilute iodine solution is dropped at the side of the coverslip and allowed to combine with the starch. The colour of the starch granules is observed under the microscope at a magnification of 400.

2:2:2 Evaluation of a Traditional Method of Starch Damage Determination, the Farrand Method[1]

Materials

1. Sodium acetate buffer solution pH 4.6-4.8. Dilute 4.1g anhydrous sodium acetate and 3ml glacial acetic acid to 1L with water.

2. Extracting Solution: 20g sodium chloride and 0.2g anhydrous calcium acetate made up to 1L with cooled freshly boiled distilled water.

3. Sodium thiosulphate solution 0.1M

4. Alkaline potassium ferricyanide solution 0.001M

5. Acetic acid salt mixture: Dissolve 70g potassium chloride and 40g zinc sulphate heptahydrate in 750ml water. Slowly add 200ml glacial acetic acid and dilute to 1L with water.

6. Potassium iodide solution 50% w/v.

7. Starch solution 2% w/v

8. Sulphuric acid 10% w/v

9. Sodium tungstate dihydrate 12% w/v

10. Acid washed pumice powder

11. Alpha amylase extract:

Mix equal volumes of buffer solution and extracting solution to a total volume sufficient for about 50ml per determination. Add 8g malt flour at 25+/- 5°C per 400ml and stir. Let it stand for about 20 minutes and filter by gravity, not by suction, through Whatman No.1 filter paper. Malt flour is stored in fridge when not in use.

Method

A sample of known starch damage e.g. oats (oats was chosen as a standard because very consistent results were obtained using it) was tested with each set of samples. Initially a standard flour was not available but later the CCFRA standard wheat flour was obtained and tested. A blank was also included in each test run.

Samples were weighed (5g) into 250ml conical flasks. A spatula spoonful of pumice powder was added to each flask. Samples were incubated at 30°C for at least 30 minutes to allow them to come to this temperature. The enzyme solution was also equilibrated to 30°C. 46ml of this solution was added to each flask. The sample was swirled to disperse the flour and then placed in the water bath.

Each sample was incubated for exactly one hour. This involves adding the samples at one minute intervals. The samples were swirled at 15 minute intervals.

10ml alkaline potassium ferricyanide solution (0.1M) was placed in boiling tubes. One was prepared for each sample and also one for the enzyme blank. 4mls buffer solution was added to each tube. Following one hour incubation of the samples, 2ml 10% sulphuric acid was added to terminate the enzymatic reaction and the samples were mixed thoroughly. 2mls of 12% sodium tungstate solution was added and the samples were shaken and allowed to stand for one to two minutes. The samples were filtered through Whatman No. 1 filter paper, discarding the first few drops of filtrate. 15 to 20mls of filtrate were collected. The filtrate was swirled and 1ml of each sample or blank was pipetted into the appropriate boiling tube. Each tube was mixed carefully and the top was covered with silver paper. The tubes were placed in a boiling water bath for 20 minutes, from the time the water bath returned to the boil. The boiling water must cover the contents of the tubes. The tubes were removed from the boiling water bath after exactly 20 minutes and were placed in a sink of cold running water to cool them to room temperature. The contents of each tube were poured into a 150ml conical flask. Each tube was rinsed with approximately 8mls acetic acid salt solution and the rinsings were added to the conical flask. The tubes were rinsed twice more using a total volume altogether of approximately 25 ml acetic acid salt solution. 1ml potassium iodide solution was added to the flasks. They were swirled and titrated with 0.1M sodium thiosulphate until the yellow colour was almost gone. 1ml starch indicator was added and the titration was continued until the blue colour had completely disappeared.

Calculations

mls alkaline potassium ferricyanide reduced by the reducing sugars present in the samples = enzyme blank titre - sample titre

% maltose = % maltose obtained from the conversion table corresponding to ml alkaline potassium ferricyanide reduced.

% starch damage (Farrand Units) = (maltose figure - 3.5) x 6

2:2:3 Evaluation of the AACC 76-31 Method[33] for Damaged Starch Determination

Materials

The AACC 76-31 Method can be obtained in the form of a kit (Megazyme) [107] containing the following materials:

Enclosed Enzymes:

1. Fungal Alpha Amylase
10ml, 1000U/ml, in 3.2 M ammonium sulphate.
Dilute an aliquot (1.0ml) to 20ml with 100mM sodium acetate buffer (pH 5.0, containing 5mM calcium chloride). Store frozen between use.

2. Amyloglucosidase
4ml, 200U/ml, in 3.2M ammonium sulphate.
Dilute an aliquot (1.0ml) to 10ml with 100mM sodium acetate buffer (pH 5.0, containing 5mM calcium chloride). Store frozen between use.

Enclosed Reagents:

1. Glucose Determination Reagent
For 1 Litre:
Reagent concentrations after dissolution in buffer:

Glucose Oxidase	> 12,000 μ/L
Peroxidase	> 650 μ/L
4-Aminoantipyrine	0.4 mM

2. Glucose Reagent Buffer (concentrate) (50ml)

Dilute the entire contents to 1 litre with distilled water and use to dissolve the Glucose Determination reagent. Divide this reagent (GOPOD reagent) into aliquots of desired volume for storage.

Stability: 2-3 months at 4°C
12 months at -20°C

Enclosed Standards

1. Glucose standard solution (150 micrograms/0.1ml in 0.2% benzoic acid)
2. Wheat Flour Standard (Starch damage 6.2%)

Reagents (not part of kit):

1. Sodium acetate buffer (100mM, pH 5.0) with calcium chloride (5mM)

Glacial acetic acid (5.7ml, 1.05g/ml) is added to 900ml of distilled water. This solution is adjusted to pH5.0 by the addition of 2M (8g/100ml) sodium hydroxide solution. Approximately 60 ml is required.

2. Calcium chloride dihydrate (0.74g) is added and dissolved. The volume is adjusted to 1 litre and the buffer is stored at 4°C.

3. Dilute sulphuric acid (0.2%v/v)

2.0mls of concentrated sulphuric acid are carefully added to 998ml of distilled water. This solution is stored at room temperature.

Method

Wheat flour samples (100 +/- 10mg) were accurately weighed into thick walled glass centrifuge tubes (12ml capacity). Before addition of fungal alpha amylase solution, the tubes plus contents were pre-equilibrated to 40°C for 2-5mins. Fungal alpha amylase solution (50U/ml) was pre-equilibrated to 40°C (5-10mins) in a small glass beaker. To each tube was added 1.0ml of pre-equilibrated fungal alpha amylase (50U/ml): the tube was stirred vigorously and immediately on a vortex mixer for 5 sec and then incubated at 40°C for exactly 10min (from time of addition of the enzyme). 5.0ml of dilute sulphuric acid (0.2%v/v) were added to terminate the reaction, and the tubes were centrifuged at 3,000 rpm (1,000g) for 5mins or filtered.

Aliquots of the supernatant solution (0.1ml) were carefully and accurately transferred to the bottom of two test tubes. To each tube was added amyloglucosidase solution (0.1ml, 2U), and the tubes were incubated at 40°C for 10mins. 4.0mls of GOPOD reagent were added to each tube (including glucose standards and reagent blank tubes) and the tubes were incubated at 40°C for 20mins. The absorbance for each sample was measured at 510nm.

With each set of determinations, reagent blanks and glucose standards of 150 micrograms are included, in duplicate. The reagent blank consists of 0.2ml of acetate buffer and 4mls glucose oxidase/peroxidase reagent. The glucose standard consists of 0.1ml acetate buffer and 0.1ml glucose standard (150 micrograms/0.1ml and 4.0mls glucose oxidase/peroxidase reagent. With each set of determinations at least one control wheat flour is also included.

Calculations

Starch damage, % $= E \times F \times 60 \times 1/1000 \times 100/W \times 162/180$

$= E \times F/W \times 5.4$

Where:

E = Absorbance (reaction) read against the reagent blank.

F = absorbance for 150 micrograms of glucose
(conversion from absorbance to micrograms)

60 = volume correction (0.1ml taken from 6.0ml)

1/1000 = conversion from micrograms to milligrams.

100/W = factor to express Starch Damage as a percentage of flour weight

W = The weight in milligrams ("as is " basis) of the flour analysed.

162/180 = Adjustment from free glucose to anhydro-glucose (as occurs in starch).

2:2:4 Evaluation of a Modified AACC 76-31 Method for Damaged Starch Determination

Materials

Enzyme Solutions

Note:

Tests were carried out to determine the concentration of enzyme required to digest a gelatinised sample of flour. Sufficient enzyme was added to digest all the starch present even though 100% starch is not damaged in these samples. As explained in the introduction, the exact end point for determination of damaged starch is uncertain.

Amylases

1. Fungal alpha amylase from *Aspergillus oryzae*
Activity: 40U/mg solid
5mg (200Uof alpha amylase) per 100mg sample of flour

To prepare:
Add 100mg enzyme to 45mls sodium acetate buffer pH5.5.

2. Porcine pancreatic alpha amylase A3176 Sigma
Activity: 29U alpha amylase / mg solid
3.6U beta amylase / mg solid
200U per 100mg sample of flour

To prepare:
Add 138mg enzyme to 45mls sodium acetate buffer pH5.5

Amyloglucosidase

Rhizopus Mould A7255 Sigma
Activity: 20800U/g solid
225U per 100mg sample of flour

To prepare
Add 100mg enzyme to 212mls sodium acetate buffer pH 5.5.

Reagents

1. Glucose oxidase peroxidase kit supplied by Boehringer Mannheim Ltd.
Includes glucose standard

2. Sodium acetate buffer solution pH 5.5
Dilute 4.1g anhydrous sodium acetate and 0.7ml glacial acetic acid to 1L with water.

3. Dilute sulphuric acid (0.2%v/v)

Method

Flour samples (100mg) and the CCFRA wheat flour standard were weighed into12 ml Pyrex test tubes. Freshly prepared alpha amylase solution (2.25 ml) was added to each tube including a blank tube and the tubes were vortex mixed to obtain an even suspension. The samples were covered with parafilm and incubated at 40°C for exactly 10 minutes. The samples were removed and 5.4mls 0.2% v/v sulphuric acid was added to terminate the enzymatic reaction. The samples were vortex mixed and centrifuged at 4000 rpm for 10 minutes. 0.5mls of filtrate was removed and added to 3.5mls sodium acetate buffer pH5.5. The solution was vortex mixed and 1.5mls amyloglucosidase solution was added and the solution was vortex mixed again. The samples were covered with parafilm and incubated at 40°C for 10 minutes. The samples were removed and cooled to room temperature. The glucose content was measured using the glucose oxidase peroxidase chromogen method. Samples were analysed at 610nm using 0.2ml of blank, standard and sample added to 5mls of glucose determination reagent.

Calculations:

mg glucose per100 ml of sample = absorption of sample x 9.1 (9.1mg per100ml glucose standard in glucose oxidase peroxidase kit) ÷ absorption of glucose standard.

mg glucose per 5.5 ml sample = mg glucose per100ml sample ÷100 x 5.5

% damaged starch = mg glucose per 5.5ml sample - blank sample (containing enzyme only) x 0.9 (to convert glucose to starch) x100 ÷ B (calculated sample weight)

B = sample weight (100mg) ÷ 7.65 x 0.5 (sample dilution)

2:3 Examination of Factors Affecting Damaged Starch Determination

2:3:1 Determination of the Optimum pH for Enzymatic Activity

Materials

Preparation of Buffers for the Determination of Optimum pH for Enzymatic Activity

The sodium acetate buffer pH 5.5 was adjusted to varying pH values using either glacial acetic acid or 0.2M sodium hydroxide solution. The pH values of the buffers were carefully checked throughout the method to ensure that the pH remained constant.

Enzyme Solutions

The enzyme solutions were prepared in the same way as in modified AACC 76-31 method using buffers of varying pH.

Method

Modified AACC 76-31 method - see section 2:2:4

This method was carried out as described above except for the fact that buffers of different pH had to be used for incubation of the flour samples with alpha amylase.

2:3:2 To Compare Filtration and Non-Filtration of the Flour/Amylase Solution to the Termination of Enzymatic Activity Using Acid.

This was a test carried out to compare termination of enzymatic digestion using acid with termination of enzymatic activity by filtration of samples and thirdly to compare both these modifications with a method whereby samples are neither filtered nor terminated with acid. In this third method the amyloglucosidase is added directly to the flour/amylase solution.

Materials

Amylase

Porcine pancreatic alpha amylase A3176 Sigma
Activity: 29U alpha amylase / mg solid
3.6U beta amylase / mg solid
200U per 100mg sample of flour

To prepare:
Add 690mg enzyme to 10mls sodium acetate buffer pH 5.5.

Amyloglucosidase

Rhizopus Mould A7255 Sigma
Activity: 20800U/g solid
225U per 100mg sample of flour

To prepare:
Add 4.3g enzyme to 40mls sodium acetate buffer pH5.5.

Reagents

1. Glucose oxidase peroxidase kit supplied by Boehringer Mannheim Ltd.
Includes glucose standard

2. Sodium acetate buffer solution pH 5.5

Dilute 4.1g anhydrous sodium acetate and 0.7ml glacial acetic acid to 1L with water.

3. Dilute sulphuric acid (0.2%v/v)

Methods

Filtration Method

1g of each flour sample and standard was weighed into 100ml conical flasks. 20mls of sodium acetate buffer was added to each flask including a blank containing no flour sample and they were stirred on a magnetic stirrer to obtain an even suspension and prevent the occurrence of lumps. 1ml of pancreatic alpha amylase solution was added to each while stirring. The samples were covered with parafilm and incubated at 40°C at a setting of 6.5 in the shaking water bath for 1 hour exactly. They were removed and filtered through Whatman No.2 filter paper into a second set of 100ml conical flasks. The original flasks were rinsed twice with de-ionised water and the rinsings added to the filtrates of each sample. 1ml of amyloglucosidase was added to each flask and mixed and incubated as before for 1 hour. The samples were removed, cooled to room temperature and filtered again into 100ml volumetric flasks. The flasks were filled to 100ml using de-ionised water. The samples were mixed well and 1ml was added to a 25mls volumetric flask and diluted to 25mls using de-ionised water. The samples were mixed well by inversion. The glucose content was determined using the glucose oxidase peroxidase method.

Calculations

A (sample weight in final sample solution) mg per 25mls= sample weight (mg) ÷ 100 (dilution of sample)

Mg of sample per 25 ml glucose in blank = absorption of blank at 610nm x 9.1 (mg per100ml glucose standard) ÷ absorption of standard at 610nm ÷ 4 (dilution factor, mg per ml glucose in final blank volume)

% damaged starch = absorption of sample at 610nm x 9.1 (mg per100ml glucose standard) ÷ absorption of standard at 610nm ÷ 4 (dilution factor, mg per ml glucose in final sample volume) – mg of glucose in 25 ml of blank x 0.9 (conversion factor for glucose to starch)x 100 ÷ (sample weight in final solution).

Non-Filtration Method

The above method was again carried out but this time instead of filtering the samples the amyloglucosidase was added directly to the buffer amylase solution and incubated.

Calculations

A (sample weight in final sample solution)= sample weight (mg) ÷ 200 x 2 (dilution of sample)

Mg of glucose in 50 ml blank = absorption of blank at 610nm x 9.1 (mg per100ml glucose standard) ÷ absorption of standard at 610nm ÷ 2 (dilution factor, mg per ml glucose in final blank volume)

% damaged starch = absorption of sample at 610nm x 9.1 (mg per100ml glucose standard) ÷ absorption of standard at 610nm ÷ 2 (dilution factor, mg per ml glucose in final sample volume) – mg per 50 ml glucose in blank x 0.9 (conversion factor for glucose to starch)x 100 ÷ (sample weight in final solution).

Acid Termination Method

Modified AACC 76-31 method – see section 2:2:4. – Samples were incubated with amylase for 60 minutes.

This method was carried out with large 1g samples as in the filtration and non-filtration methods, however, no significant difference was found between the results and those of the Modified AACC 76-31 method.

2:3:3 To Compare Termination of Enzymatic Activity using Acid with Non Enzymatic Termination.

Modified AACC 76-31 method – see section 2:2:4

This method was carried out as above for termination of enzymatic activity using acid. For non-termination with acid the method was carried out for all steps except the addition of acid to the samples. In order to maintain the same dilutions buffer was added to the samples instead of acid. The enzyme used was pancreatic alpha amylase.

2:3:4 The Effects of Microwave Heating on Percentage Damaged Starch in Flours

1. Cooking of Samples

3g samples were added to Pyrex conical flasks. 60mls acetate buffer was added. The flasks were covered with microwave film and heat treated in the following way:

160Watts for 1 minute and 2 minutes
320Watts for 1 minutes and 2 minutes

2. Percentage Damaged Starch Determination

Filtration Method – see section 2:3:3

These samples were tested for percentage damaged starch using the filtration method, however, in this case volumes of enzyme used were increased by three times. For the final sample dilution 1ml of solution was added to a 100ml volumetric flask instead of a 25ml volumetric flask.

2:4 Comparison between Percentage Damaged Starch Produced by Pancreatic Alpha Amylase and Fungal Alpha Amylase

2:4:1 Effect of Enzyme on Percentage Damaged Starch Values

Modified AACC 76-31 method – see section 2:2:4

Tests were carried out on flour samples digested by both fungal alpha amylase and pancreatic alpha amylase.

2:4:2 Examination of Variation in Percentage Damaged Starch by Pancreatic Alpha Amylase and Fungal Alpha Amylase from 0 to 60 Minutes

Modified AACC 76-31 method – see section 2:2:4

Tests were carried out on flour samples digested by both fungal alpha amylase and pancreatic alpha amylase. Incubation times were varied and samples were incubated at 10, 20, 30, 40, 50 and 60 minutes.

2:4:3. Determination of Reducing Sugars Produced by Enzymatic Degradation of Damaged Starch Using High Performance Liquid Chromatography.

HPLC HPAEPAD Instrumentation

Chromatography was carried out on a Dionex DX500 (Dionex Corp., Sunnyvale, CA) system. Separation was by means of a Carbopac PA-1 anion exchange column attached to a GP40 gradient pump. Samples were eluted using a gradient of 0-600nM NaOAc in 100mM NaOH over 30 minutes at a flow rate of 1 ml/min. The column was reequilibrated using 100mM NaOH for 10mins before successive injections. Detection was by Pulsed Amperometry (ED40 electrochemical detector), and subsequent data analysis made use of Peaknet software (Dionex Corp., Sunnyvale, CA) on a Gateway 2000 PC. 10μl was injected for both samples and standards.

Preparation of Standard Curves HPLC HPAEPAD

A known amount of carbohydrate (glucose, maltose) was weighed out and dissolved in de-ionised water (18.2M). Sample volume was made up to 100ml with de-ionised water. To 1ml of each sample was added 1ml of the rhamnose internal standard (0.110mg/ml) solution. Samples were vortex mixed to ensure a homogenous mixture. All samples were stored at -27°C when not in use. 10μl of each sample was injected on the Dionex system as described above. Peak area ratios were calculated (glucose/rhamnose, maltose/rhamnose), and standard curves were prepared plotting glucose, maltose concentration vs. peak area ratio.

Sample Preparation for the HPLC HPAEPAD

Flour samples (2g) were weighed into 100ml conical flasks in duplicate. A sample of damaged starch standard was also digested each time to allow for variation in enzyme activity. A sample blank was also run in duplicate to account for sugars present in the enzyme itself. 40mls of sodium acetate buffer pH 5.5 was added to the flour sample. (4.1g anhydrous sodium acetate in dissolved in 1 L de-ionised water and adjusted to a pH of 5.5 with 0.7mls glacial acetic acid). The sample was mixed and 5mls was removed. The 5ml sample was added to 12mls 0.2% v/v sulphuric acid in a 25ml conical flask. Freshly prepared amylase solution (5mls pancreatic alpha amylase or fungal alpha amylase) was added to each original flour sample in the 100ml conical flask and the sample stirred on a magnetic stirrer to suspend all the material. Samples were then covered with parafilm and incubated at 40°C in a shaking water bath for 10 minutes. Samples were then removed after exactly 10 minutes, stirred gently to obtain an even suspension and 5mls was removed and added to 12mls 0.2% v/v sulphuric acid in a 25ml conical flask to terminate the enzymatic reaction. The original samples were then returned to the water bath and the 10 minute samples were swirled gently and filtered through Whatman No. 2 filter paper. 1ml of filtrate was then added to 100mls sodium acetate buffer to increase the pH to 5.5.

This solution was mixed well and stored at -22°C until ready to be injected onto the column. When the HPLC was ready for sample injection 1ml of sample was added to 1ml of previously prepared internal standard solution. Each sample was vortex mixed to ensure that an homogenous mixture was obtained.

Samples were analysed as described for the preparation of standard curves and peak area ratios were calculated as before. Using the standard curves prepared for glucose and maltose it was possible to calculate the concentrations of each sugar present in each sample.

HPLC RI Instrumentation

A Shimadzu LC-10AT solvent delivery module with a Shimadzu RID-6A refractive index detector and a Shimadzu CBM-10A Communications Bus Module were used. The column was a 25cm x 4.6mm Spherisorb, Phasesep S-10 amino column. Chromatogram traces were recorded using Shimadzu LC Workstation Class LC-10 software operating on a Dell personal computer. The solvent used for elution was actonitrile/water 76:24 v/v. De-aeration was achieved by vacuum filtration. 60ul samples were injected on to the column through a Rheodyne 7125 fixed volume 20ul injector which was maintained at 30°C using a HPLC Technology column heater. A flow rate of 1.5ml/min and a data acquisition duration of 20 minutes were used each time.

HPLC RI Sample Preparation

Flour samples (500mg) were weighed into 10ml quick-fit Pyrex test tubes in duplicate. A sample of damaged starch standard was extracted each time to allow for variation in enzyme activity. A sample blank was also run in duplicate to account for sugars present in the enzyme itself. Freshly prepared fungal alpha amylase solution (2mls, 100mg/40mls sodium acetate buffer pH 5.5) was added and the tubes were vortex mixed to ensure that a homogenous mixture was obtained. The tubes were covered with parafilm and incubated at 40°C for exactly 10 minutes. The tubes were centrifuged at 4000rpm for 10 minutes. The supernatant was transferred to a second Pyrex tube and placed in a boiling water bath for 90 seconds. The tubes were removed and cooled to room temperature and centrifuged. The supernatant was transferred to small plastic screw cap storage tubes and stored at -22°C until ready to inject on to the column.

It was found by variation of the solvent concentration that the standards should be run with each test as the concentrations found for certain sugars at 76% acetonitrile were reduced when the same samples were run at 80% acetonitrile. A blank was prepared containing the enzyme with each set of samples.

Running Order for HPLC RI Samples

Standard curves for individual sugar components were determined automatically. Samples were analysed in duplicate following injection of a solution of sugar standards and a damaged starch standard sample. Chromatogram peak areas were used in the calculation of concentrations.

Effect of Enzyme on Percentage Damaged Starch Values

Tests were carried out using fungal alpha amylase and pancreatic alpha amylase and reducing sugars were determined using the HPLC HPAEPAD system. Tests were carried out with fungal alpha amylase using the HPLC RI system to determine the reducing sugars produced.

Examination of Variation in Percentage Damaged Starch Produced by Pancreatic Alpha Amylase and Fungal Alpha Amylase from 0 to 60 Minutes

Tests were carried out using fungal alpha amylase and pancreatic alpha amylase. Incubation times were varied and samples were incubated at 10, 20, 30, 40, 50 and 60 minutes. Reducing sugars were determined using HPLC HPAEPAD. Reducing sugars for incubation of flour samples with fungal alpha amylase for 10, 20 and 30 minutes were determined using HPLC RI.

2:4:4 Observation of the Effects of Digestion of Damaged Starch in Flour Samples with Fungal Alpha Amylase and Pancreatic Alpha Amylase using a Scanning Electron Microscope (SEM).

Scanning Electron Microscope Conditions

SEM's have a magnification of up to 200k. The Hitachi S2400 has two condenser lenses and an objective lens. The condenser lens system combined with the condenser lens action of the objective lens (which can be viewed as a third condenser lens) through a three step demagnification process brings the spot size down from 50 to 70 um to about 5nm. The minimum spot size achievable, which is 4-5nm dictates the resolution limit. It is recommended that the emission current which is the current leaving the electron gun should be set between 70 and 120 microamps.
The magnification displayed on the SEM screen is for a 4x5inch photograph.

Sample Preparation for the SEM

Flour samples (3g) were weighed into a 250ml conical flask and 60mls of sodium acetate buffer pH 5.5 was added. The samples were stirred on magnetic plate to obtain an even suspension. 3mls of the solution was added to 120mls of 66% ethanol in a 250ml beaker. 5mls amylase solution was added to the original flour solution in the conical flask, and stirred as before to obtain an even suspension and incubated in a shaking water bath preheated to 40°C. At 10 minute intervals the flask was removed, placed on the stirrer, stirred to obtain an even suspension and 3mls was removed as before and added to 120mls of ethanol solution. The addition of the sample to ethanol is done in order to terminate the enzymatic activity. This was continued until a sample was removed following 60 minutes incubation, and then the solution was incubated for 20 hours, and the last sample was removed at this point. The starch samples in ethanol solution were left, to allow the starch to settle on the bottom of each beaker, and most of the 66% ethanol was decanted carefully. The samples were allowed to dry in a fume hood and were re-constituted in approx. 1 ml of de-ionised water. A drop was removed and placed on a SEM disc. Up to six drops were placed on each disc. These drops were allowed to dry in air. The discs were coated with gold using a 5400 Biorad Sputter for 70 seconds and examined under a Hitachi S 2400 Scanning Electron Microscope.

2:4:6 Determination of Changes in Crystallinity of Starch Granules in Flour Digested by Amylases using X-ray Powder Diffraction

X-ray Powder Diffractometer Conditions

Siemens D-500 X-Ray Powder Diffraction System
Kristalloflex 710/710H low weight X-ray Generator
40 kV operating voltage
21 mA
Starting angle 5 degrees
Ending angle 35 degrees
Stepsize 0.05 detector diaphragm
Count time 1

Digital VT 320 Computer connected to
DACO-MP Microprocessor Controller X-Ray System
Software Diffrac 500
Tektronix 4207
Version 1.1A Siemens
Hewlett Packard 7475A Plotter

Sample Preparation

Samples of starch and flour were digested by the method of sample preparation for the SEM (section 2:4:4) except that individual samples were not removed during the course of the incubation but the entire contents were left to incubate for either 30 or 60 minutes. The sample was then cooled in cold water and left to stand, to allow the granules to sink to the bottom of the buffer solution. The buffer was decanted, and the sample dried in an oven at 30°C. The powder was placed in an x-ray diffraction sample holder (approximately 1g) and levelled off with a spatula. It was then analysed by the diffractometer.

CHAPTER 3

RESULTS

3:1. Evaluation of Damaged Starch Determination Methods

3:1:1 Damaged Starch Determination by a Dye Test using Iodine Solution

A large amount of staining was visible on the wheat flours indicating a definite presence of starch damage. Approximately one third of the wheat flour granules indicated some blue colour. However, the high protein wheat flour showed more damage than the biscuit wheat flour (low protein wheat flour). The whole meal wheat flour was again about one third damaged. The maize flour was found to be more damaged than the wheat flours and approximately half of the granules showed dye absorption. The potato flour indicated about one third of the granules were damaged similar in amount to the wheat flour. Only a few blue stains were visible on the legume flours soy and gram flour.

3:1:2 Evaluation of Enzymatic Methods for Starch Damage Determination

Table 2 shows the results obtained in Farrand units (F.U.) for the determination of percentage damaged starch in cereal flours using the Farrand method. Similar results were obtained for each of the wheat flours which ranged between 32 and 37 F.U. Wholemeal wheat, which also contains bran, gave a lower result, which was 21.8 F.U. The wholemeal wheat also showed a much larger variation in its value, (CV 43.4) than any of the other samples tested. The soft wheat flour type biscuit flour, which undergoes less damage than the other flours tested in its production process, gave a low result of 30.2 F.U. Soft and strong wheat flours showed no significant difference in their results. This indicates that the flour termed as soft flour is not as soft (as undamaged) as the biscuit flour.

Farrand (1964)[1] stated that the difference between two independent single tests results should not be greater than 2 F.U. to obtain proper repeatability. Only the oats product gave proper repeatability during this test. Whole meal wheat flour samples showed a very large standard deviation, indicating a very poor repeatability and hence poor precision in the results. As the results for the oats sample were very consistent, it was used as an initial control. If the results of the oats control sample differed by more than three Farrand units from the reference value of 50 FU then all determinations of the test run were discarded. Several test runs of all flours were discarded as the results differed too much. The CCFRA standard wheat flour was later used as the standard.

According to Farrand the method is only reliable for flours which have a total starch content in the range 68 to 72%. When the total starch value is lower than the range given, Farrand suggested that the flour weight should be adjusted by taking samples of 350/S g (S=% starch) instead of 5g. In this case samples of 10.3g were taken. However, these could not be used as a dark brown colour appeared in the tubes after they had been boiled for 20 minutes and so they were impossible to titrate.

Table 2
Percentage Starch Damage (Farrand units, F.U.) in a Selection of Wheat Flours

	Mean	*SD*	*CV*	*n*
Flour				
Oats	49.8	1.10	2.2	5
CCFRA Std.*	32.2	1.80	5.6	10
Strong wheat	34.2	3.03	8.9	5
Soft Wheat	34.6	1.95	5.6	5
Wholemeal Wheat	20.8	9.00	43.4	4
Speciality Wheat Flours				
High protein Wheat	35.7	----	----	2
Biscuit Wheat	30.2	----	----	2
Constancy Wheat	36.7	----	----	2
Quality Wheat	36.6	----	----	2
Superbaker Wheat	36.3	----	----	2

* CCFRA - standard wheat flour
---- Not calculated

Validation of the AACC 76-31 Method and Determination of Percentage Damaged Starch by this Method in Cereal and Non-Cereal flours.

The validation test for the AACC 76-31 method was carried out to determine the optimum time for incubation of the glucose samples in glucose oxidase peroxidase (GOPOD) reagent is shown in Table 3. This test was carried out on CCFRA standard wheat flour and Megazyme Standard wheat flour. It can be seen from Table 3 that although the results vary between 20, 30 and 60 minutes there is no advantage to having a longer incubation time than 20 minutes for either flour. Therefore, it was decided to incubate the samples with GOPOD for 20 minutes.

Using the AACC 76-31 damaged starch determination method a selection of cereal and non-cereal flours were tested. The results are shown in Table 4. Most of the wheat flours gave results, which varied between 6 and 7 percent damaged starch. The CCFRA standard wheat flour gave a higher value of 7.7%. The percentage damaged starch in maize and potato flour was also tested here. Maize flour had the same percentage damaged starch as the wheat flour but the percentage, damaged starch determined by this method in potato flour was very low at 1.3%. Soya and chickpea flours were tested and gave even lower results of 0.6 and 0.7 % but the results were consistent and the standard deviations were low.

The percentage damaged starch determined by the AACC 76-31 method and the Farrand Method are compared in Figure 13.

Percentage Damaged Starch Determination Using the Modified AACC 76-31 Method.

Using this method the hard wheat flours gave results of between 6.2 and 6.5%. The results are shown in Table 5. Biscuit wheat contained less damaged starch at 3.7% and the pre-cooked wheat flour was as expected higher at 10.6%. The percentage damaged starch in the maize flour determined by the AACC 76-31 method gave a result similar to the hard wheat flours and the potato flour was again low at 1.3%. In this test the percentage damaged starch in soy and chickpea flour was also tested and resulted in low values of 0.4 and 0.5% respectively. The largest variation in results was obtained in flours with low damaged starch values e.g. soy and chickpea flours.

The results for the AACC 76-31 method and the modified AACC 76-31 method are compared in Figure 14.

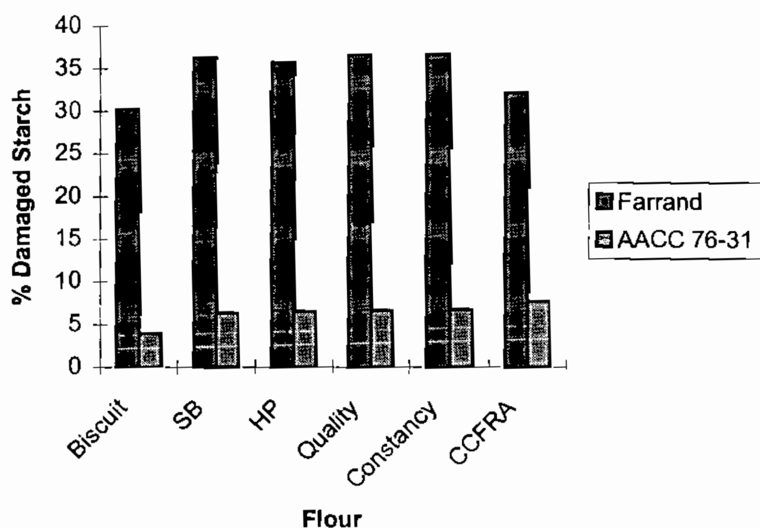
Table 3
Percentage Damaged Starch Produced following Variation in Incubation Time with GOPOD Reagent using the AACC 76-31 Method

Time/mins	20	30	60
Flour Tested: CCFRA Damaged Starch Standard Wheat			
n = 6			
Mean	7.6	7.8	7.8
SD	1.01	0.87	0.17
Flour Tested: AACC 76-31 Damaged Starch Standard Wheat			
n = 3			
Mean	6.4	6.9	6.0
SD	0.16	0.08	0.15

Table 4
Percentage Damaged Starch Released Following Incubation of Cereal and Non-Cereal Flour Glucose Samples for 20 Minutes with Glucose Oxidase/ Peroxidase Reagent using the AACC 76-31 Starch Damage Determination Method.

Flour	***Mean***	***n***	***SD***	***CV***
CCFRA Std. Wheat	7.7	12	0.18	2.34
AACC 76-31 Std. Wheat	6.2	15	0.34	5.45
Maize	6.2	6	0.15	2.41
Potato	1.3	6	0.03	2.09
High Protein Wheat	6.5	6	0.10	1.56
Soya	0.6	9	0.04	6.10
Biscuit Wheat	3.9	6	0.03	0.82
Chickpea	0.7	10	0.02	3.23
Constancy Wheat	6.7	2	0.35	5.20
Quality Wheat	6.6	2	0.05	0.76
Superbaker Wheat	6.3	2	0.13	2.05

Figure 13
Comparison between the Percentage Damaged Starch Determined by the Farrand Method and the AACC 76-31 Method in Wheat Flours.

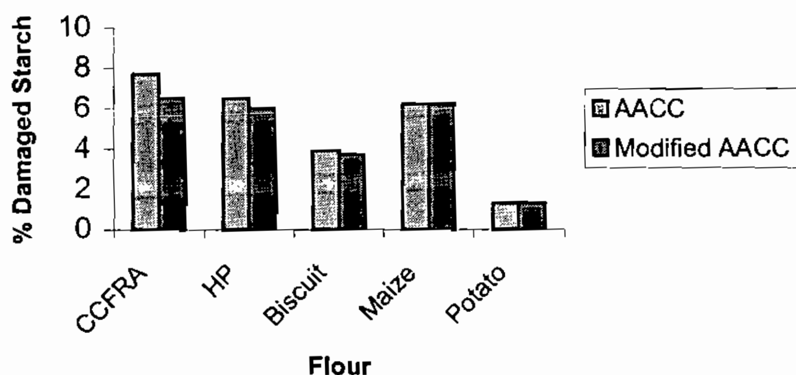


SB = Superbaker
HP = High Protein
CCFRA = CCFRA standard wheat flour

Table 5
Percentage Damaged Starch Produced in Cereal and Non-Cereal Flours using the Modified AACC 76-31 Method.

Flour	*Mean*	*n*	*SD*	*CV*
CCFRA Std. Wheat	6.5	10	0.44	6.78
Maize	6.2	9	0.13	2.09
Potato	1.3	7	0.05	3.88
High Protein Wheat	6.0	8	0.32	5.30
Soya	0.4	4	0.04	9.23
Biscuit Wheat	3.7	7	0.15	4.04
Chickpea	0.5	5	0.06	10.93
AACC 76-31 Std. Wheat	6.2	20	0.47	7.57
Precooked Wheat	10.6	3	0.19	2.

Figure 14



SB = Superbaker
HP = High Protein
CCFRA = CCFRA standard wheat flour

3:2 Examination of Factors Affecting Damaged Starch Determination

3:2:1 Determination of the Optimum pH for Activity of Amylolytic Enzymes

Initially, this test was carried out using one enzyme, either alpha amylase or amyloglucosidase only, instead of both enzymes in sequence as is normally carried out in the modified AACC 76-31 method. In other words the test was carried out without the second stage of the method where reducing sugars which have been produced by alpha amylase are converted to glucose by amyloglucosidase. Figure 15 shows the amount of glucose produced following incubation of maltodextrin with two different types of enzyme in buffers of different pH values. It can be seen that amyloglucosidase produces glucose at an optimum pH of between 5 and 5.5. It does not, however, convert all the maltodextrin to glucose. The pancreatic alpha amylase tested produced as expected very little glucose. Due to the fact that glucose is not the main sugar produced by alpha amylase this method could not be used as a determinant of optimum pH for alpha amylase activity. It was, however, found by doing this test that some glucose may be produced by alpha amylase incubation. This glucose, however, could also be present as a result of glucose already in the flour, or due to the action of naturally occurring glucoamylase, which has digested some of the starch. Later studies found that 2% reducing sugars were present in undigested high protein wheat flour.

As this test was not suitable to determine the optimum pH for the activity of alpha amylases the full modified AACC 76-31 method was used to determine the optimum pH for activity of fungal alpha amylase and pancreatic alpha amylase in a standard flour, the CCFRA standard wheat flour. This method was the same as the modified AACC 76-31 method except that the pH of the buffer for preparation of the enzyme solution was varied, and the enzymatic reaction was not terminated with sulphuric acid. Instead buffer of pH 5.5 was used in order that the amyloglucosidase was in a solution of the correct pH to subsequently convert the reducing sugars to glucose. The recommended pH for amyloglucosidase is 4.5. However, in initial tests it was found to produce the maximum amount of glucose at a pH between 5 and 5.5.

The optimum pH for fungal alpha amylase activity was found to be 5.5 and for porcine pancreatic alpha amylase was 6. It was found using fungal alpha amylase that there is a range of pH values at which either enzyme has optimum activity which is from pH 4.5 to 5.5 for fungal alpha amylase and 4.5 to 7 (which is the test range) for pancreatic alpha amylase. Therefore, there exists a wide range of pH values at which the enzymes can be used. The optimum pH values can be seen in Figure 16. The recommended pH according to the label for both fungal alpha amylase and pancreatic alpha amylase is 6.9. Therefore, the enzymes fungal alpha amylase, pancreatic alpha amylase and amyloglucosidase can all be used at a pH of 5.5. The optimum pH for activity of all three enzymes was found to be slightly different to the recommended value. If amyloglucosidase and amylase can be used at the same pH then time would be saved in not having to change the pH when using these two enzymes one after the other within the method. In the AACC 76-31 method both types of enzymes are used at the same pH which is pH 5.0.

Therefore, it was confirmed that, despite the recommended pH values for use of the enzymes, all three used in this study could be incubated with the flour sample at the same pH. It was also found that the enzymes have similar activity over a wide pH range. It was also confirmed that alpha amylase could not be used on its own with out further incubation with amyloglucosidase to convert all the starch to glucose and amyloglucosidase does not convert all the starch sample to glucose without first incubating the starch sample with alpha amylase.

3:2:2 Comparison of Methods to Terminate the Enzymatic Activity of Amylolytic Enzymes (Filtration and Non-Filtration Methods)

The modified AACC 76-31 enzymatic colorimetric damaged starch method was carried out on the flour samples, and instead of terminating the enzymatic reaction using acid, the samples were instead filtered and the method continued as before. The results (Table 6a) show no significant difference to the results of the modified AACC 76-31 method (Table 7). Therefore, there is no benefit in using the filtration method instead of the modified AACC 76-31 method. Using the filtration method it was found that the variation in percentage damaged starch values for most types of flour is quite low except for gram flour. However, this is a flour with a low damaged starch value, whose percentage damaged starch content cannot be determined with any degree of accuracy with any of the enzymatic colorimetric methods.

There are several disadvantages to using the filtration method. These include the fact that the process is quite time consuming (for the filtration process to be completed) and varies for different types of flour probably depending on the protein present which impedes the filtration process. Soya flour, which contains the most protein, relative to starch content, took the longest time to filter. However, as was done in the AACC 76-30A method a few drops of filtrate could be taken if the sample volume was kept constant throughout the method and the whole sample would then not need to be allowed to filter completely.

This test was also carried out using larger (1g) samples of flour, which require large amounts of enzyme and individual flasks rather than test tubes. This means that fewer samples can be tested in the same amount of time compared to the AACC 76-31 method and the modified AACC 76-31 method. The results obtained were no different to those obtained using small samples of flour.

In contrast to the method using the filtration step, a method whereby the enzyme amyloglucosidase was added directly to the amylase solution with no filtration or termination of the amylase enzyme was carried out. This non-filtration method gives much higher values for damaged starch using the same flours than the values obtained using the previous methods (Table 6b). The values are approximately three times the values of the filtered samples (Figure 17) and of the samples in which the amylase is terminated using acid. This is due to the fact that the amylase enzyme is allowed to continue to digest the damaged starch for an increased length of time, while at the same time the amyloglucosidase is digesting the damaged starch and possibly the undamaged starch and not just breaking down the reducing sugars produced by amylase to glucose.

Figure 15

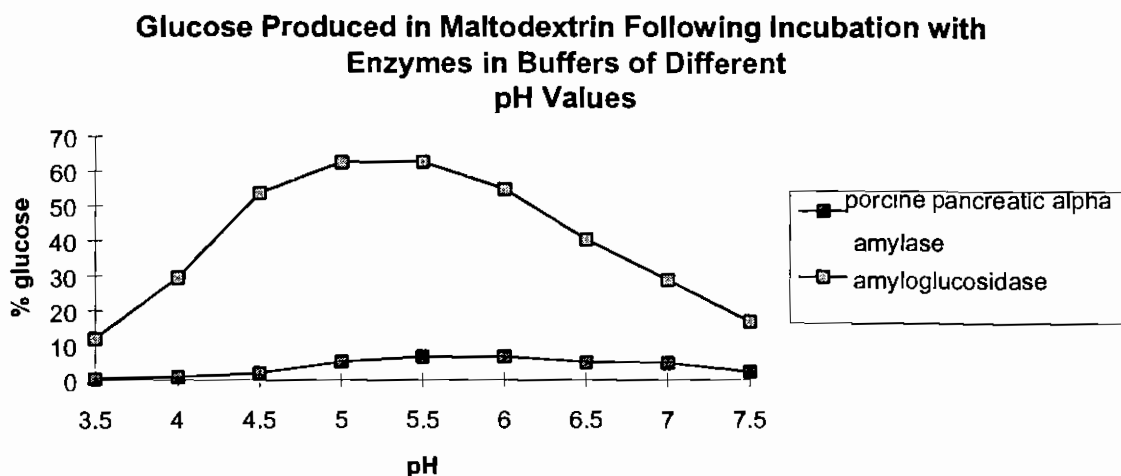
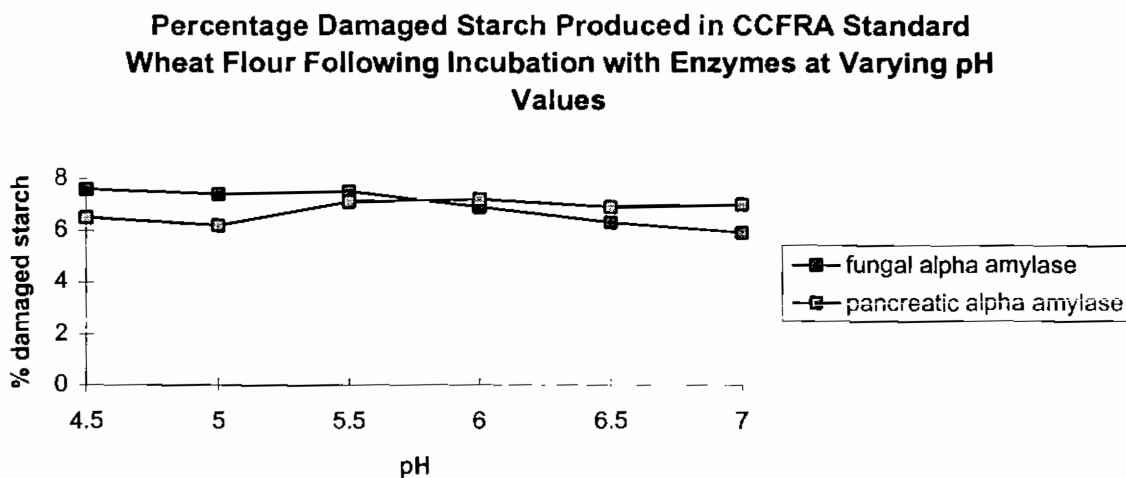


Figure 16



It was shown from the test to determine the optimum pH activity for the enzymes that the amyloglucosidase does to some extent digest the damaged starch to glucose without an intermediary step of amylolytic digestion of the damaged starch to reducing sugars. The increased digestion of the starch by the two enzymes is also related to the fact that the amylase inhibitors maltose and maltotriose are being converted to glucose and, therefore, are not being left in solution to inhibit the enzymatic digestion.

When samples are filtered variation in the damaged starch values is lower compared to samples which are unfiltered. Therefore, it would be more accurate to either terminate the alpha amylase reaction with acid or filter the samples than to add the amyloglucosidase directly to the original flour samples. For flours with a very low damaged starch value, the non-filtration method may to be a better method as a higher damaged starch value is obtained using it. However, because variation in results is greater using the non-filtration method the results would be inaccurate for flours with low damaged starch values.

General variation in results obtained by both methods may be caused by the fact that the mixing process may not thoroughly mix the samples and may not allow equal access by the enzyme to all parts of the flour sample. However, it is known from examination of the flour samples using the SEM that the enzyme does not evenly digest each of the starch granules due to clumping of the granules and also due to the fact that some of the granules are more susceptible to digestion than others.

A wheat flour sample was tested to determine the percentage damaged starch obtained when the enzymes alpha amylase and amyloglucosidase are incubated together in the same solution using the modified AACC 76-31 method. In order to do this an enzyme solution was prepared so that the same enzyme activity of both enzymes and the same enzyme solution volume could be added as before. The flour sample was incubated for 60 minutes with the enzyme solution. The result was the same as that obtained when the flour sample is incubated with the enzymes separately, one after the other, as in the non filtration method. Therefore, the two hour incubation time previously used, involving one hour with alpha amylase followed by one hour with amylase and amyloglucosidase, gave the same result as just one hour with both enzymes together.

3:2:3 To Examine the Replacement of Acid with Buffer to Terminate the Amylase Activity in the Method for the Colorimetric Determination of Percentage Damaged Starch

Tests carried out using buffer instead of acid to terminate the amylase reaction causes no general increase in variation of results. The results are shown in Table 8 and can be compared with the results in Table 9 where acid is used to terminate the activity of the amylase enzyme. The results are slightly higher when the acid is retained, however, no significant difference in results was found. The dilute acid may be slightly breaking down the damaged starch in the flour samples. The coefficient of variation was higher when acid is used. Therefore, it is not necessary to terminate the enzyme activity with acid.

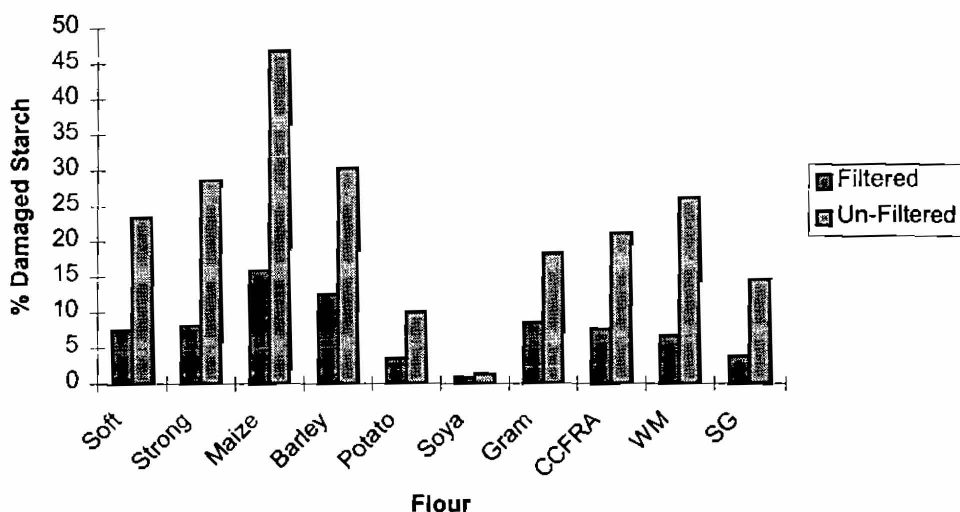
Table 6a
Percentage Damaged Starch in Cereal and Non-Cereal Flours Incubated with Pancreatic Alpha Amylase for One Hour using the Filtration Method.

Flour	*Mean*	*SD*	*CV*	*n*
Soft Wheat	7.5	0.34	4.53	5
Strong Wheat	8.1	0.26	3.23	5
Maize	15.85	0.38	2.40	3
Barley	12.5	0.16	1.31	4
Potato	3.5	0.09	2.60	3
Soya	0.8	0.04	5.00	2
Chickpea	8.5	2.04	24.00	2
CCFRA Std. Wheat	7.6	0.20	2.64	2
Whole meal Wheat	6.7	0.46	6.87	2
Stone ground Wheat	3.8	0.09	2.37	2

Table 6b
Percentage Damaged Starch in Cereal and Non-Cereal Flours Incubated with Pancreatic Alpha Amylase for One Hour using the Non- Filtration Method

Flour	*Mean*	*SD*	*CV*	*n*
Soft Wheat	23.4	1.61	6.88	7
Strong Wheat	28.65	1.22	4.26	2
Maize	46.9	1.45	3.09	2
Barley	30.3	0.90	2.97	5
Potato	10.0	0.54	5.39	4
Soya	1.3	0.04	3.08	5
Chickpea	18.3	0.30	1.64	2
Whole meal Wheat	26.2	0.23	0.88	2
Stone ground Wheat	14.7	1.19	8.11	3
CCFRA Std. Wheat	21.2	1.01	4.76	2

Figure 17
Comparison Between the Percentage Damaged Starch Values Obtained using the Filtration and Non-Filtration Methods



Soft = soft wheat flour
Strong = strong wheat flour
WM = whole meal
SG = stone ground
CCFRA = CCFRA Standard

Table 7
Percentage Damaged Starch in Cereal and Non-Cereal flours following digestion with Pancreatic Alpha Amylase for 60 Minutes Determined Using the Modified AACC 76-31 Method.

Flour	*Mean*
Soft Wheat	7.6
Strong Wheat	8.2
Maize	17.0
Barley	12.4
Potato	3.6
Soya	0.5
CCFRA Std. Wheat	8.6

Table 8
Percentage Damaged Starch Produced in Cereal and Non-Cereal Flours using Buffer Instead of Acid to Terminate the Activity of the Amylase Enzyme.

Flour	*Mean*	*n*	*SD*	*CV*
CCFRA Std. Wheat	6.9	10	0.18	2.59
AACC 76-31 Std. Wheat	6.0	2		
Biscuit Wheat	4.1	2		
High Protein Wheat	5.9	2		
Potato	1.2	2		
Pregelatinised Wheat	10.5	2		
Constancy Wheat	6.8	2		
Superbaker Wheat	6.9	2		
Quality Wheat	7.1	2		

Table 9
Percentage Damaged Starch Produced in Cereal and Non-Cereal Flours Determined Using the Modified AACC 76-31 Method.

Flour	*Mean*	*n*	*SD*	*CV*
CCFRA Std. Wheat	6.5	10	0.44	6.78
AACC 76-31 Std. Wheat	6.2	20	0.47	7.57
Biscuit Wheat	3.7	7	0.15	4.04
High Protein Wheat	6.0	8	0.32	5.30
Potato	1.3	7	0.05	3.88
Pregelatinised Wheat	10.6	3	0.19	2.39

3:2:4 Investigation of Variation in Fungal Alpha Amylase Activity on Percentage Damaged Starch in CCFRA Standard Wheat Flour.

For the AACC 76-31 method 50 units of amylase solution is added to the flour sample before incubation. In this test, alpha amylase units were varied from 25 to 100 units. The results are shown in Table 10. However, only a 1% difference in percentage damaged starch was obtained from between 25 to 100 units. Therefore, doubling the activity of the amylase from 50 units which is used in the method to 100 units does not effect the damaged starch value significantly. Therefore, there is not a direct correlation between activity of alpha amylase used and percentage damaged starch value obtained by an enzymatic, colorimetric method. A previous test was carried out on the effect of increasing the activity of alpha amylase on a 100mg sample [2]. Tests were carried out with much lower activities of alpha amylase than were used in this study. An initial rapid increase in percentage damaged starch was found after addition of less than 10 units. Following this there was a slow, steady increase. Percentage damaged starch increased by less than 1% when the activity was doubled from 50units to 100units, which is the same result that was found here.

3:2:5 The Influence of Microwave Heating on Percentage Damaged Starch in Wheat Flours.

Flour samples were heated using a microwave oven at different power settings (160Watts and 320Watts) for time periods of one and two minutes. As large samples containing buffer were incubated in the microwave using flasks the percentage damaged starch was determined using the filtration method and samples were incubated with amylase for 60 minutes. The results are shown in Tables 11 and 12 and are compared with non heat-treated samples (Table 13) in Figure 18. All wheat flour samples tested increased their percentage damaged starch values by at least 2 to 3 times compared to non heat-treated samples. This showed that the heat produced by the microwave further damaged the flour samples

Following I minute at a power setting of 160 watts the damaged starch values increased. It was found that doubling the power at which the samples were incubated roughly doubled the percentage damaged starch value for soft, wholemeal and stone-ground wheat. Therefore, the increase in power and hence the increase in heat has a direct influence on the percentage digestible starch in a flour sample. The percentage damaged starch in pre-gelatinised wheat did not increase significantly following heating at 320W compared to heating at 160W. Therefore, the initial heat treatment at 160W may have damaged most of the remaining undamaged starch in this pregelatinised flour leaving very little to be further damaged at the increased power setting.

The percentage damaged starch in the pre-gelatinised wheat flour, however, more than doubled following treatment at 160W compared to no heat treatment. Incubating the flour samples for two instead of one minute did not double the damaged starch value. In most cases there was a slight increase in percentage damaged starch following two minutes as compared to one minute of incubation time. Therefore, incubation time does not have as great an effect on digestible starch as does increase in heat.

Table 10
Percentage Damaged Starch in CCFRA standard Wheat Flour Incubated with Varying Activities of Fungal Alpha Amylase and Determined Using the Modified AACC 76-31 Method.

Enzyme Activity/Units	*Mean*	*n*	*SD*	*CV*
100	7.2	3	0.04	0.55
75	7.0	3	0.21	2.99
50	7.0	3	0.12	1.72
40	6.5	3	0.08	1.23
25	6.3	3	0.12	1.89

Table 11
Percentage Starch Damage in Wheat Flours Following Heat Treatment in a Microwave Oven at Two Different Power Settings for 1 Minute.
n=3

Power	160W	320W
Flour	% Starch Damage	% Starch Damage
Soft Wheat	23.8	53.1
Wholemeal Wheat	24.2	52.8
Pre-gelatinised Wheat	65.5	66.1
Stone-ground Wheat	11.6	22.1

Table 12
Percentage Starch Damage in Wheat Flours Following Heat Treatment in a Microwave Oven at Two Different Power Settings for 2 Minutes.

Power	160W	320W
Flour	% Starch Damage	% Starch Damage
Soft Wheat	24.3	55.2
Wholemeal Wheat	27.1	57.9

Table 13
Percentage Starch Damage in Wheat Flours Following 60 Minutes Incubation with Pancreatic Alpha Amylase following No Heat Treatment

n=3

Flour	% Starch Damage
Soft Wheat	8.0
Stoneground Wheat	3.2
Wholemeal Wheat	6.4
Pregelatinised Wheat	28.0

Figure 18

3:3 Comparison of Percentage Damaged Starch Data Produced by Fungal Alpha Amylase and Pancreatic Alpha Amylase using the Modified AACC 76-31 Damaged Starch Determination Method and High Performance Liquid Chromatography

3:3:1 The Effect of Enzyme Type on Percentage Damaged Starch

Table 14 and Figure 19 show a comparison between the percentage damaged starch produced by fungal alpha amylase and pancreatic alpha amylase using the modified AACC 76-31 method. The Table shows that for the hard wheat flour sample and the soft wheat flour the percentage damaged starch produced is the same, following incubation with both enzymes. The CCFRA standard wheat flour gave a higher result using pancreatic alpha amylase than using fungal alpha amylase. The greatest difference in results was found with the maize flour. The percentage starch damaged was almost double when pancreatic alpha amylase was used compared to use of fungal alpha amylase. Potato flour also showed an increase in digested starch when pancreatic alpha amylase was used. Soy flour gave the same result for both enzymes. However, the damaged starch value was very low compared to that of other flours.

Percentage damaged starch values were also measured following 20 minutes incubation, due to the fact that flours were incubated for 20 minutes prior to determination of the reducing sugars using HPLC. These results are shown in Table 15.

The HPLC HPAEPAD system was used to determine the reducing sugars produced by the respective enzymes in different flours. Figure 20 shows a chromatogram of the reducing sugars produced in maize flour digested using pancreatic alpha amylase for 10 minutes. Reducing sugars up to maltoheptaose could be detected. However, the three main reducing sugars produced are maltose, maltotriose and glucose in order of decreasing concentration.

Figure 21 shows the chromatograms of the reducing sugars produced from high protein wheat flour digested by fungal alpha amylase and pancreatic alpha amylase. Figure 22 shows a comparison between the reducing sugars produced in high protein wheat flour digested by the respective enzymes. It can be seen that less maltose is produced by fungal alpha amylase and more glucose compared to pancreatic alpha amylase. Pancreatic alpha amylase also produces more maltotriose. Figure 23a shows chromatograms of the reducing sugars produced by fungal alpha amylase and pancreatic alpha amylase in CCFRA standard wheat flour. The concentrations of glucose, maltose and maltotriose produced in CCFRA standard wheat flour and biscuit wheat flour are compared in Figures 23b and 23c. For CCFRA standard flour and biscuit flour more maltose is produced by pancreatic alpha amylase but in both cases more glucose is produced by fungal alpha amylase. The maltotriose produced varies and its value is inconsistent between each sample.

Table 14
Percentage Damaged Starch in Cereal and Non-Cereal Flours Incubated with Pancreatic Alpha Amylase and Fungal Alpha Amylase for 10 Minutes and Determined Using the Modified AACC 76-31 Method.

n = 3	mean	
Flour	Pancreatic Alpha Amylase	Fungal Alpha Amylase
High Protein Wheat	5.9	6.0
Biscuit Wheat	3.7	3.6
Maize	9.4	5.5
Potato	1.9	1.1
Soya	0.4	0.4
CCFRA Std. Wheat	7.2	6.2

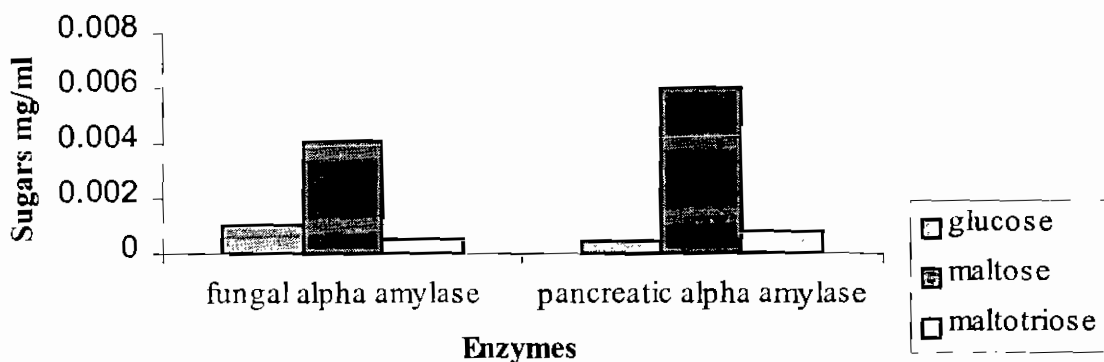
Table 15
Percentage Damaged Starch in Cereal and Non-Cereal Flours Incubated with Pancreatic Alpha Amylase and Fungal Alpha Amylase for 20 Minutes and Determined Using the Modified AACC 76-31 Method.

n = 3

Flour	Pancreatic Alpha Amylase	Fungal Alpha Amylase
High Protein Wheat	6.8	6.7
Biscuit Wheat	4.1	4.1
Maize	12.5	6.4
Potato	2.3	1.4

Figure 19

Glucose, Maltose and Maltotriose Produced During Incubation of CCFRA Standard Wheat Flour with Fungal and Pancreatic Alpha Amylases for 20 Minutes Determined using HPLC HPAEPAD



n = 2

Figure 20
HPLC HPAEPAD Chromatogram Showing the Products of Digestion of Maize Flour using Pancreatic Alpha Amylase.

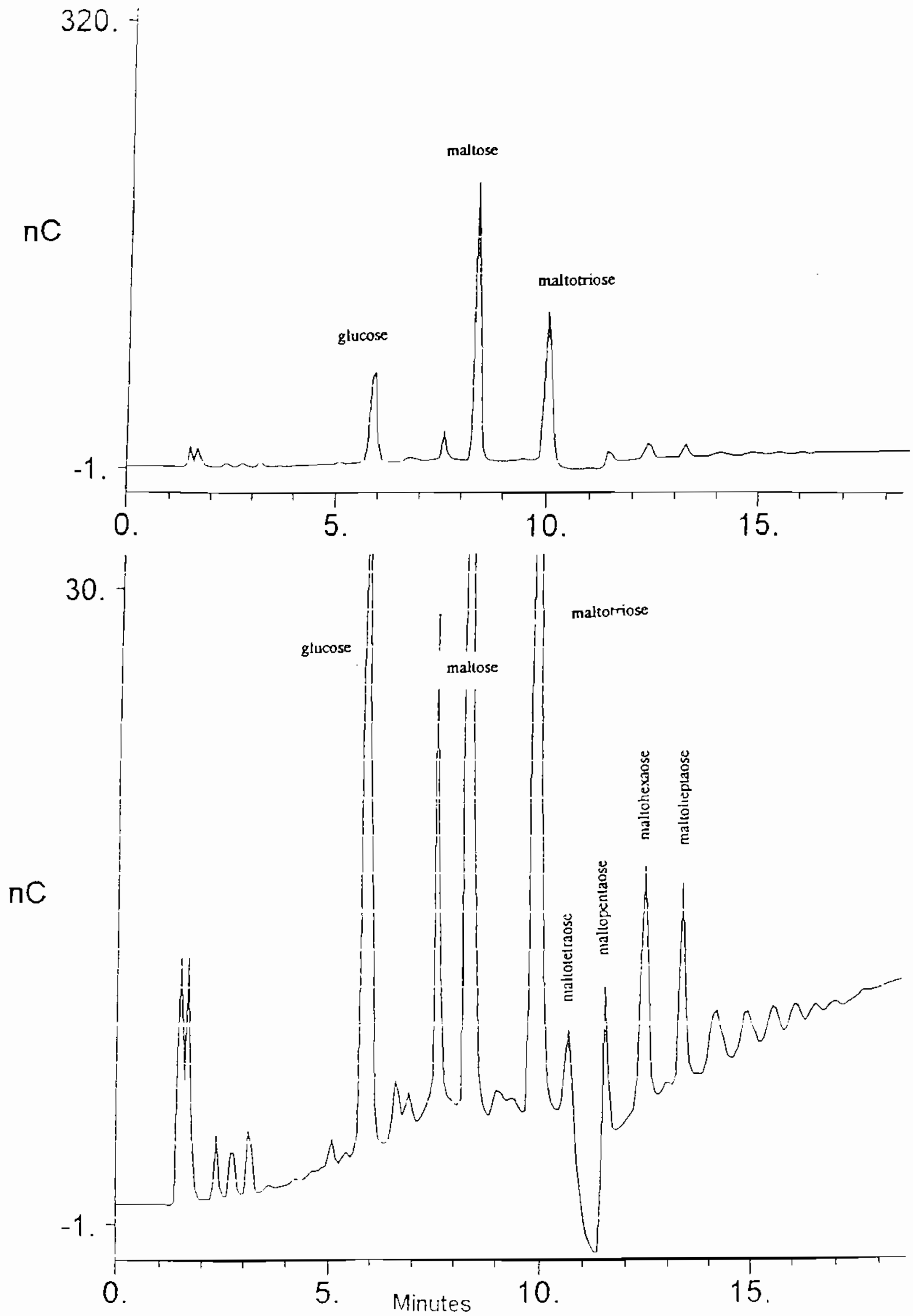
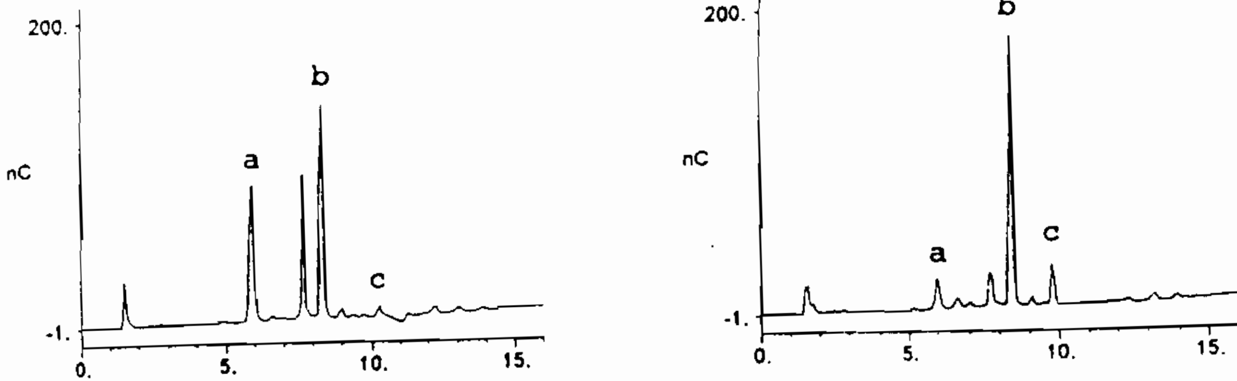


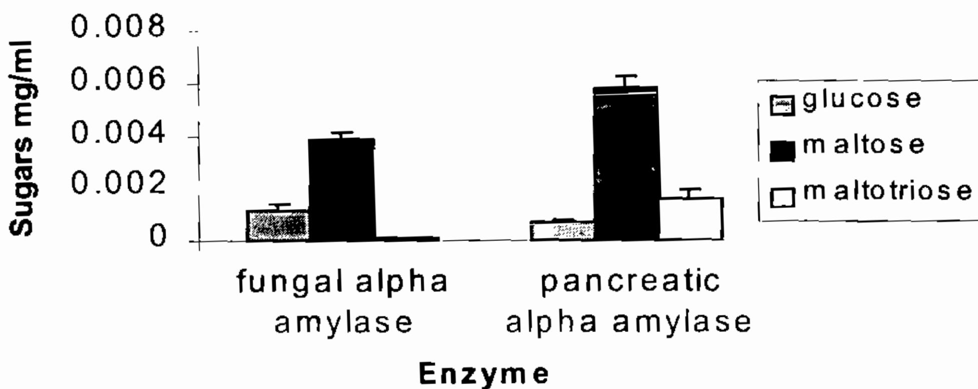
Figure 21
HPLC HPAEPAD Chromatogram Showing the Reducing Sugars Produced from High Protein Wheat Flour Incubated with Pancreatic Alpha Amylase and Fungal Alpha Amylase for 20 Minutes.

Fungal Alpha Amylase **Pancreatic Alpha Amylase**



a = glucose b = maltose c = maltotriose

Figure 22



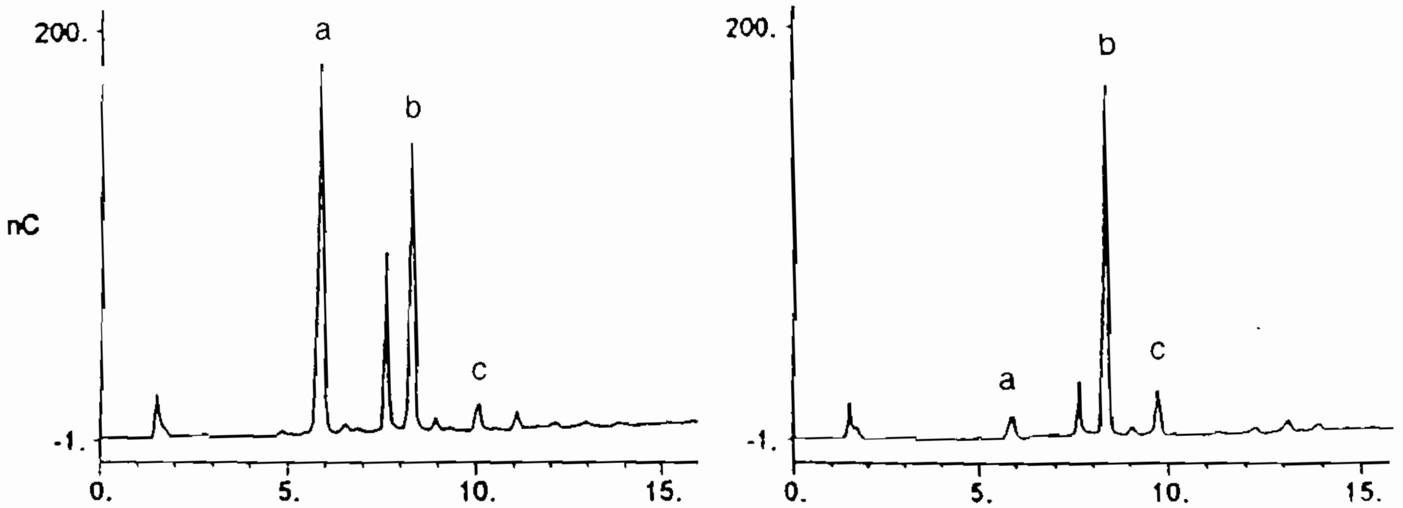
n = 3

Figure 24a shows HPLC HPAEPAD chromatograms for maize flour incubated with fungal alpha amylase and pancreatic alpha amylase. Figure 24b shows the concentrations of the reducing sugars, glucose, maltose and maltotriose in the maize flour. In this flour more glucose is produced using pancreatic alpha amylase than fungal alpha amylase and also considerably more maltotriose. It can also be seen in Figure 24c that a higher concentration of reducing sugars are produced in maize flour than in potato flour digested by pancreatic alpha amylase.

A HPLC RI system was used to determine the reducing sugars found in flours following digestion with fungal alpha amylase only. This method involved the use of an amino bonded reverse phase column and a refractive index detector. Samples were again incubated for 10 minutes. Figure 25 shows chromatograms of the reducing sugars produced in three flours and their retention times following 10 minutes incubation with fungal alpha amylase determined using the amino bonded reverse phase HPLC system. Table 16 shows the concentration of glucose, maltose and maltotriose produced in a selection of wheat flours and in maize, potato and barley flour and in pre-gelatinised wheat flour following 10 minutes digestion. The maltose produced by a selection of wheat flours including High Protein, Quality, Constancy and Superbaker is very similar in all approximately 16 mg/ml except for CCFRA damaged starch standard which is 17.1 mg/ml. The biscuit wheat flour value is lower (10.7 mg/ml) as it is a less damaged flour. The maize flour value is 14.4 mg/ml which is slightly lower than the wheat flours. Barley has a similar maltose value to maize (8.9mg/ml). The pre-gelatinised wheat flour has a value of 28.6 mg/ml maltose.

Table 17a and 17b show the percentage reducing sugars in flours determined by both HPLC systems and compared to the percentage damaged starch determined by the enzymatic colorimetric method. Table 17b also compares the percentages of glucose, maltose and maltotriose added together with the percentage damaged starch values obtained by the colorimetric method (the modified AACC 76-31 method). In almost every case the total percentage of reducing sugars exceeds the percentage of damaged starch determined colorimetrically even without the addition of the other reducing sugars when determined using HPLC RI. It was found that as using the HPLC HPAEPAD system mostly maltose is produced by fungal alpha amylase in each of the flour types. Two exceptions include maize and potato flour. The concentration of maltose derived from potato flour is very low. However, the percentage maltose values are lower than the percentage damaged starch values when determined using HPLC HPAEPAD. The percentage maltose values of non wheat flours tested are approximately half the value of the percentage damaged starch figures when the flours are digested using pancreatic alpha amylase. The percentage maltose value for high protein wheat exceeds the percentage damaged starch value determined by the enzymatic colorimetric method. The percentage maltotriose values are lower when they are detected using HPLC HPAEPAD than when they are detected using HPLC RI. The percentage maltose was compared with the percentage damaged starch values determined colorimetrically in Figure 26a. Figure 26b shows the maltose concentration determined directly by the HPLC RI system and compares it to the percentage damaged starch produced by the colorimetric method. These values are then correlated on a graph in Figure 26c. The graph shows that percentage damaged starch can be related to maltose produced in the same samples and determined using HPLC.

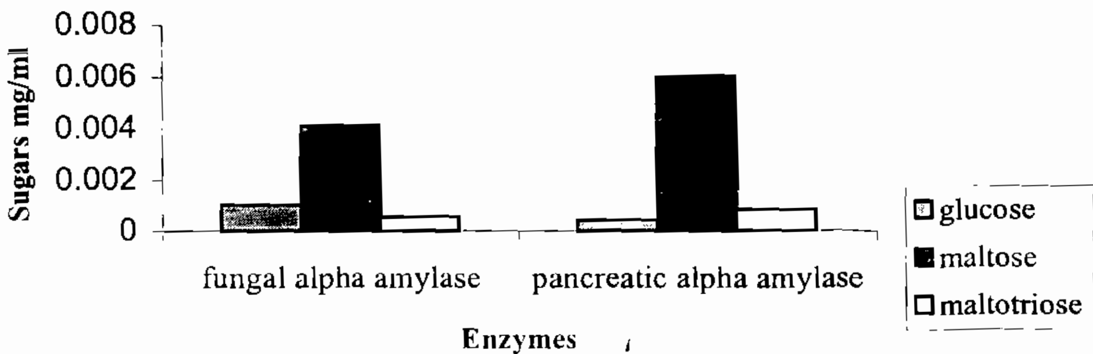
Figure 23a
HPLC HPAEPAD Chromatograms of the Reducing Sugars Produced in CCFRA Standard Wheat Flour Incubated with Fungal Alpha Amylase and Pancreatic Alpha Amylase



Fungal Alpha Amylase **Pancreatic Alpha Amylase**

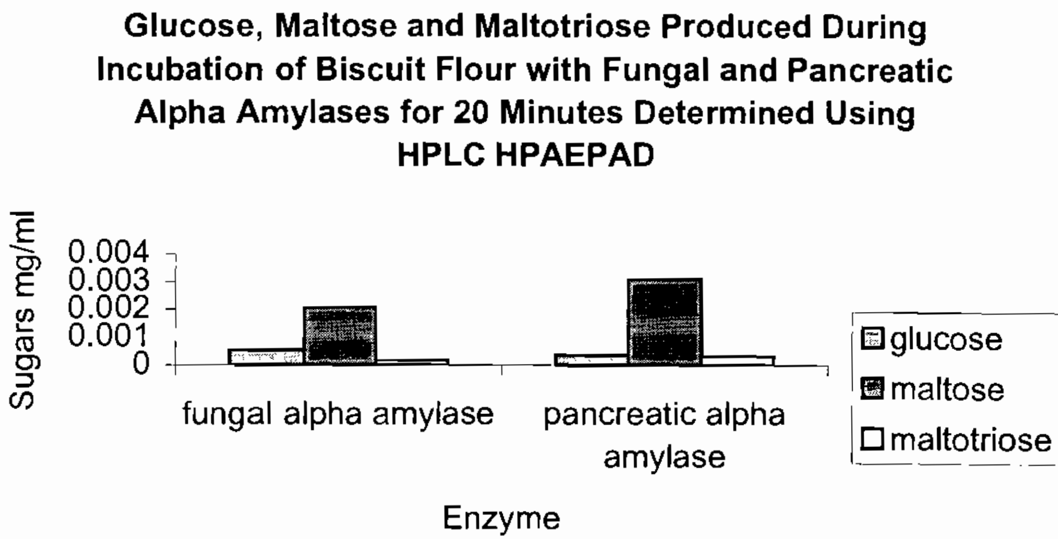
a = glucose b = maltose c = maltotriose

Figure 23b



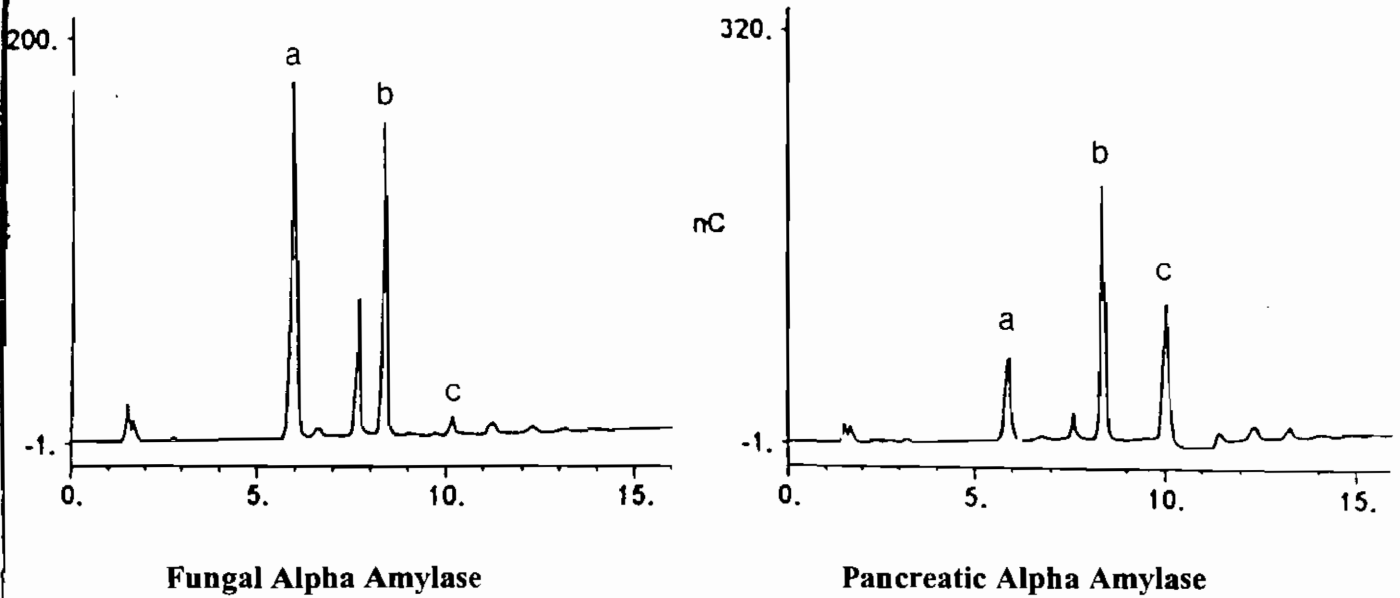
n = 2

Figure 23c



n = 2

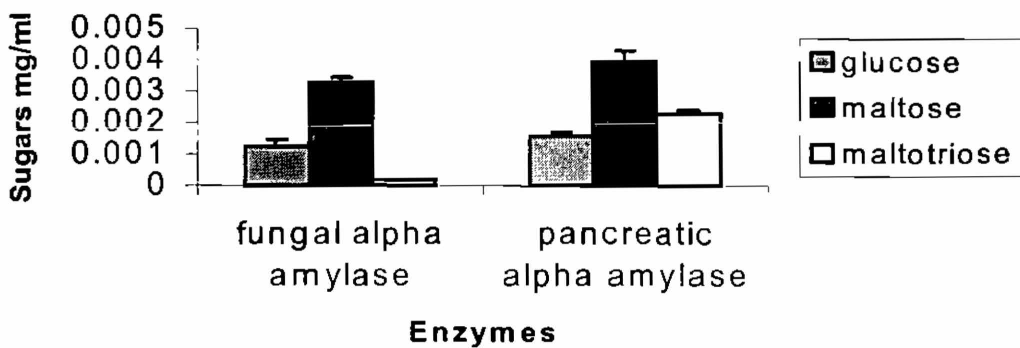
Figure 24a
HPLC HPAEPAD Chromatograms of the Reducing Sugars produced in Maize Flour Incubated with Fungal Alpha Amylase and Pancreatic Alpha Amylase



Fungal Alpha Amylase **Pancreatic Alpha Amylase**

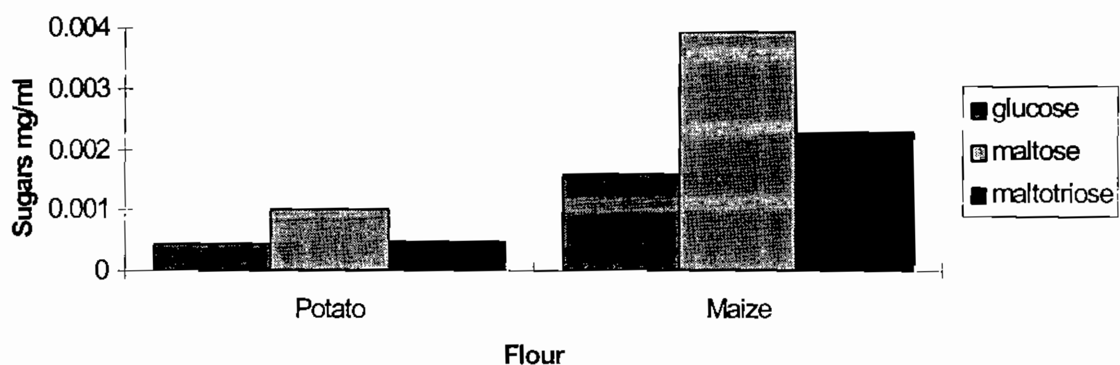
Figure 24b

Glucose, Maltose and Maltotriose Produced During Incubation of Maize Flour with Fungal and Pancreatic Alpha Amylases for 20 Minutes Determined Using HPAEPAD



n = 2

Figure 24c



n = 2

Figure 25
Reducing Sugars Produced in Flours and their Retention Times Following 10 Minutes of Incubation with Fungal Alpha Amylase Determined Using HPLC RI.

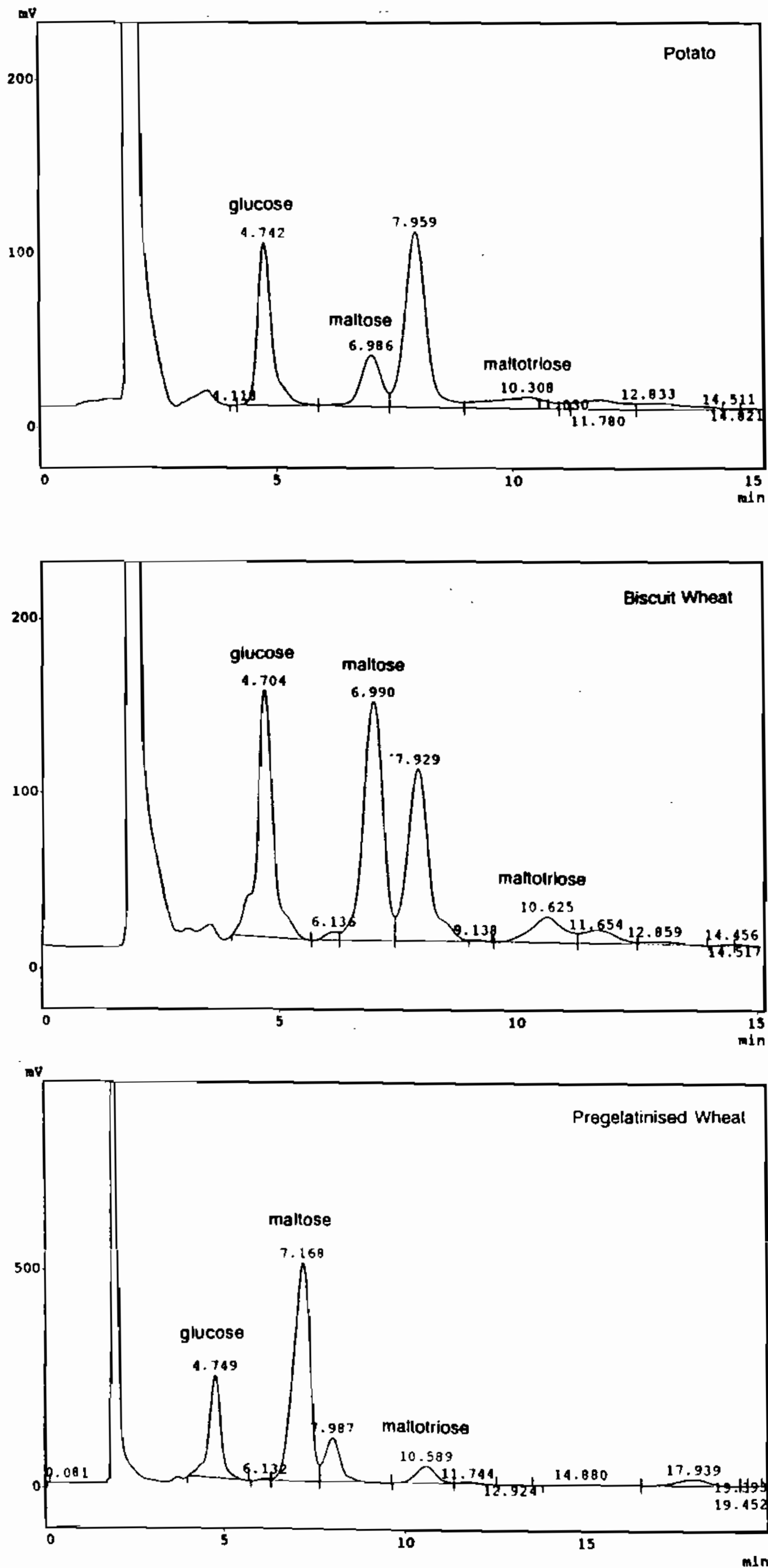


Table 16
Concentration (mg/ml) of Maltose, Glucose and Maltotriose Produced in Cereal and Non-Cereal Flours following Incubation with Fungal Alpha Amylase for 10 Minutes using HPLC RI.

	Concentration mg/ml			
Flour	Maltose	Glucose	Maltotriose	n
Potato	2.00	0.1	0	2
High protein	16.6	3.8	0.8	3
Biscuit	10.7	1.8	1.0	2
CCFRA Std.	17.3	2.7	1.7	2
Maize	14.4	3.5	1.4	2
Pre-gelatinised	28.6	4.2	4.5	2
Constancy	16.5	5.4	1.9	2
Superbaker	16.5	5.5	1.3	2
Quality	16.4	5.7	2.1	2
Barley	8.9	7.6	1.6	2

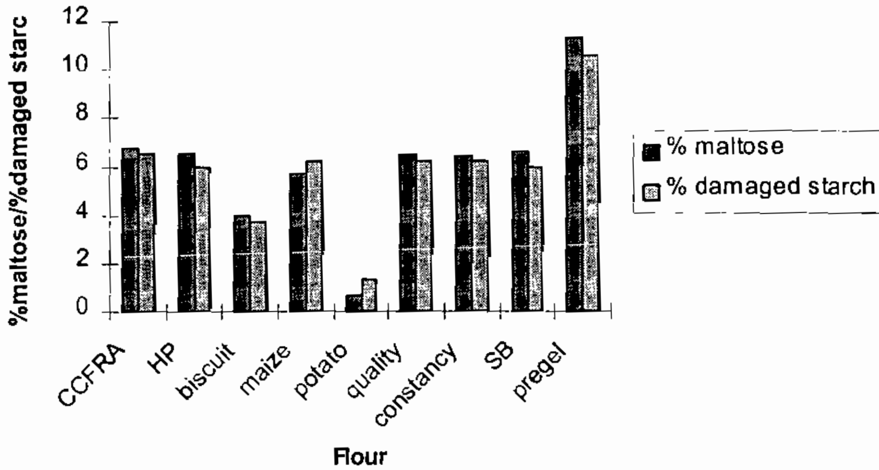
Table 17a
Percentage Reducing Sugars in Cereal and Non- Cereal Flours Following 20 Minutes Incubation and Determined Using HPLC HPAEPAD and Compared to the Percentage Damaged Starch Determined by the Modified AACC 76-31 Method.

Flour	% Maltose	% Glucose	% Maltotriose	% Damaged Starch Modified AACC 76-31 Method
Fungal Alpha Amylase				
High protein	6.1	1.7	0.1	6.7
Biscuit	3.2	0.8	0.2	4.1
Maize	5.1	1.9	0.3	6.4
Potato	0.5	0	0	1.4
Pancreatic Alpha Amylase				
High Protein	9.0	1.1	2.4	6.8
Biscuit	4.8	0.5	0.5	4.1
Maize	6.1	2.4	3.5	12.5
Potato	1.6	0.7	0.8	2.3

Table 17b
Percentage Sugars Determined in Cereal and Non-Cereal Flours using the HPLC RI Method and Compared to the Percentage Damaged Starch Determined by the Modified AACC 76-31 Method.

Flour	% Maltose	% Glucose	%Maltotriose	Total Sugars	% Damaged Starch Modified AACC Method
CCFRA Std.	6.8	1.1	0.7	8.6	6.5
High protein	6.5	1.5	0.3	7.6	6.0
Biscuit	3.9	0.7	0.4	5.1	3.7
Maize	5.7	1.4	0.6	7.6	6.2
Potato	0.6	0	0	0.6	1.3
Quality	6.5	2.3	0.8	9.6	6.2
Constancy	6.5	2.1	0.7	9.3	6.2
Superbaker	6.6	2.2	0.5	9.3	6.0
Pregelatinised	11.3	1.6	1.8	14.7	10.6

Figure 26a
Percentage Maltose Produced using HPLC RI Compared to the Percentage Damaged Starch Determined using the Modified Colorimetric Method



SB = Superbaker
HP = High Protein
CCFRA = CCFRA standard wheat flour
Pregel = Pregelatinised wheat flour

Figure 26b

Concentration (mg/ml) of Maltose Determined by HPLC RI and Compared to Percentage Damaged Starch Produced by the Colorimetric Method

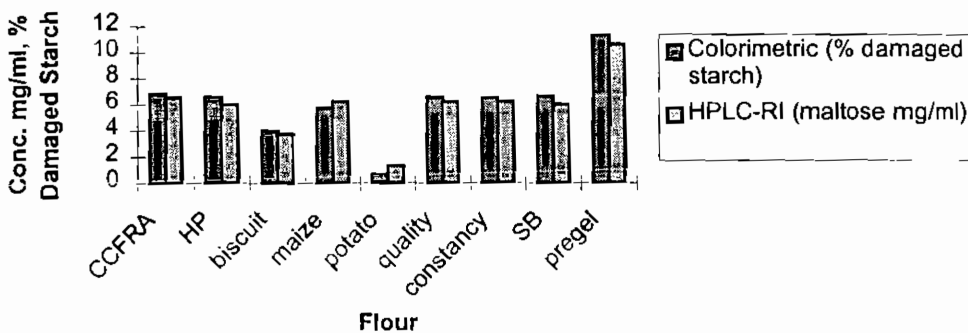
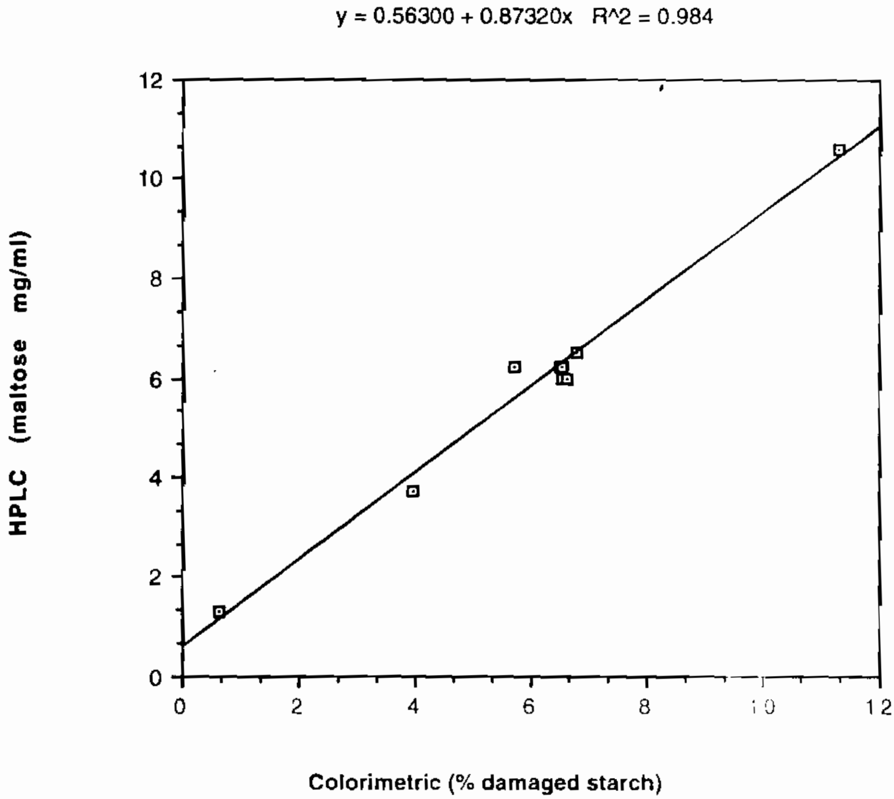


Figure 26c
The Correlation between Maltose (mg/ml) Determined Using HPLC RI and Percentage Damaged Starch Determined Using the Modified AACC 76-31 Method.



3:3:2 Effect of Variation of Incubation Time on Percentage Damaged Starch Values

Two wheat and two non-wheat flours were tested for percentage damaged starch by the modified AACC 76-31 method at ten minute intervals over a period of sixty minutes. The results are shown in Figure 27. Figure 27a shows the change in percentage damaged starch over a period of 60 minutes in high protein and biscuit wheat flours digested by fungal alpha amylase. Figure 27b shows the rate of change of percentage damaged starch produced in high protein and biscuit wheat flours digested by pancreatic alpha amylase. The greatest rate of increase in digestion of damaged starch occurs during the first ten minutes of incubation.

The percentage damaged starch in high protein wheat flour increases from 5.9% after ten minutes to 7.9% after 60 minutes incubation with either enzyme. Biscuit wheat undergoes even less of a percentage damaged starch increase which is from 3.7 to 4.9 %. There is no significant difference between the rate of increase in digestion of wheat flours by either enzyme tested.

The results shown at 0 time are samples taken prior to the addition of enzyme. It shows that some flours contain a small amount of reducing sugar prior to digestion by an enzyme. The maltose present in flour samples prior to incubation with an added enzyme and determined by the HPLC RI system are shown in Table 18. It is shown that more maltose is present in the hard wheat flours (High Protein wheat flour and CCFRA standard wheat flour) than in the softer wheat flour (Biscuit wheat) or the maize flour. Pre-gelatinised and potato flour contain the least amount of naturally occurring maltose.

Figure 27c shows the rate of increase of percentage damaged starch determined in maize flour following incubation with pancreatic alpha amylase and fungal alpha amylase for sixty minutes. Maize flour contains 9.4% damaged starch after ten minutes incubation with pancreatic alpha amylase and this increases to twice this value after 60 minutes incubation. Therefore, the increase in damaged starch is not directly proportional to the time left incubating. Figure 27d shows the percentage damaged starch produced in potato flour following incubation with pancreatic alpha amylase and fungal alpha amylase for 60 minutes. The percentage damaged starch in potato flour digested by pancreatic alpha amylase increases from 1.9% to 3.2% the increase being slow and gradual. However, fungal alpha amylase does not digest maize and potato flour to the same extent as pancreatic alpha amylase. There is virtually no increase in percentage damaged starch in potato flour following increased incubation with fungal alpha amylase. Maize flour undergoes a similar amount of digestion as high protein wheat flour following incubation with fungal alpha amylase. However, maize flour is digested to a greater extent over the period of sixty minutes with pancreatic alpha amylase.

Table 18
Concentration (mg/ml) of Maltose Produced in Cereal and Non-Cereal Flours following Incubation with Fungal Alpha Amylase for 0 Minutes and Determined Using HPLC RI.
Time /0 minutes

Flour	Maltose mg/ml
Potato	1.05
High protein	4.75
Biscuit	2.28
CCFRA Std.	5.70
Maize	2.63
Pre-gelatinised	1.61

Figure 27a

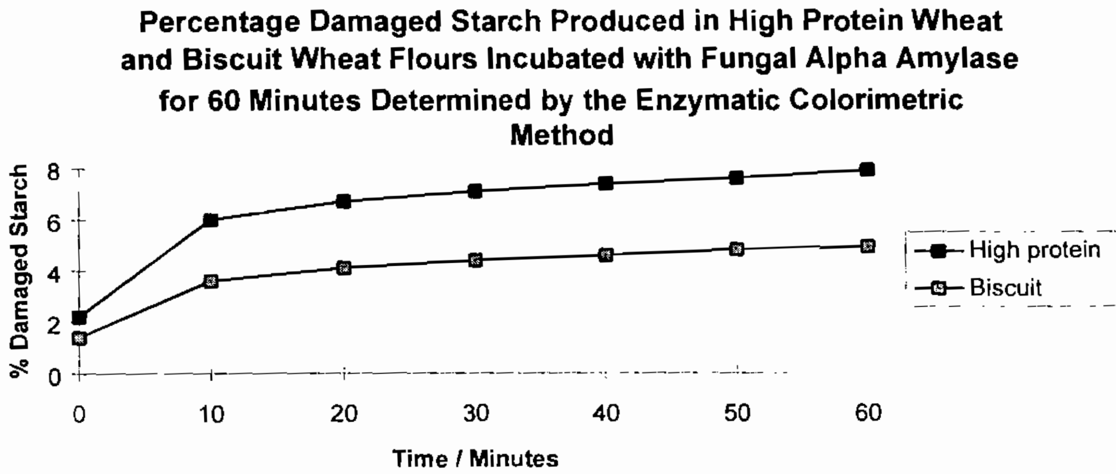


Figure 27b

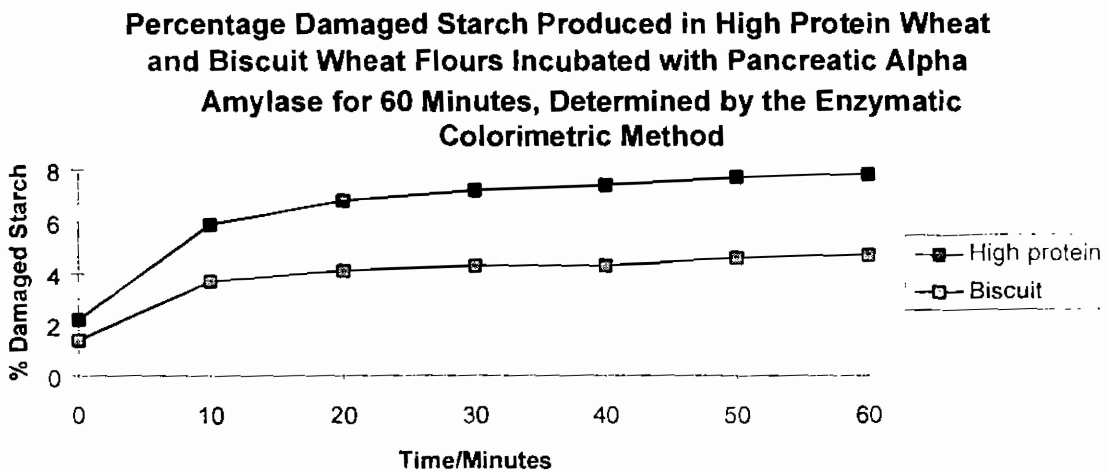


Figure 27c

Percentage Damaged Starch in Maize Flour Incubated with Pancreatic Alpha Amylase and Fungal Alpha Amylase for 60 Minutes Determined by the Enzymatic Colorimetric Method

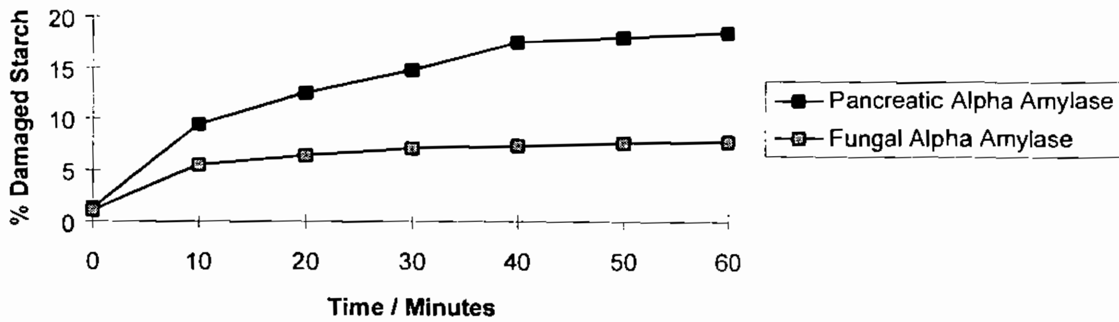
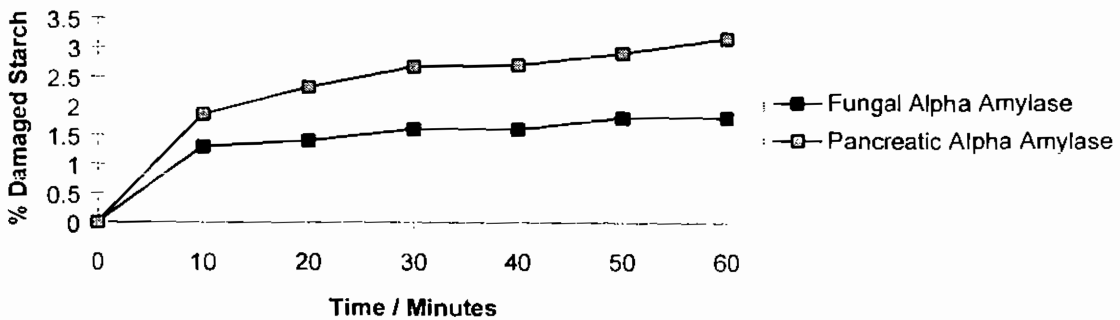


Figure 27d

Percentage Damaged Starch Produced in Potato Flour Incubated with Fungal Alpha Amylase and Pancreatic Alpha Amylase for 60 Minutes Determined by the Enzymatic Colorimetric Method



The reducing sugars produced following the digestion of each of these flours up to sixty minutes were also determined. Figure 28 shows the chromatograms of the reducing sugars produced from high protein wheat flour by fungal alpha amylase and pancreatic alpha amylase for a period of sixty minutes. Figure 29a shows the maltose produced in high protein wheat flour up to sixty minutes. It shows that there is a greater rate of increase in maltose produced when it is digested by pancreatic alpha amylase than when it is digested by fungal alpha amylase. This occurs even though the total percentage damaged starch determined by the colorimetric method indicates that equal amounts of damaged starch are produced by both enzymes. Figure 29b shows an increase in maltose produced in biscuit flour incubated from 0 to 10 minutes, very little increase up to a period of 40 minutes and a second increase after 40 minutes.

The glucose concentrations in Figure 29c and 29d however, show that some of the difference in the rate of change of percentage damaged starch values may be accounted for by glucose rather than maltose. It can be seen that there is a high rate of increase in the production of glucose by fungal alpha amylase compared to pancreatic alpha amylase over a period of 60 minutes.

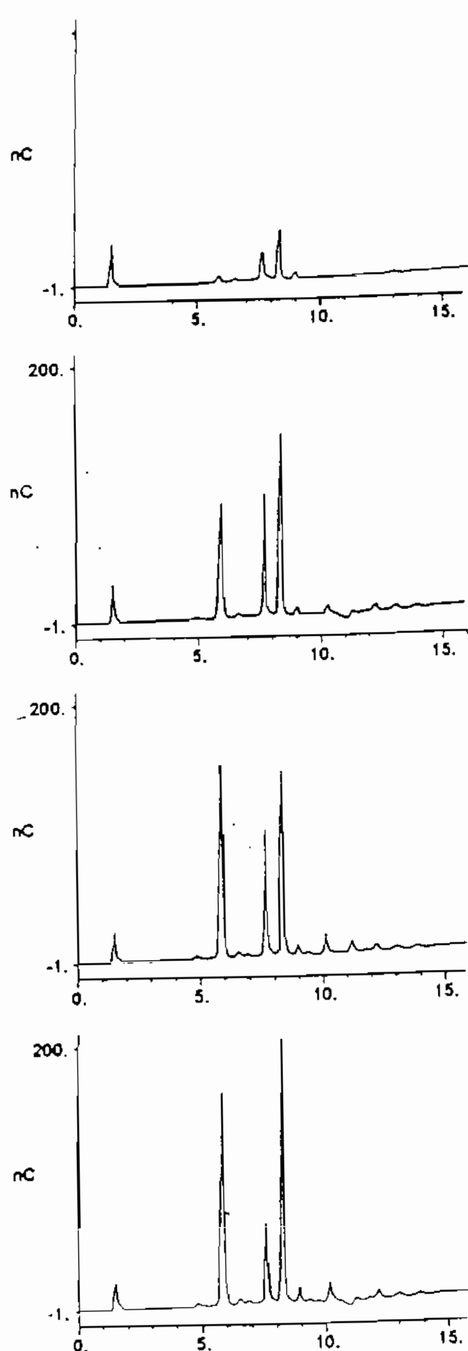
Figure 30 shows chromatograms of the reducing sugars produced from maize and potato flour following incubation with pancreatic alpha amylase for 60 minutes. The rate of production of glucose maltose and maltotriose in maize flour incubated with pancreatic alpha amylase and fungal alpha amylase are shown in Figure 31 and 32 respectively. Using pancreatic alpha amylase to incubate for ten minutes maltose is the main sugar produced followed by maltotriose and then glucose. Following ten minutes incubation the concentrations of each sugar increase at similar rates. When fungal alpha amylase is used the profile of reducing sugars produced over sixty minutes is different. Maltose is again the main sugar produced. After 20 minutes its rate of production decreases. A steady increase in glucose production occurs so that its concentration is close to that of maltose after sixty minutes incubation. Very little maltotriose is produced in maize flour by fungal alpha amylase.

The rate of production of maltose in potato flour following digestion with pancreatic alpha amylase was found to be slow and steady and progress at the same rate as the percentage damaged starch determined by the colorimetric method. As can be seen from Figure 33 glucose and maltotriose are produced at the same rate throughout the incubation period. Figure 34 shows the comparison between the rate of increase in production of maltose in maize and potato flour digested by pancreatic alpha amylase. Figure 35 shows the rate of increase in glucose produced in two wheat and two non-wheat flours by fungal alpha amylase and determined by HPLC-RI.

It was found that there was no increase in the maltose produced in the cereal and potato flours by fungal alpha amylase tested between 10 and 30 minutes determined using the HPLC RI.

Figure 28
Chromatograms of the Reducing Sugars Determined using HPLC HPAEPAD Produced from High Protein Wheat Flour following Incubation with Pancreatic Alpha Amylase and Fungal Alpha Amylase for 60 minutes.

Fungal Alpha Amylase **Pancreatic Alpha Amylase**



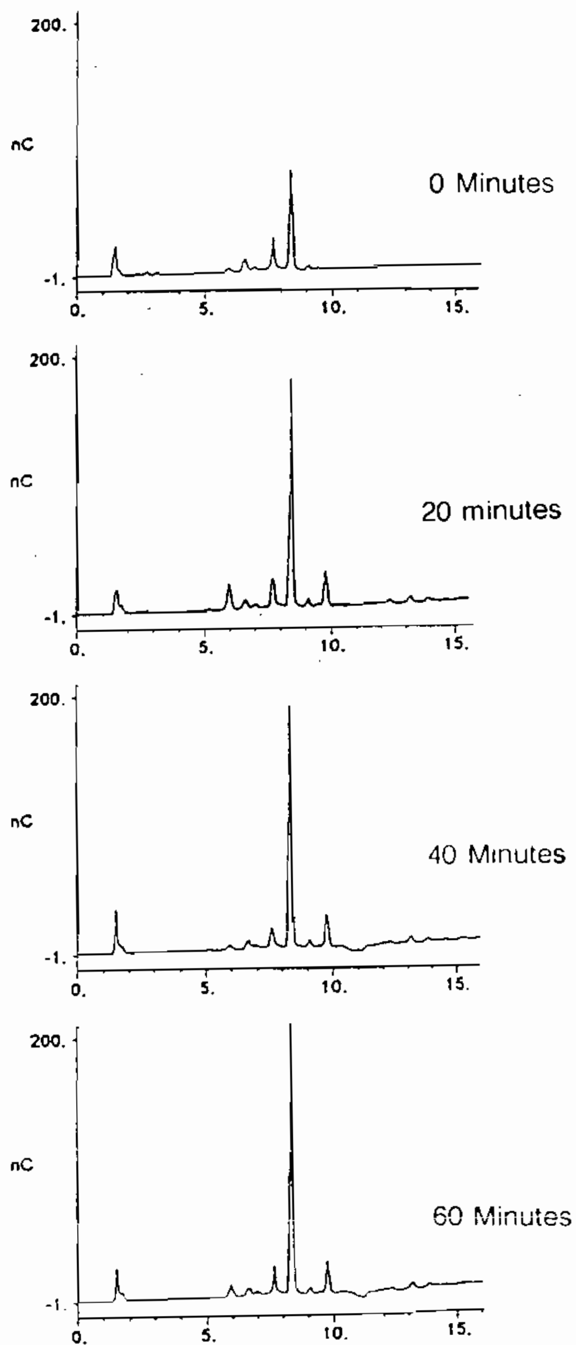


Figure 29a

Production of Maltose following Incubation of High Protein Wheat Flour with Fungal Alpha Amylase and Pancreatic Alpha Amylase for 60 Minutes Determined Using HPLC HPAEPAD

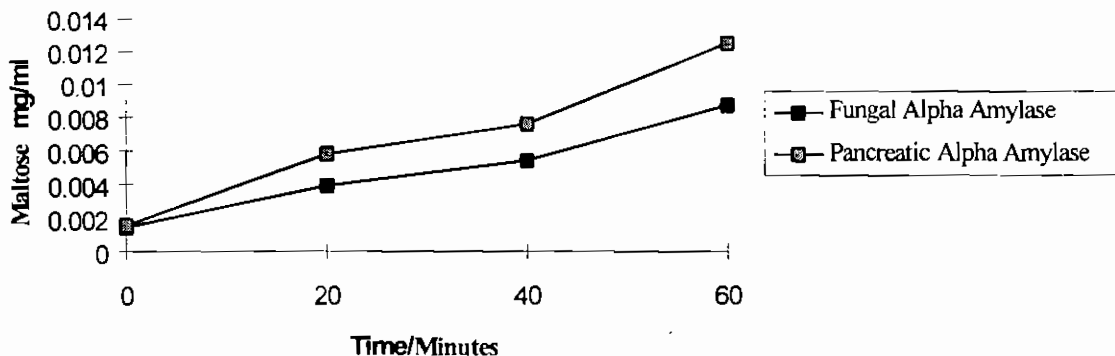


Figure 29b

Production of Maltose Following Incubation of Biscuit Flour with Fungal Alpha Amylase for 60 Minutes Determined using HPLC HPAEPAD

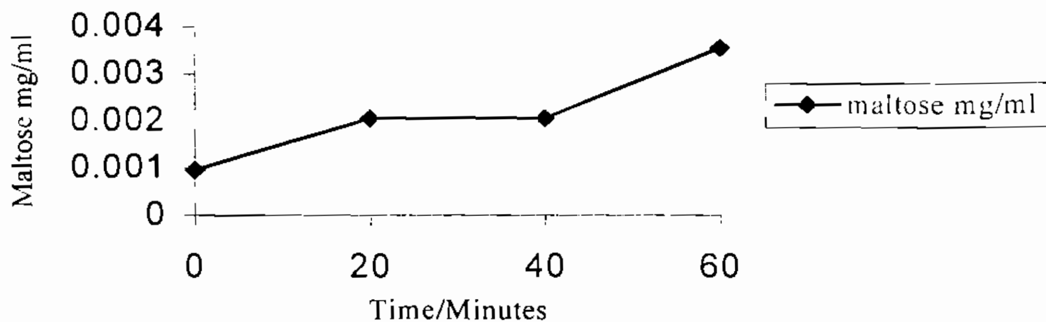


Figure 29c

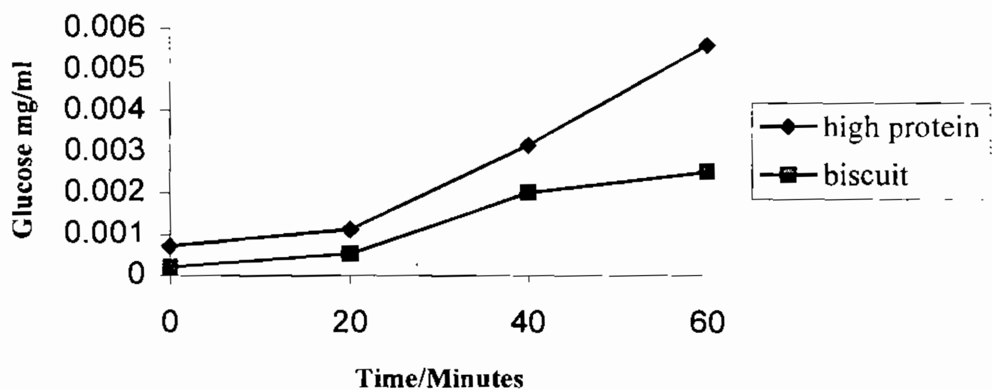


Figure 29d

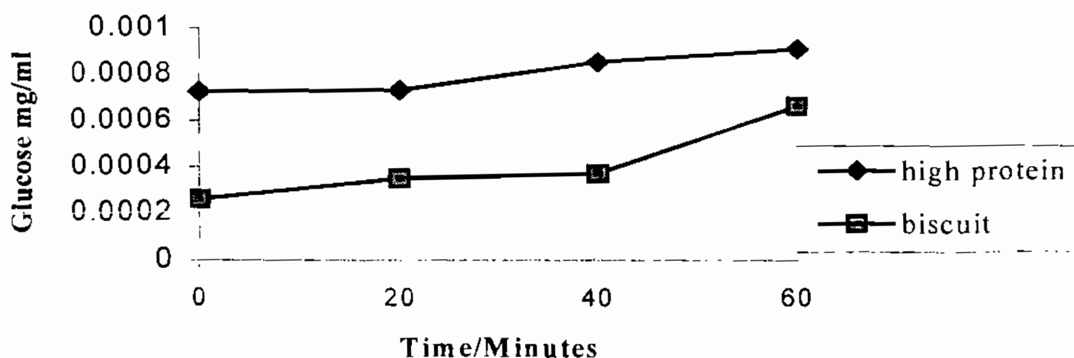


Figure 30
Chromatograms of the Reducing Sugars Produced from Maize and Potato Flour following Incubation with Pancreatic Alpha Amylase for 60 minutes and Determined Using HPLC HPAEPAD.

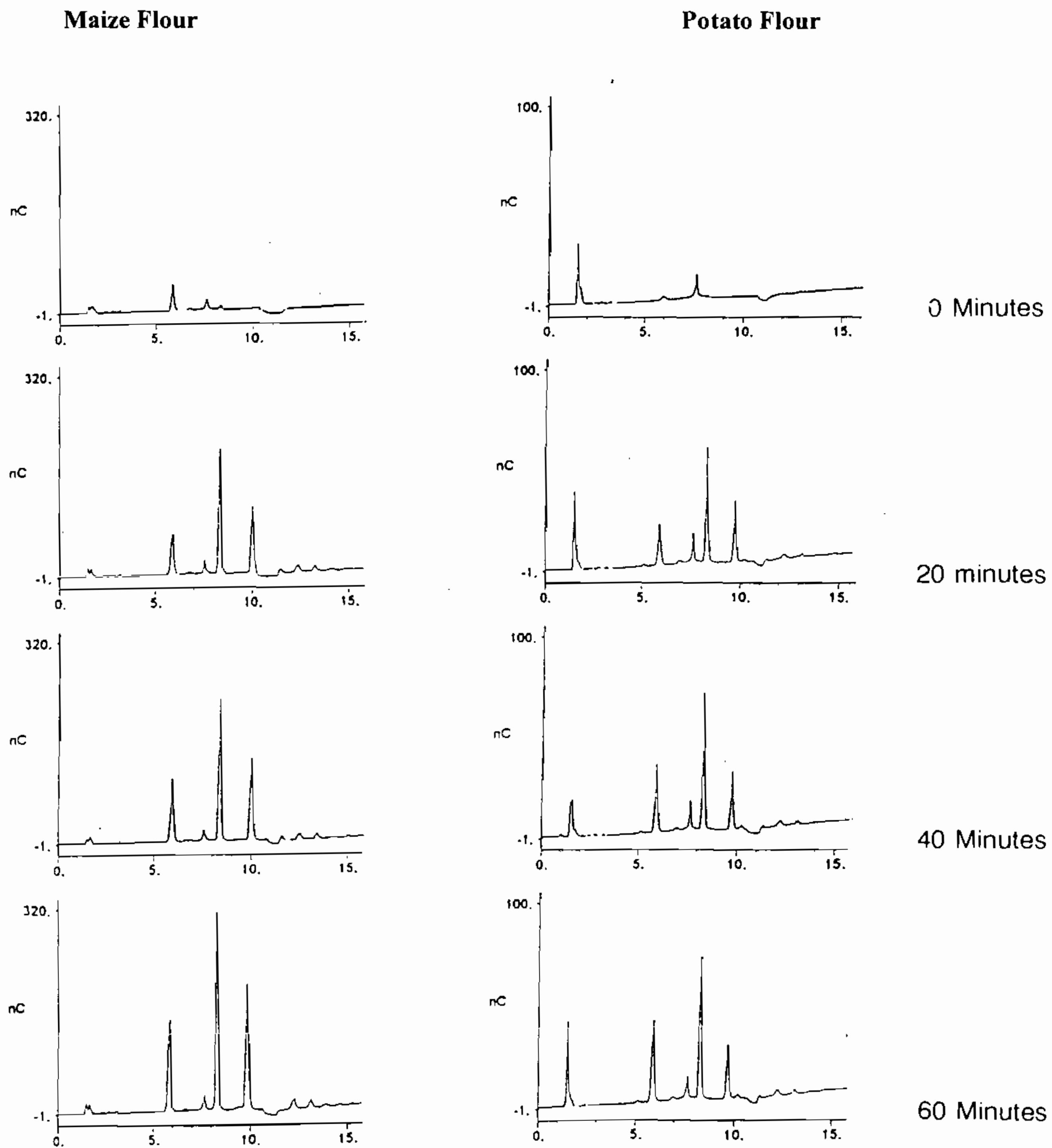


Figure 31

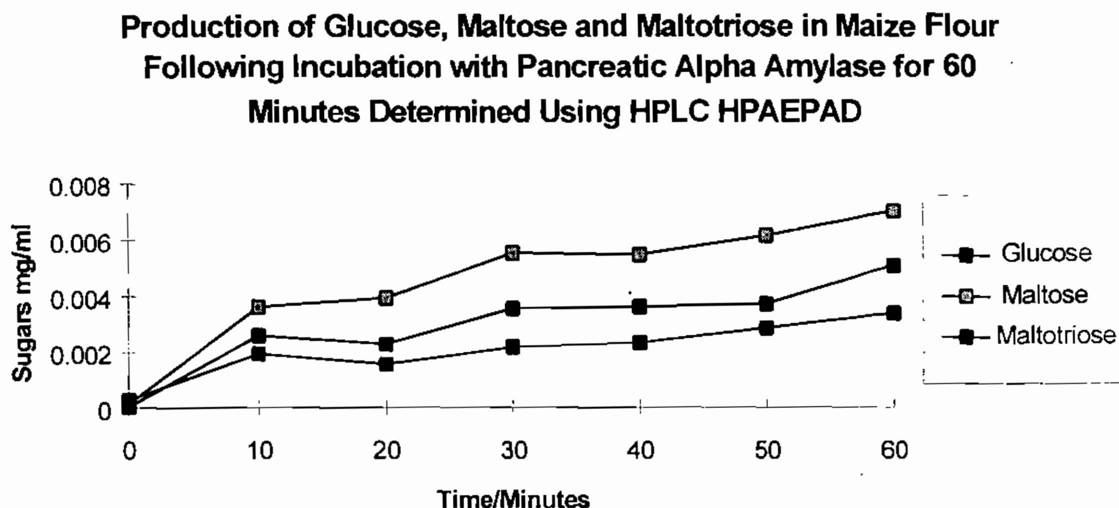


Figure 32

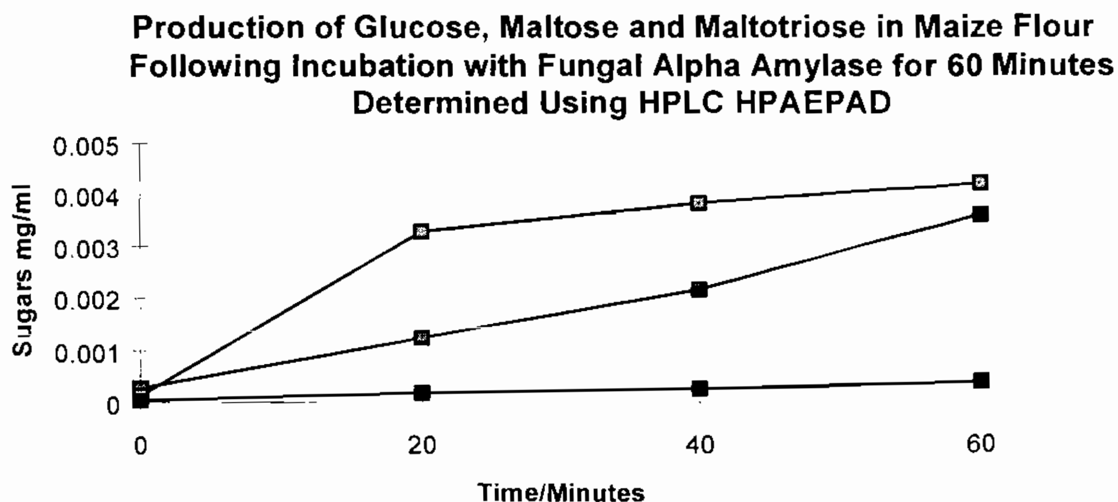


Figure 33

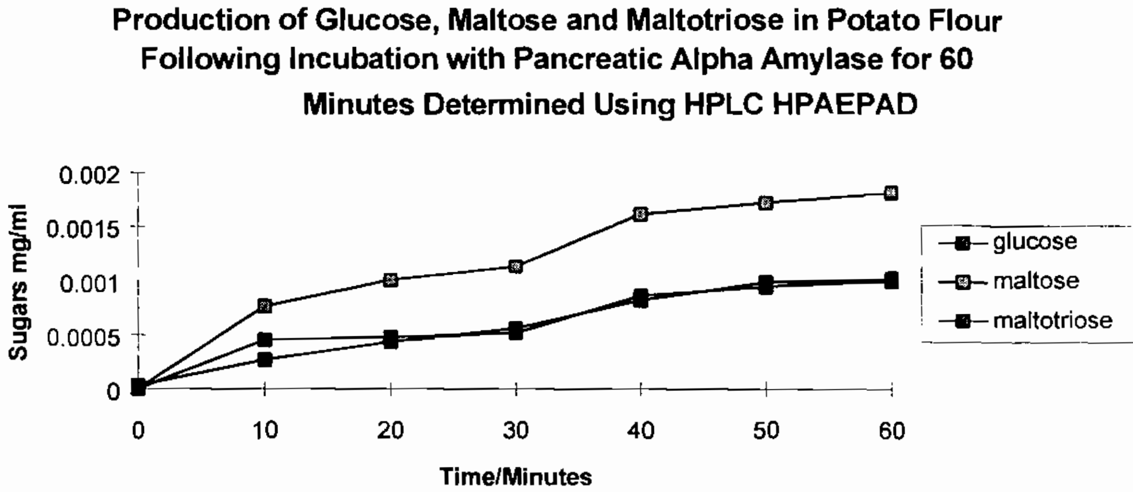


Figure 34

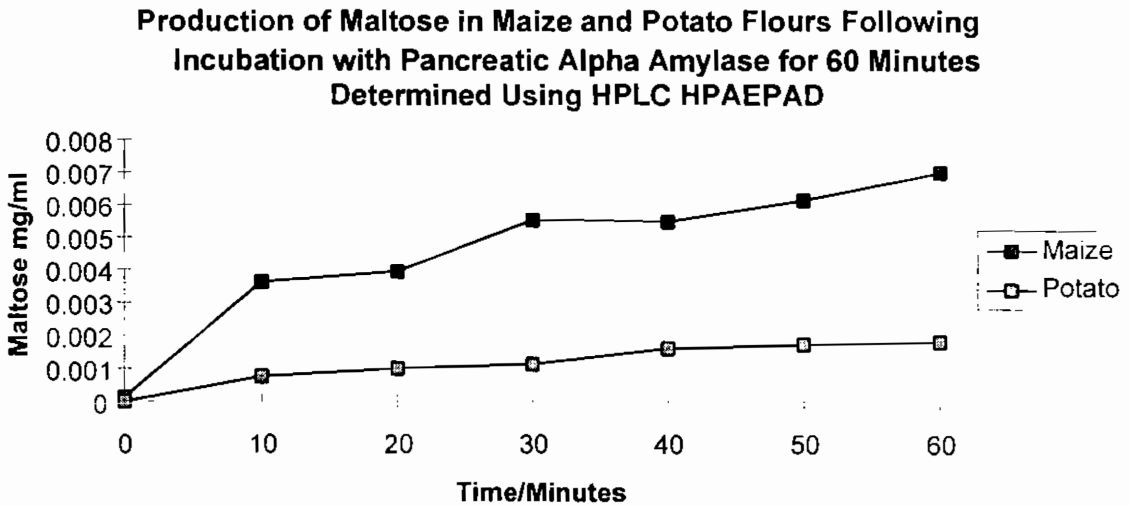
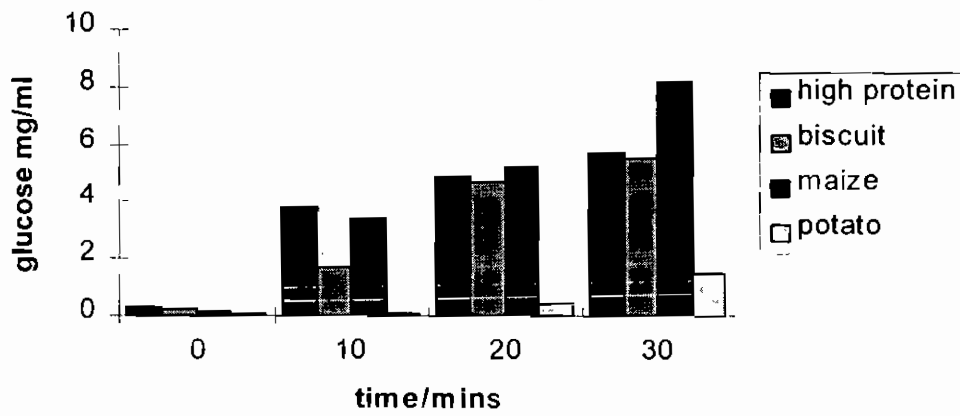


Figure 35



3:3:3 Observation of the Progressive Digestion of Starch Granules using the Scanning Electron Microscope

<u>**Maize Starch Granules in Flour**</u>

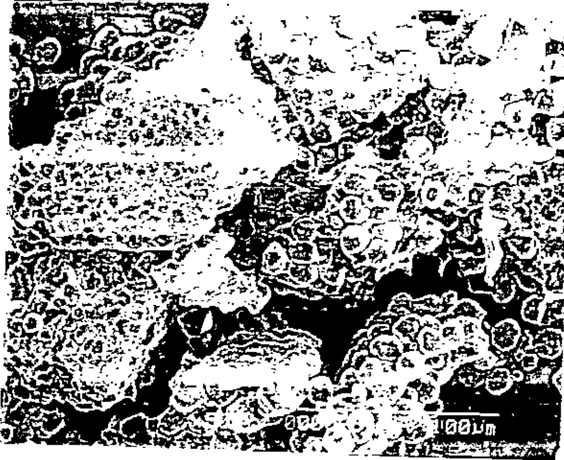
Figure 36 shows micrographs of the maize granules prior to incubation with an enzyme. Figure 36a shows the way in which the granules stick together in clumps. Figure 36b shows the variety of shapes, sizes and surface textures of milled, undigested maize starch. Figure 36c shows how small particles or pieces of a substance are also visible. It was found that some granules have been slightly digested by endocorrosion seen by the appearance of tiny holes on the granule's surface due to naturally occurring enzymes in the flour sample.

Figure 37 shows the progressive breakdown of the maize starch granules in the flour sample at a magnification of 1.0K following digestion using pancreatic alpha amylase. In these micrographs the progressive digestion of the granules cannot be clearly seen from one stage to the next. There does appear, however, to be a general increase in digestion of the granules. The starch granules in Figure 37d following 40 minutes of incubation are much more digested than those in Figure 37b following 20 minutes of incubation.

Figure 38 shows micrographs of granules at higher magnifications. Endocorrosion is clearly visible at each stage of digestion. After 10 minutes of incubation with the pancreatic alpha amylase enzyme a few small holes are already visible on the granule surface. The granule also has a roughened surface. Figure 38b shows that after only 20 minutes much greater endocorrosion has occurred, however, not to the same extent on each granule. The granule on the right is corroded but the one on the left is still undigested externally. Figure 38c shows a different type of endocorrosion. Smaller finer holes are produced which do not penetrate into the granule in the same way as above in 36b. Figure 38d shows more granules which following 40 minutes of digestion have been corroded in the same way as before. Figure 38e shows a progression to the previous endocorrosion, which is a granule, which has begun to be hollowed showing the crystalline layers inside. Several granules which have most likely been cut across during the milling process are visible in Figure 38f. Two granules are also visible which have been hollowed out. A large gap is produced on their surface followed by digestion of the interior of the granule rather than the surface. Some digestion holes are also visible on the surface.

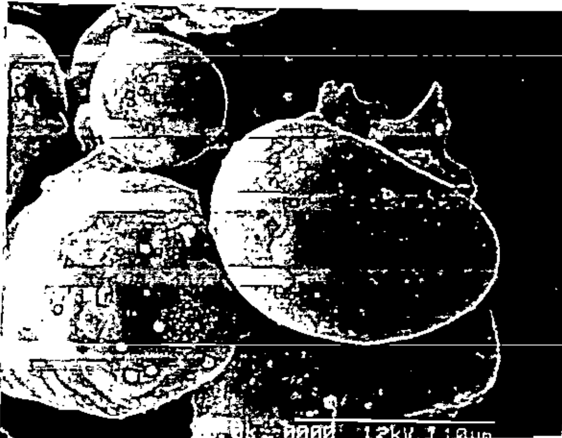
Figure 39 again shows the crystallinity present in the maize starch granules. Due to the prominence of the rings on the insides of the granules it is probable that digestion of the softer amylose is occurring between these lines. Figures 39c and 39d show what the starch granules look like following 20 hours of digestion. Digestion has clearly progressed much further than before. Much larger holes are now clearly visible on an increased number of granules.

Figure 36
Undigested maize starch granules in flour.



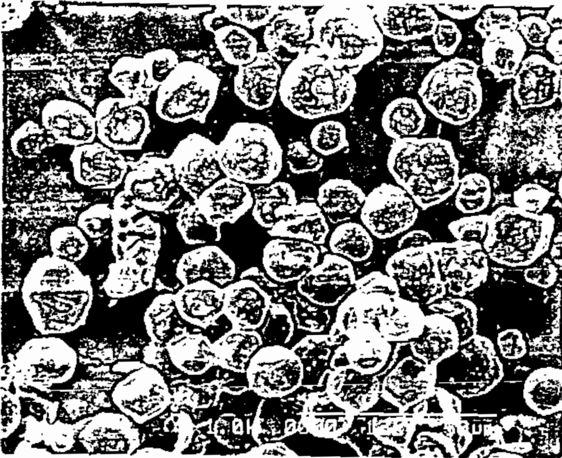
a

b

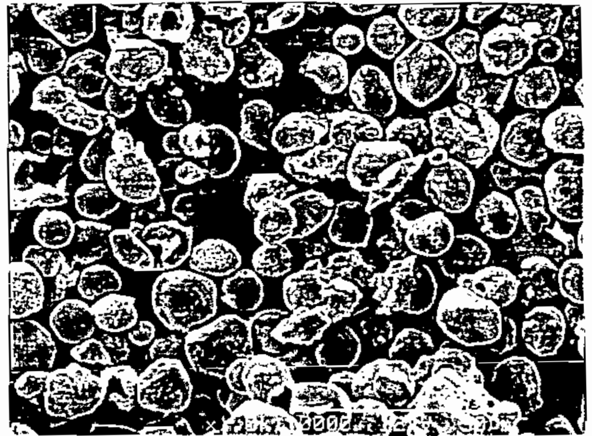


c

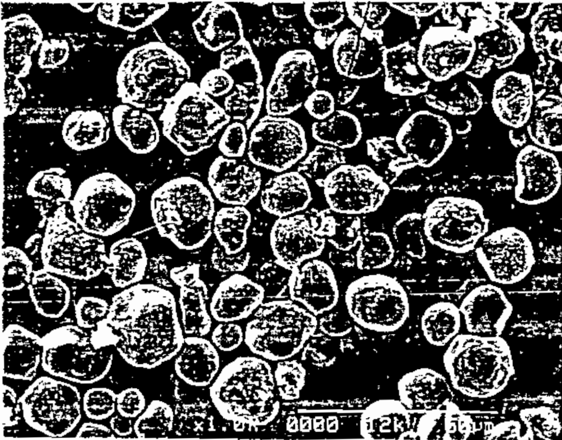
Figure 37
Maize starch granules digested by pancreatic alpha amylase at 10 minute intervals from 10 to 60 minutes.



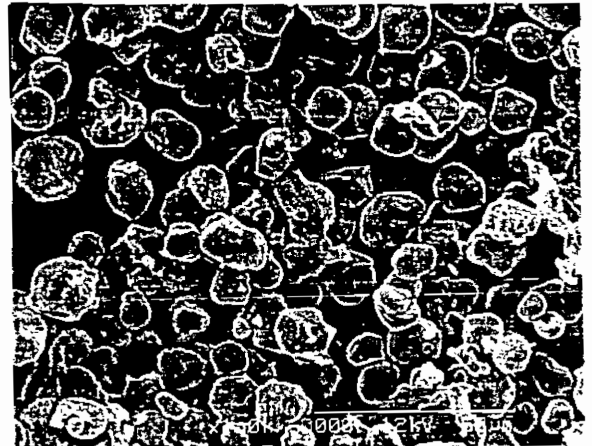
a
10 minutes



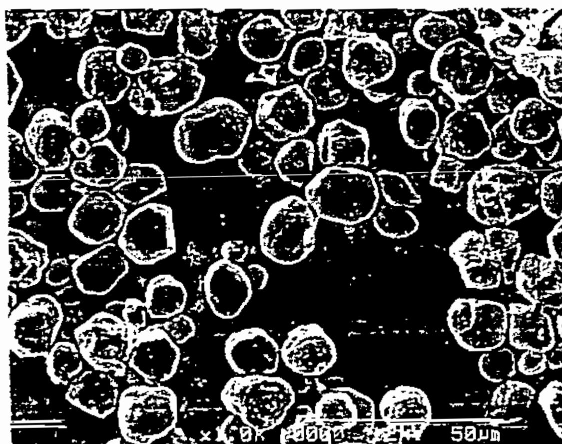
d
40 minutes



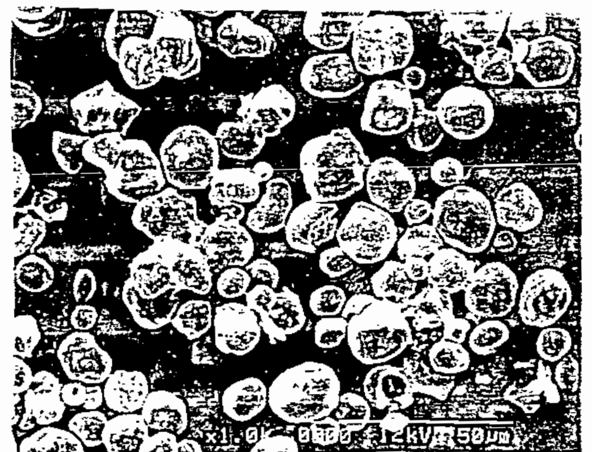
b
20 minutes



e
50 minutes

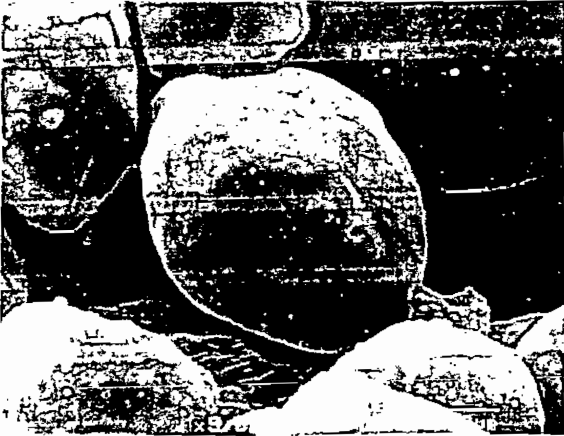


c
30 minutes



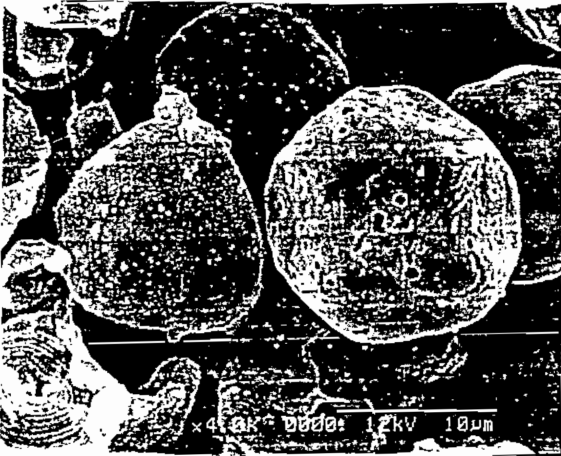
f
60 minutes

Figure 38
Starch granules in maize flour digested by pancreatic alpha amylase.

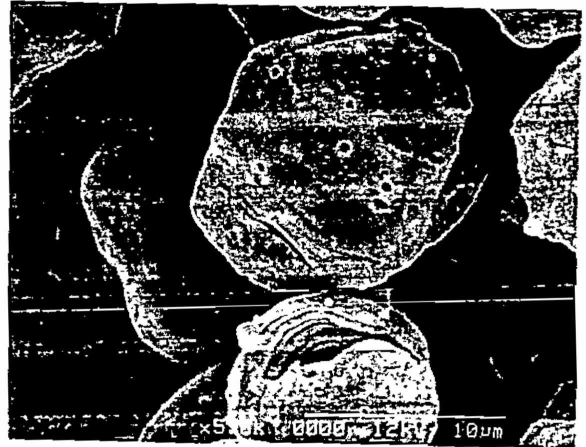


a
10 minutes

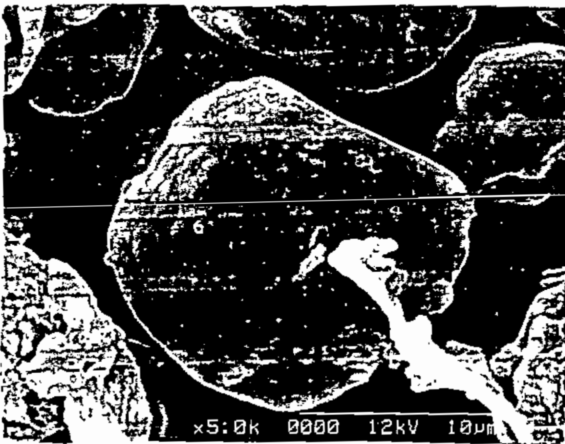
d
40 minutes



b
20 minutes



e
50 minutes



c
30 minutes

f
60 minutes

Figure 40 shows the maize granules following digestion with fungal alpha amylase. Following 20 minutes of incubation the granules are all digested to some extent as can be seen by endocorrosion on the surface in Figure 40a. However, other granules which are also present in close proximity to these granules show no digestion by endocorrosion. The surface of these granules is rough. This may be because of the milling process or due to another form of enzymatic digestion. The same comparison can be seen following 40 minutes digestion in Figure 40b where a digested granule is situated alongside undigested granules. It may be that the digested granule has lost a protective layer in the production process. Figure 40c shows a different type of digestion whereby the small holes are coming together on certain areas of the granule and generally roughening the surface. Figure 40d shows that digestion is still taking place following two hours of incubation and larger holes as well as smaller pinholes seen previously are visible. These larger holes are scattered all over the surface of the granule.

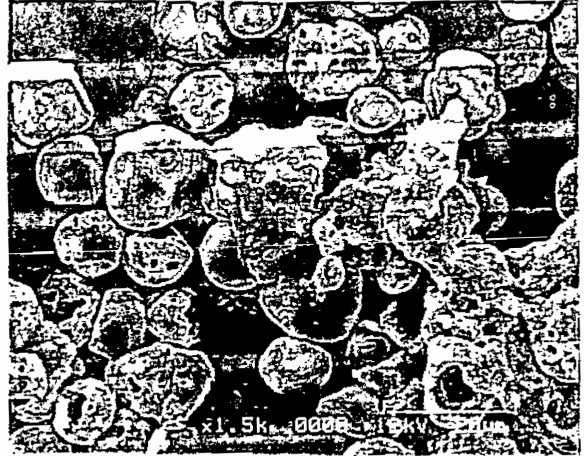
Potato Starch Granules in Flour

Figure 41a, 41b and 41c show potato starch granules in flour prior to enzymatic digestion. Figure 41a shows the diversity in size of the potato granules and that despite the flour preparation process, the granules appear to be intact. Figure 41b shows the granules at a higher magnification of X 1500. It can be seen that the ends of the smooth granules are slightly roughened. Figure 41c shows a granule that has been cracked possibly by the flour preparation process. Figure 42 shows the progressive breakdown of the potato starch granules over 60 minutes. Again as with the maize granules the progression of breakdown cannot be clearly defined every 10 minutes. Following 10 minutes digestion very little endocorrosion can be seen. However, after 20 minutes digestion, endocorrosion appears to be present. The processes of digestion can be more clearly seen in Figure 41 at a higher magnification. In 41d the granule has a roughened surface with some open cracks, which appear to go deep into the granule. Figure 41e shows a hollowed granule with a thick shell containing what appear to be smaller granules inside. Figure 41f shows one hollowed granule inside another one.

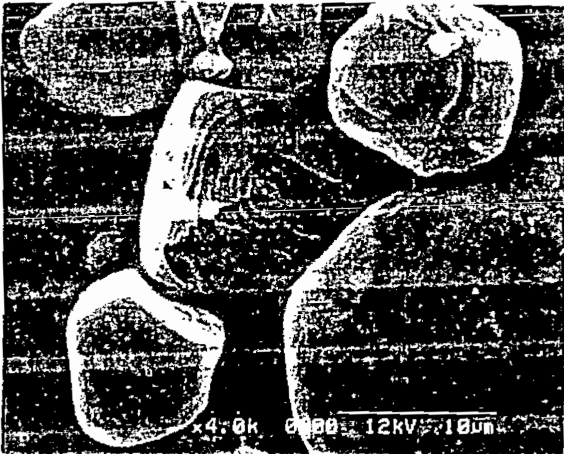
Again as was found with the maize granules no distinguishable difference can be seen between the granules digested with fungal alpha amylase and those digested with pancreatic alpha amylase. Figure 43 shows granules digested using fungal alpha amylase. Some more examples of effects of digestion are visible here. Figure 43a shows a cracked granule with one large hole at one end of the crack and what could be a hollowed interior. Figure 43b shows a granule inside the shell of either the same or another starch granule. Figure 43c shows a thick shell of a granule. Circular lines are faintly visible on the inner surface. Figure 43d shows a rare example of surface corrosion. This is shown by the appearance of faint cracks across the surface.

Figure 39
Starch granules in maize flour digested by pancreatic alpha amylase.

a
10 minutes



c
20 hours

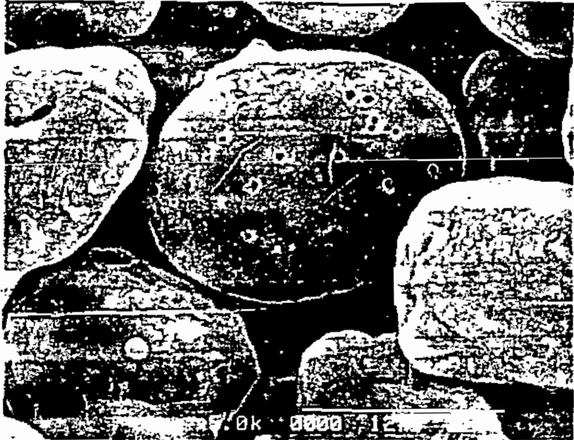


b
60 minutes

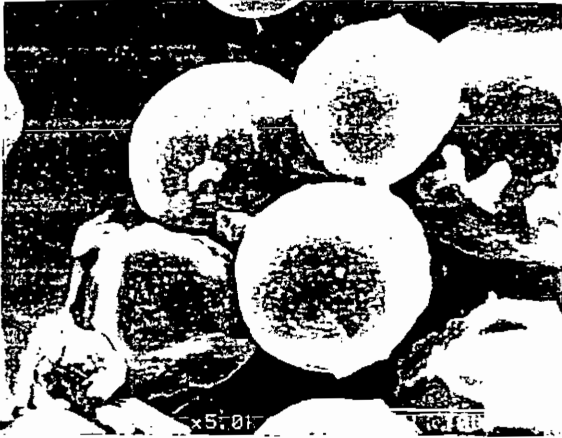
d
20 hours

Figure 40
Starch granules in maize flour digested by fungal alpha amylase.

a
60 minutes



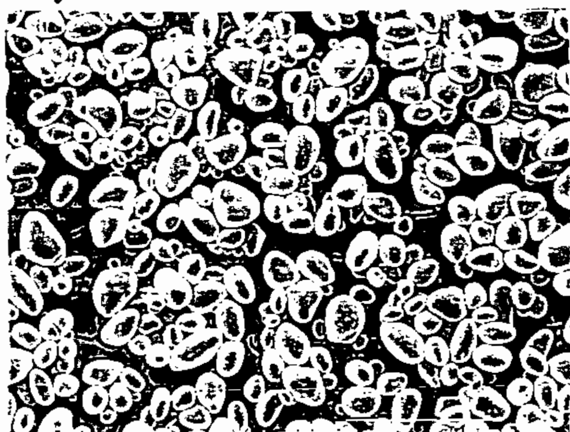
c
40 minutes



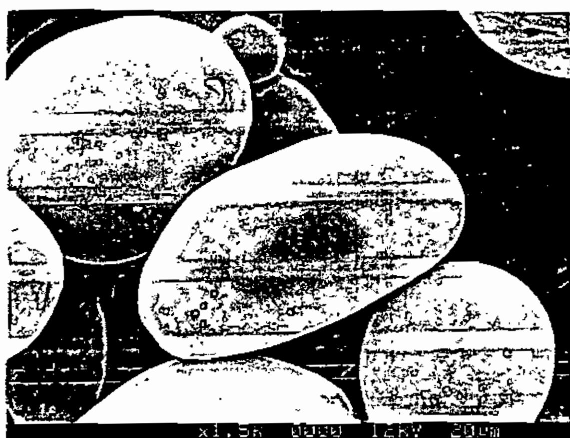
b
20 minutes

d
2 hours

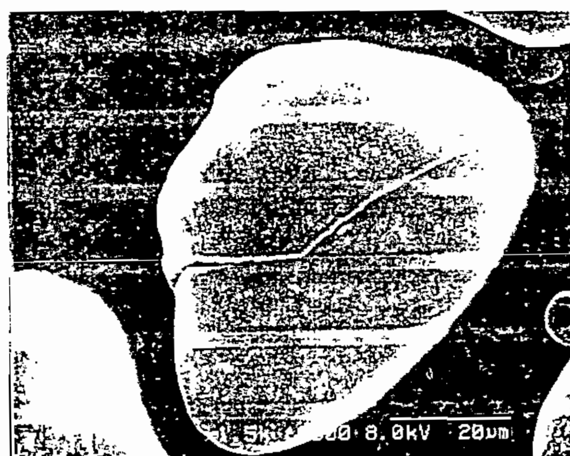
Figure 41
Starch granules in potato flour undigested and digested by pancreatic alpha amylase.



a
0 minutes

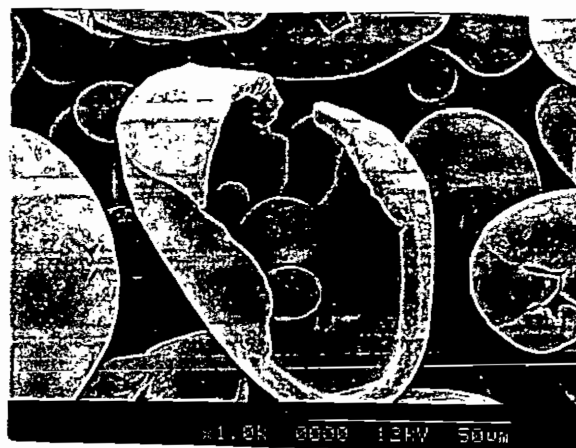


b
0 minutes



c
0 minutes

d
30 minutes

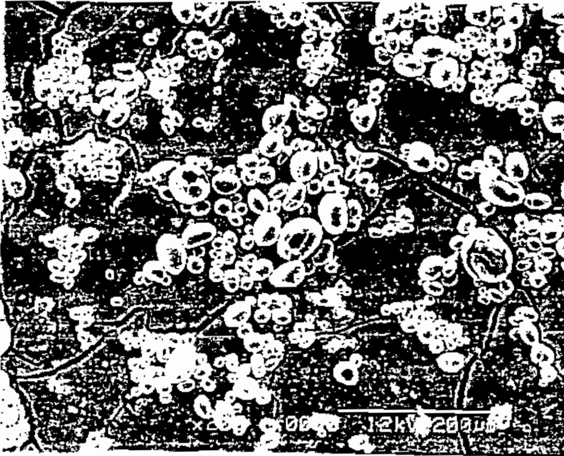


e
40 minutes

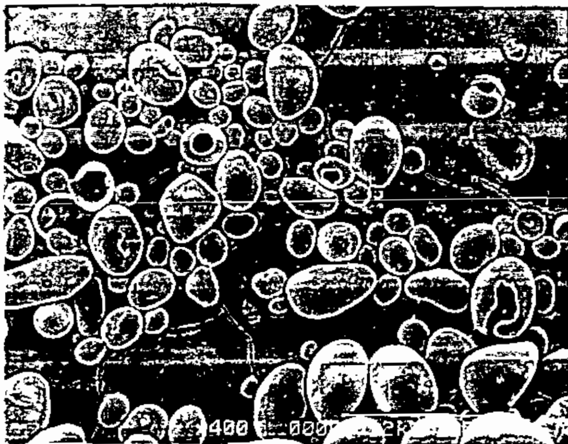
f
40 minutes

Figure 42
Starch granules in potato flour digested by pancreatic alpha amylase.

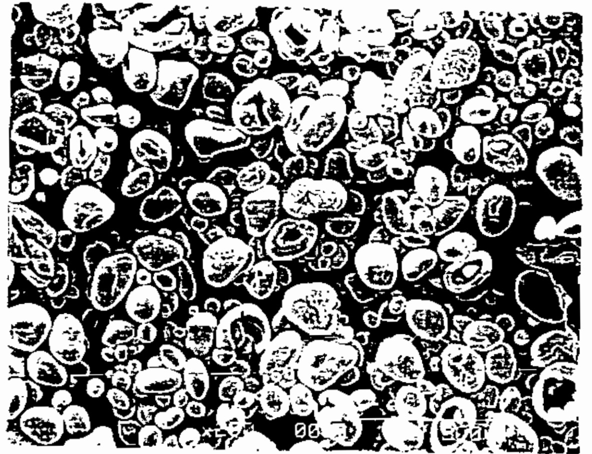
a
10 minutes



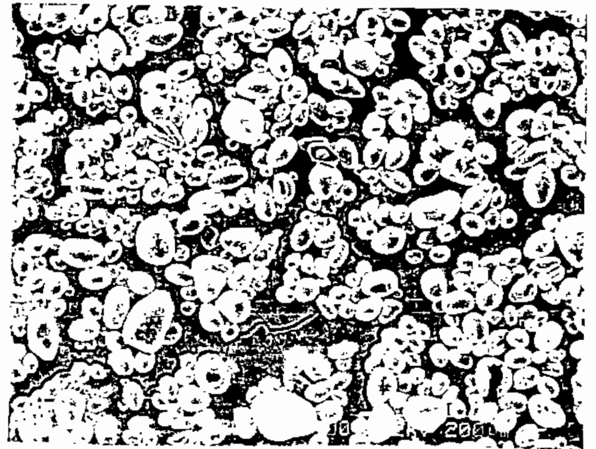
b
20 minutes



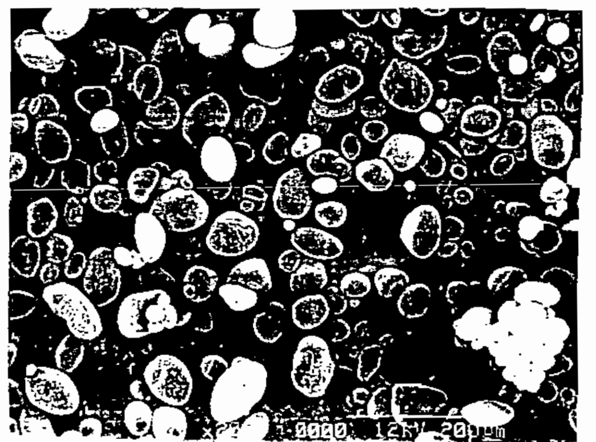
c
30 minutes



d
40 minutes

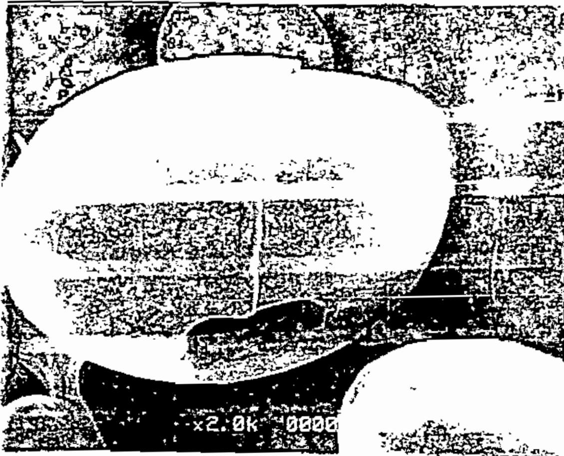


e
50 minutes

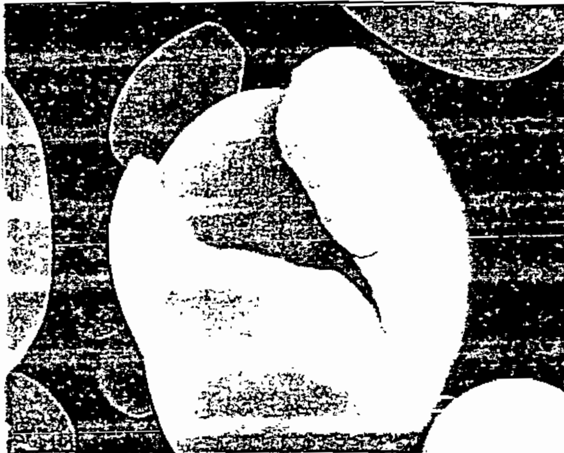


f
60 minutes

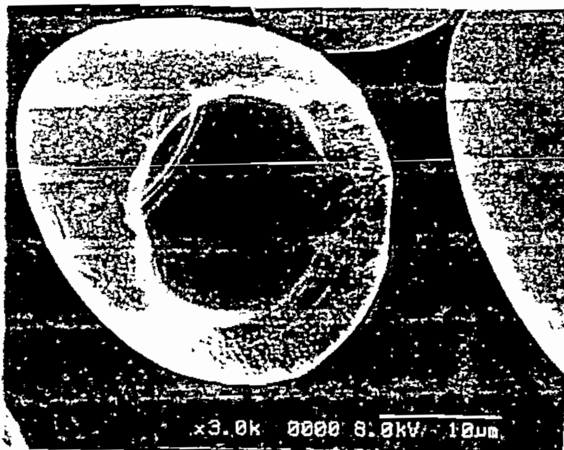
Figure 43
Starch granules in potato flour digested by fungal alpha amylase.



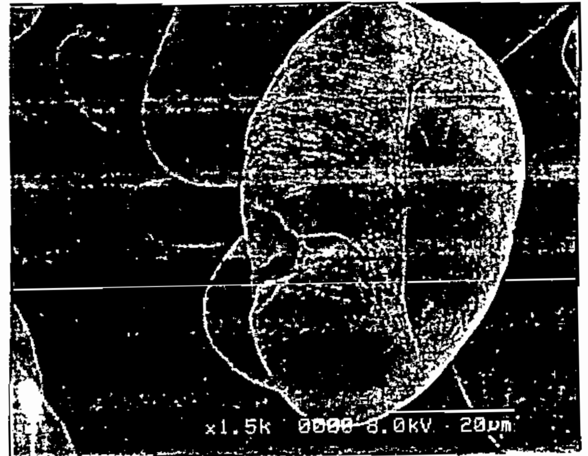
a
10 minutes



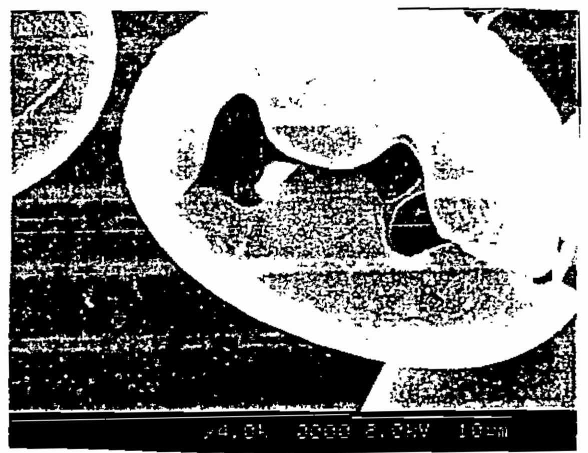
b
10 minutes



c
60 minutes



d
30 minutes



e
2 hours

3:3:4 Determination of the Effect of Digestion of Starch Granules on Crystallinity using X-ray Powder Diffraction

X-ray powder diffraction patterns were taken of selected flour and starch samples. They were then compared with x-ray patterns taken of pure starch samples. The angles at which peaks were found were compared with those of the reference diffractograms (Figure 8 in the Introduction) to observe the comparison in crystallinities. Flour samples were digested for 30 minutes and 60 minutes and compared with the crystallinity of undigested samples. This was carried out to see if there was any decrease in crystallinity following digestion of the flour samples with alpha amylase.

For the biscuit flour sample tested (Figure 44) in this study peaks were observed at 15, 17, 18.1 and 23 degrees. A small peak can be observed at 19 degrees. Lower peaks can also be seen at between 9 and 13 degrees. The same pattern can be seen for maize flour (Figure 45) except that the peak between 10 an 13 degrees is absent.

The diffractogram for potato flour (Figure 46) shows a large peak at 17 and a double peak at 14 and a small peak at 22. There is a small peak at 6 degrees. Less peaks are seen here to correspond with the reference (Figure 8) than in the case of the biscuit flour. For potato starch (Figure 47) peaks are visible at 18, 20, 23 and 25 degrees. Peaks are visible at 17, 20, 22 and 24 degrees for the resistant starch standard in Figure 48.

As can be seen from Figure 49 the preparation process for digestion of the flour sample, which in this case had no enzyme added, had very little effect on the crystallinity of the biscuit flour. Figure 50 shows that digestion also had no effect on the cystallinity of the biscuit flour. The same results were found for potato flour whereby neither the preparation process (Figure 51) nor the digestion with added enzyme (Figure 52) reduced the crystallinity of the flour sample. The crystallinity of potato starch is shown in Figure 53 following digestion for 60 minutes and was compared with undigested potato starch. No difference was found between the two profiles.

Figure 44
X-ray powder diffraction pattern of Biscuit Flour

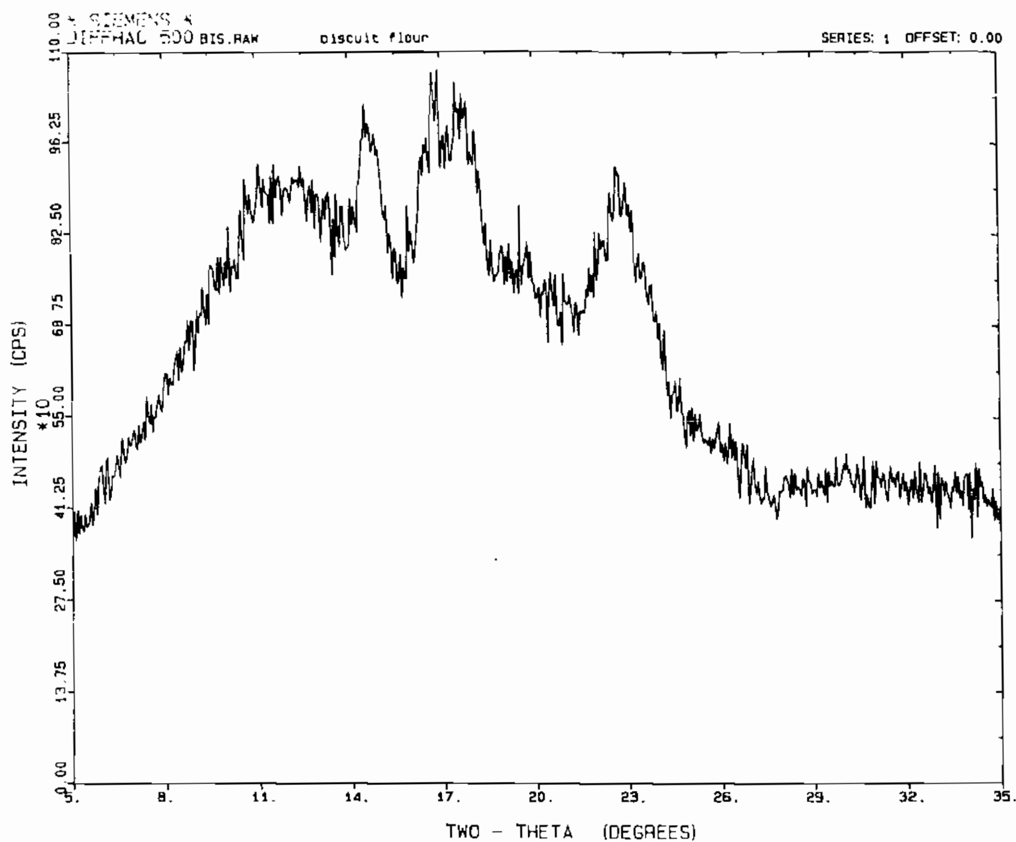


Figure 45
X-ray powder diffraction pattern of maize flour compared to biscuit flour.

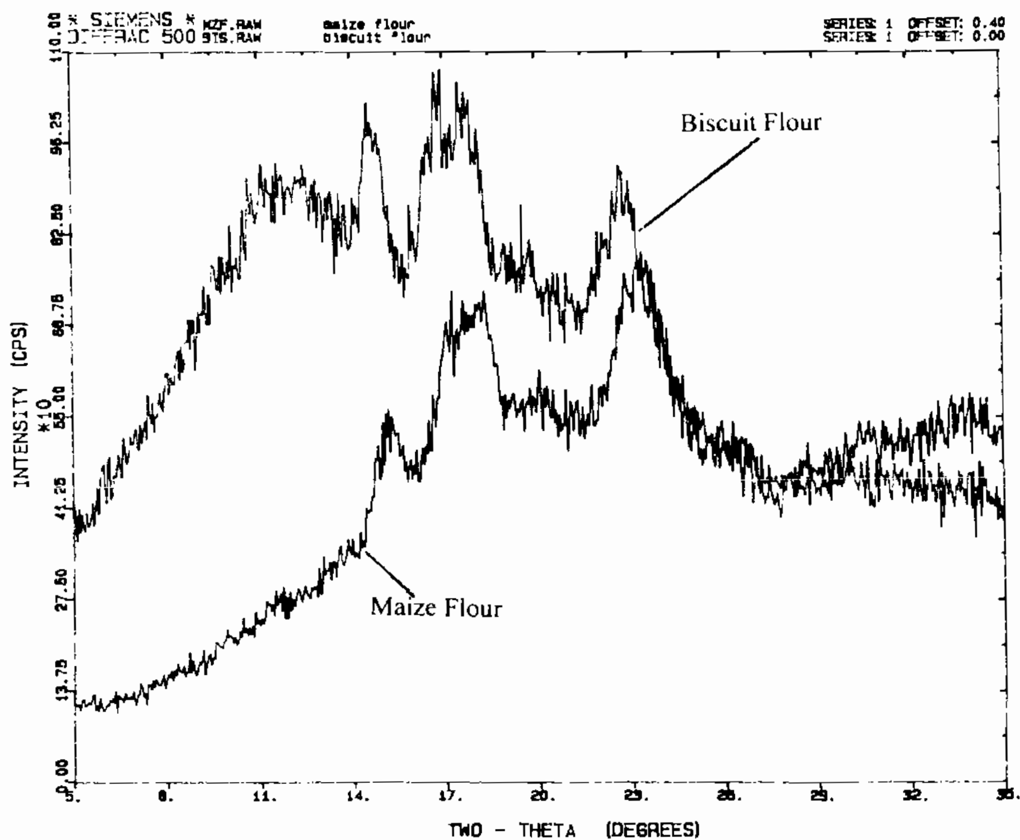


Figure 46
X-ray powder diffraction pattern for potato flour

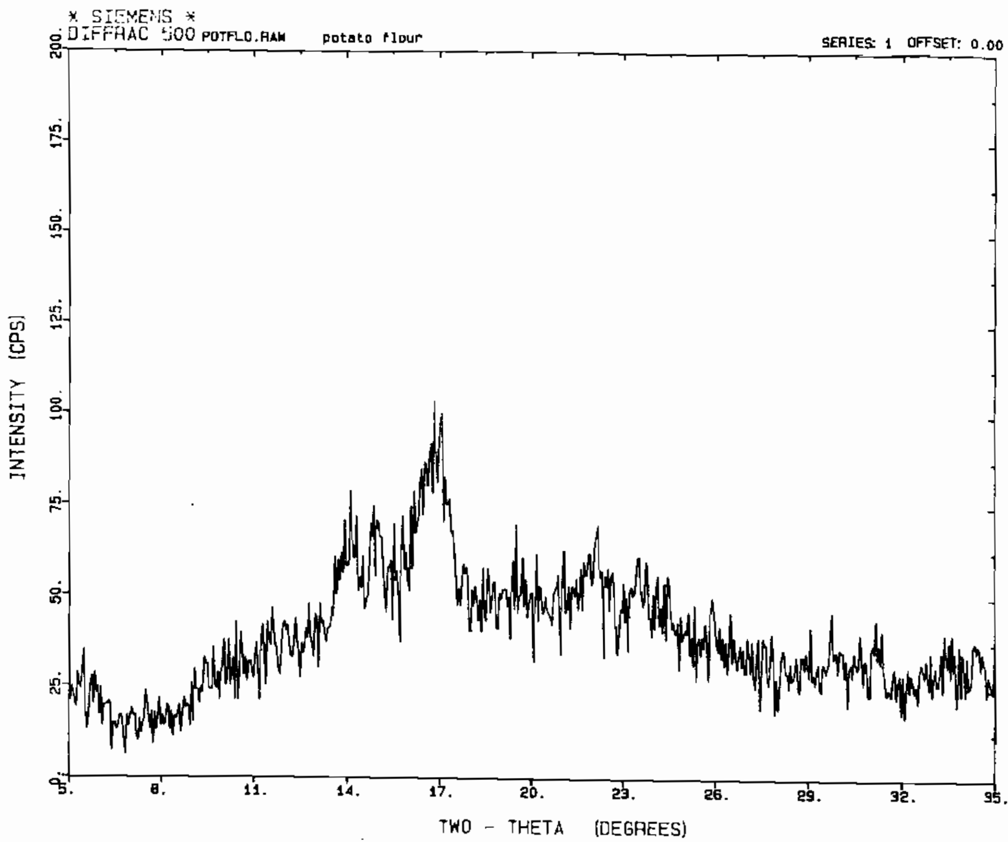


Figure 47
X-ray powder diffraction pattern for potato flour compared to potato starch.

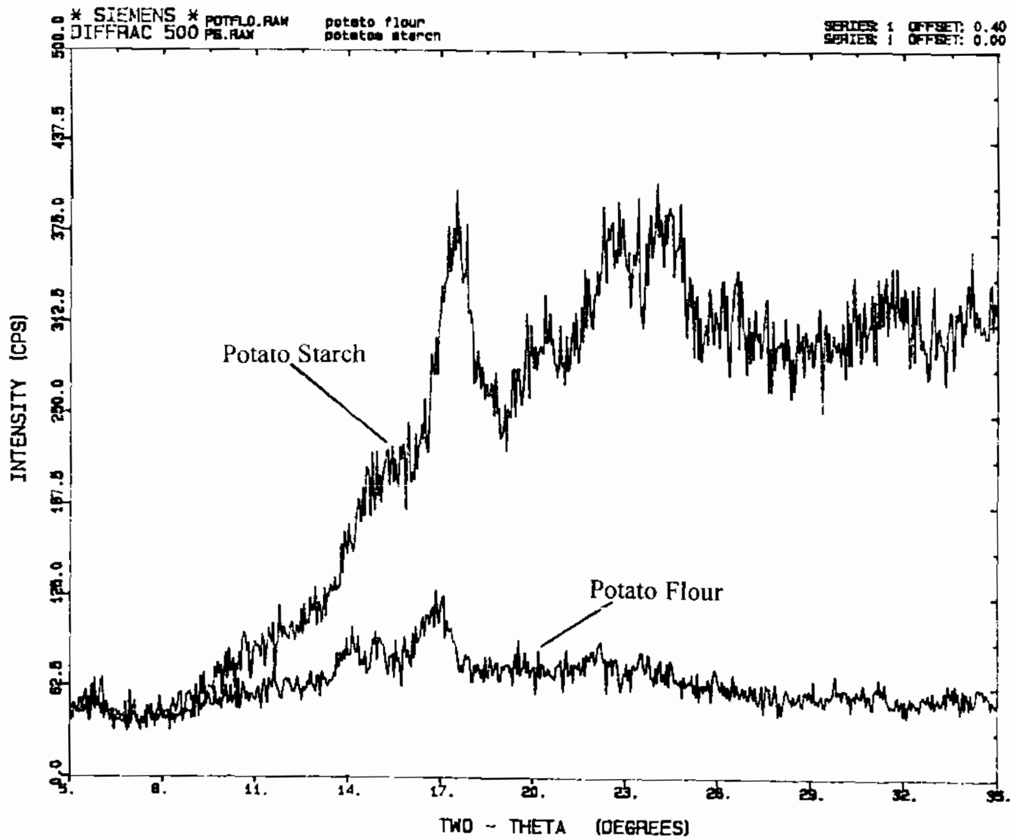


Figure 48
X-ray powder diffraction pattern for resistant starch standard flour.

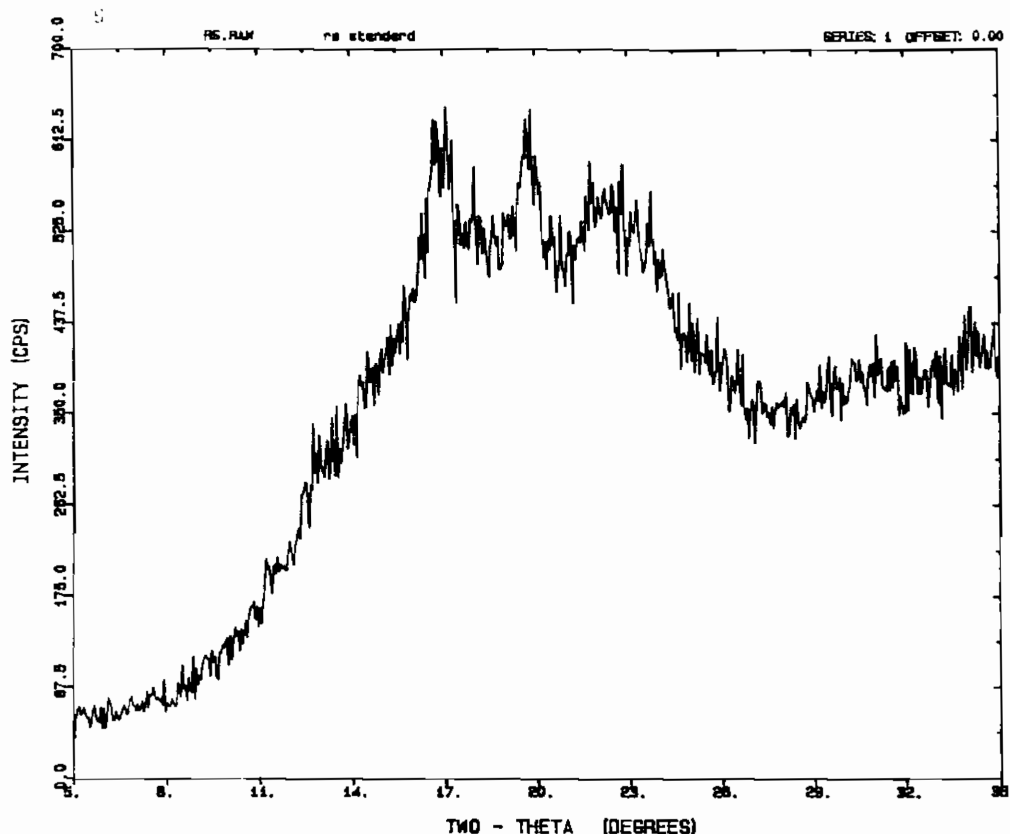


Figure 49
X-ray powder diffraction pattern for biscuit flour compared to biscuit flour which has under gone preparation process for digestion but without any enzyme added.

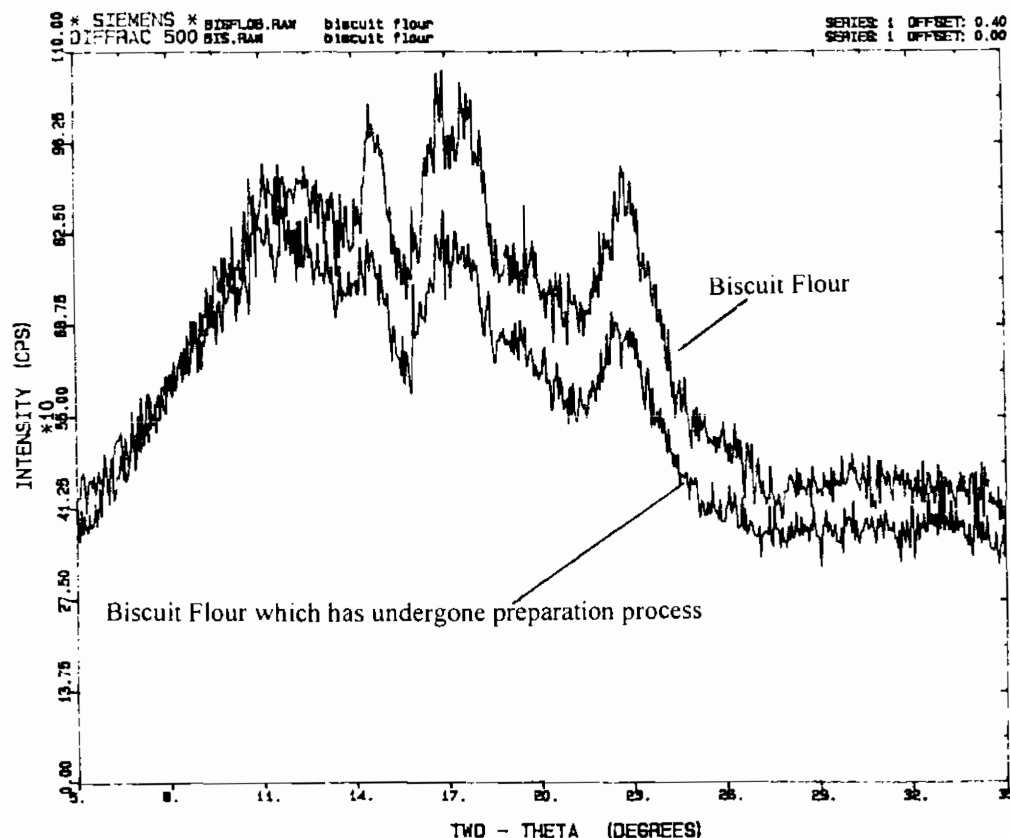


Figure 50
X-ray powder diffraction pattern for biscuit flour following digestion with alpha amylase for 30 minutes.

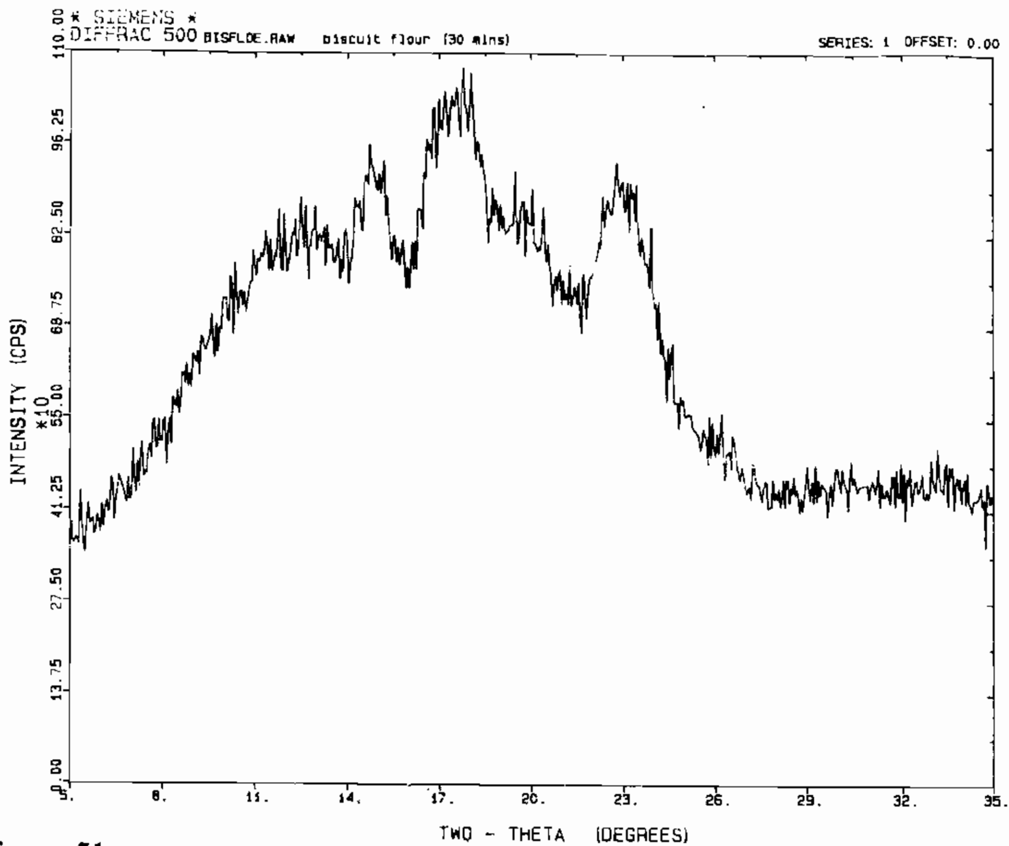


Figure 51
X-ray powder diffraction pattern for potato flour compared to potato flour which has under gone the preparation process for digestion but without any enzyme added.

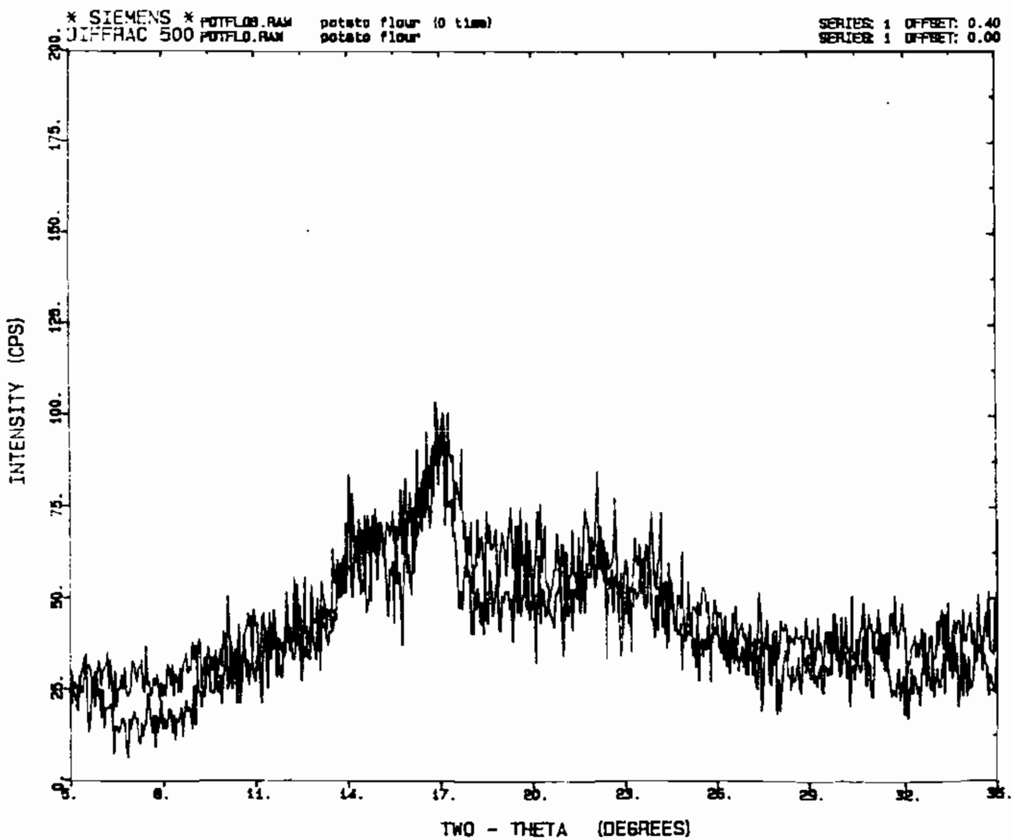


Figure 52
X-ray powder diffraction pattern for potato flour following digestion with alpha amylase for 30 minutes and compared to un-digested potato flour.

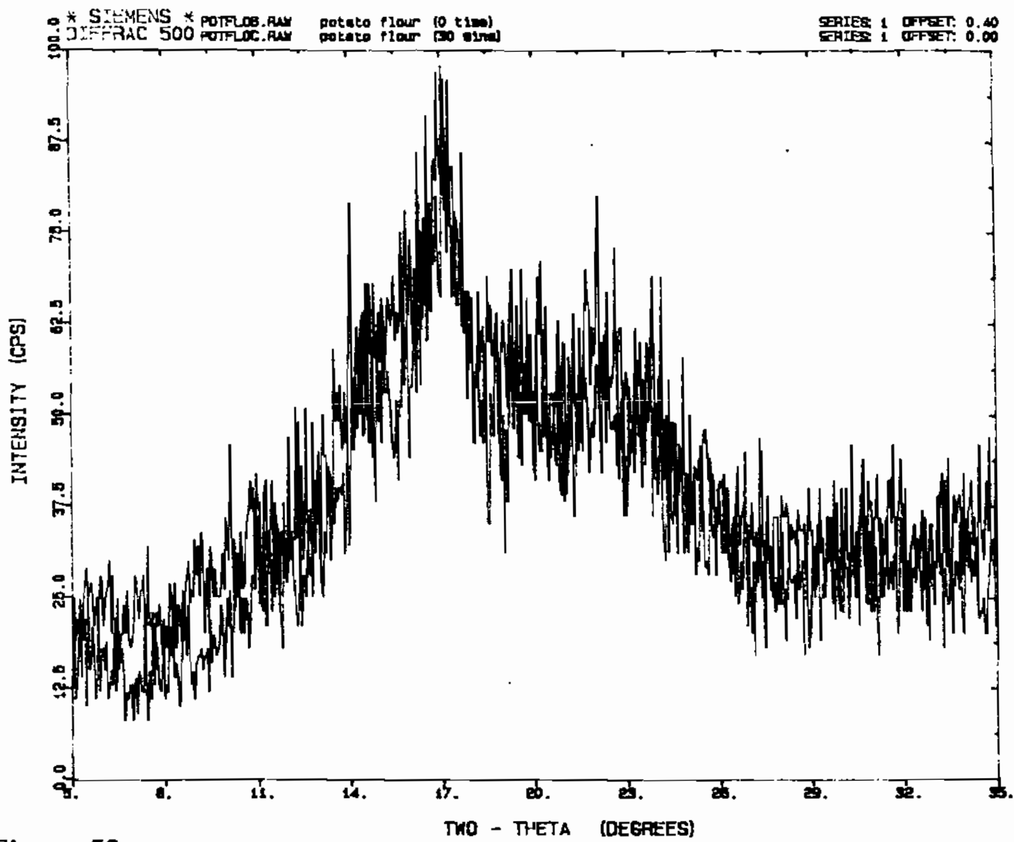
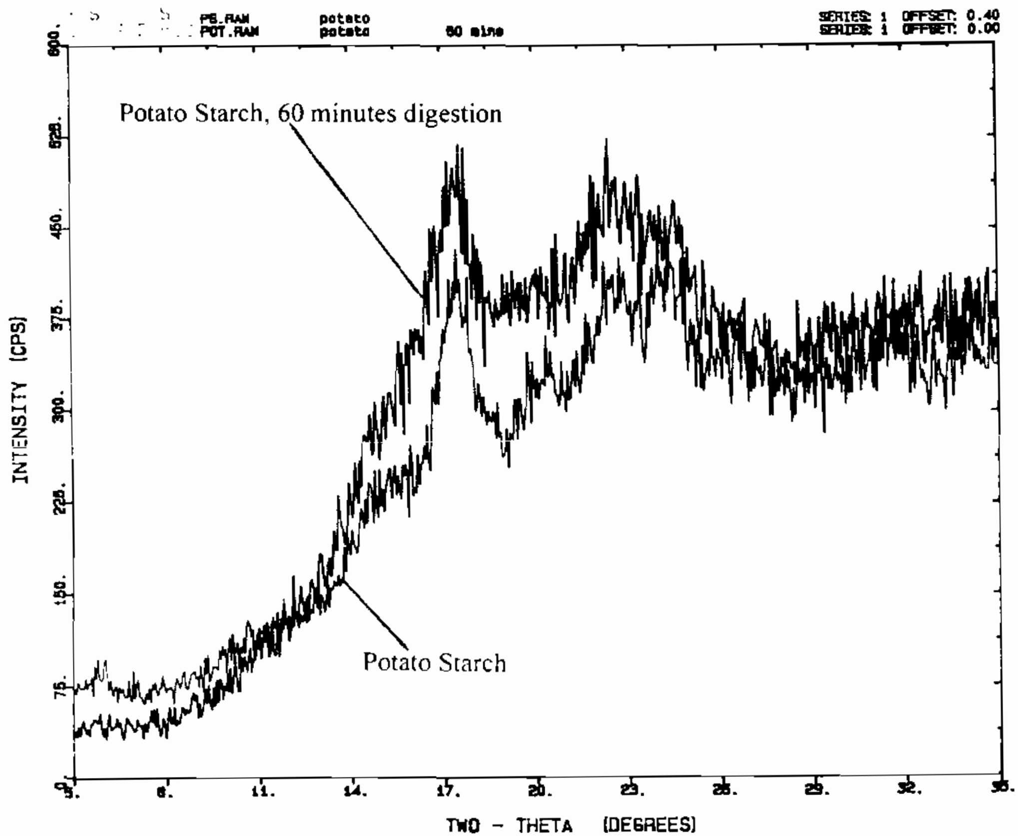


Figure 53
X-ray powder diffraction pattern for potato starch following digestion with alpha amylase for 60 minutes and compared to un-digested potato starch.



CHAPTER 4

DISCUSSION

4:1 Evaluation of Damaged Starch Determination Methods

4:1:1 Damaged Starch Determination by a Dye Test using Iodine Solution

Dye staining with iodine gives an estimation of the amount of damaged starch in a sample of starch granules. It was found in this study that approximately one third of the wheat flour granules were damaged as observed by dye staining. However, the enzymatic colorimetric method used in this study (modified AACC 76-31 method) gave a result of approximately 6% digestible (apparent damaged) starch for wheat flours. Observing dye absorption on maize flour it was seen that approximately half the granules absorbed dye. Maize flour, however, exhibited the same amount of enzymatic digestion as wheat flours. It was found that in potato flour, which enzymatically has a very low amount of digestible starch (1.3%) according to the same colorimetric method, the ability of the starch to absorb iodine is similar to that of wheat flours. This may be related to the fact that the potato flour is physically well damaged but still not readily available for enzymatic digestion. This is either because of the flour preparation method or because of the structure of this particular type of starch. Chickpea and soy flour samples showed very little dye absorption and their digestibility by enzymes was also very low (0.5% and 0.4% respectively). Therefore, the enzymatic, colorimetric test is generally not a good indication of the level of damage in starch. Colorimetric dye absorption tests are, therefore, more indicative of starch damage levels and enzymatic tests give an indication of digestible starch levels. The dye absorption tests used here are, however, qualitative rather than quantitative and only approximate estimations of damaged starch levels could be taken.

4:1:2 Evaluation of Enzymatic Methods for Starch Damage Determination in Cereal and Non-Cereal Flours

The amylolytic methods used in this study to determine percentage digestible (estimate damaged) starch found that the harder wheat flours were, as expected, more highly damaged than the soft wheat flours. Most of the wheat flours tested here were, although of different types, mainly high protein flours which are used for bread making e.g. High Protein wheat, Constancy wheat, Quality wheat and Superbaker wheat. Using the AACC 76-31 method the percentage damaged starch values of the four flours were not significantly different to each other. The results of the Farrand method did give higher coefficients of variation to the AACC 76-31 method but the results for the different types of hard wheat flours were again not significantly different to each other.

Intrinsic Factors Affecting Damaged Starch in Cereal and Non -Cereal Flours

Cereal Flours

Cereal flours tend to have an A-type crystalline structure. This type of structure is most susceptible to digestion by amylolytic enzymes. Wheat and maize have an A-type starch structure. However, barley, which is also a cereal flour, has a mostly B-type crystalline structure.

Other factors aside from crystallinity, however, also contribute to the digestibility of flours. For example porosity of enzymes may be influenced by other substances in the food such as the gluten in wheat [105].

Stone ground wheat flour contains much less damaged starch than the other wheat flours tested. This is due to the fact that it has a high fibre content, which lowers the total percentage of starch in the sample and the fact that it is probably milled less thoroughly and efficiently than roll milled samples. The wholemeal wheat flour had slightly less damage than the white wheat flours which is again probably due to its fibre content.

The pre-gelatinised wheat flour is produced by pre-cooking wheat flour. Its purpose is that it should be added to instant foods so that the instant food is readily digestible for human beings as a source of energy and readily soluble in water without the need for cooking. Using the AACC 76-31 method only 11.0% damaged starch was found in this flour. This is clearly more than other wheat flours but is still well below complete damage as wheat flours contain approximately 70% starch.

The maize flour used in these methods was finely milled. Milling maize involves de-germing of moisture-conditioned corn, de-hulling by friction, partial drying and then grinding of the endosperm. De-germing is the main difference between the production of maize and wheat flour. For the Farrand and AACC 76-31 methods similar damaged starch results were obtained for maize flour to the hard wheat flours. Maize flour contains 8-9% protein and wheat flour contains 11.5 % protein. Maize contains 92% starch and wheat flour contains approximately 74% starch. Therefore, there is significantly more starch in a sample of maize flour than a sample of wheat flour. Therefore, less starch was damaged in the maize flour out of the total starch present than was damaged in wheat flour.

Potato Flour

The potato flour used in these methods was prepared by peeling, trimming and cooking potatoes, flaking on a drier and grinding. It would be assumed that, by these intensive damaging processes, a high level of damaged starch would be obtained. However, this is not the case. As was found with the dye absorption tests, damage does not automatically imply digestibility. The Farrand method could not be used to determine percentage damaged starch in the potato flour, as the result was too low and would have given a negative value. The AACC 76-31 method gave a value of 1.3% damaged starch. Potato starch naturally contains a mainly B-type structure which is the least susceptible type of starch to amylolytic digestion. It is believed that increased resistance of the B-crystalline structure is due to the higher actual content of crystalline structure in potato starch [62] or a greater number of crystallites on the granule surface [10]. Whether this crystallinity is affected by digestion by alpha amylases was observed using x-ray powder diffraction studies and it was found that digestion of potato flour by alpha amylase did not change the crystallinity of the flour sample.

Potato starch also has a larger granule size, which results in a smaller surface area available to enzymes. Particle size affects accessibility of enzymes to starch and the larger the surface area which is available to enzymes the higher the initial rate of hydrolysis [10].

Potato starch granules have a smoother surface than cereal starches. A rougher surface would also increase surface area. It was not excluded as a possibility that some of the measured apparent starch damage in undamaged granules may result from the removal of exposed clusters of amylopectin protruding from the granule surface [2, 24]. The hairy structure or dangling chains at the starch granule surface is necessary for alpha amylase absorption to occur. These are probably amylopectin chains protruding from the granule surface. Their removal leads to a smoother surface [10].

It may also be the case that following the cooking process used in the production of potato flour the starch has retrograded. Re-crystallisation occurrence is enhanced by low temperature, long chains of amylose and amylopectin and high starch concentration. Retrograded starch is not digestible by amylololytic enzymes unless it is incubated for a very long period of time, much longer than that which would be carried out in these tests. It is important to note that potato flour is composed of almost 100% starch (% dry weight).

With regard to the potato flour preparation process the starch granules may have been too resistant to be affected by the grinding process. The cooking process may have involved insufficient heating or insufficient water to be able to gelatinise the starch resulting in intact granules. Previous tests carried out in this study on determination of total starch in potato flour found that the flour gelatinised easily and became highly digestible to amylolytic enzymes following gelatinisation.

Legume Flour

Both soy and chickpea starches have a C-type crystalline structure. It is uncertain as to whether the C-type crystalline structure is intermediate between or a combination of A and B-type crystalline structures. The dye absorption test found that very few starch granules were damaged in either soy or chickpea flour.

Soya flour is not used in bread making due to the fact that it contains no gluten, which is necessary to hold steam and gas in the dough and allows the loaf to rise. Its use, therefore, results in lower loaf volume. It may, however, be used in small quantities to improve the crumb texture. Soy flour is produced by steam cooking, followed by drying and milling. Soya beans must also be treated prior to consumption generally for 10 minutes at 100°C to inactivate trypsin inhibitors that would otherwise reduce the nutritive value of the protein in the flour. These, and enzymes which cause flavour problems, are inactivated by application of moist heat treatment to soy flakes from which flours are produced. Soya flour contains a low level of carbohydrate (34 to 26%) and has a high protein content (59 to 47% dry basis) compared to cereal and potato flours. Soy flour contains 12.3% starch. The proteins present in soya flour do not include gliadin and glutenin. It has been found that proteins present in legume flours inhibit alpha amylase activity. Soya flour also contains 23.5% fat. Amylose in starch is known to complex with fat rendering it more resistant to digestion than free solubilised amylose [106]. Therefore, this may be a factor in the low percentage damaged starch value found here. Starch lipid complexes will eventually be degraded if sufficient enzyme is used [108]. However, there would not be sufficient time or enzyme for this level of degradation to occur in the above methods.

As the flour has been cooked during production the retrogradation process following cooking would aid in complexation with fat. Digestion may also be affected by entrapment of starch in the cell walls of un-ground seeds [10]. In legumes, cell walls limit accessibility of enzymes to starch [38]. In one previous study hydrolysis increased when pre-treatment with proteolytic enzymes was carried out [10]. As the maximum level of damage expected cannot be more than the total starch content the maximum value that could obtained would be 12.3%. Due to other circumstances mentioned above it is likely to be less. In fact only 0.6% of the starch in soy flour was recorded as digestible by the AACC 76-31 method used in this study.

Chickpea or gram flour as it is more commonly known was also tested as it is another example of a legume flour and also has a C-type crystalline structure. Chickpeas and gram flour when consumed raw cause a disease called lathyrism due to a toxic substance, which is a derivative of beta-amino-propionitrile (BAPN). Therefore, the chickpeas must be detoxified. This involves cooking in excess water, draining, soaking overnight in cold water and steeping the de-husked seed in hot water. The chickpeas are dried on a sieve in air and milled. Therefore, cooking in excess water would result in gelatinisation and cooling could result in retrogradation and complexation with fat. Dried chickpeas contain 21.3% protein, 5.4% fat and 49.6% carbohydrate not including oligosaccharides. Therefore, chickpea flour contains twice as much carbohydrate as soy flour and less fat. Proteins present in legumes inhibit alpha amylase [109] and chemically associate with some starch, which becomes inaccessible to digestive enzymes [110]. The damaged starch value was found to be the same as that of soy flour using the AACC 76-31 method. As gram flour contains more starch (43.8% in dry chickpeas) than soy flour, it must be less damaged than soy flour.

Conclusions

Hence potato, soy and chickpea flour are unsuitable for bread production from the point of view of damaged starch content unless mixed with cereal flour, or reducing sugars (which could be added to the flour in the form of glucose syrups).

The amount of damaged starch produced is related to the type of crystalline structures, the granular structure and the existence of lipids or proteins, amylose: amylopectin ratio and the arrangement of crystalline structures in the particular flour being tested. Substances also exist which inhibit the activity of enzymes. The main ones are proteins or glycoproteins and they inhibit the alpha amylase in in-vitro conditions [109]. The inhibitors are more important in legumes than in cereal [10] but are destroyed during cooking [25]. Oligosaccharides e.g. maltose and maltotriose are competitive inhibitors of alpha amylase [10]. Glucose and maltotetraose do not affect activity significantly [10]. Formation of an enzyme product complex, which cannot absorb the substrate may reduce the rate of absorption. This gives an explanation for the synergistic action between alpha amylases and amyloglucosidase, whereby amyloglucosidase can hydrolyse maltose and maltotriose into glucose, which are the reaction products of alpha amylolysis [10].

Analytical factors Affecting Damaged Starch Determination

The results for the modified AACC 76-31 method were very similar to those of the AACC 76-31 Method, despite the variation in enzyme type and concentration used. The coefficients of variation were higher for the modified AACC 76-31 method. This is probably because the enzyme used must be prepared fresh for each sample run and is subject to slight differences when weighing it. This may be controlled by running a standard, damaged starch flour (CCFRA standard damaged starch wheat flour) each time. The modified method is a good replacement for the AACC 76-31 method. However, the kit provides the user with pure enzymes in solution and a damaged starch standard. The kit, however, works out more expensive than the modified AACC 76-31 method, and each kit is limited to 100 assays.

A test was carried out based on the same principles as the modified AACC 76-31 method but using larger initial samples. The results obtained show no significant difference from those determined using smaller samples. The variation in results was also found to be similar to the modified AACC 76-31 method. Therefore, there is no benefit to testing larger initial samples of flour.

If the coefficients of variation are examined, it is shown that all of these damaged starch methods are less suited to samples containing low amounts of damaged starch than to samples containing larger amounts e.g. wheat flours. Certain damaged starch methods involve the removal of beta amylase prior to damaged starch determination [80, 94]. This is done so that the amount of enzyme digesting the damaged starch can be strictly controlled.

The Farrand method was far more time consuming than the AACC 76-31 method per sample analysed. The Farrand method takes approximately 2 hours and 45 minutes whereas the AACC 76-31 method takes just over 1 hour. The Farrand method is also limited by the fact that only flours having a certain total starch value may be determined (68 to 72 % total starch). The precision of the results for the Farrand Method is low compared to the other two methods tested. The Farrand units refer to the percentage maltose in the flour sample, however, as this is a representation of percentage damaged starch it far exceeded the results found for the AACC76-31 method and its modified version. It has been found in previous studies that the results of the Farrand method were three times higher than the official AACC method 76-30A, which indicates an approximate result of 10% [111] for wheat flour. The standard flour tested here was CCFRA standard wheat flour. This has a damaged starch value of 36 FU. However, in this study a value of 32FU was obtained. Therefore, the damaged starch values for each of the wheat flours tested by the Farrand method should be slightly higher than those obtained. A value of 7.7 % was obtained with the modified AACC 76-31 method and 6.5% with the modified method for CCFRA standard wheat flour. Therefore, the results for the AACC 76-31 method and its modified version are approximately five times lower than the value obtained from the Farrand method. It is also known that negative results can be obtained using the Farrand method and results of over 100%. The conditions of digestion using the Farrand method are different to the modified AACC 76-31 method - the enzyme used is barley amylase and the incubation time is 1 hour for the Farrand method. Farrand uses a scale in which the least damaged commercial flour available has a value of 0 and 100 represents the level in a flour that absorbed its own weight of water.

Farrand has shown that digestibility does correlate well with water absorption. In an intact granule water will only enter amorphous regions. When crystalline regions are disrupted access to the whole granule becomes possible. Hydrogen bonding occurs between exposed hydroxyl groups on starch molecules and water molecules. Protein also contributes to water absorption. The method also assumes that wheat flour contains an average of 70% starch. Damaged starch is quantified as maltose but maltose makes up only 64 to 70% of the starch digested by alpha amylase.

4:2 Comparison Between Percentage Damaged Starch Produced By Fungal Alpha Amylase and Pancreatic Alpha Amylase Using The Modified AACC 76-31 Damaged Starch Determination Method and High Performance Liquid Chromatography.

4:2:1 Effect of Enzyme Type on Percentage Damaged Starch Values

The colorimetric assay used to compare the percentage damaged starch produced by two different types of enzymes was a modified version of the AACC 76-31 method. HPLC HPAEPAD and HPLC RI systems (described in materials section) were then used to determine the actual reducing sugars produced by the enzymes in the respective flours. Incubation was carried out under the same conditions for the preparation of samples for HPLC analysis as that carried out in the colorimetric damaged starch method except that in the case of the colorimetric damaged starch assay a further incubation of samples was carried out using amyloglucosidase to convert the reducing sugars produced by amylases to glucose. Generally the sugars determined using the HPLC HPAEPAD system are quantitatively very low and this together with the fact that the concentrations obtained for each sugar were variable mean that these results are an indication only of the concentration of sugars obtained as breakdown products of starch by amylases. In this section fungal alpha amylase and pancreatic alpha amylase were compared. The same activity of alpha amylase was used for both enzymes.

Firstly a comparison was carried out on the percentage, damaged starch produced by pancreatic alpha amylase and fungal alpha amylase following 10 minutes incubation of each of the six flours tested. These included high protein wheat flour, biscuit wheat flour, maize flour, potato flour, CCFRA standard wheat flour and soy flour. The percentage, damaged starch was then compared with the reducing sugars produced.

Wheat Flours

Percentage damaged starch in high protein wheat flour was the same no matter which enzyme was used (Figure 19). The sugars produced following incubation of the high protein wheat flour with pancreatic alpha amylase and fungal alpha amylase for 20 minutes are compared in Figure 21 and 22. Using pancreatic alpha amylase 0.732×10^{-3}mg/ml of glucose was produced and using fungal alpha amylase 1.122×10^{-3}mg/ml of glucose. Both enzymes also produce maltotriose, $0.0843\ 10^{-3}$mg/ml by fungal alpha amylase and $1.564\ 10^{-3}$mg/ml by pancreatic alpha amylase.

Maltose is the main reducing sugar produced in starch by digestion with either fungal (3.903 x 10-3mg/ml) or pancreatic alpha amylase (5.782 x 10-3mg/ml). Therefore, the glucose value is higher when the flour is digested with fungal alpha amylase but the maltose concentration is slightly higher and maltotriose concentrations are much higher when digested by pancreatic alpha amylase compared to fungal alpha amylase. The glucose peak produced by fungal alpha amylase, which is shown in Figure 21, also contains glucose derived from the enzyme itself. This glucose was discoved when the enzyme was incubated with buffer without a flour sample being added. This glucose is not included in the histogram in Figure 22 and the glucose value derived from the enzyme has been subtracted from all flours to find the true value of glucose derived from the sample only. Therefore, even though the percentage damaged starch values are the same, more reducing sugars were detected by HPLC when the flour had been digested with pancreatic alpha amylase.

Again as with high protein wheat flour the percentage damaged starch value for biscuit flour was the same following digestion with both pancreatic alpha amylase and fungal alpha amylase. Figure 23c shows the reducing sugars produced in biscuit flour incubated with fungal and pancreatic alpha amylases. Slightly more maltose and more maltotriose are produced when pancreatic alpha amylase is digesting the sample. However, a greater amount of glucose is produced using fungal alpha amylase than pancreatic alpha amylase. Overall, in the case of glucose, maltose and maltotriose, more reducing sugars would seem to be produced from biscuit flour following digestion with pancreatic alpha amylase than with fungal alpha amylase even though the colorimetric method indicates that the same amount of digestion is carried out by both enzymes. A similar ratio of sugars was found in the case of biscuit flour as was found with high protein wheat flour, however, less total reducing sugars were produced in biscuit flour. This is due to the fact that biscuit wheat flour is a softer wheat flour and, therefore, less damaged than high protein wheat flour.

Figure 23a and 23b show a chromatogram and histogram of the peaks of a hydrolysate of starch obtained from the incubation of the CCFRA damaged starch standard with pancreatic alpha amylase and fungal alpha amylase for 20 minutes. From the chromatogram it can be seen that maltose was the main hydrolytic product with smaller amounts of glucose and maltotriose also produced. Again when a more highly damaged flour than high protein wheat flour is tested such as the CCFRA standard wheat flour a larger maltose value than biscuit wheat flour and a slightly larger value than high protein wheat flour can been seen. Therefore, the more damaged the flour the more maltose is produced which is as would be expected as more starch is available for digestion. This compares well with the colorimetric percentage damaged starch tests. It has been found before that the maltose concentration (as opposed to other reducing sugars) of flour samples varied most with changes in damaged starch level[86]. Those tests involved the use of the same fungal alpha amylase as was used here[86]. In those previous studies, the concentrations of other simple sugars varied little or unsystematically with damaged starch level. It was found in this study that glucose values were lower in the biscuit flour than the other two flours. The variation was unsystematic for maltotriose. Using pancreatic alpha amylase the most maltotriose was produced in high protein wheat flour, followed by the CCFRA standard wheat flour and the least was produced in biscuit flour. For fungal alpha amylase the most maltotriose was produced in CCFRA standard wheat flour, followed by biscuit flour and the least was produced in high protein wheat flour.

A higher concentration of maltose is expected following digestion with pancreatic alpha amylase than following digestion with fungal alpha amylase due to the presence of beta amylases in the former enzyme solution. Fungal alpha amylase contains only alpha amylase according to its label but the pancreatic alpha amylase used in these tests also contains some beta amylase. Alpha amylase an endo enzyme breaks down glycosidic bonds in amylose and amylopectin to produce alpha limit dextrins, maltose and glucose. Beta amylase is more restricted in its ability to digest starch and produces mainly maltose and beta limit dextrins due to the fact that it is an exo enzyme. This was found from the results of this study and shown by the fact that pancreatic alpha amylase generally produced less glucose and more maltose (Figures 23b and 23c) than fungal alpha amylase in wheat flours.

These HPLC results indicate that the AACC 76-31 and modified AACC 76-31 methods cannot determine the total concentration of reducing sugars produced in the same way as the HPLC. This is shown by the fact that damaged starch colorimetric tests show no difference in percentage damaged starch produced in these flours when different enzymes were used. However, HPLC shows that one enzyme is producing more reducing sugars than the other is. Another explanation is that the slight variation in results obtained for the enzymatic colorimetric method may not allow an increased amount of damaged starch produced by pancreatic alpha amylase to be distinguishable from results obtained following digestion with fungal alpha amylase in the same way as the HPLC can. Also fungal alpha amylase may have digested the sample resulting in the production of more dextrins than the pancreatic alpha amylase. Reducing sugars other than glucose, maltose and maltotriose were not quantitatively determined in this study using HPLC.

Non-Wheat Flours

Maize and potato flour were digested with fungal and pancreatic alpha amylase. Maize flour was tested because it is a non-wheat cereal flour with a mainly A-type crystalline structure. Potato flour was tested as it contains starch which is mainly B-type in structure.

The percentage, damaged starch in maize flour was 5.5% when fungal alpha amylase was used to digest it and 9.4% when pancreatic alpha amylase was used following 10 minutes incubation. The pancreatic alpha amylase value is nearly twice the value found for fungal alpha amylase. The same amount of each enzyme is used and incubation times are also the same, therefore, the variation in damage must be related to the behaviour of each enzyme on digestion of the particular flour sample. Therefore, pancreatic alpha amylase digests maize flour to a greater extent than the fungal alpha amylase. This may be because the maize flour is highly milled and this may allow for further digestion by a particular enzyme. Pancreatic alpha amylase may also digest in a different way to fungal alpha amylase. It is now known that following digestion of the wheat flours, different amounts of the same reducing sugars are produced by different amylases.

Figure 24a and 24b show the reducing sugars produced in maize flour following digestion with fungal and pancreatic alpha amylases. A large amount of glucose is produced by both enzymes relative to the amount of maltose produced, as compared to the results for wheat flours. With fungal alpha amylase, maltose accounts for over 80% of the sugars released. However, incubation with pancreatic alpha amylase appears to produce appreciable amounts of glucose and maltotriose also, relative to the maltose produced.

The pancreatic alpha amylase particularly produces a very large amount of maltotriose relative to that produced by fungal alpha amylase and to that produced in wheat flours. This may account for the significant difference in total damaged starch produced by each of the enzymes whereby using the modified AACC 76-31 method just under twice the percentage damaged starch is produced by pancreatic alpha amylase than is produced by fungal alpha amylase. However, in the case of maize flour the pancreatic alpha amylase produced more of all three sugars than fungal alpha amylase. Also any longer chain sugars than maltotriose have not been accounted for by HPLC. These could slightly alter the percentage damaged starch values for both non-wheat and wheat flours.

Neither enzyme used in this study will break down alpha (1-6) bonds and beta amylase cannot digest to within two to three D-glycosyl residues of an alpha 1-6 glycosidic bond in amylopectin or beyond these bonds and so is more restricted in its ability to digest the starch than alpha amylase. In the case of amylopectin glucose, maltose and alpha limit dextrins (oligosaccharides of 4 or more glucose units) are produced. Beta amylase and alpha amylase do, however, work together to enable beta amylase to digest beyond the branch points.

A previous study showed that while using porcine pancreatic alpha amylase to digest different types of starch wheat starch is degraded over 20 times faster than potato starch [61]. The percentage damaged starch values for potato flour are very low. Following 20 minutes incubation, 2.3% damaged starch is produced by pancreatic alpha amylase in potato flour and 1.4% by fungal alpha amylase. The percentage damaged starch values for potato flour as well as maize flour were both significantly higher when pancreatic alpha amylase was used. With potato starch, for the reasons discussed above, the initial breakdown is expected to be slow and damaged starch values for this flour so far have been very low. Again as was found with maize flour the structure of the starch may allow further penetration into the starch granule by pancreatic alpha amylase, a process which was not possible using fungal alpha amylase. It has been previously found that B-type starches within granules are hydrolysed more rapidly than A-type even though potato starch is generally hydrolysed more slowly than wheat starch [62]. This may be due to more accessible surface area of wheat starch compared to potato starch. In the potato starch the granules may become more exposed and more easily digestible as damage progresses. However, this does not occur up to 60 minutes. Studies on digestibility of starches using pancreatic alpha amylase have shown that canna and potato starches are the least digested and high amylose, corn starches are also poorly digested. As potato starch contains less amylose than the high amylose corn starch (23% compared to 55 to 60 %) the degree of digestibility is not, however, related to the amylose content [112].

Figure 24c compares the profiles of the sugars produced in the potato and maize flours when digested by pancreatic alpha amylase. It shows how the potato flour is generally much less damaged compared to maize flour and potato flour does produce much less total reducing sugars than other flours which is in accordance with its damaged starch value. The ratio of glucose, maltose and maltotriose was approximately 20:50:30 for both maize and potato flours digested by pancreatic alpha amylase. This differs from the wheat flours. The reducing sugars produced both for maize and potato may be due to the structure of the starch granules or the way in which these two flours are produced. The amylose: amylopectin ratios are slightly different in that potato starch generally speaking contains 80% amylopectin whereas maize starch contains 76% amylopectin [113]. As amylopectin gives the starch its crystallinity this would mean that the potato contains more of the crystalline component. The difference is, however, very small. Maize is a cereal starch and should have the same basic crystalline structure (A-type) as wheat starches, however, the wheat flours have shown a different sugar profile consisting of mostly maltose. Potato starch has mainly a B-type crystalline structure.

Therefore, the sugars produced do not depend on the crystalline structures of the starch granules and starches with different crystalline structures cannot be differentiated by observation of the sugars produced following the starch's digestion by enzymes, and determination of those reducing sugars produced by the HPLC method. The sugars produced generally are more likely to do with the type of enzyme used in combination with the type of flour it is digesting.

Soya flour following digestion with pancreatic alpha amylase also produces mainly maltose. However, the amount is much less than that of the wheat flours, because soy flour contains less total starch and consequently less damaged starch than wheat flours. Soya flour may also be complexed with lipids or prevented from being digested by the presence of the large amount of protein, which is associated with it.

As was found with the HPLC HPAEPAD system the main reducing sugar produced by fungal alpha amylase in flours and determined by the HPLC RI system is maltose followed by glucose and then by maltotriose (Table 16).

Differential Amylolytic Digestion

The fungal alpha amylase used in this analysis contains alpha amylase (40 U /mg solid). However, the pancreatic alpha amylase has an activity of 3.6U beta amylase as well as 29U alpha amylase per mg of solid. Beta amylase is not used on its own in any of these tests. Its only use in these tests is in the pancreatic alpha amylase with alpha amylase. Considering both enzymes are in excess the combination of alpha and beta amylase in the pancreatic alpha amylase exerts greater degradation than the alpha amylase alone (in the fungal alpha amylase). This is clearly shown here. It is either due to the enzyme source or the presence of beta amylase in pancreatic alpha amylase. The extent of digestion may also be affected by the different types of flours being digested. In previous studies fungal alpha amylases were found to be less active on starch granules than pancreatic, salivary, malt and bacterial alpha amylases.

It is not the case that all reducing sugars are equally greater in concentration in maize flour compared to high protein wheat flour. More maltose relative to glucose and maltotriose are produced in high protein wheat flour indicating that a different ratio of glucose: maltose: maltotriose are found in the two different flours digested by the same enzyme.

Evidence for the fact that different enzymes produce different amounts of each reducing sugar can be found from the fact that enzymes may attack starch in different ways [10]. Two mechanisms have been proposed for the hydrolysis of amylose (non-crystalline structure) by alpha amylase in solution, the multiple attack and the preferential attack [114, 115, 116]. Porcine pancreatic alpha amylase carries out the multiple attack whereas *Aspergillus oryzae* (fungal) alpha amylase carries out the preferential attack. In the multiple attack all bonds have the same probability of being disrupted (digested). After hydrolysis one part of the macromolecular chain is retained in the enzyme and one of the two-substrate fragments dissociates and moves into the active centre enabling a new hydrolysis [114]. This may occur several times before both products leave the active centre of the enzyme. In the preferential attack a single hydrolysis occurs and the two parts are then released. The internal links are then hydrolysed in a random fashion. As the chain becomes smaller, specific end effects associated with each type of alpha amylase become dominant [10]. This breakdown pattern could account for the greater percentage of maltotriose produced from flours by pancreatic alpha amylase compared to fungal alpha amylase.

When chain lengths have been reduced to 10 glucose molecules or less the reaction rate decreases. Also the type of attack pattern changes. Products with more specified lengths predominate. This is explained by the existence of sub-sites. They are distributed on both sides of the catalytically active site of the enzyme. The number of sub-sites differs between the alpha amylases. Therefore, end products of different lengths depend on the enzyme used. An example is barley alpha amylase an enzyme which is used in the Farrand method and which has nine sub-sites and produces preferentially maltoheptaose and maltohexaose [10]. A chromatogram was not obtained in this study for the digestion products of barley alpha amylase.

The action of alpha amylases on amylopectin produces alpha limit dextrins in addition to glucose, maltose and maltotriose [117]. The sub-sites effect the efficiency by which an enzyme attacks the linkage close to a branch point [10]. Depending on the source of the amylase, branches attached to alpha 1-6 bonds are three (*B.sutilis*), four (pancreatic, *Aspergillus oryzae*) or five (barley amylase) glucosyl units long [117,96]. Therefore, as pancreatic alpha amylase and fungal alpha amylase are tested here, the alpha limit dextrins they will produce have four glucose units attached to them.

In a previous study soluble potato starch and pre-gelatinised roller dried wheat starch were examined using HPLC [85]. Using the AACC 76-30A method (fungal alpha amylase) the ratio of glucose: maltose: maltotriose was 2.5:100:61.5 and with the Farrand method (barley alpha amylase) it was 0.9:100:7.8. Therefore, the different enzymes used in these two methods are resulting in the production of different ratios of the reducing sugars. The combined recovery of these three sugars using the two methods was about 77%. Other peaks obtained were thought to be as a result of branched alpha limit dextrins which would be expected to make a contribution to the reducing value of the digests.

Maltose as an Indication of Percentage Damaged Starch

If the values for maltose were taken as representing percentage damaged starch as has been done in the Farrand and the AACC 76-20A methods, the maltose values, particularly for the maize flour used in this study (which produces large amounts of maltotriose and glucose also), would not be a good representation of the amount of actual digested starch. On closer analysis of a chromatogram of reducing sugars digested from maize in Figure 20 the longer chain sugars produced by pancreatic alpha amylase can be seen. Reducing sugars up to maltoheptaose were qualitatively detected using standards. These longer chain sugars may also be contributing to the percentage damaged starch values. In all flours tested small amounts of longer chain sugars are being produced and may contribute to the total damaged starch values.

Glucose Produced by Alpha Amylases

The glucose concentrations produced from the flour samples digested by fungal alpha amylase are all quite low and may result from breakdown by amylase or alternatively, by glucoamylase, which is already present in the flour. This would be unlikely in the case of the pre-gelatinised flour as it is heat treated which would destroy any enzymes present. The enzymes in the flour may have produced, however, reducing sugars before the heat treatment was carried out. A heat treatment has also been used in the preparation of potato flour. Potato flour digestion has produced very little glucose. The values produced may be too low to be detected. It may also be because potato flour is known to be resistant to digestion. Barley flour has a very high glucose value. This may be due to the fact that it has a different structure to the other cereal flours tested (contains more B-crystalline structure). This is unlikely, however, to be the reason, as the same result was not obtained in the case of potato flour, which also has a B-type crystalline structure. It may be that barley has a lot of naturally present enzyme. This may be why malted barley was used as an enzyme source in the more traditional damaged starch determination method, the Farrand method, and why barley is so suitable for producing reducing sugars for fermentation.

Reducing Sugars Produced by Enzymes and Compared with Percentage Damaged Starch

The concentration of each reducing sugar detected was calculated as a percentage of the starting flour sample injected onto the HPLC RI following digestion of a selection of flour samples with fungal alpha amylase. The percentage of each sugar was then added together to give a final percentage damaged starch value. Glucose, maltose and maltotriose were detected. These values were compared with the percentage damaged starch values from the modified AACC 76-31 method. It was found that in almost all cases the value obtained from HPLC was greater than that obtained from the enzymatic, colorimetric damaged starch method. Therefore, the modified AACC 76-31 damaged starch method is underestimating the actual reducing sugars present in the enzymatically digested flour samples.

The same test was carried out using the HPLC HPAEPAD following digestion with fungal alpha amylase. It was found that the percentage maltose values were lower than the percentage damaged starch values obtained using the modified AACC 76-31 method. Therefore, the HPLC HPAEPAD method is either underestimating the maltose concentrations of the flours or the HPLC RI system is overestimating the maltose concentrations. Due to the fact that the percentage damaged starch value should take into account all reducing sugars and not just maltose it is more likely that the HPLC RI system is overestimating the percentage maltose derived from the flour samples. The maltotriose concentrations determined by the HPLC HPAEPAD system were less than those determined by the HPLC RI system although their values increase at similar rate for the three flours tested when determined by either method. Again it may be that HPLC RI is overestimating the concentrations. However, it may also be because the samples for the HPLC HPAEPAD system are of very low concentration, and it may he difficult to detect a sugar present in such a low concentration. The percentage glucose and percentage maltotriose concentrations were too low to be detected in potato flour.

The reducing sugars produced by pancreatic alpha amylase were also converted to percentages, and compared with the percentage damaged starch values determined by the enzymatic colorimetric method. For maize and potato flour the percentage maltose is about half the percentage damaged starch value. This is because greater amounts of glucose and maltotriose are produced by pancreatic alpha amylase than are produced by fungal alpha amylase in these two flours. For biscuit flour the maltose value is approximately he same as the percentage damaged starch value. This can be explained by the fact that very little glucose or maltotriose was produced by pancreatic alpha amylase in biscuit flour. Therefore, the pancreatic alpha amylase is digesting different types of flour to produce different ratios of reducing sugars. High protein wheat flour, however, contains a higher percentage maltose than the percentage damaged starch value. This was also found to be the case with CCFRA standard wheat flour from which a similar amount of maltose (9.3%) is obtained as the high protein wheat flour (9.0%). It is, therefore, not possible to correlate the percentage maltose produced by pancreatic alpha amylase with the percentage damaged starch results. The enzymatic colorimetric method is, therefore, not detecting all the reducing sugars produced by pancreatic alpha amylase in the wheat flours. If it were it would be found that pancreatic alpha amylase does in fact digest wheat flours as well as non wheat flours to a greater extent than fungal alpha amylase.

HPLC as a Method for Determination of Damaged Starch

Two different HPLC systems were used to quantify the reducing sugars produced following amylolytic breakdown of flours with fungal alpha amylase. The system using refractive index detection is more traditionally used for damaged starch analysis research than the HPLC HPAEPAD system. As was found in a previous study one of the main advantages of using the HPLC RI system is increased throughput of samples compared to an enzymatic colorimetric method[86]. With an automatic injector samples could be injected overnight and throughout the day while other work is being carried out. In this previous study the use of HPLC was being compared with the AACC 76-30A method which takes approximately one hour per sample[86]. This is longer than more recent methods such as the AACC 76-31 method, which takes approximately 10-15 minutes per sample.

It was found that the HPLC had greater sample capacity than the AACC 76-30 method. However, it involved greater capital cost and required more technical expertise. It was decided in previous tests [86] that a standard would be necessary to redefine damaged starch quantitatively. Enzyme activity must also be taken into account as it varies from batch to batch [86].

The HPLC RI system requires much more concentrated samples than the HPLC HPAEPAD system. However, it takes the same amount of time to prepare samples for either system. The HPLC HPAEPAD system has the advantage of detecting lower concentration sugars and also mono, di and oligosaccharides under the same conditions. Using the HPLC RI system the acetonitrile concentration (mobile phase) must be reduced to measure longer chain sugars. It may not, however, be necessary to determine the oligosaccharides as they are present in only very small concentrations. Although the HPLC HPAEPAD system has been used for analysing the breakdown products of pure starch it has not been used in this type of damaged starch determination study before.

In a previous study [86] the maltose value was determined by the HPLC RI system and compared with the damaged starch figure determined by the AACC 76-30 method. A high coefficient of determination was found between them ($r^2 = 0.97$). A good correlation ($r^2 = 0.98$) was found between the maltose values obtained by HPLC RI and percentage starch damage determined by the colorimetric assay in this study (Figure 26c). Therefore, as was shown in a previous study[86] the HPLC RI system or the HPLC HPAEPAD system could effectively be used instead of an enzymatic colorimetric method for the determination of damaged starch.

4:2:2 The Effect of Variation of Incubation Time on Percentage Damaged Starch Values

Previous studies have shown that prolonged incubation time with amylase increased apparent starch damage values [2]. The word apparent, which is used here, refers to the fact that damage cannot be definitively determined using enzymes but is empirical and depends on various factors. According to Enzymes in Food Processing [118] initially maltose and maltotriose are produced by alpha amylase from amylose and at a later stage oligosaccharides are hydrolysed to glucose and maltose. Therefore, in a digestion process showing the sugars produced over a period of time the ratio of sugars produced will tend towards the production of mainly glucose followed by maltose. Over time the amount of larger reducing sugars detectable within the method should decrease. This study investigated this theory over a certain period of time (60 minutes) in cereal and non-cereal flours.

In these tests the incubation time of wheat and non-wheat flours with pancreatic alpha amylase and fungal alpha amylase was increased to see if a further increase in percentage starch damage would be obtained. The second purpose of these tests was to observe the reducing sugars produced over that incubation time. The damaged starch test carried out here was the modified AACC 76-31 method. Flours were incubated for 10 minutes as in the modified AACC 76-31 method and at 10 or 20 minute intervals up to 60 minutes. HPLC HPAEPAD and HPLC RI were used to determine the reducing sugars produced by both enzymes at different incubation times.

Reducing Sugars Present in the Flour Samples Prior to the Addition of Amylase

0 minutes refers to the fact that no added enzyme is present and the damaged starch value refers to the reducing sugars already present in the flour. Starch damage values of flour samples to which no alpha amylase has been added are due to the action of endogenous enzymes (mainly beta amylase) on damaged granules [2] or may be produced by the actual process of damage itself. Cereal beta amylases have no activity on undamaged granules [11, 120]. However, the starch granules used here have been damaged to produce flour. Table 18 shows the maltose present in flour samples before the addition of enzymes.

Previous damaged starch determination methods have involved a stage whereby these enzymes are destroyed before proceeding with the method. This is to control exactly the amount of enzyme digesting the flours. All of the flours tested in this study had some reducing sugars present before the addition of enzymes. However, the amounts were very low, were difficult to detect, and were inconsistent.

Wheat Flour

Percentage Damaged Starch in Wheat Flour Following 60 Minutes of Incubation

The apparent percentage damaged starch in wheat flours digested by fungal alpha amylase and pancreatic alpha amylase from 0 to 60 minutes was compared (Figures 27a and 27b respectively). Using pancreatic alpha amylase, following 60 minutes digestion, apparent percentage damaged starch in both high protein and biscuit flour has increased by one to two percent (5.9% to 7.8%) and (3.7% to 4.7%) respectively. It was found that the increase in percentage damaged starch up to 10 minutes is rapid and after 10 minutes is slow and steady with generally higher damaged starch values for high protein wheat flour than biscuit wheat flour as was found before. This is expected as high protein wheat undergoes more damage by the milling process. However, the rate of increase obtained from fungal alpha amylase was not significantly different to pancreatic alpha amylase.

Reducing Sugars Produced in Wheat Flour Following 60 Minutes of Enzymatic Incubation

Figure 29a shows the effect of incubation time with pancreatic and fungal alpha amylases respectively on the concentration of maltose hydrolysed from damaged starch in high protein wheat flour. The rate of production of maltose over 60 minutes is similar using either enzyme. This results in a high rate of increase in production of maltose between 0 and 10 minutes, a slightly slower rate of increase between 20 and 40 minutes and an increase in the rate again after 40 minutes and up to 60 minutes. The production of maltose in high protein wheat flour by pancreatic alpha amylase is greater than fungal alpha amylase as was found in the previous section. The initial rate of production of maltose is probably due to the most available damaged starch up to 10 or 20 minutes. After 20 minutes endocorrosion is probably taking place.

The rate of production of maltose in biscuit flour was also determined. The overall rate of production of maltose using pancreatic alpha amylase was similar to the rate of production of maltose in high protein wheat flour. It consisted of an initial increase in rate between 0 and 20 minutes, virtually no change between 20 and 40 minutes and a second increase in rate between 40 and 60 minutes. Overall, however, much less maltose is produced from biscuit flour than high protein wheat flour. The rate of production of maltose from biscuit flour using fungal alpha amylase showed a low increase between 0 and 40 minutes and then a slightly greater subsequent increase between 40 and 60 minutes. Therefore, when percentage damaged starch is being determined in biscuit flour after 10 minutes incubation with fungal alpha amylase by the enzymatic colorimetric method, the damaged starch value is not entirely derived from maltose. Therefore, it must be as a result of glucose production or as a result of the production of longer chain reducing sugars.

Glucose production in biscuit flour and high protein wheat flour was also determined. Using fungal alpha amylase, production of glucose from biscuit flour increased between 0 and 60 minutes (Figure 29c). This shows that glucose rather than maltose can account for the overall increase in damaged starch between 0 and 40 minutes. Between 40 and 60 minutes the maltose increased at a greater rate. Using pancreatic alpha amylase the production of glucose showed its greatest rate of increase between 40 and 60 minutes in biscuit flour (Figure 29d). Up to 20 minutes very little glucose is produced. However, much less glucose is produced in wheat flour by pancreatic alpha amylase compared to fungal alpha amylase and the overall increase is very slight.

In high protein wheat flour very little glucose is produced by fungal alpha amylase in the first 20 minutes also. Following this there is a rapid increase in production of glucose up to 60 minutes. Overall using pancreatic alpha amylase the rate of production of glucose is much lower. Very small amounts of maltotriose were produced in either flour following enzymatic incubation.

The fact that more glucose is produced in both flours by fungal alpha amylase may be due to the fact that pancreatic alpha amylase contains a proportion of beta amylase that is more likely to produce maltose than glucose. Therefore, the percentage, damaged starch does not always relate to maltose produced but may depend on glucose and maltotriose and possibly some other longer chain sugars also.

The Bi-phasic Effect

Previous studies have been carried out on the rate of digestion of wheat flours. In one study digestion of wheat flour was found to occur rapidly during the first few minutes of incubation after which sound starch is digested at a slower more constant rate over a more prolonged period of time. This second stage of digestion is due to increased enzymatic access to damaged sites and hydrolysis of undamaged granules [81,2]. This is known as the bi-phasic effect [2]. The second phase is where the rate of increase in percentage damaged starch for flour samples is similar to the rate of increase in percentage damaged starch in undamaged starch samples with increased incubation time [2].

If a sound flour is analysed for diastatic activity, using the Blish-Sandstedt [119] damaged starch method over periods for 5 minutes to five hours, a rapid initial rise in maltose is got for the first 30 to 45 minutes after which it is produced in regular increments. Damaged starch is determined using the Blish-Sandstedt [119] damaged starch method by alkaline potassium ferricyanide reducing sugar method and the value got is assumed to be maltose. In this study, however, there is an initial rapid increase in the rate of digestion for the first 10 minutes followed by a slower increase up to 60 minutes. The initial rapid rise is due to autolytic digestion of the damaged starch present by beta amylase and the subsequent straight line increase is due to digestion of sound starch at a slower, but constant rate.

Differences in slopes are due to differences in susceptibilities of sound starch to attack by the amylases. Therefore, if wheat itself is high in alpha amylase activity considerably higher amounts of maltose will be produced initially and the results of the damaged starch test may not be reliable [82].

These rates of increase correspond to the damaged starch values produced by the colorimetric method in a previous study [2], which refers to an initial rapid increase in hydrolysis of damaged wheat starch granules up to 2 minutes, followed by a gradual continued increase of less accessible sites on partially damaged granules following this. This previous study [2] was only carried out over the course of 20 minutes. In those tests 50 U fungal alpha amylase was added to 100mg of wheat flour whereas in this study 200U of fungal alpha amylase per 100mg of flour was used. The sustained second phase increase in apparent starch damage suggests either that undamaged granules are susceptible to a limited rate of hydrolysis by alpha amylase or that within individual granules damaged sites exist which differ in their susceptibility to amylolytic hydrolysis [2]. The increase is due to the fact that in the measurement of starch damage using alpha amylase there is no absolute endpoint of hydrolysis [2, 81, 80]. Digestion will tend towards the eventual breakdown of all the starch present.

Oligosaccharides e.g. maltose and maltotriose are competitive inhibitors of alpha amylase [10]. Glucose and maltotetraose do not affect activity significantly [10]. Formation of an enzyme-product complex, which cannot absorb the substrate may reduce the rate of absorption. This gives an explanation for the synergistic action between alpha amylases and amyloglucosidase, whereby amyloglucosidase can hydrolyse maltose and maltotriose into glucose and at the same time continuously transform the reaction products of alpha amylolysis [10]. Therefore, the continued production of maltose and maltotriose over an increased period of incubation may in fact be hindering the further production of maltose and other reducing sugars.

Maize Flour

Percentage Damaged Starch in Maize Flour Following 60 Minutes of Incubation

The rate of change in apparent damaged starch values over 60 minutes was also determined for maize flour. The profile of sugars for maize flour in Figure 30 shows the type of sugars present before any breakdown by an enzyme. These sugars may have been produced by damage as maize is a finely, milled flour or may have been produced by the enzymes which were already present in the flour.

A rapid increase was found in the percentage, damaged starch in maize flour during the period from 0 to 10 minutes using both types of enzyme tested. It was found that, as with the wheat flours, most of the digestion occurs up to 10 minutes. The percentage damaged starch in maize flour digested by pancreatic alpha amylase (Figure 27c) increased considerably to just under double its value between 10 and 60 minutes. Digestion of maize flour by fungal alpha amylase shows a lower increase in damaged starch value after 10 minutes (1.1 to 5.5 %) compared to pancreatic alpha amylase. It can be seen, therefore, that the amount of damaged starch digested is greater in the case of pancreatic alpha amylase as was seen in the previous section and also has a higher rate of increase than fungal alpha amylase. This can be seen all the way up to 60 minutes. However, in the case of maize flour in which pancreatic alpha amylase causes increased digestion compared to fungal alpha amylase, both types of enzyme give the same rate of increase in digestion of damaged starch in wheat flour.

Reducing Sugars Produced in Maize Flour Following 60 Minutes of Incubation

Figure 31 shows the glucose, maltose and maltotriose produced from 10 to 60 minutes from maize flour following incubation with pancreatic alpha amylase. It shows an initial rapid increase of glucose, maltose and maltotriose up to 10 minutes followed by a slower, steadier increase up to 60 minutes. There is an initial rapid rate of increase in maltose production in maize flour up to 10 minutes when digested by pancreatic alpha amylase and 20 minutes in the case of fungal alpha amylase (Figures 31 and 32 respectively). This is purely to do with the fact that a reading was taken at 10 minutes for one enzyme and at 20 minutes for the other enzyme. If Figure 27c is examined which shows percentage damaged starch determinations for both enzymes it can be seen that the same rate of increase would be likely to found up to 10 minutes for fungal alpha amylase as was seen up to 20 minutes. It is uncertain from these studies at what time most of the first stage of digestion of the damaged starch occurs, but it was somewhere between 0 and 10 minutes. The rate of maltose production compares well with the rate of percentage damaged starch production up to a period of 60 minutes.

Rate of Change in Production of Individual Reducing Sugars Following Incubation For 10 to 60 Minutes.

A similar rate of glucose production up to 60 minutes was found with both enzymes, following digestion of maize flour (Figures 31 and 32). By digesting with fungal alpha amylase a higher rate of glucose is produced after 20 minutes than maltose and maltotriose in maize flour. The amount of glucose produced increases more than maltose production as digestion proceeds. It increases steadily instead of in stages as maltose does. Prolonged incubation of maize flour with pancreatic alpha amylase revealed that following 22 hours incubation, the glucose peak had become the largest peak in the chromatogram so that eventually the amylases used are going to proceed towards breaking down all the damaged starch to glucose. This is as expected [118].

The rate of increase in maltotriose production is greater when the maize flour is digested by pancreatic alpha amylase than when it is digested by fungal alpha amylase.

Overall the production of reducing sugars corresponds well with the increase in percentage damaged starch determined by the colorimetric method over a period of 60 minutes in maize flour. Therefore, pancreatic alpha amylase is digesting more starch than fungal alpha amylase over the same period of time (60 minutes) in maize flour causing further damage and endocorrosion.

Potato Flour

Potato flour, following incubation with fungal alpha amylase, has increased from 1.3% damaged starch after ten minutes incubation to 1.8% after 60 minutes incubation (Figure 27d). The digestion of potato starch, therefore, does increase after time. However, the rate of increase is very low and is not significant. This is because the starch, which is left, has a more resistant crystalline structure and the slower rate of breakdown is due to [62] the higher content of crystalline structure in potato starch and to the size of the granule [64]. For potato flour there is a lower rate of increase in the production of maltose by pancreatic alpha amylase from 0 to 20 minutes compared to maize flour and a very slow rate of increase up to 60 minutes following this (Figure 34). This is probably due to the fact that potato starch is more resistant to digestion than cereal starch and also contains much less damaged starch, which may be digested very early on leaving behind intact starch, which is difficult to digest further. The rate of production of maltotriose by pancreatic alpha amylase for potato flour is very similar to that of the rate of production of glucose (Figure 33).

However, it was also found that B structures within starch granules are attacked more rapidly than A type[62]. This was not found here as evidenced by the fact that there was no increase in the rate of production of percentage damaged starch values after 10 minutes incubation. A possible explanation is that the B-structures were all digested during the first 10 minutes, leaving A-type crystalline structures behind. This may also be because the claim regarding B structures being more rapidly digested is based on studies using very long digestion periods. It is also possible that the flour production processes which the starches have undergone before being used in this study have affected the digestibility of the starch.

HPLC RI

Chromatograms were also obtained of digested flour samples using the HPLC RI system following enzymatic incubation for 10, 20 and 30 minutes. As expected from the Dionex results the maltose value did not increase greatly between 10 and 30 minutes, however, the glucose values do increase (Figure 35) as was also seen with Dionex HPLC results. This is due to a trend towards complete breakdown of the starch to glucose. Therefore, maltose is not a good indication of percentage damaged starch at earlier stages of digestion as an increase in incubation time will result in a greater percentage of glucose in the total reducing sugars present.

High protein wheat flour was incubated for increased incubation times of 2,3 and 4 hours. It was found that there was very little change in percentage starch damage up to three hours. It can be concluded that very little starch in these flours was available for digestion after 60 minutes. However, following 4 hours incubation the amount of damaged starch increased from approximately 8 to 10 %. The enzyme is now digesting further into the intact or partially damaged starch granules and has reached a new phase of digestion.

The Effects of Increased Incubation Periods on Damaged Starch Determination

Using HPLC the type of sugars produced in a particular flour by a particular enzyme can be determined. By quantitation using enzymatic colorimetric methods the amount of damaged starch can be determined which is constant under constant conditions while remaining an empirical value. The colorimetric assay takes into account all reducing sugars produced and not just maltose. The maltose produced can be related to the overall damaged starch value and used as a damaged starch determination method but quantitatively it does not account for the same amount of damaged starch as is obtained by the colorimetric method. The rate of increase of the combined total of all reducing sugars was found to be different to the rate of increase of maltose.

Starch damage values increased with increasing time when using either enzyme. As both enzymes were used in excess it can be assumed here that endo-corrosion is occurring into less damaged or undamaged starch granules. This breaks up partially damaged or undamaged granules and as the surface area of the granules increases the rate of starch digestion increases again. This increase in rate did not increase significantly in the first 30 minutes; however, if digested up to 60 minutes the percentage damaged starch in the flour samples increases again.

4:2:3 Observation of the Progressive Digestion of Starch Granules using the Scanning Electron Microscope.

The purpose of this section of the study was to determine whether the digestion of the starch granules in flours, by the enzymatic, colorimetric method carried out above, over a period of 60 minutes could be observed through the breakdown of the starch granules and related to the determination of percentage damaged starch using the HPLC or the colorimetric method. The two flour samples tested were maize and potato, one cereal flour with an A-type crystalline structure and a non-cereal flour with a B-type crystalline structure.

Maize Flour

It was firstly found that, by using the method for preparation of the samples for examination using the SEM, (which is similar to the modified AACC 76-31 method), the granules stick together in clumps (Figure 36a) possibly due to the protein and solubilised starch present. This would consequently result in uneven digestion of the starch granules, using the enzymatic colorimetric method, due to the fact that the granules on the inside of the clumps would have much less access to the enzyme than the ones on the surface. Therefore, even though extensive mixing of the sample is carried out during determination of percentage damaged starch, the sample and enzyme are still not thoroughly and evenly mixed.

The flour samples were firstly examined undigested. On observation of the maize flour samples using the scanning electron microscope it is important to note that the starch granules have already been damaged by the milling process resulting in, as expected, granules of varying size and appearance. Naturally the granules also have a very varied morphology. The size of the granules varies from approximately 7 to 21 microns in length. They have an angular structure and a surface which is seen to be either smooth, or uneven and rough (Figures 36b and 36c). Tiny holes were also visible on the granule surfaces. This may be due to the enzyme naturally present in the flour. It was found that while determining percentage damaged starch by the enzymatic colorimetric method and by using HPLC, that some reducing sugars were detected before any enzyme is added to the flour samples. Therefore, digestion is occurring even before incubation with added enzyme during the process of damaged starch determination. Some granules have been cut into pieces and some are cracked on the surface. This is as a result of the milling process. The variation in size and appearance of the granules made the process of observation of breakdown over the selected period of time quite difficult.

Figure 37 shows micrographs taken of maize flour digested by pancreatic alpha amylase at 10 minute intervals from 10 to 60 minutes. As it can be observed from these, it is not possible to detect any progressive process of breakdown every 10 minutes.

On closer analysis, under higher magnification (Figure 38), some of the effects of digestion on the granule structure can be seen. The enzyme appears to be eating into the surface of the granule, possibly at weak points, which were cracked by the milling process, breaking down the surface in a random fashion resulting in an uneven, cracked, pitty appearance (Figures 38b and 38e) and leaving some pieces of the surface practically untouched, while roughening the surface in other places. Previous studies[9, 122] have stated that generally the surface of the granule is eroded first which can also be observed in this study. This is slight in the case of potato and other B- type starches [5]. In this study the surface has been degraded giving the granule a rough, uneven surface possibly the effect of removing amylose and leaving amylopectin. It was also found previously that in maize starch treated with porcine pancreatic amylase the radial rate of degradation was faster than the tangential one with numerous rapidly developing pin holes randomly distributed on the surface with corrosion canals showing internal saw tooth patterns inside the granules[123]. These previous observations compare well with the ones found in this study.

After 10 minutes very little endo-corrosion can be seen at higher magnifications (Figure 38a) (a small amount is visible) but after 20 minutes much more endo-corrosion has occurred due to the appearance of small holes scattered over the surface of certain granules (Figure 38b). Tiny holes (these would be characteristic of beta amylase), as well as larger ones, can be seen, indicating that endocorrosion occurs even following incubation for 10 minutes. Figure 38c shows much smaller pinholes, which are more widespread on the surface rather than fewer larger holes. There also appears to be a very general increase in the amount of endo-corrosion as digestion proceeds. This increase, however, is not visually clear cut after every 10 minute increase in incubation time.

After 50 and 60 minutes incubation (fig 38e and 38f) the digestion process is continuing in much the same way as before, with individual granules being broken down at varying rates and degrees, and in different ways. It was found that while some granules are being digested by a number of small holes, others look untouched even at a magnification of X 4000 (Figures 38b and 38d). Some granules are heavily digested while others appear smooth and undigested even after 60 minutes of incubation (Figure 38f). It was found in previous studies that for native wheat granules, hydrolysis occurs granule by granule, and an attacked granule is completely hydrolysed, the limiting step being penetration of enzymes into the granules by successive formation of pits or larger pores [121]. The number of adsorption sites for an enzyme depends on the porosity and accessibility of the substrate [10].

As the digestion process progresses, the holes produced initially increase in size and appear to penetrate towards the centre of the granule in some cases eventually resulting in a hollow granule. Some starches including wheat, barley, rye and some tropical tubers have susceptible areas, which become pitted due to endo-corrosion[5]. These pits become enlarged and sink canals of endo-corrosion into the granule, which are randomly formed around the surface[5]. This is shown in this study in Figure 38e. Previous studies have found that under the effects of pancreatic alpha amylase attack [5] maize starch granules are hydrolysed randomly and the digestion tunnels down towards the centre of the granule increasing the width of these pathways as digestion proceeds.

Previous studies have found that granules from maize taro, rice and yam are randomly hydrolysed and deep pitting canals enlarge through the granules at each shell level. In waxy maize (high-amylopectin maize) pitting is amplified by tangential corrosion cutting out the soft part of each shell. Amylomaize on the other hand is undigested externally. Only small protuberances containing a pore on the top are visible, through which the enzyme penetrates around the granule, some granules being completely empty [5]. This effect does not occur in this study, due to the fact that ordinary maize is being digested.

Previous studies have shown that during digestion of starch granules by starch degrading enzymes the softer amorphous part of the granule is more easily digested than the hard crystalline part [5]. This can be seen by looking at the rings on the starch granule in Figure 39a. In this micrograph granules have been cut into pieces to reveal the inside and the enzymes appear to be digesting between the ordered concentric rings. These rings are the crystalline part of the starch granule and the enzyme is digesting the amorphous component between these rings, presumably amylose. This process of digestion of amylose rather than amylopectin can also be observed in the case of the hollowed out granule where the crystalline layers can be clearly seen (Figure 38e). This hollowing process may be due to the fact that the surface of the granule is structurally stronger and more crystalline than the interior. According to previous studies[9, 122] starch granules appeared to be composed of successive layers or shells of more or less crystalline material. In Figures 39a and 39b the granule was most likely cut across the centre during the milling process revealing the internal crystal structure.

To observe what happens over a prolonged period of time the flour granules were digested for 20 hours. The small holes in the granule surface have become much larger, in some cases joining together to form larger gaps (Figures 39c and 39d). The pits, which are formed, can also now be clearly seen to be penetrating through the granule layers towards the granule centre (Figure 39d). Digestion is more widespread throughout the solution but is still not evenly distributed. This can be seen by the fact that some granules have a large hole on their surface and a hollowed out granule and other granules look similar to those which have been digested after only 10 minutes with small pin holes on their surface (Figure 39c). The crystalline layers can be clearly seen as the gaps in the granule enlarge. However, the general trend is towards continued endo-corrosion of the starch granules constantly increasing the percentage damaged starch.

It would be expected that as the granules are digested a greater surface area would be available for further digestion and the rate of digestion would increase. It can be seen from the above percentage damaged starch results that there is a large difference in reducing sugars present between flour incubated with no enzyme and following enzyme incubation for 10 minutes. After 20 minutes there is a slower rate of increase in reducing sugars produced due to the fact that readily available starch has already been digested. The slower rate of increase in reducing sugars is probably also due to further endo-corrosion. This would correspond to the maltose produced by the colorimetric method relating to the hydrolysis of damaged granules and the later second phase of slow hydrolysis of undamaged granules or the hydrolysis of less accessible sites on partially damaged granules. Between 20 and 40 minutes little difference can be seen between the amount of reducing sugars produced.

After 40 or 50 minutes the process of digestion may result in increased surface area as granules are persistently hollowed and broken up. Therefore, at this stage an increase in rate of production of reducing sugars may again occur. At 60 minutes a second increase in the rate of reducing sugars produced can be seen. Corresponding change in the appearance of the maize granules cannot be observed using the SEM due to the variation in rate of digestion of individual granules and variation in the types of digestion of each granule.

Overall this type of starch, which is from a cereal source, and has an A-type crystalline structure seems to have been digested by several different processes here. An overview of granule digestion shows that some granules are highly digested whereas others in close proximity appear to be untouched. This is as a result of readily available damaged sites on the granules and the initial onset of endo-corrosion, which does start to occur before the addition of any enzyme. The enzymes are then relying solely on the process of endo-corrosion for the production of reducing sugars following 10 minutes and possibly earlier.

There appears to be no distinct difference found in this study between digestion by fungal alpha amylase and by pancreatic alpha amylase. Fungal alpha amylases were, however, previously found to be less active on starch granules than pancreatic, salivary, malt and bacterial alpha amylases [12]. It has been shown microscopically that the alpha amylase of *Aspergillus oryzae* produced larger pores on the surface of maize starch than other amylases and produced maltose as the main product [13].

In this study the same processes of breakdown occur with fungal alpha amylase and pancreatic alpha amylase, with extreme digestion of certain granules occurring alongside no digestion of other granules, within the same time frame (Figure 40b). The same pin hole digestion, together with larger hole digestion, can also be observed. When two sets of granules were equally well exposed to 10 minutes digestion, one set show virtually no digestion except for a roughened surface, whereas the second set of granules show large digestion holes on their surface digesting towards the inside of the granule.

In conclusion it was found that there are three main ways in which the damaged maize starch granule may be broken down by alpha and beta amylase. However, individual granules behave differently depending on how much damage they have been subjected to before digestion and possibly also the variation in their individual structures. Results were found to be similar to previous studies, which involved the observation of undamaged granules. However, time related digestion had not been studied before.

Potato Flour

Potato flour may be produced in a variety of ways, for example the USA and Germany produce it by two very different methods. The USA produces farina. By this method starch is separated by a wet washing process, allowed to settle, centrifuged and dried. In Germany potatoes are scrubbed, peeled and sliced and the slices dried by heat, ground and sieved [124]. The potato flour used here is Canadian and is prepared by pealing, trimming, cooking and flaking in a drier and finally grinding.

It has been found previously that potato starch is more difficult to digest than other types of starch. Potato starch has a higher content of crystalline structure. The resistance of the B-type structure of potato starch may be due to a greater number of crystallites on the granule surface [10]. Previous studies have found that at a higher magnification under the SEM, granules treated by enzymes appear to be made up of small, almost spherical blocklets coming together tangentially[10]. In the B-type pattern larger blocklets are superimposed at the peripheral level. These are also seen in the C pattern. This is thought to be why the B and C crystalline structures of starch are more resistant to hydrolysis than the A-type crystalline structures. Unpublished work of Gallant and Bouchet has found that the blocklets are packs of crystals composed of small crystalline sheets stacked tangentially to the granule surface[10]. Potato starch granules have a larger granule surface area and, therefore, a lower total surface area, which is generally smoother than cereal starches. Previous studies have shown that generally the surface of starch granules are eroded first during digestion and that this is slight in the case of potato and other B- type starches [5].

On first observation of the micrographs in this study the potato granule as expected is much larger than the maize granule with a much greater variation in size, approximately from 9 to 63 microns in length. The surface is smoother and rounder and generally has an egg or ovoid shape (Figure 41b). Very few granules appear to be broken but some broken ones are visible (Figure 41c). Despite the preparation process which they have undergone their appearance after no addition of enzyme is in most cases intact. Some of the surfaces on closer inspection are cracked (Figure 41c) or scratched. Mainly the granules are smooth with slightly rough areas at the pointed ends (Figure 41b).

An overview of the digestion process every 10 minutes can be observed in Figure 42. As was found with the maize flour there is no regulated, clearly visible increase in digestion every 10 minutes. Several views were taken of the starch granules as was done with maize flour and examples are shown in the results section. Therefore, the different types of digestion processes were instead examined.

Previous studies have found that potato starch and other B-type crystalline starches are slightly surface eroded by amylase [2]. This can be seen in this study in Figure 41d. This roughened, cracked surface may be due to the flour preparation process. After 20 minutes of digestion there is more striking evidence of breakdown by enzymes. A cracking process is becoming increasingly visible and also a hollowing out process can be seen (Figure 42c). These appear to be as a result of enzymatic digestion, as smooth round holes can be seen in some granules, which then enlarge and open out to eventually leave thick, hollow shells. In some cases a thick shell only remains (Figure 41e). Therefore, the inside of the granule is obviously much more readily digestible than the outer shell. It also appears to be more easily digestible than the inside of a maize granule. Also rather than damaging using a series of holes as in the case of maize flour the enzyme seems to accentuate the crack and form one break in the surface which gets larger eventually opening out the granule. The granule may be being hollowed at the stage when only a crack or a small hole is visible. It was found in a previous study that potato starch granules digested by alpha amylase showed " collapses, cracks and erosions"[125]. It was also found in another study that alpha amylase caused several large pits to be formed in B type granules and small pits were formed by beta amylase[16]. Small pits were not seen in this study on potato flour following digestion even though the pancreatic alpha amylase does contain beta amylase. It was also found in another study that as soon as the enzyme attack moved towards the central parts of the potato starch granule digestion occurred at an increased speed so that there were no fragments (small pieces of starch granule), which were present following the hydrolysis of cereal starches[62]. This can be seen here also, as the fragments, which were seen in the maize flour micrographs are not seen here with potato flour. Therefore, the observations of previous studies do in some way relate to what is being seen here, however, in those cases intact starch rather than damaged starch granules were being observed.

Figure 41d shows initial stages of cracking on the granule surface. These cracks may have been formed during the production of the flour and may be the area where the beginning of the digestion process occurs. Figure 41e shows a granule, which has opened out revealing a thick shell and a centre containing what appear to be smaller granules. Figure 41f shows that in fact the granules may be hollowed to reveal an inner granule. As the granules are hollowing they seem to be doing so in layers producing what looks like a smaller potato granule inside and in some cases more than one smaller granule again. The granule appears to have a strongly crystalline layer followed by an amorphous layer which is easier to break down followed by another crystalline layer and so on. It may, however, also be the case that the smaller granules, through the process of shaking, have positioned themselves inside the larger ones. This hollowing process continues to be visible right up to 60 minutes of digestion.

Again the rate of digestion and determination of percentage damaged starch by colorimetric damaged starch methods in the first 20 minutes is quite high compared to the rate following 20 minutes which is shown in the above chromatograms. This cannot be clearly observed using micrographs. General increased endo-corrosion can only be seen (Figure 42).

Again use of fungal alpha amylase as compared to pancreatic alpha amylase shows no real difference that can be detected in this study. Figure 43a again shows the initial stages of formation of a hole on the granule surface. This occurs alongside a crack but the digestion is occurring in a circular form rather than along the crack. Figure 43b demonstrates that the granule may have an inner granular layer, which is removed when the shell opens. Figure 43c shows part of a granule which has been hollowed leaving a thick shell and what appears to be rings on the inside indicating the crystalline structure of the starch granule. In only one case surface digestion was observed as an eroding process on the granule. Tangential cracks can be seen although the granule may also be hollow as well (Figure 43d).

The processes of breakdown for the two different types of flour examined in this study (maize and potato) vary considerably. This is due to the different types of crystallinity in the different starches, mostly A in the case of maize and B in the case of potato. The amylose: amylopectin ratios are slightly different in that potato starch generally speaking contains 80% amylopectin whereas maize starch contains 76% amylopectin [113]. As amylopectin gives the starch its crystallinity this would mean that the potato contains the more crystalline component, which implies that it may be more difficult to digest. However, the ease of digestion is more likely to be related to the arrangement of the starch in its crystal structure rather than the amount of amylose and amylopectin. Potato also has less surface area available for digestion than maize, and the two flours have also been prepared in different ways. Another reason for variation in breakdown is the presence of lipids or proteins with the starch. Potato flour contains almost all starch, whereas maize flour also contains significantly larger amounts of protein and some lipid.

It can be concluded that the digestion process of starch granules cannot be observed every 10 minutes by the observation method used here. However, the general process of breakdown and the types of breakdown expected can be observed. As was found by previous studies each individual granule is digested differently. Further work could be done in this area. In order to more closely observe the stages of breakdown of the starch granules a small number of granules could be isolated and monitored while they are being digested. In this study some granules were highly digested and others were untouched in the same solution of starch following incubation with enzymes for a fixed period of time.

4:2:4 Determination of the Effect of Digestion of Starch Granules on Crystallinity using X-ray Powder Diffraction

X-rays are diffracted by the crystalline structure of starch granules. There are two principle types of diffraction diagrams obtained corresponding to A and B type crystalline structures. Starch granules contain a crystalline structure that may be broken down by damage. Whether this crystalline structure is affected by digestion with alpha amylase is what is to be examined here.

Diffractometry spectra for A and B crystalline patterns are shown in the introduction in Figure 8. The first flour sample to be examined here had an A-type crystalline structure and was biscuit wheat flour. In the reference diagram for the A-type crystalline structure Figure 8, peaks were observed at 23.3 degrees and 18.1, 17 and 15 degrees. Peaks should also be present at 9.9 and 11.2 degrees. For the biscuit flour sample tested in this study peaks were observed at 15, 17, 18.1 and 23 degrees. This is shown in Figure 44 in the results section. A small peak can be observed at 19 degrees. Lower peaks can also be seen at between 9 and 13 degrees.

Therefore, the crystalline pattern of the biscuit flour corresponds very well with the reference diffractogram even though the starch used here is from a flour which contains damaged starch. For the biscuit flour diffractogram the peaks are not as well resolved as those of the reference diagram. This is due to the fact that for the biscuit flour the sample is not pure starch and the milling process has also damaged the starch. The levels of hydration of the sample also affect the intensity of the peaks. The more hydrated the sample the more intense the individual peaks. The flour sample used in this study may have been drier than the reference sample. The biscuit flour used here had a moisture content of 9.6%. In Figure 9 it can be seen that the moisture content was between B - 8% and C – 11%. These diagrams in Figure 9 show that the lower the moisture content of the samples the less well defined are the peaks.

Maize flour, which also has an A-type crystalline structure was compared with biscuit flour and the corresponding crystallinity can be clearly seen from Figure 45 which shows maize flour and biscuit flour chromatograms slightly offset from one another. The peak between 10 and 13 degrees is absent in the case of maize flour. This may be due to the fact that maize flour is a finely, milled flour which may have resulted in loss of some of the crystallinity. It may also be because maize flour is a different type of flour than wheat flour and has a slightly different type of crystalline structure.

Potato flour was also examined in this study as it has a B-type crystalline pattern. The reference diffractogram in Figure 8 shows peaks at 24, 22 a large peak at 17, a double peak at 15 and a large peak at 5.6 degrees. The diffractogram for potato flour (Figure 46) shows a large peak at 17 and a double peak at 14 and a slight peak at 22. There is a small peak at 6 degrees. Less of the peaks are seen here to correspond with the reference diffractogram than was found in the case of the biscuit flour and its reference, showing possibly a greater loss of crystallinity in the potato flour preparation process. However, certain corresponding peaks are again comparable with the reference chromatogram. Figure 47 shows potato starch compared to potato flour. For potato starch peaks can be clearly seen at 18, 20, 23 and 25 degrees. All the peaks are much larger in the case of potato starch than with potato flour although peaks at the same degrees are seen in both. Therefore, these peaks may be reduced in size by the flour preparation process. An alternative reason is that the starch sample may be more hydrated than the flour sample. However, this is not the case as potato flour sample contains 13.2% moisture and the potato starch 8.09% moisture. There are two peaks at 14 and 15 degrees, which are larger in the case of the flour than in the case of the starch. These may have been formed as a result of retrogradation of starch during the flour making process.

Figure 48 shows the crystallinity in a resistant starch standard. The resistant starch standard is made of amylomaize, which is a cereal flour, which has been gelatinised and cooled to produce resistant starch. Peaks are seen at 17, 20, 22 and 24 degrees. The peaks are very similar to those of potato starch and the reference B-crystalline chromatogram except for the large peak at 20 degrees. If the resistant starch chromatogram is compared with that of ordinary maize flour, the maize flour has an extra peak at 15 degrees which has been lost during production of resistant starch and the resistant starch standard has an extra peak at 20 degrees created during production of resistant starch. The difference in peaks found might also be due to the fact that the resistant starch is made up of amylomaize, which contains more amylopectin than ordinary maize. As amylopectin gives the granule its crystallinity the extra amylopectin in the resistant starch sample may account for the different peaks observed.

Digestion of Flours and Its Effect on Crystallinity

Firstly the method for the preparation of samples was tested without the addition of any enzyme to see if the process itself had any effect on the crystallinity of the flour. Very little effect was had on biscuit flour (Figure 49). The biscuit flour was then digested for 30 and 60 minutes to see if the digestion process resulted in reduction in crystallinity. The moisture content of the digested flour samples was 10.1% moisture which is very similar to the undigested sample. Therefore, the moisture content would have little effect on the result in this case. Figure 50 shows biscuit flour digested for 1 hour. Very little change in crystallinity can be seen from this diagram. There is a slight reduction in the size of some of the peaks. This is probably due to slight difference in hydration of the two samples.

Figure 51 shows how there was no change in crystallinity following the preparation process for the potato flour sample. It also shows potato flour and potato flour which has undergone the preparation process, without any enzyme added. Figure 52 shows that digestion of potato flour for 30 minutes had no effect on crystallinity. Figure 53 shows that digestion of potato flour for 60 minutes had no effect on crystallinity.

Therefore, no reduction in crystallinity of starch granules digested by alpha amylase can be detected by this method of x-ray powder diffraction.

4:3 CONCLUSIONS

1. Enzymatic, colorimetric methods are generally not a good indication of the level of damage in starch. Colorimetric dye absorption tests are more indicative of starch damage levels and enzymatic, colorimetric methods give an indication of digestible starch levels.

2. The results obtained by using the modified AACC 76-31 method were very similar to those of the AACC 76-31 Method despite the variation in enzyme purity and concentration used. The results for the AACC 76-31 method and its modified version are approximately five times lower than the value found for the Farrand method. The Farrand method was far more time consuming than the AACC 76-31 method. It was found that all methods tested in this study are less suited to samples containing low amounts of damaged starch than to samples containing larger amounts. It can, however, be concluded that the AACC 76-31 method or the modified version used in this study are faster, more economical and more accurate that the traditionally used Farrand method.

3. Percentage damaged starch determined using the colorimetric assay in the two selected wheat flours tested (biscuit wheat and high protein wheat) was the same when either pancreatic alpha amylase or fungal alpha amylase were used. A similar profile of reducing sugars was found from biscuit flour digested using either pancreatic alpha amylase or fungal alpha amylase as was found with high protein wheat flour. However, a greater percentage of reducing sugars were produced in high protein wheat flour by pancreatic alpha amylase than by fungal alpha amylase. It is, therefore, likely that the pancreatic alpha amylase is digesting a greater amount of high protein wheat flour than fungal alpha amlylase is. Less total reducing sugars were produced in biscuit flour. This is due to the fact that biscuit wheat flour is softer and, therefore, less damaged that high protein wheat flour. Maltose was the main reducing sugar produced and the more damaged the flour the more maltose was released.

The percentage glucose was found to be slightly higher when the flour samples were digested with fungal alpha amylase. However, the maltose concentrations were slightly higher and the maltotriose concentrations were much higher when the flours were digested by pancreatic alpha amylase. Therefore, the colorimetric method is probably not detecting all the reducing sugars produced by pancreatic alpha amylase. The glucose values in the flour samples digested by fungal alpha amylase are, however, all quite low and may result from breakdown by alpha amylase, or alternatively, by glucoamylase which is already present in the flour.

Pancreatic alpha amylase digested both maize flour and potato flour to a greater extent than the fungal alpha amylase. Therefore, the combination of alpha and beta amylase in the pancreatic alpha amylase exerts greater degradation than the alpha amylase alone in the fungal alpha amylase on both maize and potato flours as compared to wheat flours. This is either due to the enzyme source, the presence of beta amylase in pancreatic alpha amylase or the different types of flours being digested. The reducing sugars are greater in concentration in maize flour compared to high protein wheat flour, which is because maize flour is more highly damaged. However, more maltose relative to glucose and maltotriose was produced in high protein wheat flour compared to maize flour.

Therefore, for each of the different flours tested the different enzymes used in these tests are resulting in the production of different ratios of the reducing sugars. The maltose values particularly for maize flour would, therefore, not be a good representation of the amount of digested starch as significant amounts of other reducing sugars were also produced. Therefore, the percentage damaged starch does not always relate to maltose produced but may depend on glucose and maltotriose and possibly some other longer chain sugars also. Therefore, maltose is not a good indication of actual percentage digestible starch values. Therefore, it was found by damaged starch tests that the pancreatic alpha amylase digested more starch than fungal alpha amylase. In the case of both maize and potato flours the increase is mainly derived from the increased production of maltotriose.

The sugars produced do not depend on the crystalline structures of the starch granules and starches of different crystalline structures cannot be distinguished between by observation of the sugars produced following their digestion by enzymes and determination of these sugars by this HPLC method. The sugars produced generally are to do with the type of enzyme used in combination with the flour it is digesting. Reducing sugars up to maltoheptaose were qualitatively detected using standards. A greater amount of starch was digested when alpha amylase was incubated in combination with amyloglucosidase. This is because amyloglucosidase can digest a certain amount of damaged starch to glucose without the assistance of amylase and also because certain reducing sugars including maltose inhibit further digestion by amylase. This inhibition is prevented by the maltose being converted to glucose, which does not inhibit amylase digestion. The HPLC RI system requires much more concentrated samples than the HPLC HPAEPAD system, however, it takes the same amount of time to prepare samples for either system. The HPAEPAD HPLC system has the advantage of detecting lower concentration sugars and also mon, di and oligosaccharides under the same conditions. The HPLC RI system requires much less capital cost.

It was found that the enzymes used in this study were active over a wide range of pH values but had an optimum activity at approximately pH 5.5.

4. It was found that during up to 10 minutes incubation with an enzyme most of the digestion of starch in the flour samples occurs. A rapid increase in glucose, maltose and maltotriose was produced up to 10 minutes followed by a slower, steadier increase up to 60 minutes. As both enzymes were used in excess it can be assumed here that endocorrosion is occurring into less damaged or undamaged starch granules. The enzyme is now digesting further into the intact or partially damaged starch granules and has reached a new phase of digestion. Overall the production of reducing sugars corresponds well with the increase in percentage damaged starch determined by the colorimetric method over a period of 60 minutes. The rate of maltose production also compares well with the rate of percentage damaged starch production up to 60 minutes. A good correlation (R = 0.98) was found between maltose values obtained by HPLC RI and percentage starch damage determined by the colorimetric assay in this study. The maltose produced can be related to the overall damaged starch value and used as a damaged starch determination method but quantitatively it does not account for the same amount of damaged starch as is obtained by the colorimetric method. Therefore, as was shown in a previous study this HPLC system could effectively be used instead of an enzymatic colorimetric method for the determination of damaged starch.

It was found that a higher rate of increase in the amount of damaged starch digested from maize flour occurs in the case of pancreatic alpha than when fungal alpha amylase is used. The rate of increase in percentage damaged starch produced in wheat flours was the same when either enzyme was used. It was found that the incubation time has a greater effect on starch digestion than alpha amylase activity.

5. Using the SEM tiny holes were found to be visible on the undigested maize starch granule surface in the flour sample, which may be due to enzyme naturally present in the flour. As was found before while determining percentage damaged starch, some reducing sugars were detected before any enzyme was added to the flour samples.

It was found that, while some maize starch granules are being digested to form a number of small holes, others look untouched even at a magnification of X 4000. Individual granules are digested differently depending on how much damage they have been subjected to before digestion and possibly also variation in their individual structures. Also the softer amorphous part of the granule is more easily digested than the hard crystalline part. This can be seen by observation of the rings inside the starch granule. A hollowing process, which also occurs, may be due to the fact that the centre of the granule may have weaker crystallinity than the surface. Generally the surface of the granule was eroded first. Therefore, it is at this stage that an increase in rate of production of reducing sugars may again occur.

As the digestion process of the maize starch progresses, the holes produced initially, increase in size and appear to penetrate towards the centre of the granule. In some cases this eventually results in a hollow granule. After 20 minutes there is a slower rate of increase in reducing sugars produced due to the fact that readily available starch has already been digested. This is probably also due to further endocorrosion of undamaged granules or the hydrolysis of less accessible sites on partially damaged granules.

Despite the preparation process which the potato granules have undergone their appearance after no addition of enzyme is in most cases intact. Some of the surfaces on closer inspection are cracked or scratched A hollowing out process can be seen which, appears to be as a result of enzymatic digestion as smooth round holes can be seen in some granules, which then enlarge and open out to eventually leave thick, hollow shells. As the granules are hollowing they seem to be doing so in layers producing what looks like a smaller potato granule inside and in some cases more than one smaller granule again. These digestion processes are likely to be related to the arrangement of the starch in its crystal structure.

It can be concluded that the digestion process of starch granules cannot be observed every 10 minutes by the preparation method used here. However, the general process of breakdown and the types of breakdown expected can be observed.

6. No reduction in crystallinity of starch granules digested by alpha amylase can be detected by x-ray powder diffraction.

7. It was found that increase in heat has a greater effect on digestible starch than increase in time exposed to heat.

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