

# **The stability of enzymes during breadmaking and their influence on staling characteristics**

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for the degree of

*Master of Science (Food Science and Technology)*

By

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The stability of enzymes  
during breadmaking and their  
influence on staling

## Declaration

I hereby declare that all work carried out in this project was performed while I was enrolled as a Masters student in the School of Life Sciences and Technology, Victoria University of Technology, Werribee Campus. To the best of my knowledge, this work has not been submitted in whole or part for any other degree or diploma in any University and no material contained in this thesis has been previously written or published by another person, except where due reference is made in the text.



Hatinder Sodhi

March 2003

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## Abstract

Baked products continue to enjoy popularity in many parts of the world. This reflects the appealing sensory attributes of the fresh products, including the soft textural characteristics of the freshly baked goods. Staling remains a significant cause of economic loss in breads due to the loss of saleability resulting from crumb firming. The aims of the current project have been to investigate some of the factors which may impact on staling, particularly the stability of amylolytic enzymes in bakery formulations. In addition, the role enzymic hydrolysis of starch in producing partial hydrolysis products and the potential of microencapsulation as a means of studying these effects is investigated.

Recently developed assay methods which are able to specifically measure  $\alpha$ -amylase and  $\beta$ -amylase, in the presence of the other potentially interfering activity, have been evaluated. These have been applied to an investigation of enzyme levels at various stages during the breadmaking process, using a cereal source of the enzyme  $\alpha$ -amylase. For this a rapid dough formulation and processing procedure were used.  $\alpha$ -Amylase activity levels appeared to gradually increase during the proofing stages and then to decline upon heating of the dough. However, the activity remaining in the final baked loaf was readily measurable indicating that not all of the enzyme had been inactivated. Free and total  $\beta$ -amylase activities were also measured. Most of the enzyme was found to be in the free form although a higher proportion was bound in the dough than in the wheat flour used for baking.  $\beta$ -Amylase was heat labile with only relatively low activities remaining in the final baked loaf. It appears that of the two amylolytic enzymes,  $\alpha$ -amylase is sufficiently stable that it may exert some impact on the crumb characteristics in the freshly baked product and during subsequent storage.

In order to assess the likelihood that amyolysis is of significance in influencing crumb characteristics, samples were also extracted and analysed for the levels of low molecular weight carbohydrates. For this purpose high-performance liquid chromatography was used to analyse the aqueous extracts for maltose, glucose, fructose, lactose and sucrose. Commercial flours were found to contain low levels of sugars with maltose being the predominant sugar present. For comparative purposes, a number of commercial breads

were also analysed and the composition found to vary between the different samples. Typically maltose was present at higher levels than the other sugars. When experimental loaves were analysed, the patterns showed that other sugars declined during proofing whereas maltose remained at readily measurable levels. Upon baking and subsequent storage the amounts of maltose increased. These results are consistent with the findings that some amylolytic activity remains in the baked product.

In the third phase of this study, a potential means of investigating the role of particular carbohydrates in product textures and staling rates was examined. The approach of spray drying was used to prepare microencapsulated maltodextrin. The encapsulating agents used were based upon rice starch and guar galactomannan. When these microcapsules were incorporated into the breadmaking formulation and baked, it appeared that softer crumb characteristics were achieved. The data also indicates an effect of delay in the staling rates.

From these studies it is concluded that cereal grain  $\alpha$ -amylase may be more stable during breadmaking than previously thought. There appears to be an increase in the level of some low molecular weight sugars in the final, baked product.

Microencapsulation may offer a useful technique for the study of the role of specific low molecular weight carbohydrates and dextrin fractions during baking and storage of breads. These findings could form the basis of further research into staling of breads.

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## Abbreviations

|                      |  |
|----------------------|--|
| <b>AACC</b>          | American Association of Cereal Chemists  |
| <b>AOAC</b>          | AOAC International (formerly known as Association of Official Analytical Chemists)                                     |
| <b>AWB</b>           | Australian Wheat Board   |
| <b>ASW</b>           | Australian Standard White  |
| <b>A\$</b>           | Australian dollars   |
| <b>BRI</b>           | Bread Research Institute of Australia  |
| <b>BSA</b>           | Bovine serum albumin   |
| <b>DP</b>            | Degree of polymerisation   |
| <b>EU</b>            | European Union   |
| <b>HPLC</b>          | High-performance liquid chromatography   |
| <b>IFT</b>           | Institute of Food Technologists (Chicago)  |
| <b>na</b>            | not applicable   |
| <b>NHMRC</b>         | National Health and Medical Research Council (Australia)   |
| <b>r<sup>2</sup></b> | coefficient of determination for a regression line   |
| <b>rpm</b>           | Revolutions per minute   |
| <b>sd</b>            | standard deviation   |
| <b>SKB</b>           | Sandstedt, Kneen and Blish units of $\alpha$ -amylase activity (further described in Chapter 2, Section 2.15. page 20) |
| <b>U</b>             | Enzyme Units (defined in Chapter 3)  |
| <b>UK</b>            | United Kingdom   |
| <b>USA</b>           | United States of America   |

## Explanatory notes

The purpose of these notes is to briefly describe the approaches adopted during the preparation of this thesis. They relate to issues including the expression of enzyme assay and other analytical results, as well as the referencing of literature sources:

1. Where alternative spellings are in common use then the British rather than the American approach has been adopted in the text. Examples include the term colour (rather than color), words ending with –ise (rather than –ize) and some technical terms.
2. Experimental data is presented on a variety of moisture bases. The approach applied for particular data depends upon on the purpose. For example, a dry weight (or dry matter) basis was used rather than a fresh weight (or “as is”) basis was used for comparison of the levels of enzyme activities at various stages of the breadmaking process. This approach was adopted in order to facilitate direct comparisons of results obtained at different processing stages. Values for the contents of sugar components are presented on an “as is” basis to allow convenient comparison with literature data. The calculation of data is described in Section 3.9.
3. In the citation and listing of references and information sources, the current recommendations of the Institute of Food Technologists (IFT) for the Journal of Food Science (IFT 2002) have been applied throughout (see page 87).

# Chapter 1

## Introduction

The purpose of this chapter is to briefly describe the background to the research program described in this thesis. This project has been developed on the basis of the following issues:

- Baked goods are staples and sources of enjoyment around the world;
- Baked products typically are made from ingredients which include a variety of enzymes which are important in determining the quality and appeal of these foods;
- The levels of simple sugars in flours and baked goods are often low. Although sugars are produced by hydrolysis of starches these are usually utilised by yeast during fermentation;
- Three alternative types of  $\alpha$ -amylase are available for use in bread formulations and these are from cereal grain, fungal and bacterial sources;
- It has been reported that the  $\alpha$ -amylases from the three different sources have quite different stabilities to heat;
- Most of the studies into the stability of these enzymes have involved extraction of the enzyme into dilute aqueous solutions. Whether these conditions replicate the effects of *in vivo* heating processes within the dough and particularly during baking is unclear;
- The assay of amylolytic enzymes has generally been limited by the lack of specific procedures that would allow the assay of one particular enzyme in the presence of others which also hydrolyse starch molecules. Recently new substrates that overcome this problem have become readily available;
- In addition there is evidence that the enzyme may also produce partial hydrolysis products which are potentially of significance in influencing the rate of staling of breads;
- It remains unclear as to what the significance of different enzyme sources may be and the relative impact on bread characteristics of the many different partial hydrolysis products of starch remain to be fully elucidated;

- One of the newer techniques which might be of value to investigations of these issues is microencapsulation. If some of the products could be encapsulated into capsules which were ultimately released into a dough during processing, then it may be possible to clarify the role and significance of these molecules.

Accordingly, the research described in this thesis has been based upon the hypothesis that enzymes may actually remain active despite the heating of the baking process and may therefore be able to contribute partial hydrolysis products that, in turn, change the sensory attributes of baked goods and their staling characteristics.

This project seeks to investigate the stability of the main amylolytic enzymes during baking and to assess the potential of microencapsulation as a tool to further our understanding of the baking and staling processes.

## Chapter 2

### Background and literature review

The purpose of this chapter is to provide background and review the relevant scientific literature on the importance of breads and the baking process. The areas covered are the formulation and processing of bread, the roles of enzymes, particularly the amylases.

#### 2.1 The significance of wheat

Wheat is grown throughout the world. It is adaptable to a wide range of environmental conditions from xerophytic to littoral. Wheat flourishes in subtropical, warm temperature and cool temperate climates. An annual rainfall of 229-762 mm, falling more in spring than in summer, suits it best. The mean summer temperature should be 13 °C or more.

#### *Area, production and yield of wheat*

Between 1965 and 1989 the world wheat area showed a small increase (from 215 to 227 Mha) while wheat production doubled, from 261 to 537 Mtonnes per annum, reflecting the world average yield over the period, from 1.2 to 2.36 kg per ha (Kent and Evers 1994).

The capacity for cereal production continues to increase due to the use of higher yielding varieties, and by changes in husbandry. The use and spread of the high yielding varieties brought a revolution known as the Green Revolution. This is an expression used to describe the rapid spread of high yielding dwarf varieties of wheat. The effect of the Green Revolution in India, for example, has been to increase the amount of wheat available to the total population from 23.4 kg per person in 1967 to 52.5 kg per person in 1984-1986 (Kent and Evers 1994).

#### *The Australian grain industry*

The Australian grain industry is a major contributor to the output of the rural sector. The five - year average annual value of the grain production is about A\$4.27 billion. By far the largest enterprise in the Australian grain industry is wheat production, with the

annual gross value of production averaging around A\$2.3 billion over the past five years. This amounts to around 54% of the total value of grain production (AWB 1998).

Today, Australia harvests up to 22 million tonnes of wheat when favourable conditions occur. Average annual wheat exports have been 17 million tonnes, having a total value of A\$3 billion and in some years wheat has been the leading export earner (O'Brien and Blakeney 1998). Globally, Australia ranks as one of the five leading exporters of wheat; generally after the United States, Canada and the European Union (EU). More than 80% of the wheat produced in Australia is exported and in recent years the figure has approached 90% (Simmonds 1989; Wrigley 1994). Australian wheat is shipped to more than 50 countries (AWB 1998) as summarised in Table 2.1.

As the domestic demand is limited due to Australia's small population, consumption is relatively stable from year to year (around 2 million tonnes) except in the drought year's when stockfeed demand increases significantly. The major sector of the domestic wheat market is for wheat sold to flour mills to produce flour for human consumption. In an average year, this accounts for about 1.5 million tonnes of wheat. A further 360,000 tonnes of wheat are milled for production of starch and gluten (Table 2.2). Total wheat usage in stockfeed rations can range from 500,000 tonnes to 1.9 millions tonnes annually (AWB 1998).

**Table 2.1 Major destinations for Australian wheat exports (three year averages 1992-93 to 1994-95)**

| <b>Destination</b> | <b>Tonnes</b> |
|--------------------|---------------|
| Abu Dhabi          | 110,942       |
| China              | 835,074       |
| Dubai              | 95,574        |
| Egypt              | 892,787       |
| Former USSR        | 478,621       |
| Indonesia          | 1,107,715     |
| Iran               | 1,411,754     |
| Iraq               | 180,434       |
| Japan              | 1,181,105     |
| Malaysia           | 640,759       |
| New Zealand        | 195,044       |
| Oman               | 163,505       |
| Pakistan           | 110,641       |
| PNG                | 116,826       |
| Singapore          | 126,162       |
| South Korea        | 949,738       |
| Yemen Republic     | 185,114       |
| Others             | 1,269,982     |

Source: AWB 1998

**Table 2.2 Annual domestic Australian wheat usage**

| <b>Enduse</b> | <b>Tonnes</b>     |
|---------------|-------------------|
| Flour         | 1,500,000         |
| Starch/gluten | 360,000           |
| Stockfeed     | 500,000-1,900,000 |

Source: AWB 1998

## 2.2 The classes of wheat

Australian wheat is classified into various classes. These classes are further subdivided into a number of grades according to protein content, variety and state of origin. The major classes are shown in Table 2.3.

**Table 2.3 Major Australian wheat classes and their typical end-uses**

| Class                      | Minium protein content       | End uses  |
|----------------------------|------------------------------|---|
| Australian Prime hard      | 13-15%                       | To produce flours for Chinese-style noodles and high volume breads.                                       |
| Australian Hard            | 11-14%                       | To produce wide range of breads including Middle Eastern Style flat breads and European volume breads.    |
| Australian Premium White   | 10-12%                       | Milled to flour suitable for Middle Eastern flat breads and Asian foods such as noodles and spring rolls. |
| ASW                        | 9-11.5%                      | Suitable for wide variety of flour products able to produce loaf and flat breads, steam bread and noodles |
| Australian Soft            | Max. protein content<br>9.5% | Flours for biscuits, cakes, pastries, steamed buns and snack foods.                                       |
| Australian Durum           | 13%                          | To produce semolina for a wide range of products such as spaghetti, macaroni and fettuccine.              |
| Australian Noodle          | 9.5%                         | To produce Japanese Udon and Korean dry noodles   |
| Australian General Purpose |                              | Suitable for blending with other wheats to produce flour.   |
| Australian Feed            |                              | Used for animal livestock consumption   |

Source: AWB 1998

### 2.3 Wheat flour usage

Currently, aggregate flour milled for domestic human consumption is approximately 1,500,000 tonnes a year. Use of flour for industrial purposes such as starch, gluten and their derivatives, has tended to increase in recent years and now amounts to about 300,000 tonnes a year.

By far the largest use for flour in Australia is in the manufacture of bread. It is also used for cakes, biscuits, pasta products, starch and gluten. (Bread Research Institute of Australia (BRI) 1989). In 1990, approximately 1.6 million tonnes of flour was produced of which 70,866 tonnes was exported. The Australian market is regarded as a "mature" market. However, flour production increased by approximately 196,850 tonnes over the five-year period 1986-1990 (McMaster and Gould 1995).

### 2.4 The Australian bread making industry

The period of 1956 saw emergence of 4 major bakery companies; each allied to a flour milling group, which supplied the majority of bread in Australian big cities. These companies were built by buying smaller bakeries. Important developments in the production and marketing of bread occurred at the same time and helped to make the consideration process possible (AWB 1998). The Australian bread industry is currently made of three segments: wholesale bakeries, hot bread shops and "in-store" bakeries.

*(a) Wholesale bakeries:* These bakeries are large, automated and account for approximately 65%. The companies who own the large plant bakeries are vertically integrated with interests in flour milling, bakery ingredient production and related industries.

*(b) Hot bread shops:* Hot bread shops have proliferated in Australia through the 1980s and have made significant inroads into the market share of larger plant bakeries. The aroma of freshly baked breads has proved to be an extremely powerful marketing strategy. In 1988 in New South Wales there were only 13 hot bread shops. In Victoria the number is to be estimated to be on the order of 600 hot bread shops. A more recent

phenomenon has been the emergence of franchise chains of hot bread shops offering a wide range of bread and pastry products.

**(c) In-store bakeries:** In-store bakeries have become a significant market force in Australia. The bakeries are located in supermarkets and major drawing cards for customers seeking a fresh, hygienic image. Both major Australian supermarkets chains (Coles and Woolworths/Safeway) now have a policy of including a bakery in each new supermarket store. There are estimated to be 280-300 in-store bakeries in Australia (McMaster and Gould 1995).

Today it is estimated that the large bakery companies currently supply about 65% of the national bread market (Maguire 1999).

Hot bread shops-25%

In-store bakeries-5% and

Independent bakeries-5%

## **2.5 The Indian bread making industry**

Although less than 5 million tonnes of white flour is used for the production of western style baked goods; it represents one of the single largest organized processed food industries of India. Approximately 53,900 bakery units operate in the country with a total annual turnover of 7 billion rupees. The leading producers of bakery goods are registered under the Director General Technical Development. The size distribution of units is large, 22; medium, 383; small, 3,728; and household, 49,767. Because of the growing population and increasing urbanization, there is great potential for growth in the Indian baking industry in the coming years (Radhakrishnan 1987). A large number of bakeries in the organized sector are now mechanized. As a result, the production of bakery goods in India is projected to a dramatically increase in the 1990s (Anon 1989).

## **2.6 The importance of bread**

The most popular yeast leavened product by far is bread (Table 2.4). The amount of bread consumed in the world is truly staggering. Also staggering is the wide array of sizes, shapes, and textures and tastes that bread comes in. Australian food regulations define bread as the product obtained by baking a yeast – leavened dough prepared from flour, water, yeast and with or without a number of other ingredients. Bread has often

been referred to as the “staff of life”, and even today is the major form of carbohydrate consumption in Australia.

Most Australian bread has a subtle flavor which combines well with other foods and spreads. Whilst the range of baked products has increased, white sliced bread remains the dominant segment of the market. There has been a trend in more recent years towards “high fibre” and “multigrain” breads in line with the positioning of bread as a component of healthy diet (McMaster and Gould 1995). The range and variety of bread products made in Australia has increased over the past few decades. Bread products; with a retail value of approximately \$1.6 billion, are the most significant baked products in Australia (McMaster and Gould 1995). Never-the-less bread consumption per capita has been declining in the more highly urbanised communities of Australia as affluence has increased.

**Table 2.4 A comparison of annual bread consumption in various countries**

| <b>Country</b> | <b>Consumption<br/>(kg per person)</b> |
|----------------|--|
| Australia      | 44                                     |
| Britain        | 44                                     |
| France         | 60                                     |
| Greece         | 125                                    |
| Italy          | 78                                     |
| Ireland        | 64                                     |
| New Zealand    | 52                                     |
| Russia         | 140                                    |
| USA            | 37                                     |
| West Germany   | 65                                     |

Source: BRI 1998

## 2.7 Nutritional value of bread

All types of bread supply significant portions of energy, protein, B-vitamins and fibre to the Australian diet (Table 2.5). Bread also provides the minerals, calcium, phosphorous and iron, but it is not a source of vitamins A, D and C.

**Table 2.5 The nutritional value of bread**

| Nutrient/component | Average Composition of Bread<br>per 100 g as consumed (40% moisture) |                 |
|--------------------|--|-----------------|
|                    | White bread  | Wholemeal bread |
| Protein (g)        | 8.0  | 8.5             |
| Fat (g)            | 2.0  | 2.4             |
| Carbohydrate (g)   | 49   | 47              |
| Crude fibre (g)    | 0.2  | 1.1-1.5         |
| Energy (kJ)        | 1017   | 986             |
| Thiamin (mg)       | 0.12   | 0.24            |
| Riboflavin (mg)    | 0.08   | 0.09            |
| Niacin (mg)        | 0.9  | 2.6             |
| Calcium (mg)       | 20   | 33              |
| Iron (mg)          | 1.5  | 3.9             |
| Sodium (mg)        | 507  | 529             |
| Potassium (mg)     | 85   | 204             |

Source: Saxelby and Una 1980

White bread is a nutritious food, although it contains less vitamin B, minerals and dietary fibre than wholemeal and brown breads. It is nevertheless, a significant source of these nutrients and of protein in the typical Australian diet. There has been little scientific support for the notion that wholemeal is superior nutritionally to white bread for humans consuming a mixed diet (Saxelby and Una 1980).

## 2.8 Attitudes to bread

Consumer surveys indicate that consumers generally have a low opinion of bread and are poorly informed about its nutritional value. In addition, it has been reported that most people have an opinion of bread that was not favourable (Butcher 1977).

Approximately 60% of females questioned rated bread as having slight nutritional value, whilst 40% of men and 30% boys rated it the same way. In Australia, as in most affluent countries, per capita bread consumption has been declining for the past 50 years. Whilst the increase in population over the period has meant an increase in the total production, per capita consumption has decreased significantly to 48.4 kg per head per yr (Saxelby and Una 1980).

Throughout history, white bread has been preferred to wholemeal and brown bread and, in the free market situation existing today, white bread constitutes 80-85% of the bread sold in Australia. Consumer surveys conducted by the BRI indicate that whilst white bread is preferred by the younger age groups but this preference declines with age. Adults choose more wholemeal and brown bread varieties. White bread accounts for only 26% of the bread eaten by people aged 50 and over (Saxelby and Una 1980).

The following dietary guidelines for Australians (also referred to as goals for Australians) have been adopted nationally (National Health and Medical Research Council (NHMRC) 1992):

1. Enjoy a wide variety of nutritious foods.
2. Eat plenty of **breads and cereals** (preferably wholegrain), vegetables and fruits.
3. Eat a low diet in fat and particular, low in saturated fat.
4. Maintain a healthy body weight by balancing physical activity and food intake.
5. If you drink, limit your alcohol.
6. Eat a moderate amount of sugars and foods containing added sugars.
7. Choose low salt foods and use salt sparingly.
8. Encourage and support breast-feeding.

## 2.9 Bread manufacture

Bread was first manufactured in a crude form in Egyptian times. Today, bread manufacture is a complex blend of science and art, and many forms of bread manufacture are used from traditional slow long methods to highly automated ‘no-time’ methods. In the modern bakery system the same basic principles are applied but technological developments have occurred in production techniques, bakery equipment and the quality of ingredients used to produce breads with shorter fermentation times. This was possible with the use of a rapid or no-time dough system, such as that developed by the BRI or by mechanical dough development such as the Chorleywood Breadmaking Process, developed by the British Baking Industry Research Association (Saxelby and Una 1980).

Bread is made by different procedures. The particular procedure used depends upon many factors, including tradition, the amount (cost) and type of energy available, the type and consistency of the flour available, the type of bread desired, and the time between baking and eating. There are numerous bread making procedures that are used in different countries. The procedures have been accustomed till date due to the consumer preferences and, new technological procedures have been made in bread making to fulfill customer demands. (Simmonds 1989; Wrigley 1994).

### *Various bread making processes*

1. Straight dough system.
2. Sponge and dough.
3. Mechanical dough development-batch/continuous bread making system.
4. Chemical dough development.

### *Australian bread making process*

The rapid dough system is used mainly in commercial bakeries in Australia. (BRI 1998). In recent times, some new commercial bakeries have installed high-density “Tweedy” mixers. Smaller bakeries tend to employ medium intensity “spiral” mixers. (McMaster and Gould 1995). In contrast to the Australian bread making process, some Asian countries including India continue to use the two traditional types of bread making procedures:

1. Straight dough system.
2. Sponge and dough system.

**Table 2.6** Typical Australian bread formulations (expressed as parts by weight compared to flour weight)

| Ingredient               | White Sandwich Bread | Wholemeal Bread |
|--------------------------|----------------------|-----------------|
| Flour                    | 100                  | -               |
| Wholemeal                | -                    | 100             |
| Water (approx.)          | 58                   | 62              |
| Yeast                    | 2.5                  | 2.5             |
| Salt                     | 2.0                  | 2.0             |
| Fat                      | 2.0                  | 2.0 a           |
| Sugar                    | 1.0 a                | 1.0 a           |
| Dry gluten               | -                    | 4.0             |
| Composite bread improver | as required          |                 |

(a) Optional  
Source: BRI 1998

Despite the range of processes applied, the basic stages of manufacture remain the same. There are three main stages in bread manufacture:

- (a) Mixing and development of dough.
- (b) Aeration of the dough.
- (c) Baking.

No matter what formulation is used, bread may be produced in a large-scale commercial bakery or your local bread shop. The difference in production levels is great. A typical major bakery produces about 300 tonnes of bread each day while local bread shops produce approximately 2 tonnes.

## 2.10 Changes occurring in bread during baking

To see a dough come out of an oven in the form of bread seems to be a very simple process, however it is not so easy to understand how dough is converted or transformed into bread. Several characteristic changes occur in dough when formed into baked bread. These changes are mainly due to physical/chemical reactions that take place during baking. Baking is a heating process in which many reactions occur at different rates. Some of these reactions bring the characteristic changes from a dough into baked product (Drapron and Godon 1987). These reactions are:

1. Evolution and expansion of gases.
2. Coagulation of gluten and gelatinization of starch.
3. Partial dehydration from evaporation of water.
4. Development of flavors.
5. Changes of color due to Maillard browning reactions between gluten, proteins with sugars, as well as other chemical colour changes.
6. Crust formation from surface dehydration and,
7. Crust darkening from Maillard browning reactions and caramelization of sugars.

The rate of these reactions and the order in which they occur depend to a large extent on the rate of transfer through the dough.

## 2.11 Changes after baking – retrogradation and staling

It has been said that “all bread fresh from the oven is good bread”. Bread loses its desirability progressively with the time it is out of the oven. These undesirable changes that occur with time are collectively referred to as staling (Zobel and Kulp 1996). They include toughening of the crust, firming of the crumb, and a decrease in soluble starch levels.

The term retrogradation describes the recrystallization of starch following heating and gelatinisation. It appears to be primarily the amylopectin fraction that recrystallises. The increase in opacity of the crumb is presumably caused by the growth of the crystallites, which change the refractive index (Zobel and Kulp 1996). The changes that

occur in the crust are clearly different from those that occur in the crumb. The toughening of the crust appears to mainly associate with migration of water from crumb to crust. The changes that occur in crumb appear to be much more complex. It was shown, almost 150 years ago, that firming of the bread-crumbs is not a drying process. Firming occurs even though no moisture is lost. Occurring over the same general time span as the firming is a recrystallization of the starch (Zobel and Kulp 1996).

The basic cause of staling is the transformation of starch from one form to another at temperature below 55 °C. The starch becomes more crystalline below this temperature. The change in the form of the starch results in the binding of less water leading to rapid hardening, and to the shrinkage of the starch granules away from gluten skeleton with which they are associated, with the consequent development of crumbliness. These changes occur in the bread even though it is sealed in a moisture/vapour-tight wrap (Zobel and Kulp 1996).

### ***Microbiological spoilage***

Breads undergo microbiological spoilage due to the growth of molds and undesirable bacteria. Excessive growth of proteolytic bacteria may destroy some of the gas-holding capacity so essential during of the dough and produce sticky dough (Bread Research Institute of Australia 1989). These also are the possibility of the production by microorganisms of undesirable flavors other than sourness (Frazier and Westhoff 1996). The microbial spoilage may be:

1. By mould- the species primarily involved in the spoilage of bread are *Rhizopus stolonifer* (syn, *R. nigricans*), *Monila sitophila* (pink mould) and members of the *Aspergillus* and *Penicillium* groups.
2. By bacteria- *B. subtilis* type are the main bacteria, which cause spoilage. Ropiness is the result of the above species. Red bread has a striking appearance but rare in occurrence. The red colour results from the growth of pigmented bacteria, usually *Serratia marcescens*.

## 2.12 Introduction to enzymes

Enzymes are biological catalysts. A catalyst is an agent affecting the velocity of a chemical reaction without appearing among the final products of the reaction. Enzymes are proteins with catalytic properties due to their power of specific activation. It is the protein nature of the enzymes for their characteristic behaviour and properties.

Among the factors affecting the rate of enzymically catalysed reactions are:

1. enzyme concentration.
2. substrate concentration.
3. temperature.
4. pH.
5. presence and concentration of electrolytes.

### *Functions of enzymes*

Enzymes function by lowering the activation energy of specific substrate. They do this by temporarily combining with the substrate to form an enzyme-substrate-complex that is less stable than the substrate alone. This overcomes the resistance to reaction. The substrate thus excited plunges to a still lower energy level by forming new products of reaction. In the course of action the enzyme remains unchanged.

### *Enzymes in bread manufacture*

Because cereals store their excess energy as starch and thus contain relatively high levels of starch, it is not surprising that the starch-degrading enzymes have been studied extensively in cereals. The starch degrading enzymes are of importance when cereals are used in bread making.

## 2.13 Roles of enzymes in bread making

Enzymes are widely used in food processing (Table 2.7). In relation to bread, they have been used non-intentionally in bread making already for centuries because enzymes are present in wheat. The deliberate addition of enzymes to bread making was described for the first time more than 100 years ago. In 1886 malted barley flour was mentioned for use in bread (Oort and others 1995).

**Table 2.7** Some uses of enzymes within the food processing industries

| <b>Enzyme</b>                 | <b>Application</b>   |
|-------------------------------|--|
| Amylases and Amyloglucosidase | Production of fermentable sugars in baking and brewing<br>Starch liquification |
| Cellulase                     | Solubilisation of pentosan in baking   |
| Protease                      | Bread quality in baking  |
| Lipases                       | Foam stabilisation in baking   |
| Glucose oxidase               | Prevention of Maillard browning reaction<br>Bromate replacer in baking         |

Source: Tucker 1996

Due to amylose degrading action of enzymes in the malt, sugars were formed from starch, which could serve as a yeast substrate during fermentation. Approximately 50yrs later the use of lipoxygenase was described for the first time as a tool for bleaching the breadcrumb and to improve machinability. Since then the use of different enzymes in bread making has been increasing rapidly and is now well established (Table 2.8).

In general the enzymes act on one of the biopolymers present in the wheat (for example polysaccharides, proteins) or sometimes combinations thereof. Each enzyme has a specific application. The roles the enzymes play in bread making are very important due to the large variation in manufacturing processes.

**Table 2.8 Enzymes currently used in breadmaking together with the main function of each of those enzymes**

| Function                 | Key enzyme           |
|--------------------------|----------------------|
| Mixing time              | Protease             |
| Dough development        | (per) Oxidase        |
| Proofing time, tolerance | Xylanase             |
| Bread volume             | Xylanase, amylase    |
| Shape                    | Xylanase, peroxidase |
| Anti-staling             | Bacterial amylase    |
| Flavour                  | Protease, peptidase  |
| Colour                   | Amylase, xylanase    |

Source: Oort and others 1995

The vast majority, if not all, of the enzymes employed in the industry are catabolic in nature that is they carry out reactions, which are essentially degradative.

- Amylases, attacking damaged or gelatinised starch, are used for generating sugars for the yeast fermentation and for slowing down the firming of the breadcrumb i.e. the aging of the bread.
- Proteases are used for shortening mixing times and for obtaining for flexible doughs.
- Pentosanases (hemi-cellulases, xylanases) acting on non-starch polysaccharides is able to change and to control water absorption and improve loaf volume (Oort and others 1995).

## 2.14 Amylases in bread making

Amylases are enzymes that catalyze the hydrolysis of starch. They are extensively distributed in nature and widely employed in the industry. Amylases may be classified as either endoamylases or exoamylases.

### *Endoamylases*

$\alpha$ -Amylase, ( $\alpha$ -1, 4-glucano-hydrolases) belong to this class, which randomly hydrolyze the  $\alpha$ -1, 4-glucoside linkages of amylose or amylopectin but not the  $\alpha$ -1, 6-glucosidic linkages of amylopectin. They are also known as dextrinogenic or liquifying-amylases.

### *Exoamylases*

The exoamylases are also known as Saccharifying or Saccharogenic amylases.  $\beta$ -Amylase, which splits only the second  $\alpha$ -1, 4-glucoside linkage from the non-reducing end chain, detaching one molecule of maltose at a time from the chain and finally leaving limit dextrins. Some characteristics of amylases are presented in Table 2.9.

**Table 2.9 Properties of  $\alpha$ -amylases from different sources**

| Enzyme                                    | Source   | pH range for activity<br>(Optimum pH) | Inactivation temp<br>(Optimum temperature)<br>(°C) | Average DP of product |
|---|--|---------------------------------------|--|-----------------------|
| $\alpha$ -amylase                         | Cereal (wheat malt)  | 5.0-5.5                               | 65-80  | 3.5                   |
| Bacterial $\alpha$ -amylase               | <i>Bacillus subtilis</i><br><i>Bacillus amylo-liquefaciens</i> | 4.5-9.0<br>(6.5-7.5)                  | 70-85<br>(70-95)                                   | Approx<br>14          |
| Thermo-stable bacterial $\alpha$ -amylase | <i>Bacillus licheniformis</i>                                  | 5.8-8.0<br>(7.0)                      | 90-105<br>(120)                                    | 14                    |
| Fungal $\alpha$ -amylase                  | <i>Aspergillus oryzae</i><br><i>Aspergillus niger</i>          | 4.0-7.0<br>(5.0-6.0)                  | 55-60<br>(80)                                      | 13                    |

Note: DP refers to degree of polymerisation

Source: Walden 1955

### 2.15 Amylases from different sources

*Bacillus subtilis* has been the principal bacterium used for the production of amylases, although other species of bacteria are known to yield these enzymes. Most bacteria produce more  $\alpha$ -amylase than  $\beta$ -amylase (Takagi and others 1971).  $\alpha$ -Amylase preparations are available in either liquid or powder form. Bacterial  $\alpha$ -amylases are divided into standard amylases and heat stable amylases, depending upon their stability to heat. Standard bacterial  $\alpha$ -amylase from *Bacillus subtilis* or *B. amyloliquefaciens* requires temperature of 70-85 °C for optimal activity. Heat stable bacterial  $\alpha$ -amylase from eg. *B. Licheniformis* has a temperature optimum between 90 and 105 °C. However the pH, the concentration of calcium ions and substrate concentrations are important (Wong and Robertson 2003a).

Fungal amylases are obtained from *Aspergillus niger* or *Aspergillus oryzae*. They are less resistant to heat than bacterial  $\alpha$ -amylases and are inactivated before starch gelatinizes. This is important in the making of bread. Fungal  $\alpha$ - amylase preparations used in the food industry is standardized with edible substance sugar or starch and have activities between 500 and 100,000 SKB units per g. It is noted that the abbreviation SKB refers to the units described by Sandstedt, Kneen and Blish in their pioneering work on assay procedures for the enzyme (Sandstedt and others 1939). Proteinases are also present in these preparations.

$\beta$ -Amylase is naturally present in the grain and effects successive removal of maltose units from the non-reducing ends (Thoma and others 1971; (Wong and Robertson 2003b). In the case of amylopectin, this process stops 2-3 glucose residues from the  $\alpha$ -1,6-branching points. The optimal temperature of this enzyme is 55 °C at pH 5.1-5.5.

### 2.16 Roles of amylases in baking

The amount of fermentable monosaccharides and disaccharides in wheat flour is quite small, generally not exceeding 0.5%. The amount is not sufficient to sustain the fermentation required to give good loaf volume, and consequently bread quality depends on the generation of maltose by  $\alpha$ - and  $\beta$ -amylase activities of the flour.

However, these enzymes may induce undesirable changes when their activities are too high or unbalanced (Kruger and others 1987). Alternatively the addition of sufficient sugar to the formulation may support yeast growth.

$\alpha$ - and  $\beta$ -Amylase activities within the dough are independent of each other. Walden (1955) showed that the effects of the two amylases are additive when their activities are low but at high concentrations, the quantity hydrolyzed and the hydrolysis rate are higher than the sum of those produced when the enzymes are working separately.

The capacity of amylases to break down starch depends on the penetration and diffusion of amylolytic enzymes into the granule, in relation to its hydration and surface state. Only  $\alpha$ -amylase is able to hydrolyze native damaged starch, although the degradation rate is low. The amylolytic activity is more important when granular starch is damaged. During bread making, the amylolytic enzymes act within a particular medium; the starch is trapped within a complex protein network, and the mobility of the components is limited by relatively low dehydration. Starch amylolysis is consequently dependent on the texture of this medium and also on other enzymatic reactions such as those of proteases as well as on fermentations.

Amylolytic activity starts as soon as water is added to the flour. During dough making, the granular structure of the starch is preserved by low and constant temperature, and the rate of enzyme action is not affected. Starch hydration is low, that is 50% of the available water is associated with starch and 50% is distributed among insoluble proteins, pentosans, yeast and a concentrated solution leading to the relative mobility of components (Kruger and others 1987).

Under these conditions, only the  $\alpha$ -amylase is able to hydrolyze the damaged starch granules but the rate of degradation is low. On the other hand, both enzymes attack the damaged granules. Normally flour contains 5-9% damaged starch. Thus damaged starch is very susceptible to amylase attack during dough making, and amylolysis can be schematised as follows:

Damaged starch + water + amylases  $\rightarrow$  dextrins + maltose + glucose

### 2.17 Amylolytic activity during fermentation and baking

Since the formation of maltose from starch is essential for adequate growth of yeast in an unsweetened dough, the level of these enzymes in the dough is of paramount importance. If amylase activity is sufficient, the production of maltose will keep up with the demands of the rapidly multiplying yeast, and carbon dioxide formation will be also be rapid. Where amylase activity is low, the addition of dextrans and soluble starch will increase gas formation.

Amylase enzymes have optimal pH values in the range of 5.0 to 7.5 (Table 2.9, page 19). With sound wheat flours, the maltose yield mainly results from the action of  $\beta$ -amylase on damaged starch granules; Walden (1959) estimated that 6% of the starch was hydrolyzed. In the case of flours from germinated wheat, some dextrans are formed by  $\alpha$ - and  $\beta$ -amylase activity and maltose production is higher. The limiting factor for maltose production is the presence of a sufficient level of damaged starch granules. Thus with flours with weak  $\alpha$ -amylase activity, carbon dioxide development can be increased/accelerated by adding damaged starch or exogenous  $\alpha$ -amylase.

The study of amylolytic activity during is difficult because dough is a complex medium and interactions take place between yeast activity, carbon dioxide, starch gelatinization, gluten denaturation and crust formation.

When the temperature of the dough exceeds 56 °C, starch granules begin to gelatinise and their hydration and amylose dispersion are increased. During the events, the amylase diffusion rate and activities reach a maximum. At the same time, the amylolysis activation is counter balanced by thermal inactivation. At 80 °C, the inactivation of amylases prevails and starches hydrolysis stops. However the activities inside the crumb varies with the site considered, bread type and baking modalities.

Cereal  $\beta$ -amylase, destroyed near 70 °C, generates appreciable amounts of maltose from the dextrin produced by  $\alpha$ -amylase from gelatinized starch. Cereal  $\alpha$ -amylase destroyed at about 80 °C, transform starch into dextrans and oligosaccharides. The simultaneous

presence of  $\alpha$ - and  $\beta$ -amylases has synergetic effect the reaction intensity depending on the amount of each enzyme and the ratio of one to the other. It is desirable that some degradation of starch by  $\alpha$ -amylase should continue during baking to allow expansion of the dough mass. Inadequate amylase activity restricts loaf volume. The crumb qualities of softness, resilience and the rate of firming are directly affected by amylase activity during baking. (Kruger and others 1987).

### 2.18 Amylases used in baking

Both of types of amylases,  $\alpha$ - and  $\beta$ -amylase, are used in baking.  $\beta$ -Amylase is generally available in sufficient quantity in the grain, but  $\alpha$ -amylase is often deficient and must be supplemented. The amount of additional enzyme must be sufficient for gas production, volume control and colour. It must not be added in excess because this can result in excessive dextrin formation, leading to loaf stickiness, dark colour and possibly insufficient product strength. The  $\alpha$ -amylase supplement can be derived from malted flour; however fungal amylase is commonly used because, this  $\alpha$ -amylase is believed to be inactivated before the temperature approaches the gelatinization point of starch. This provides a means to control dextrin formation. The malt amylase is thermally more stable and can lead to excessive break down of starch. The enzymes derived from *Aspergillus oryzae* act in the pH range 4.5-5.5. The pH of dough can vary with the product, so a pH of 7.0 may result and alter the enzyme dose. In the formation of bread, the temperature may increase from 30 to 90 °C over a period of 20 min. Dextrin formation begins to decrease at around 70 °C as enzyme activity declines. Expansion is also limited by the activation of yeast as the temperature is reached. Increasing evidence has become available on the different influences of amylases from the different sources on crumb firming.

### **2.19 Bread firming and starch crystallisation**

Early work by Zobel and Senti (1959) found that recrystallisation increased when bread was supplemented with bacterial  $\alpha$ -amylase. In this study, bread was supplemented with bacterial  $\alpha$ -amylase. Bread containing cross-linked corn starch was also prepared. All the breads were then studied using the technique of x-ray diffraction. Gels which contained either wheat or cross-linked starches were used to investigate the effect of bacterial  $\alpha$ -amylase on starch crystallisation. It was observed that gels with addition of enzyme crystallised quicker than those without enzyme addition. Similar observations were also found in bread samples. During storage, both gels showed an increase in starch crystallinity. The authors suggested that the increase was due to the hydrolytic cleavage by amylases at the amorphous region. This provides more freedom for the crystallites to move independently and align themselves properly hence increase in crystallinity. The cleavage of long starch chains would weaken the strength of the three-dimensional network hence causes decrease in rigidity. It was concluded that starch crystallisation is not synonymous with bread firming. This conclusion was supported by the work of Dragsdorf and Varriano-Marston (1980).

### **2.20 Impact of amylase sources on bread firming rates**

More recent studies by Martin and Hosney (1991) and Akers and Hosney (1994) indicate that different amylase sources have some impact on the rate of bread staling. Both studies found that bread supplemented with barley malt flour firmed at a faster rate when compared to unsupplemented bread. Use of bacterial or fungal  $\alpha$ -amylases decreased crumb firming rates with the effect of bacterial amylases most significant. The differences in bread staling rates may be partly due to the differences in thermal stability of enzymes. The most heat-stable  $\alpha$ -amylase is from bacterial sources, followed by cereal then fungal sources (Table 2.9, page 19).

Thermal stability of  $\alpha$ -amylases will determine the amount of dextrans present in bread crumb. The stability of bacterial  $\alpha$ -amylase enables them to survive the baking process and remain active after baking. This causes extensive starch hydrolysis during baking

and results in production of sticky and gummy bread crumb (Herz 1965). Hence the amount of  $\alpha$ -amylases particularly those from bacterial sources added to bread formulations must be controlled carefully.

There is evidence that the three sources of  $\alpha$ -amylases have varying hydrolysis patterns and produce fragments of different sizes. However the significance of this to bread staling rates remains unclear. Many studies have found that high molecular weight carbohydrates have no antistaling effects on bread crumb (Lin and Lineback 1990; Martin and Hosney 1991).

Breads supplemented with bacterial  $\alpha$ -amylase, fungal  $\alpha$ -amylase or malted barley flour were compared by Martin and Hosney (1991). It was observed that bread supplemented with bacterial  $\alpha$ -amylase at a rate of 100 SKB units per loaf did not firm during 5 days of storage. Malted barley flour supplemented bread firmed at a slightly faster rate than the control bread while fungal  $\alpha$ -amylase supplemented bread firmed at a moderate rate. Since  $\alpha$ -amylase degrades starch to dextrins, the window of amylase activity can be determined by measuring the amount of dextrins present in bread. For the study, doughs and bread crumbs were extracted with distilled water then analysed using high performance liquid chromatography (HPLC). No measurable dextrins between degree of polymerisation (DP) range from 3 to 9 were found in either control doughs or those supplied samples with bacterial  $\alpha$ -amylase (50 SKB units per dough). As for bread samples, dextrins in the range of DP 3 to 9 were not found in control bread but were detected in bread supplemented with bacterial or fungal  $\alpha$ -amylase. The dextrins found in bread supplemented with malted barley flour larger than DP 9. Based on this observation, it was clearly shown that  $\alpha$ -amylase remained active during the baking process.

In this study, bacterial  $\alpha$ -amylase was also added to bread doughs at various levels. The results showed that the firmness decreases as the amount of bacterial  $\alpha$ -amylase

supplement increases. An addition level of 100 SKB units per loaf was found to be most effective since the bread did not firm during 5 days of storage.

## **2.21 The relationships between dextrins and staling rates**

### ***Linear dextrins and staling rates***

As previously mentioned, cereal  $\alpha$ -amylase is less thermostable hence larger quantities might be required in order to achieve the same antistaling effects as bacterial  $\alpha$ -amylase to decrease bread firmness. Bread supplemented with 10% malted barley flour, an addition levels that was much higher than the recommended values was found to firm. Hence Martin and Hosney (1991) suggested that the antistaling effect of bacterial  $\alpha$ -amylase was not only due to the wide window of activity of this enzyme. The type of dextrins produced by the action of  $\alpha$ -amylase was further investigated by adding pullulanase. It was observed that the antifirming effect of bacterial  $\alpha$ -amylase might be related to the particular size of dextrins produced. That was the production of linear low molecular weight oligosaccharides rather than the presence of branched dextrins. The size of dextrins produced was in the range of DP 3 to 9. It has been postulated that these low molecular weight carbohydrates interfere with the cross linkages formed between starch molecules and protein fibrils. Hence they weaken the interactions and reduce bread firming rates.

Martin and Hosney (1991) also found that the addition of malted barley flour had increased the bread firming rate. This may due to the production of large dextrins. These molecules would then interact with the protein fibrils and hence the numbers of cross linkages increased thereby producing firmer bread.

### ***Branched-chain dextrins and staling rates***

Findings by Martin and Hosney (1991) were at variance to the earlier work of Lin and Lineback (1990). They reported that branched-chain products of low molecular weight with average chain length of 19 to 24 have antistaling effects. If retrogradation of starch causes staling as proposed by Schoch and French (1947), the branched-chain products

reduced bread firming rates either by interfering with other interactions or by decreasing the retrogradation of amylopectin. These observations might be explained as  $\alpha$ -amylase acts on amylopectin and degrades it to more soluble low molecular weight branched chain dextrans. Hence less amylopectin is available to retrograde and so the firming rate is lower.

Lin and Lineback (1990) also found that there was a greater proportion of low molecular weight carbohydrates in enzyme supplemented bread compared to control bread. This was due to the action of bacterial  $\alpha$ -amylase on starch. The dextrans formed were predominantly low molecular weight branched chain polymer. More branched-chain carbohydrates were detected in bread immediately after baking as the linear carbohydrates had already retrograded. The levels of branched-chain carbohydrates were found to decrease during storage. This was due to their interference with the association of amylopectin.

### *Dextrans size and staling rates*

Findings by Martin and Hosney (1991) supported by Akers and Hosney (1994) also highlighted the conclusion that the presence of particular sizes of dextrans produced by amylases are responsible for controlling bread firming rate. Suggested dextrin sizes were DP 3 to 9 as determined by HPLC (Martin and Hosney 1991). The analytical method used by Akers and Hosney (1994) was slightly different where high performance anion-exchange chromatography with pulsed amperometric detector was used to analyse water soluble dextrans. Bacterial  $\alpha$ -amylase supplemented bread contained more total carbohydrates compared to fungal  $\alpha$ -amylase or malted barley flour supplemented bread. However bread firming rate did not seem to be related to the level of the carbohydrates found. Some samples have the same total amount of carbohydrates but varied in firming rates. The results also showed that different bacterial  $\alpha$ -amylases produce different amount of the same dextrans hence the bread firming effects varied.

### *The modification of starch and staling rates*

In contrast, Gerrard and colleagues (1997) suggested that the decrease of bread firming rate was not the direct result of the presence of dextrans but rather associated with the changes to the starch. Modification of starch by amylases causes decreased interactions between starch and protein fibrils. In their experiments, breads were added with either bacterial  $\alpha$ -amylase or glucoamylase or both. The results showed that addition of all three enzymes decrease bread firmness. However bread firmness with addition of both enzymes did not show significant difference to bread supplemented with  $\alpha$ -amylase alone. Since dextrans found in both treatments were different, dextrin profiles in the range from DP 1 to 7 were analysed using HPLC. Bread loaves supplemented with  $\alpha$ -amylase were found to contain more DP 3 to 8. Addition of glucoamylase increased the levels of DP 1 while DP 2 levels decreased. It would therefore be expected that DP 1 (glucose) exerted an antistaling effect. However it was not the case where bread supplemented with both enzymes, contained higher DP 1 levels still firmed at the same rates as bread supplemented with  $\alpha$ -amylase alone. Therefore it was suggested that modification of starch rather than the presence of specific dextrans causes decreased in bread firming rates. The results also indicated that  $\alpha$ -amylase is more effective in starch modification compared to glucoamylase.

Considerable evidence had accumulated indicating that dextrans produced by the action of  $\alpha$ -amylase have antistaling effect (Zobel and Senti 1959, Dragsdorf and Varriano Marston 1980 and Martin and Hoseney 1991). In recent studies by Gerrard and colleagues (1997), dextrans oligosaccharides extracted from  $\alpha$ -amylase treated loaves were added into experimental bread to mimic the dextrans produced by  $\alpha$ -amylase. It was found that following the addition of dextrans to a standard bread formulation, the added dextrans did not survive the baking process. It is presumed that the added dextrans were hydrolysed by amylases that are naturally present in flour and the products are metabolised by yeast. In addition, no significant difference was observed in staling rates on these bread loaves.

## 2.22 Other enzymes used in baking

### *Proteases*

The proteolytic enzymes, or proteases, include the proteinases, which catalyse the hydrolysis of the protein molecule into large fragments, and the peptidases, which hydrolyse these polypeptide fragments as far down as amino acids. The proteolytic enzyme preparations from microorganisms are proteases, ie. mixtures of proteinases and peptidases. Proteases also are prepared from plant or animal sources. All proteases have characteristic properties with regard to pH and temperature, ion required, specificity, activity and stability. The specificity depends on the amino acids involved in peptide bond to be hydrolysed. These biochemical parameters determine the application of proteases.

### *Use in the baking industry*

Since the strength of the gluten depends upon the intact proteins, any reaction which hydrolyses a part of the protein reduces the amount of the gluten. If too much of these proteolytic enzymes are present, too much hydrolysis occurs and the dough becomes sticky, difficult to machine the mixers, and yield bread of poor volume and quality. However some protease activity is desirable since it improves the gluten. Dough of low proteolytic activity is called “bucky” because the gluten is tough and inelastic. It does not machine well and it produces loaves of poor volume because the dough not will stretch around the gas bubbles. The complexity of the problem “what is gluten” is well demonstrated here.

Protease can reduce the dough viscosity caused by gluten and, thereby, improving the desired softening effect of proteases. The use of fungal proteinases has assumed a more important role than amylases. There are several reasons for this. Flour contains some native  $\alpha$ -amylase, and other sources of amylase are readily available. But the native proteinases of flour play no part in the bread baking, and, therefore, proteolytic supplementation must be supplied from other sources. Newer methods of bakery production require strict control of the mixing times and the production of doughs with optimum handling properties. The latter need is of greatest importance. The use of fungal protease permits a reduction of mixing time by as much as 30% without

producing a detrimental effect on the dough when used at the proper level fungal proteinase improves the handling and machining properties of the dough. Excessive amounts of the enzyme make the dough too slack and extensible, even to the point of undesirable stickiness of the dough.

The action of proteases on plastic properties of flour proteins effects the finished product. When the flour used in baking is rich in proteins, adding proteases allows better gas retention and greater dough extensibility, which explains why crumb texture is more regular and aerated (Belloc and others 1974). The volume of bread is increased, with positive effects on crumb structure and loaf symmetry (Singh and Katragadda 1980). However these workers reported the opposite reaction between the proteolytic activity of the dough and the volume of bread. Proteases improve the texture and organoleptic properties of baked goods if their use controlled: too high a protease dosage is detrimental to volume as well as to other products

### *Pentosanases*

There are now a number of commercial enzyme preparations, which contain enzyme activities allowing at least partial breakdown of the non-starch polysaccharides in wheat flour. The general names commonly used for these activities include pentosanases and hemicelluloses. These names derive from the fact that the non-starch polysaccharides present contain predominantly pentose units and they occur in the residual cell wall structures from the wheat endosperm. The specific activities, which may contribute to the hydrolysis of the pentosans, include  $\alpha$ -L arabinosidase,  $\beta$ -xylanase and  $\beta$ -xylosidase.

The effect of pentosanase on the insoluble pentosans of wheat flour has been studied, and a process based on pentosanase activity may be of value. The endosperm of wheat contains about 2.4% pentosans or hemicellulose materials. This can be isolated in the so-called tailing fraction from flour, which has been freed from gluten. Flour contains about 1% of insoluble pentosans and 0.5% of soluble pentosan. These fractions may include Arabinoxylans, hemicellulose, pentosanases etc.

The non-starch polysaccharides in particular can have enormous influence on the processing characteristic and the quality of the final product. The Arabinoxylans are the most important non-starch polysaccharides of wheat flour. Arabinoxylans can be

divided in soluble (pentosans) and insoluble (hemi-cellulose) fractions. In general; Arabinoxylans represents 1-3% of wheat flour. About 20-30% are water soluble (Oort and others 1995).

### ***Pentosans in baking***

Enzymatic hydrolysis has been used to investigate the functional role of pentosans in dough and bread. Endo-xylanase is the most effective hemicellulase in the bread making process. It hydrolyses both water-soluble and water insoluble Arabinoxylans into relatively large oligomers. Water-soluble pentosans of wheat exhibit some unique physical properties that have a direct functional implication during mixing, dough development and baking of bread. The increase in viscosity may probably be related to the mechanism behind the observed dough softening. Water binding and formation of high viscosity gels are important attributes. Besides water binding and gelling capacity, pentosans are also thought to have influence on the gas holding capacity of the wheat doughs.

It was found that both Arabinoxylans and Arabinogalactans are able to reduce the surface tensions of water. But only the Arabinoxylans are able to effectively stabilise protein foams upon heating and may therefore help in stabilising gas cells which contain the yeast generated gas and therefore are very important for volume increase of bread doughs, the crumb structure and final volume of the baked bread.

Further addition of Arabinoxylans fractions to the flour leads to increased water absorption and decreased dough-mixing time. Loaf volume increased in some studies, but not in others. Interestingly crumb firmness was reduced during bread storage after addition of pentosans. The latter phenomenon has been explained in an experiment, which demonstrated that the water bound by pentosans can migrate to starch, thus slowing down the rate of starch degradation (Oort and others 1995).

### ***Oxido-reductases***

Oxido-reducing enzymes have been widely studied because of their role in wheat flour dough qualities. Oxidising agents have a beneficial effect on the dough development and dough quality. Several flour oxidases have been used to improve dough handling and

bread volume and have Oxidoreducing properties. Several enzymes, like lipoxygenase, glucose-oxidase and ascorbic acid oxidase are reported as enzymatical dough oxidisers (Oort 1996).

### *Lipoxygenase*

Lipoxygenase is one of the most important and predominant Oxido-reducing enzymes used in bread production. Lipoxygenase is naturally present in the grain and flours, where it is found preponderantly in the germ fraction (Reed and Thorn 1971). This use of the enzyme was patented in the 1930s (Hass and Bohn 1934).

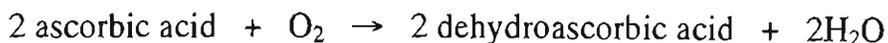
Lipoxygenase acts on unsaturated fatty acids to produce initially fatty-acid peroxy free radicals. Soybean lipoxygenase has been used in bread making for bleaching flours since the 1930s (Hass and Bohn 1934). Lipoxygenase oxidises the natural yellow pigment of flour and results in bread with a very white crumb. Lipoxygenase has an effect on the dough mixing properties of flour and on the internal structure of the bread. Also, it has been noted to have an effect on the flavour of the bread. Whether the contributions to bread flavour is desirable or undesirable is a matter of personal judgement.

### *Glucose oxidase*

As early as 1957, very low amounts of glucose oxidase had been used and demonstrated to have an improving effect on the maturation of the flour and on the quality of baked products. Glucose oxidase is commercially available, usually produced from micro-organisms. Several *Aspergillus* and *Penicillium* strains have been used in the production of glucose oxidase. The role of the glucose oxidase has not been described very clearly, since very low amounts of it has shown some oxidising effects on the flour and dough properties. Furthermore, the dough has increased stability (Oort 1996).

### *Ascorbic acid oxidase*

This enzyme occurs naturally in a variety of plant materials including grain and flour. Its physiological role remains a matter of conjecture, but in flour the following reaction is catalysed:



This reaction is coupled to the reduction of two sulphhydryl groups attached to the gluten molecules within the dough matrix. This effectively develops sulphhydryl bridges as a covalent link and provides coherence to the structure during breadmaking. Furthermore there is a natural system at work in the dough with dehydroascorbic acid reductase able to disrupt the disulphide bonds and allow expansion of the dough matrix as gas is produced by the yeast during fermentation.

The commercial production of bread in Australia takes advantage of these natural reactions and the endogenous enzymes present in the flour. To facilitate the development of a coherent dough matrix, ascorbic acid is routinely added as part of the improver. The oxidative system described here has effectively replaced the chemical oxidation that had been traditionally used in Australia particularly based on potassium bromate. The use of this latter additive was phased out early in the 1990s.

### **2.23 Summary of significance of project**

Staling represents an issue of considerable commercial significance and various mechanisms have been suggested to explain the changes that occur after baking. Mechanisms proposed include the loss of moisture from baked loaves; the retrogradation of starch and the development of cross linkages between starch and protein molecules. There is also evidence that other factors including the moisture of bread as well as the amount and quality of protein present will influence the staling rate of bread.

The enzyme  $\alpha$ -amylase has long been used in bakery formulations. Considerable research has been applied to elucidate the role of amylases from different sources and to explain the apparent differences in the impact of bacterial, cereal and fungal enzymes on bread quality. A variety of conclusions have been drawn by different researchers and these include the production of dextrans having specific characteristics: particular sizes or linear or branched-chain structures. Alternatively it has been suggested that the

modification of starch by amylases produces the antistaling effects. It remains unclear as to whether the amylases exert effects through the products of hydrolysis or by their impact on the starch present in the baked loaf.

Accordingly this project has been developed in the context of our current knowledge and the need to enhance our understanding of the role of enzymes in bread making. The specific issues include:

- Various forms of bread are produced around the world and represent a major staple in the diets of many consumers;
- In Australia bread production annually is valued at over 2.5 billion dollars;
- Consumer preferences are generally for breads which are soft in texture and freshness is an important issue;
- Considerable wastage occurs as staling rapidly changes the acceptability of baked products;
- Various types of enzymes are present in the flour and other ingredients used in breadmaking. Other enzymes are added into bakery formulations as processing aids to perform a range of functions;
- There is evidence that some enzymes in bread doughs may influence texture and keeping characteristics of these products; and
- Generally enzymes are assumed to act in the dough prior to being inactivated as a result of the elevated temperatures during baking.

Relatively little published data is available on the stability of enzymes during baking and subsequent storage of these products. Accordingly, in the current study, the stability of bakery enzymes will be investigated in relation to staling and product quality.

## 2.24 Aims of the project

The broad aim of this proposed project is to investigate the stability of enzymes during the baking process and assess their significance for product quality and storage potential.

The specific objectives are to:

1. Monitor the levels of enzyme activities through each step of the breadmaking process from dough preparation, through proofing and baking. The enzymes to be assayed are the amylases;
2. Study and observe the technological role of enzymes in baking particularly those that are stable and remain active following the oven baking stage of the process. This will involve studies of the significant effects of different enzymes on the concentrations of sugars in the bread as well as the characteristics of the finished product and keeping qualities of baked products; and
3. To investigate the potential of microencapsulation as a tool for investigating the role of partial hydrolysis products of starches in baking.

## Chapter 3

### Materials and methods

#### 3.1 Chemical reagents

All laboratory chemicals were of analytical reagent grade unless otherwise indicated. The details of the chemicals used, together with the respective suppliers are listed in Table 3.1. The enzyme substrates used are described in Table 3.2.

**Table 3.1 List of chemicals**

| Supplier                           | Chemical   |
|------------------------------------|--|
| Sigma Chemical Co, USA             | Tris(hydroxymethyl)aminomethane base (T-1503), Di Sodium Ethylene diamine tetra acetic acid (EDTA) (ED2SS), Cysteine (C - 7755), Maleic acid (M-0375), Bovine serum albumin (BSA) (A-9647) |
| BDH Laboratory Supplies, Melbourne | Sodium dihydrogen orthophosphate (AR), Disodium hydrogen orthophosphate (AR), Calcium chloride dihydrate (AR), Citric acid (AR) (20999)  |
| Ajax Chemicals, Melbourne          | Di Sodium EDTA 181 (007837)  |

Note: Information presented as supplier (product number, batch or lot number)

**Table 3.2 List of enzyme substrates and assay kits**

| Supplier                                    | Substrate/kit  |
|---|--|
| Megazyme International Ireland Ltd. Ireland | For $\alpha$ -amylase measurements Amylazyme tablets were used as substrate (lot 50504)                      |
| Megazyme International Ireland Ltd. Ireland | Control sample for $\alpha$ -amylase measurements  |
| Megazyme International Ireland Ltd. Ireland | For $\beta$ -amylase measurements Betamyl reagent was used as substrate (lot numbers 00902, 00903 and 00905) |
| Megazyme International Ireland Ltd. Ireland | Control malt flour 820 Units per g (lot 60502)   |

Note: Information presented as supplier (product number, batch or lot number)

### 3.2 Equipment

The items of general equipment used, together with the details of manufacturers and model numbers are presented in Table 3.3. The equipment used in baking studies is described in Table 3.4.

**Table 3.3 List of general laboratory equipment and consumable items**

| Equipment                                | Manufacturer   | Model No. |
|--|--|-----------|
| pH meter                                 | Hanna Instrument Ltd, Italy                          | 8417      |
| Water bath (thermostatically controlled) | Thermoline Scientific Instruments Pty Ltd, Melbourne | BTC 9090  |
| Water bath (thermostatted)               | Ratek Instruments                                    |           |
| Stop clock                               | Quartz   |           |
| Pipettors                                | Accupipett Unilab                                    | A1 303    |
| Filter paper                             | Whatman Type No 1                                    |           |
| Auto vortex mixer                        | Ratek Instruments                                    |           |
| Auto vortex mixer                        | Chiltern   | MT19      |
| Magnetic stirrer                         | Industrial Equipments & Controls Pty Ltd, Melbourne  | CS76083V  |
| Bench centrifuge (refrigerated)          | Sorvall. Dupont                                      | RT-7      |
| Bench centrifuge                         | Beckman.J2-HS Centrifuge                             | JA 2C05   |
| Spectrophotometer (uv/vis)               | Pharmacia Nova Spec LKB and                          | 11        |
|  | Biochrom Ultra Spec Plus LKB                         | 4054      |
| Moisture oven                            | Gallenkamp vacuum oven                               |           |
| Balance (two decimal places)             | Phoenix  | AA 200    |
| Analytical balance (four decimal places) | Denver Instrument Company                            | 3000D     |

**Table 3.4 List of equipment used in baking and assessment of baked loaves**

| <b>Equipment</b>     | <b>Manufacturer</b>                   | <b>Model No</b> |
|----------------------|---------------------------------------|-----------------|
| Baking machines      | Panasonic                             | SD-206          |
| Minolta Chroma Meter | Minolta Camera Co Ltd, Japan          | CR 300          |
| Texture assessment   | Stable Micro Systems Texture Analyser | TA-XT2          |

### **3.3 Bread ingredients**

#### **3.3.1 Flour type and storage**

The flour used for all of the baking experiments in this study was a commercial product described by the manufacturer as strong bakers flour. The type of flour was “Maximus” flour obtained from Weston Milling, North Melbourne and milled from the Victorian Hard Class of Wheat. All samples were stored in sealed containers at 2-3 °C pending further use.

#### **3.3.2 Salt**

The salt used was the standard table salt of premium grade manufactured by Olssons and obtained from a retail supermarket.

#### **3.3.3 Fat**

The fat used was blended hydrogenated liquid vegetable oil obtained as a generic product from a local supermarket.

#### **3.3.4 Improver**

The improver used was a product from a retail supermarket manufactured by Lowan Whole Foods Flour Milling, Nhill, Victoria. The improver was a commercial preparation. The dough improver ingredient list from the product package includes: Soya flour; mineral-calcium carbonate; emulsifier - stearyl lactylate; wheat malt flour; flour treatment agents (ascorbic acid and L-cysteine monohydrochloride).

### 3.3.5 Yeast

The yeast used was a product from a retail supermarket manufactured by Lowan Foods, Victoria. The yeast was a commercial preparation and the type was instant dry yeast. This was used in order to minimise variability in fresh yeast as experiments were carried out over a period of months. The instant yeast was stored frozen between uses, in line with the manufacturers recommendations.

### 3.4 Bread formulation

The baking formula (Table 3.5) is presented here in the way generally adopted in the baking industry (Bread Research Institute of Australia 1989). This is in relative terms in comparison to the flour in the formulation and is expressed in comparison to the flour proportion set to one hundred parts. The quantities used in this investigation were found to give a suitable dough weight, which was appropriate to the size and shape of the baking pan used.

**Table 3.5 Basic ingredient formulation of white bread**

| <b>Ingredient</b> | <b>Relative amount (%)</b> | <b>Amount</b> |
|-------------------|----------------------------|---------------|
| Flour             | 100                        | 450 g         |
| Yeast             | 3.0                        | 6.8 g         |
| Sugar             | 4.0                        | 9.0 g         |
| Salt              | 3.2                        | 7.4 g         |
| Shortening        | 4.0                        | 9.0 g         |
| Improver          | 2.0                        | 4.4 g         |
| Water             | 62.5                       | 292 mL        |

### 3.5 Baking process

Experimental doughs and baked loaves were prepared from the flour using a series of breadmaker units (Panasonic Bread Bakery- Model SD-206). The loaves were baked under controlled conditions, which are similar to current commercial practice. The bread

makers have a range of settings, with the “basic” process (4 hr normal operation) being most suitable for this comparison. The machines automatically operate a set cycle once the start button has been pressed. The sequence is as follows:

1. Weighing the ingredients and commencing the cycle.
2. Resting period (to allow ingredients to equilibrate) - 30 minutes.
3. Mixing and kneading - 30 minutes.
4. 1<sup>st</sup> proof - 1.25 hours.
5. Knock back - 15 minutes.
6. 2<sup>nd</sup> proof - 45 minutes.
7. Baking - 45 minutes.

### 3.6 Analysis of compositional parameters

The procedure followed was AOAC Method 934.01. Samples of baked bread (3-4 g) were taken from the central crumb after removal of crusts and placed in predried and weighed aluminium dishes and dried in a Gallenkamp vacuum oven at 70 °C under vacuum (1020 mbar) for 3 hours or until constant weight was obtained. The moisture content was calculated from the weight loss on drying to constant weight and expressed as g per 100 g on wet weight basis.

$$\text{Moisture content (per cent)} = \frac{\text{Loss in weight of dish, lid and sample upon drying (g)}}{\text{Initial weight of sample (g)}} \times 100$$

### 3.7 Procedures for preparation of extracts and measurement of $\beta$ -amylase activity

The procedure followed for the  $\beta$ -amylase assay was that using Betamyl method from Megazyme (Megazyme International 1996b), which was originally described and published by McCleary and Codd (1989). The procedure is based upon the use of a prepared substrate in which a *p*-nitrophenyl group is covalently linked to  $\alpha$ -D-maltopentaose. The activity of the enzyme is determined from the liberation of free *p*-nitrophenol that is measured by absorbance spectrophotometrically.

### 3.7.1 Preparation of reagents and solutions

#### *Substrate (Betamyl reagent)*

The Betamyl reagent was supplied in sealed vials with each vial containing:

*p*-nitrophenyl  $\alpha$ -D-maltopentaose (47.5 mg)

$\alpha$ -glucosidase (1000 Units)

Stabilizers

The substrate solution was prepared by dissolving the entire contents of the vial in 10.0 mL of distilled water. The supplier recommends that to ensure maximum stability of the substrate, the contents of the vials (on dissolution) be divided into 2-3 mL aliquots and stored frozen in polypropylene containers. This approach was followed and the substrate was stored frozen between use and on ice after thawing.

#### *Extraction buffer (Buffer A)*

The extraction buffer A was prepared by dissolving 6.06 g of Trizma® base with 0.37 g of di sodium EDTA and adjusting to pH 8.0 with 1 M HCl, and the volume to 1 litre.

The buffer was then stored at 4 °C in the refrigerator.

The  $\beta$ -amylase activity was measured with and without the inclusion of cysteine in the extraction buffer. Where required, cysteine was dissolved in buffer A immediately before use (1.75 g per 100 mL). This buffer was then re-adjusted to pH 8.0 using 1 M NaOH solution.

#### *Dilution/assay buffer (Buffer B)*

Buffer B was prepared by adding maleic acid 11.6 g and di-sodium EDTA 0.37 g to 800 mL distilled water and the pH was adjusted to 6.2 with 4 M sodium hydroxide (16 g per 100 mL). The volume was adjusted to 1.0 litre, and then BSA (1.0 g) was added and dissolved. The buffer was then stored at 4 °C in the refrigerator.

#### *Stopping reagent*

A stopping reagent was prepared by dissolving 1% (weight/volume) Trizma® base in distilled water and the solution was kept at room temperature until further use.

### 3.7.2 Extraction of enzyme from flour and bread samples

In the initial trials malt flour was used for determining and calculating  $\beta$ -amylase activity in order to check the repeatability and consistency of the assay. In the subsequent experiments the enzyme was extracted from malt flour, wheat flour, and dough and then at different stages of bread baking using the same procedure. This was based upon the recommended method from Megazyme, which is described as follows:

1. To exactly 0.5 g of sample (flour, dough or bread) 5.0 mL of extraction buffer was added (Buffer A) either with or without cysteine.
2. The enzyme was extracted over a 1 hr period at room temperature, with frequent vigorous stirring on a vortex mixer (5 times over the one hour period).
3. An aliquot of the enzyme preparation was filtered through a filter paper, or centrifuged at  $1,000 \times g$  or 3,000 rpm for 10 mins at 20 °C.
4. Exactly 0.2 mL of filtrate was diluted to 10.0 mL with Buffer B, and then an aliquot of this diluted solution (0.2 mL) was further diluted to 5.0 mL in Buffer B.

### 3.7.3 Assay of extracts for $\beta$ -amylase activity

For each set of the assays, a reagent blank value was determined. To obtain this value, 3.0 mL of Stopping Reagent was added to 0.2 mL of pre-equilibrated Betamyl reagent solution and then 0.2 mL of diluted  $\beta$ -amylase solution was added. A single blank determination was found to be sufficient for each batch of assays. Assays of enzyme extracts were carried out as follows:

1. Aliquots (0.2 mL) of Betamyl substrate solution were dispensed into the bottom of the glass test tubes and the contents were pre-incubated at 40 °C for approximately 10 mins.
2. The enzyme preparation was pre-incubated at 40 °C for approximately 5 mins.
3. To each tube containing the Betamyl substrate solution, an aliquot (0.2 mL) of pre-equilibrated (and suitably diluted) enzyme preparation was added directly to the bottom of the test tube, and then incubated at 40 °C for exactly 10 mins (from time of addition).

4. At the end of the 10 mins incubation period, stopping reagent (exactly 3.0 mL) was added into the test tube and the contents stirred thoroughly on a vortex mixer.
5. The absorbance of each of the reaction solutions as well as the reaction blank were read against distilled water at 410 nm by the spectrophotometer.

### 3.7.4 Calculation of $\beta$ -amylase activity

$$\beta\text{-amylase activity (Units per g)} = \frac{\text{total volume in cell}}{\text{aliquot assayed}} \times \frac{\text{extraction volume}}{\text{sample weight}} \times \frac{\Delta E_{410}}{\epsilon} \times \frac{\text{dilution}}{\text{incubation time}}$$

**where:**

|                      |   |   |
|----------------------|---|---|
| total volume in cell | = | 3.4 mL  |
| aliquot assayed      | = | 0.2 mL  |
| extraction volume    | = | 10 mL per g (flour, dough or baked bread)   |
| $\Delta E_{410}$     | = | absorbance (reaction) – absorbance (blank)  |
| $\epsilon$           | = | 17.8, which is the extinction coefficient of nitrophenol in 1% Trizma® base expressed in units of millimolarity |
| dilution             | = | 1250 fold   |
| incubation time      | = | 10 mins   |

**Thus:**

$$\beta\text{-amylase activity (Units per g)} = \frac{3.4}{0.2} \times \frac{10}{1} \times \frac{\Delta E_{410}}{17.8} \times \frac{1250}{10}$$

$$\beta\text{-amylase activity (Units per g)} = \Delta E_{410} \times 1194$$

One Unit of activity is defined as the amount of enzyme required, in the presence of excess  $\alpha$ -glucosidase, to release one micromole of *p*-nitrophenol from PNPG5 in one min under the defined assay conditions; and is termed a Betamyl Unit.

### 3.8 Assay of $\alpha$ -amylase activity

The procedure followed for the  $\alpha$ -amylase assay was that using Amylzyme method from Megazyme (1996a), which was originally described and published by McCleary and Codd (1989). The procedure is based upon the use of a prepared substrate Azurine-crosslinked amylose (AZCL-Amylose) and is dyed to produce a material, which hydrates in water but is water insoluble. Hydrolysis by  $\alpha$ -amylase produces water soluble dyed fragments, and the rate of release of these is found from the measurement of absorbance spectrophotometrically using a wavelength 590 nm. These readings can then be related directly to enzyme activity.

#### 3.8.1 Preparation of reagents and solutions

##### *Amylzyme substrate (in tablets)*

Amylzyme tablets were supplied by Megazyme International (Bray, Ireland) in pack sizes of 200 or 1000 tablets (catalogue numbers T-AMZ 200 and T-AMZ 1000). The tablets were unbuffered, allowing their use in conjunction with the following buffer selected to suit the  $\alpha$ -amylase present during baking.

##### *Buffer A (for cereal $\alpha$ -amylase)*

Maleic acid (11.6 g) was dissolved in 900 mL of distilled water and the pH was adjusted to 6.0 with sodium hydroxide solution (2 M) (approximately 80 mL was found to be required). Calcium chloride dihydrate (0.74 g) was added and dissolved. The pH was readjusted to 6.0 and the volume of the solution made up to 1 litre. This solution was stored at room temperature (Megazyme International 1996a)

##### *Stopping Reagent*

A stopping reagent was prepared by dissolving 1% weight by volume Tris (hydroxymethyl) aminomethane base in distilled water and the solution stored at room temperature until further use.

### 3.8.2 Extraction of $\alpha$ -amylase from flour and bread samples

Malt flour was used in the initial trials for determining and calculating  $\alpha$ -amylase activity in order to check the repeatability and consistency of the assay. In the subsequent experiments the enzyme was extracted from malt flour, wheat flour, and dough and then at different stages of bread baking using the same procedure. This was based upon the recommended method from Megazyme (Megazyme International 1996a).

#### *Enzyme Extraction*

The sample was weighed (exactly 1.0 g) and suspended in Buffer A (20.0 mL), mixed by inversion and allowed to extract over 15 min. At five minute intervals the tubes were thoroughly vortexed. The slurry was then centrifuged ( $1,000 \times g$  corresponding to 3,000 rpm) for 10 mins. An aliquot (0.5 mL) of the filtrate was then diluted 100-fold by addition to 49.5 mL of Buffer A. Assays for enzyme activity were performed within 2 hr of extract preparation.

### 3.8.3 Assay procedure for $\alpha$ -amylase

With each set of analyses a single reaction blank was run. This was performed by adding an Amylazyme tablet to the buffer and proceeding as for the enzyme assays. The blank was used to zero the spectrophotometer and the absorbance of the reaction solutions was then measured against the reaction blank.

1. Aliquots (1.0 mL) of diluted enzyme extracts were pre-equilibrated at 40 °C for 10 mins.
2. It is noted that in handling Amylazyme tablets, forceps were used and care was taken to avoid any contact of fingers, skin or saliva with the tablets to minimise contamination of the tablets with human  $\alpha$ -amylase.
3. A substrate /enzyme blank was prepared by adding stopping reagent (described in Section 3.8.1, page 44) to an aliquot of the enzyme solution prior to addition of an Amylazyme tablet.
4. For each assay tube, an Amylazyme tablet was added (without stirring). The substrate hydrates rapidly and absorbs most of the free liquid. Following the

instructions provided, the suspension was not stirred. The tubes were incubated at 40 °C for 10 mins.

5. Tris(hydroxymethyl) aminomethane base solution (10 mL, 2% w/v) was added after exactly 10 mins (from the addition of the tablet) to terminate the reaction. The tubes were stirred vigorously on a vortex mixer and then left to stand at room temperature.
6. After approximately 5 min the tubes were stirred again and the contents filtered through Whatman No.1 (9 cm) filter paper.
7. The absorbance at 590 nm of each filtrate was measured against the reaction blank.

### 3.8.4 Calculation of $\alpha$ -amylase activity

$\alpha$ -Amylase activity was determined by reference to the standard curve supplied by Megazyme with the substrate and shown in Appendix 1. For this the absorbance reading (590 nm) was used read from the graph to give a figure for the activity in units of milli-Ceralpha Units per assay (that is per 1.0 mL). These values were then calculated back to the flour originally used for enzyme extraction as follows:

$$\text{Units per g of original sample} = \text{Milliunits per assay} \times \frac{1}{1000} \times 100 \times \text{dilution}$$

Where:

$$\frac{1}{1000} = \text{Conversion from milliunits to Units}$$

$$100 = \text{Intial extraction volume (i.e. 100 mL per g of solid)}$$

$$\text{dilution} = \text{Further dilution of the intial extraction solution}$$

### 3.9 Calculation of enzyme activities to a dry weight basis

The results obtained for enzyme activities were routinely adjusted by calculation to a dry weight basis. The purpose was to facilitate the direct comparison of the results particularly for different sample types. All samples analysed for enzyme activity were also tested for moisture content. These were then used in the following formula:

$$\text{Enzyme activity (Corrected to a constant moisture basis)} = \text{Enzyme activity (as is basis)} \times \frac{100 - \text{constant moisture figure}}{100 - \text{actual moisture of sample}}$$

Where the constant moisture figure used is zero so that all values are expressed on a dry weight basis.

### 3.10 Water extraction of carbohydrates for HPLC analysis

The procedure for extraction using water was according to AACC Method 80-04 (AACC 1994d). In this method, dough and bread samples were homogenized with Milli-Q water using a homogeniser (Janke and Kunkle Type UltraTurrax) at the speed of 9,500 revolutions per min. All the samples were then stirred for sugar extraction on magnetic stirrers for 30 minutes at room temperature. The solids were then allowed to settle and removed by filtering the solution through Advantec 2 filter paper. The aliquots were kept in the freezer until analysis of carbohydrates by HPLC. Samples (2 to 8 g) were extracted depending on the sample to be tested.

### 3.11 Analysis of carbohydrates using HPLC

The High Performance Liquid Chromatography (HPLC) was employed to quantify and identify the simple sugars that were present in dough and bread samples by the following procedures.

#### *Standards preparation*

Five different sugars were selected as reference sugars. Fructose and maltose were both from Sigma Co. Sucrose, lactose and glucose were obtained from BDH. All these sugars

were of Analar grade. One gram of each sugar was dissolved in 100 mL of Milli-Q water (Millipore Co.) individually. Sugars solution at this concentration were then diluted and mixed to prepare standards curves in the concentration range of 0.1 to 1.0%. The retention times of each individual sugar was identified.

### ***Instrumentation***

The Varian Star HPLC system was used. The system consisted of a Varian Star 9012 pump, a Varian Star 9100 autosampler and a Varian RI - 4 refractive index detector. The area counts response were integrated by Interactive Graphics Varian Software Version 5.31. The samples were analysed using a lead form column, Aminex HPX - 87P column (300 × 7.8 mm) equipped with a Carbo-P Refill Cartridges (30 × 4.6 mm) guard column both from Bio-Rad Laboratories. The mobile phase was 100% degas HPLC grade Milli-Q water (Millipore Co.). HPLC was set at a flow rate of 0.6 mL per min and 10 $\mu$ L of samples were injected each time for analyses. The separations were performed at 85 °C using a Bio-Rad HPLC column heater (220 V) for 40 min. All the samples were filtered using a 0.2  $\mu$ m filter (Schleicher and Schuell) before injection into the HPLC column. In some cases results were presented either on a dry moisture or a 14% adjusted basis using the same approach as described for enzyme activity data in Section 3.9.

### **3.12 Preparation of microcapsules by spray drying**

Microcapsules were prepared using procedures based upon the published methods of Zhao and Whistler (1994), Trindade and Grosso (2000) and Uddin and others (2001). The procedure adopted used a pilot scale spray drying system and a carrier material consisting primarily of rice starch.

The Maltodextrin used was Fieldose 17, supplied by Starch Australasia, Sydney (see Appendix 2). The purified rice starch, guar galactomannan and citric acid used in preparing the slurry feed that was spray dried were all supplied by Sigma Chemical Co, St Louis, Missouri. The preparation was based on the dissolution of 2.5 g galactomannan in distilled water (500 mL) by stirring on a magnetic stirrer with gentle heating (final temp less than 50 °C). Rice starch (100 g) was gradually added with continued stirring, after which the pH was adjusted to 3.5 by dropwise addition of 1.0 M citric acid. Maltodextrin was incorporated at a rate of 5.0 g per batch.

A Niro Atomiser (Copenhagen) type minor spray drier was used. The conditions used were: inlet temperature 120 °C, setting I, 3.5 percent setting; outlet temperature 75 °C; flow rate 7 mL per minute; total final volume of feed sample approx 600 mL.

The spray drying of the sample followed a warm-up and equilibration stage of around one and a half hr. The samples took approximately seventy minutes to complete. The product was weighed to establish the yield and stored in an air-tight container.

### **3.13 Examination of microcapsules by scanning electron microscopy**

The microencapsulated maltodextrin powder was inspected by environmental scanning electron microscopy (Type Philips XL-30).

### **3.14 Differential scanning calorimetry of maltodextrin samples**

The maltodextrin sample was analysed by differential scanning calorimetry using a Perkin-Elmer DSC 7 instrument. The sample was prepared by mixing in the proportion of one part of maltodextrin to three of water. Maltodextrin (0.5 g) and 1.5 mL of water were weighed in a beaker and stirred thoroughly. The mixture was stored overnight prior to sub-sampling and analysis.

The sample was stirred in the beaker thoroughly with a spatula. Approximately 0.01 g of sample was pipetted onto a mini aluminium pan and forceps used to transfer pan with the sample onto a pan sealer. A lid was placed over the aluminium pan and sealed. The thermogram was recorded using a heating program of 10 °C per minute over the range of 25 °C to 100 °C.

### **3.15 Physical analyses of bread samples**

Experimental breads were analysed for their moisture content and assessed for loaf volume and textural characteristics.

### *Determination of loaf volume*

Bread loaf volume was determined using the standard method of the Royal Australian Chemical Institute Cereal Chemistry Division (RACI) (RACI 1995). For this, two measurements were taken: the first one was the circumference of the baked loaves and the other measurement was the circumference that was perpendicular to the first measurement and the sum of the measurements was recorded. All measurements were taken after one hr of cooling at ambient temperature. An average of two baked loaves for the same treatment were taken and expressed in units of cm.

### *Texture analysis*

Bread firmness was measured with a Texture Analyser (TA-XT2, Stable Micro Systems, England) following AACC method 74-09 (1994c). This measures the firmness of bread analysing the force required to compress bread crumb to a preset distance. The instrument consists of a 5 kN compression load cell and a aluminium plunger of 36 mm diameter. The crosshead speed was set at 100 mm per min. The compression of was approximately 10 mm into the bread crumb samples. The samples were of 25 mm thickness.

A slicing guide (Breville) was used to ensure that the slices of the baked loaves were consistently of 25 mm thickness. Firmness was recorded as the maximum force (N) after each compression. Two readings were taken for one sample slice where compression was taken on the centre of each side of the bread slice. Total of at least 4 bread slices were measured from one baked loaf and the average of duplicate loaves were calculated.

## Chapter 4

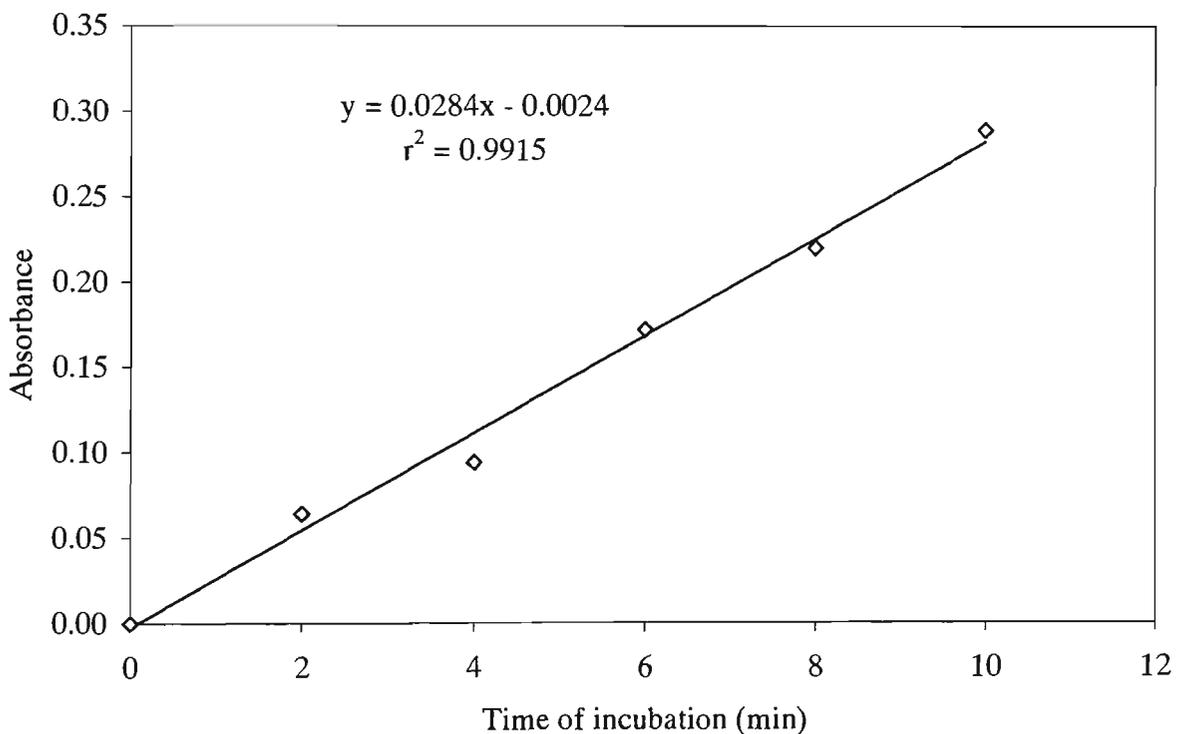
### Results and discussion – the assay and levels of amylolytic enzyme activities during baking

In the preliminary phases of this research, consideration was given to the selection of suitable methods for assay of the enzyme  $\alpha$ -amylase. This enzyme has been widely studied and there are a variety of approaches available for its routine estimation for clinical diagnostic purposes as well as for quality control in cereal grain grading, handling and storage, in brewing and for baking purposes. Some of these are based upon viscometric approaches and others utilise dye labelled starch substrates. The viscometric procedures do not readily facilitate comparisons, as they do not have a linear relationship between either the time of incubation or the amount of enzyme extract and the measured parameter of viscosity. More recently various substrates and procedures have become available and gained acceptance. A dyed starch substrate was used to measure the wheat enzyme by Barnes and Blakeney (1974). For the current study a newer dyed starch substrate known as Amylazyme was adopted (Megazyme International 1996a). In the first instance it was decided to evaluate the suitability and the consistency obtained using this product according to the procedure recommended by the manufacturer.

#### 4.1 Selection and validation of assay method for $\alpha$ -amylase

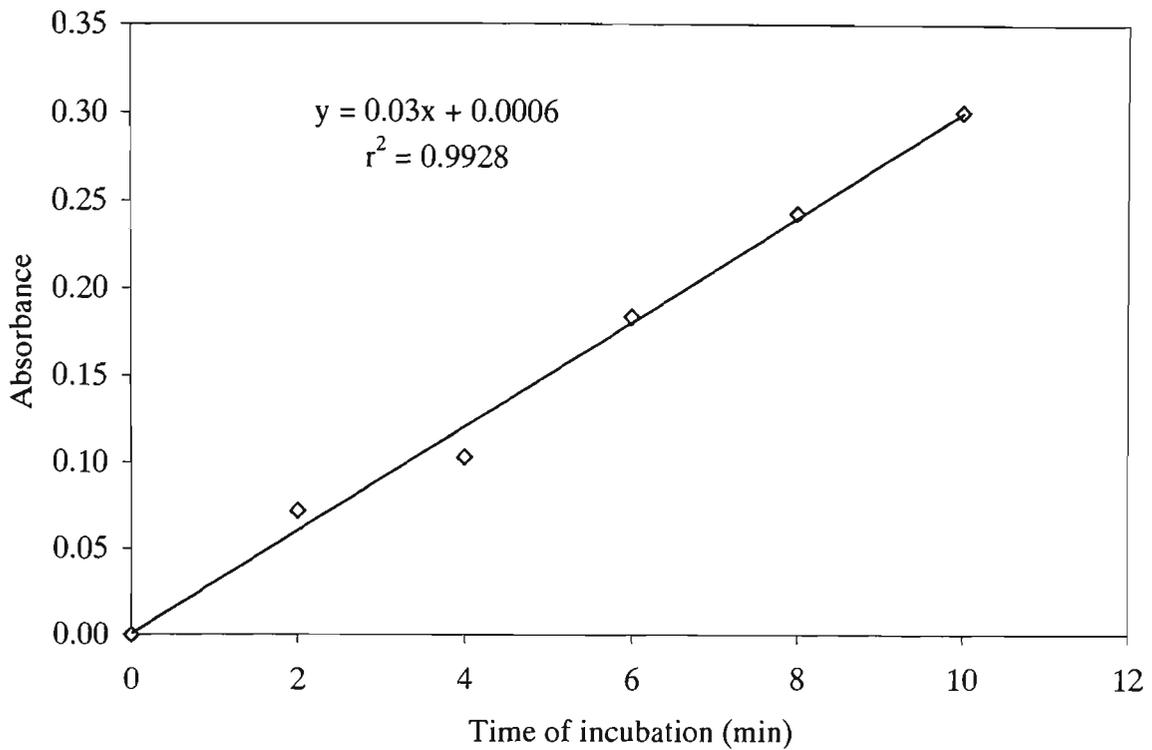
A series of experiments were carried out to establish the suitability of the Amylazyme assay method. For this, reagents were prepared according to the procedure suggested for malt flour. The buffer system was prepared using maleate adjusted to pH 6.0. The trials utilised malt flour supplied by Megazyme as a control sample. The enzyme extraction for the assay was prepared by weighing 1 g of malt flour suspended in buffer A (20.0 mL), mixed by inversion and allowed to extract for 15 min at every 5 min interval by vortex. The slurry was then centrifuged ( $1,000 \times g$  corresponding to 3,000 rpm) for 10 min at 20 °C. An aliquot (0.5 mL) of the filtrate was then diluted 100-fold by addition to 49.5 mL of Buffer A. The assay was performed by taking aliquots (1.0 mL) of diluted malt flour extract and pre-equilibrated at 40 °C for 5 min. With the help of forceps an Amylazyme tablet was added to each tube without stirring and incubated at 40 °C for exactly 10 min.

On incubation Trizma® base solution (10 mL, 2% w/v) was added after exactly 10 min to terminate the reaction. The tubes were stirred vigorously on a vortex mixer. After 5 min the tubes were stirred again and filtered through a filter paper to obtain a clear aliquot. The absorbance at 590 nm of the filtrate was measured against the reaction blank. The substrate/enzyme blank was prepared by adding Trizma® base to the enzyme solution before adding the amylzyme tablet. The blank was used to zero the spectrophotometer and the absorbance of the reaction solutions was then measured against the reaction blank. Typical results obtained for the assay of  $\alpha$ -amylase are shown in Figures 4.1, 4.2 and 4.3 where the time courses of reaction are shown for three different extracts of the malt flour supplied by Megazyme.

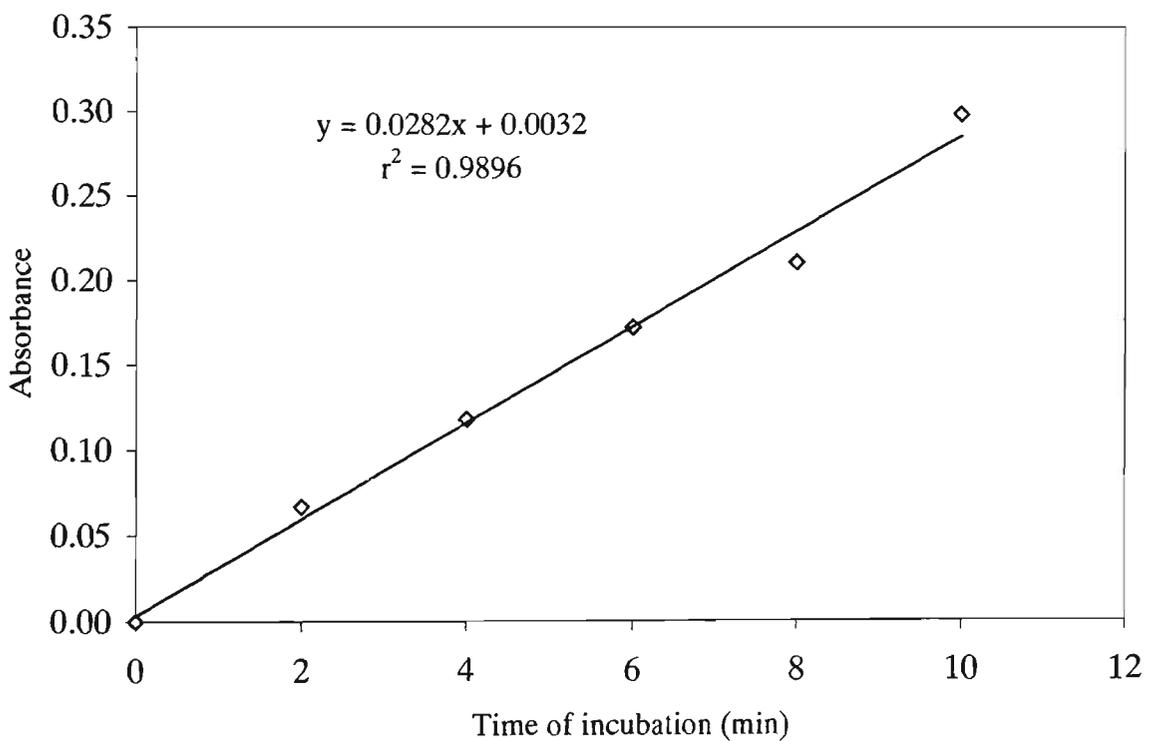


**Figure 4.1** The time course of reaction when  $\alpha$ -amylase extract from malt flour was incubated with substrate for varying times (extraction 1)

The results in Figure 4.1 show a reasonably linear time course of reaction. Similarly for the duplicate extractions and analyses (Figures 4.2 and 4.3) the curves were similar to those shown in Figure 4.1. The results of these experiments are summarised in Table 4.1. The values for absorbance obtained at ten minutes were compared because this is the time recommended in the procedure for a single point reading.



**Figure 4.2** The time course of reaction when  $\alpha$ -amylase extract from malt flour was incubated with substrate for varying times (extraction 2)



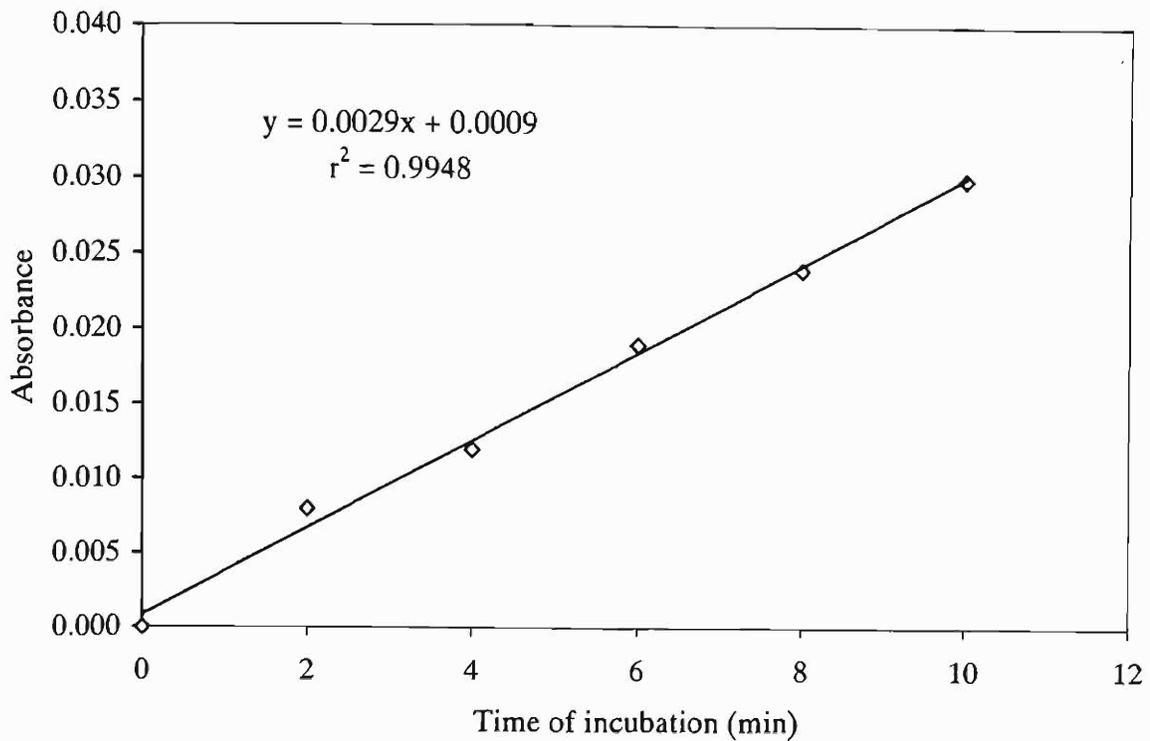
**Figure 4.3** The time course of reaction when  $\alpha$ -amylase from malt flour extract was incubated with substrate for varying times (extraction 3)

**Table 4.1** Summary of  $\alpha$ -amylase results obtained when three separate extracts from malt flour were assayed. The absorbance values at ten minutes are compared with those estimated from the time course data (Figures 4.1-4.3) using the lines of best fit

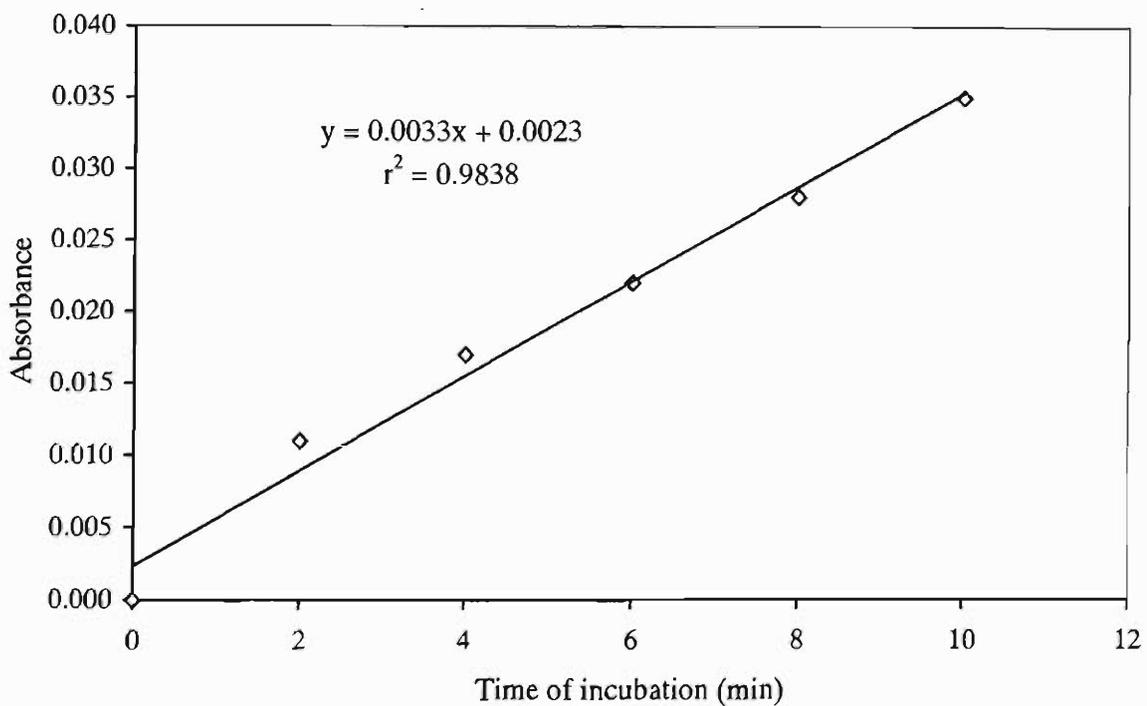
|                                | Absorbance at 10 min | Absorbance at 10 min estimated from the line of best fit |
|--------------------------------|----------------------|--|
| Assay 1                        | 0.289                | 0.282  |
| Assay 2                        | 0.301                | 0.300  |
| Assay 3                        | 0.298                | 0.285  |
| Mean                           | 0.296                | 0.289  |
| Standard deviation             | 0.0062               | 0.0096   |
| Coefficient of variability (%) | 2.1                  | 3.3  |

From these results it was found that the ten min reading obtained from the single point and also from the linear regression value were very similar. The extraction and assay method for the Amylazyme procedure using malt flour gave repeatable and consistent data. It was concluded that this method was suitable for the measurement of  $\alpha$ -amylase activity in the malt flour used here to evaluate the procedure. The next step was then to apply the method to dough samples for which lower activities might be expected.

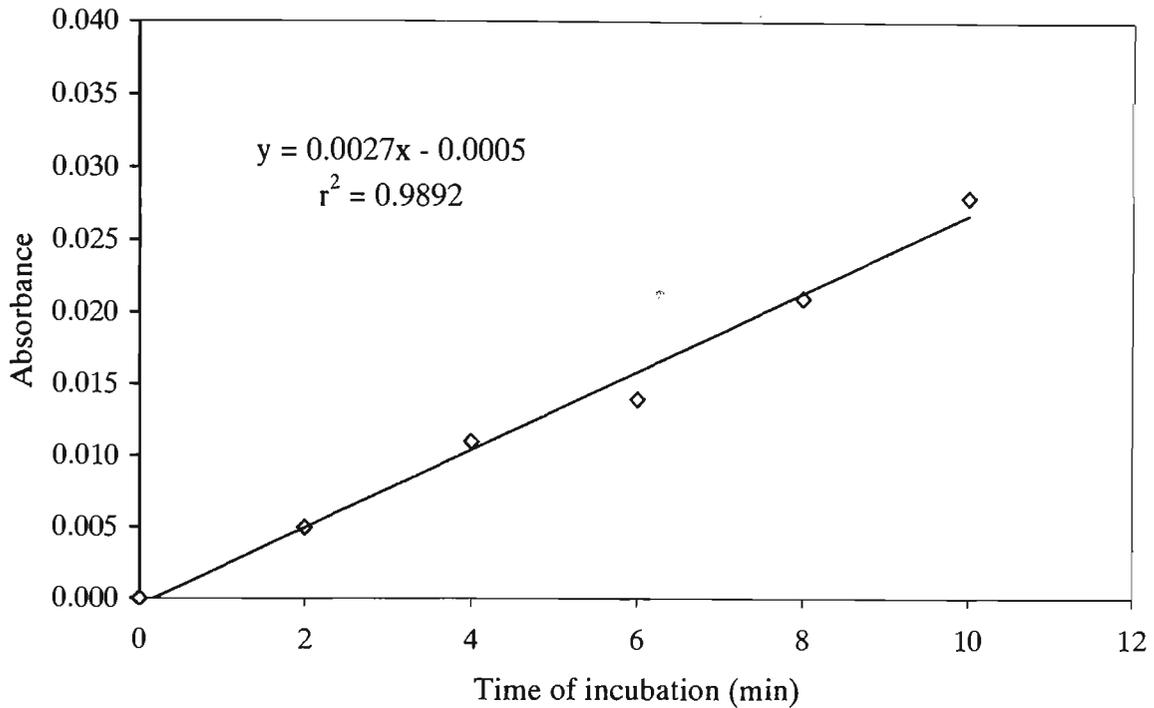
The results of replicate extractions and assays of wheat flour doughs prepared with the breadmaking formula and procedures described in Chapter 3 are presented in Figures 4.4 to 4.6 and also in Table 4.2.



**Figure 4.4** The time course of reaction when  $\alpha$ -amylase extracted from dough was incubated with substrate for varying times (dough 1)



**Figure 4.5** The time course of reaction when  $\alpha$ -amylase extracted from dough was incubated with substrate for varying times (dough 2)



**Figure 4.6** The time course of reaction when  $\alpha$ -amylase extracted from dough was incubated with substrate for varying times (dough 3)

**Table 4.2** Summary of  $\alpha$ -amylase results obtained when extracts of three separate doughs were assayed. The absorbance values at ten min are compared with those estimated from the time course data (Figures 4.4-4.6) using the lines of best fit.

|         | Absorbance at 10 min | Absorbance at 10 min estimated from the line of best fit |
|---------|----------------------|--|
| Assay 1 | 0.030                | 0.030  |
| Assay 2 | 0.035                | 0.035  |
| Assay 3 | 0.028                | 0.032  |

From the analyses of the enzyme in dough extracts the results were again repeatable and good correspondence was seen between the single ten min point and those estimated from the linear regression equation. On this basis, the procedure was adopted and applied in further studies of the levels of the enzyme during the stages of the baking process.

## 4.2 Results for $\alpha$ -amylase activity assayed at various stages of the breadmaking process

In establishing a baking procedure, it was decided to utilise commercial breadmakers as these allow the readily repeatable production of small loaves for evaluation and comparison purposes. This approach has been utilised in a relatively recent study of the potential of hydrocolloid gums as ingredients in breadmaking formulations (Schwarzlaff and others 1996). The formulation described in Chapter 3 was used in combination with a rapid program as these approximate the rapid dough method applied commercially in the Australian baking industry.

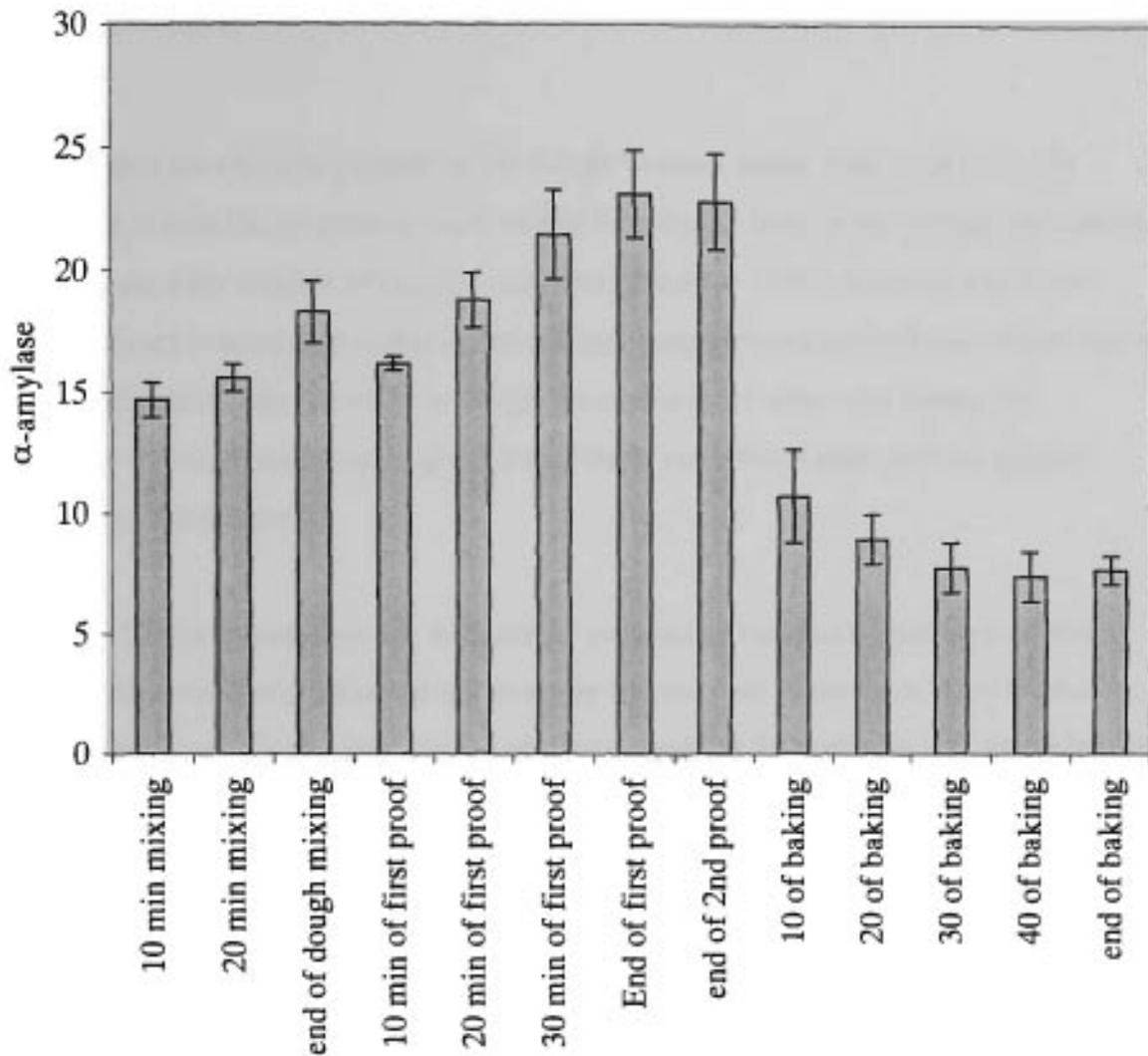
At various stages of the breadmaking process, small samples of dough and bread crumb were taken. Each of these samples was analysed for moisture. The results of moisture testing are presented in Table 4.3. In addition, dough and pieces were sampled and for this purpose the region of the doughs and loaf selected was as close to the centre as possible. The reason for this was to assess the extent of loss in activity in the part of the dough/loaf which would have been subjected to the least increase in temperatures during the process. Sample extracts were prepared and assayed for  $\alpha$ -amylase using the Megazyme Amylazyme method. Based upon the earlier evaluations of the assay procedure, the single point assay approach was used. In addition, the data presented have been calculated on a dry matter (or dry weight) basis so that they can be directly compared. The results are presented in Figure 4.7.

The primary value of moisture data was for use in the calculations of the enzyme values to a constant moisture basis. Selected dough samples were analysed in duplicate showed good repeatability. The moistures were as expected and show a decline during the baking procedure. For amylase, each result represents the mean of at least duplicate determinations and the data demonstrate that the repeatability was again good, as shown by the standard deviation values determined for each mean value. In comparing the activities measured at each stage considered, the first observation is that there is a major drop in activity when the proofed dough is heated during baking. This is as expected as the source of  $\alpha$ -amylase as part of the commercial improver in the formulation was of fungal origin. It is reported that typically the enzymes from fungi are inactivated at temperatures of around sixty degrees (Takagi and others 1971; Guy 2001; Wong and

Robertson 2003a). The data in Figure 4.7 indicates that there is considerable activity remaining in the dough as it bakes and in the final loaf at the end of the breadmaking process. The data also indicates that the level of activity appears to increase during the proofing of the dough. Whilst there seems to be no comparable data in the literature, these results are not as might have been expected. It is noted that the whole trial was repeated on at least six different occasions and the patterns obtained were the same.

**Table 4.3** Moisture contents of strong bakers flour, dough samples taken at various stages during the breadmaking process as well as the final baked loaf

| Sample description/stage of baking | Moisture content (g per 100 g) |
|------------------------------------|--------------------------------|
| Flour                              | 13.13 ± 0.07                   |
| 10 min of mixing                   | 44.94 ± 0.08                   |
| 20 min of mixing                   | 44.63 ± 0.05                   |
| 30 min of mixing                   | 44.92 ± 0.07                   |
| 10 min of proof                    | 44.65 ± 0.08                   |
| 20 min of proof                    | 44.27 ± 0.09                   |
| 30 min of proof                    | 44.14 ± 0.07                   |
| End of first proof                 | 43.93 ± 0.05                   |
| End of second proof                | 43.75 ± 0.05                   |
| 10 min of baking                   | 43.69 ± 0.06                   |
| 20 min of baking                   | 43.53 ± 0.05                   |
| 30 min of baking                   | 42.36 ± 0.04                   |
| 40 min of baking                   | 41.50 ± 0.07                   |
| End of baking                      | 40.67 ± 0.04                   |



**Figure 4.7** The activity of  $\alpha$ -amylase in samples taken during the breadmaking process

Notes: Units of activity are Ceralpha units per gram expressed on a dry weight basis

Results are presented as mean value  $\pm$  standard deviation

In interpreting these amylase results it appears as though the activity increases gradually during proofing. This may be due to some form of activation of the enzyme although further studies would be required to clarify these findings. Of more interest are the apparently low rates of loss in enzyme during baking. It might have been predicted that virtually no enzyme activity would have been found at the end of the baking period. It has been documented that the temperature at different points in the loaf vary depending upon the distance of the dough material from the loaf surface, reflecting the resistance to transfer of heat from the surface to the centre of the dough. Despite this the evidence

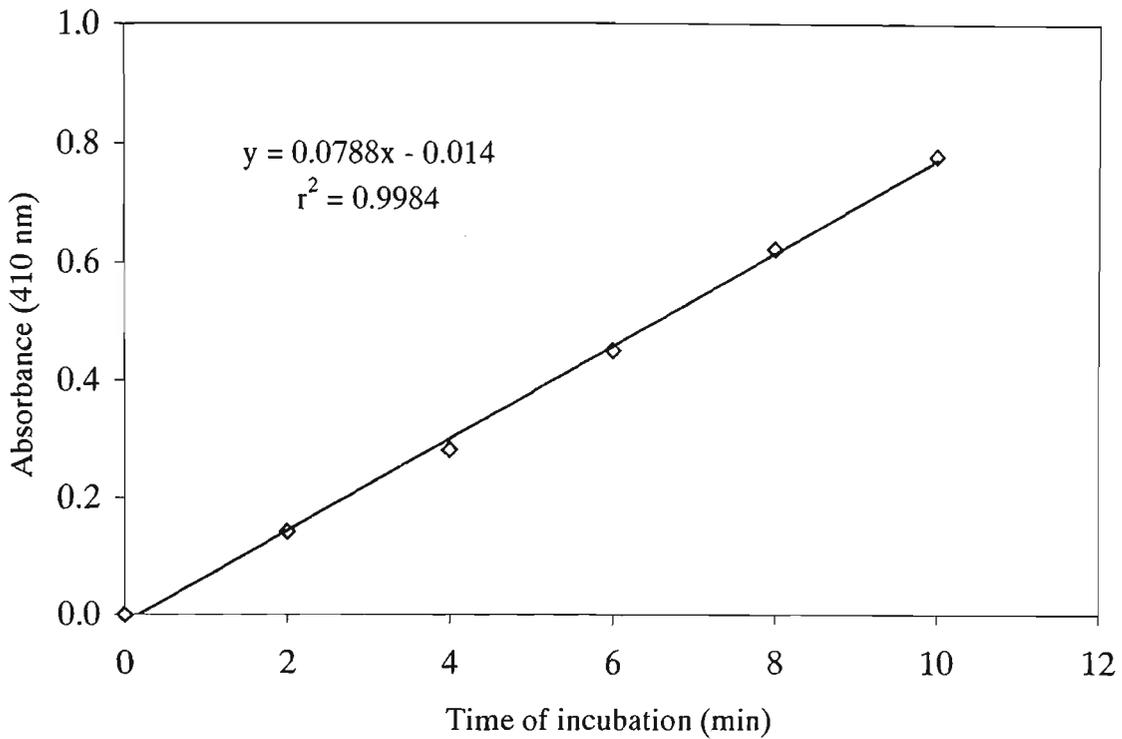
indicates that temperatures in the centre of the dough approach 95 °C at the end of baking.

It appears that the enzyme present in the dough is more stable than expected. An explanation is that the enzyme is more stable in a dough than in the typical experimental conditions used for studies of enzyme stability (Stauffer 1989). Usually a buffered aqueous extract is used and subjected to various temperatures around and above that at which inactivation may occur. In a dough the amounts of water are lower, the availability of starch substrate is greater and these conditions may provide greater protection to the enzyme.

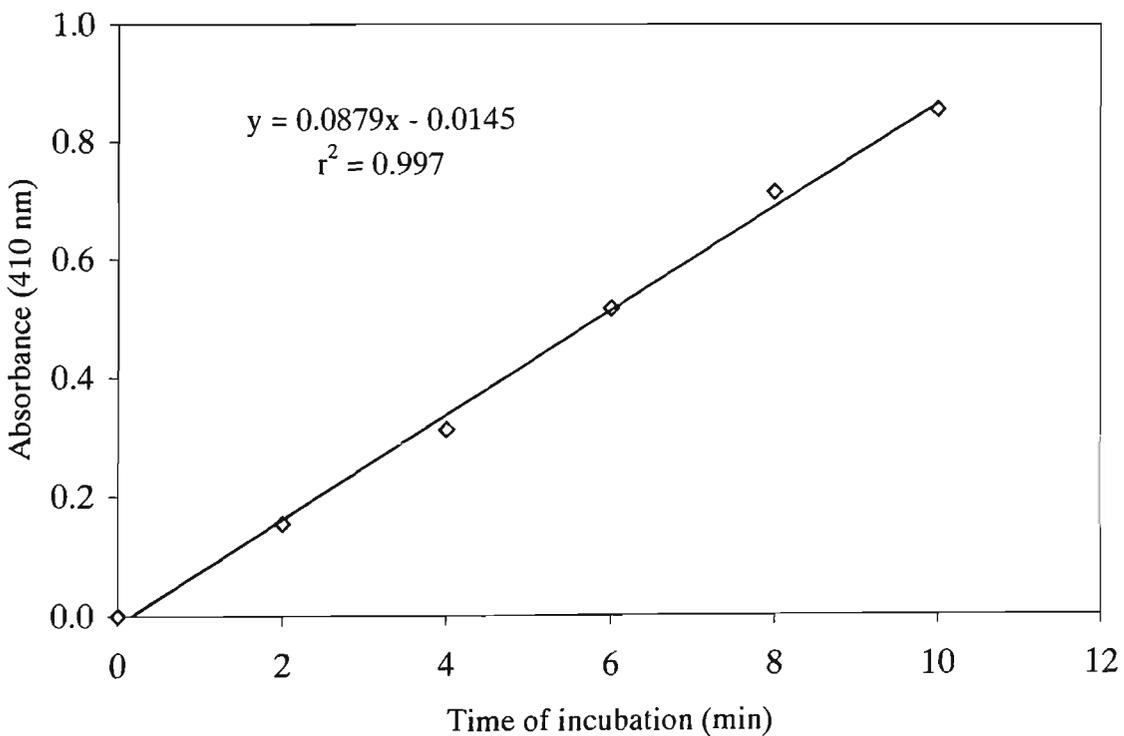
In order to further investigate the impacts of processing on starch hydrolysis during baking, further trials were carried out to study the enzyme  $\beta$ -amylase. This is also known to be present in the ingredients of a breadmaking formulation and may ultimately lead to the production of partial hydrolysis products of starch that in turn may contribute to the texture characteristics of bread during storage.

### **4.3 Selection and validation of assay method for $\beta$ -amylase**

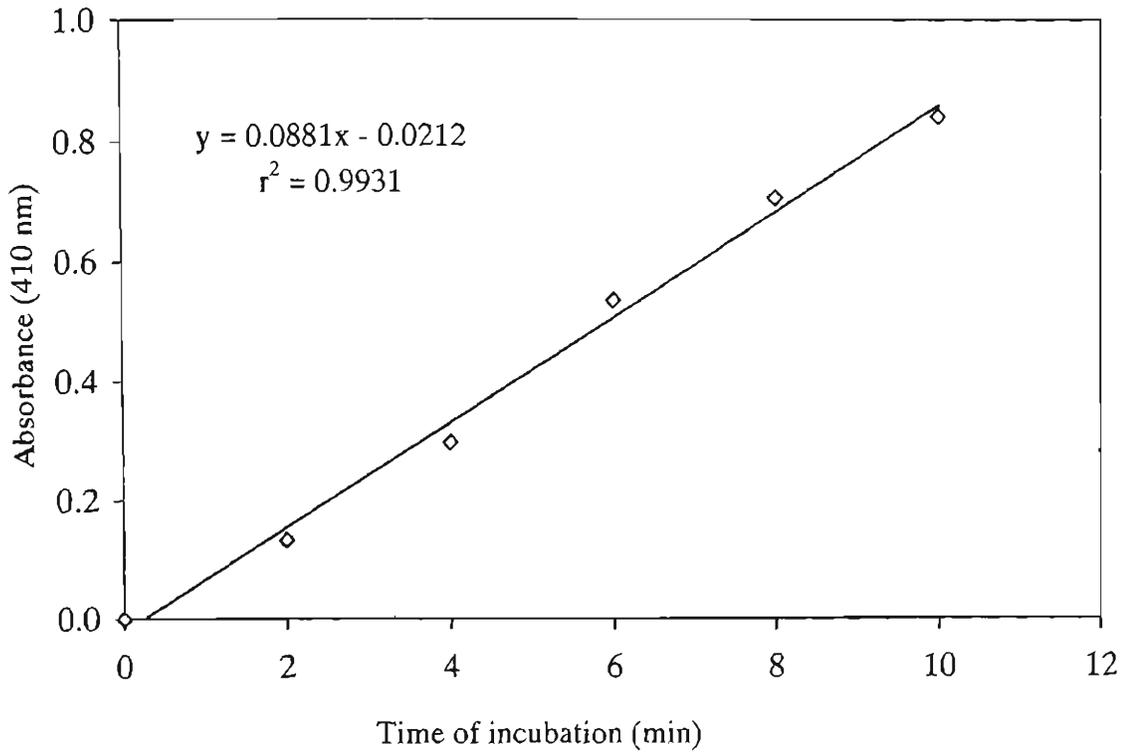
The initial step was to evaluate the Megazyme Betamyl® procedure. This utilises a carefully defined substrate so that it is possible to measure the enzyme activity in the presence of  $\alpha$ -amylase. The approach to evaluation was similar to that outlined earlier for  $\alpha$ -amylase. A further aspect of the method which required evaluation was the fact that the levels of activity measured by this method are influenced by the inclusion of the reagent cysteine (Megazyme 1996a; Kruger and others 1987). This results in the measurement of higher levels of activity as some of the enzyme is normally in a bound form in a flour or dough sample. Cysteine results in the release of the bound form of the enzymes allowing the measurement of total activity of enzyme. The results for five experiments on the time course of reactions for three different extractions and assays are shown in Figures 4.8 to 4.12. The first of these was for the Megazyme reference sample and when the results were calculated and compared with the known value provided then the expected value was obtained. The data for the flour samples are summarised in Table 4.4



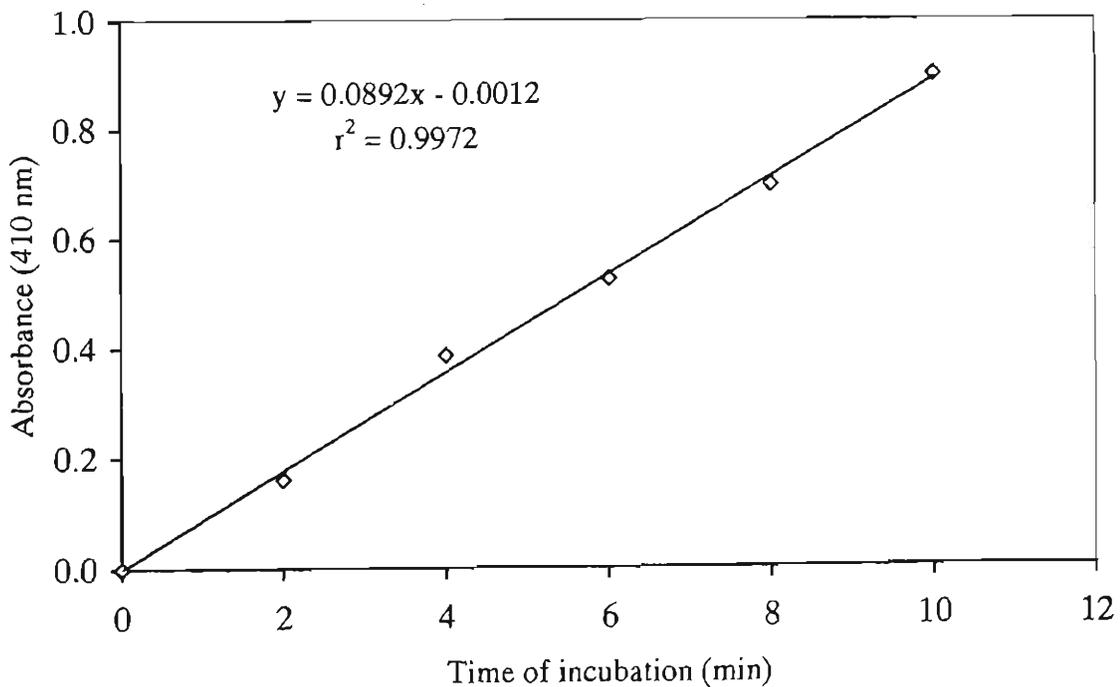
**Figure 4.8** The time course of reaction when  $\beta$ -amylase extracted from the Megazyme reference malt flour sample without added cysteine in the extracting buffer was incubated with substrate for varying times



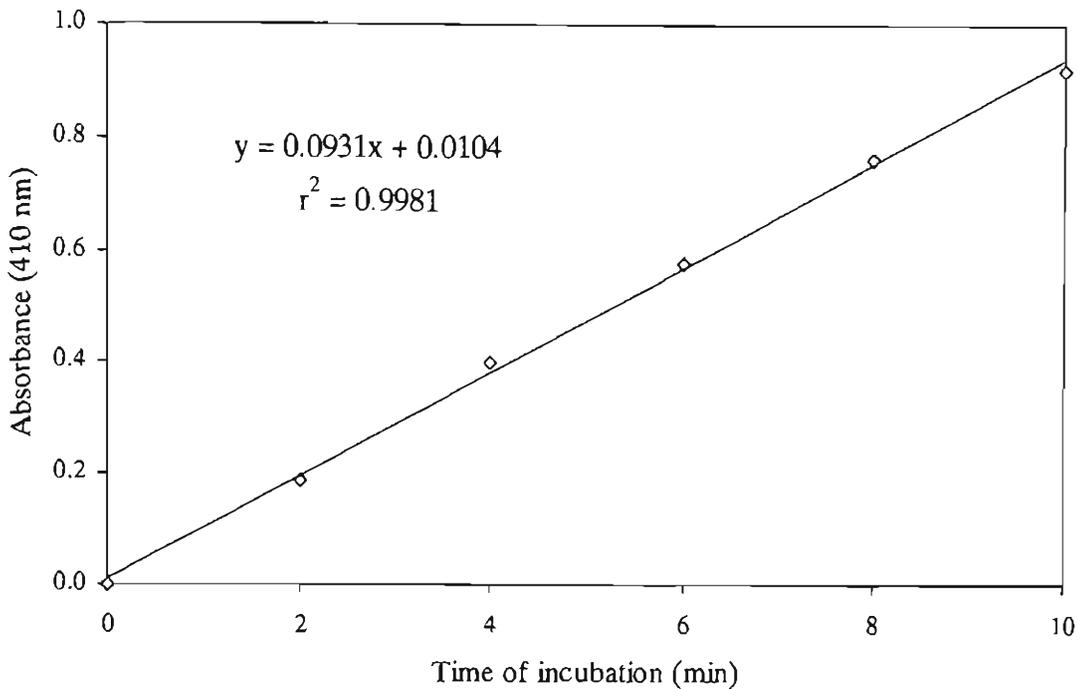
**Figure 4.9** The time course of reaction when  $\beta$ -amylase extracted from strong bakers flour without added cysteine in the extracting buffer was incubated with substrate for varying times (sample 1)



**Figure 4.10** The time course of reaction when  $\beta$ -amylase extracted from strong bakers flour without added cysteine in the extracting buffer was incubated with substrate for varying times (sample 2)



**Figure 4.11** The time course of reaction when  $\beta$ -amylase extracted from strong bakers flour without added cysteine in the extracting buffer was incubated with substrate for varying times (sample 3)



**Figure 4.12** The time course of reaction when  $\beta$ -amylase extracted from strong bakers flour with added cysteine in the extracting buffer was incubated with substrate for varying times

**Table 4.4** Summary of correlation values for  $\beta$ -amylase assay results obtained when four separate extracts of strong bakers flour were assayed for varying times

|                       | Equation of line of best fit | Correlation coefficient ( $r^2$ ) of line of best fit |
|-----------------------|------------------------------|---|
| Assay 1 (Figure 4.9)  | $y = 0.0879x - 0.0145$       | 0.997   |
| Assay 2 (Figure 4.10) | $y = 0.0881x - 0.0212$       | 0.9931  |
| Assay 3 (Figure 4.11) | $y = 0.0892x - 0.0012$       | 0.9972  |
| Assay 4 (Figure 4.12) | $y = 0.0931x + 0.0104$       | 0.9981  |

Note: Assays 1-3 are for samples extracted without added cysteine in the extracting  
For assay 4 the flour was extracted with added cysteine in the extracting solution

In considering these assay data, the correlation coefficients were excellent in all cases. Therefore for further studies of the levels of the enzyme activities during baking, it was appropriate to again utilise the single point assay approach recommended by the

manufacturer of the substrate. A further observation is that the incorporation of cysteine into the extracting solution did result in a higher apparent activity level of  $\beta$ -amylase. When the results were calculated in enzyme units for the bakers flour sample the mean activity was ten percent higher when cysteine was used (Table 4.5). In order to study the activity of the enzyme during baking, extracts were prepared both with and without cysteine.

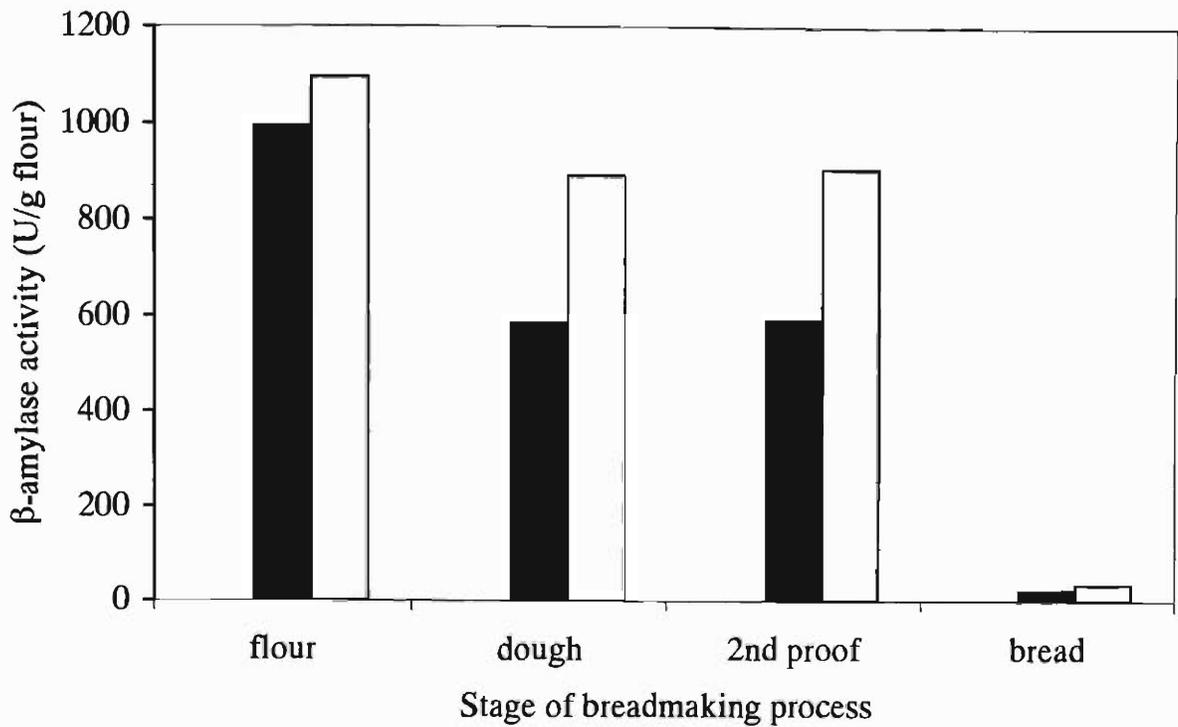
**Table 4.5**  $\beta$ -Amylase results for strong bakers flour comparing the impact of assaying with and without added cysteine in the extracting buffer

|                         | <b>With<br/>added Cysteine</b> | <b>Without<br/>added Cysteine</b> |
|-------------------------|--------------------------------|-----------------------------------|
| Mean activity           | 1095                           | 994                               |
| Standard deviation      | 38                             | 23                                |
| Coefficient of variance | 3.4%                           | 2.0%                              |

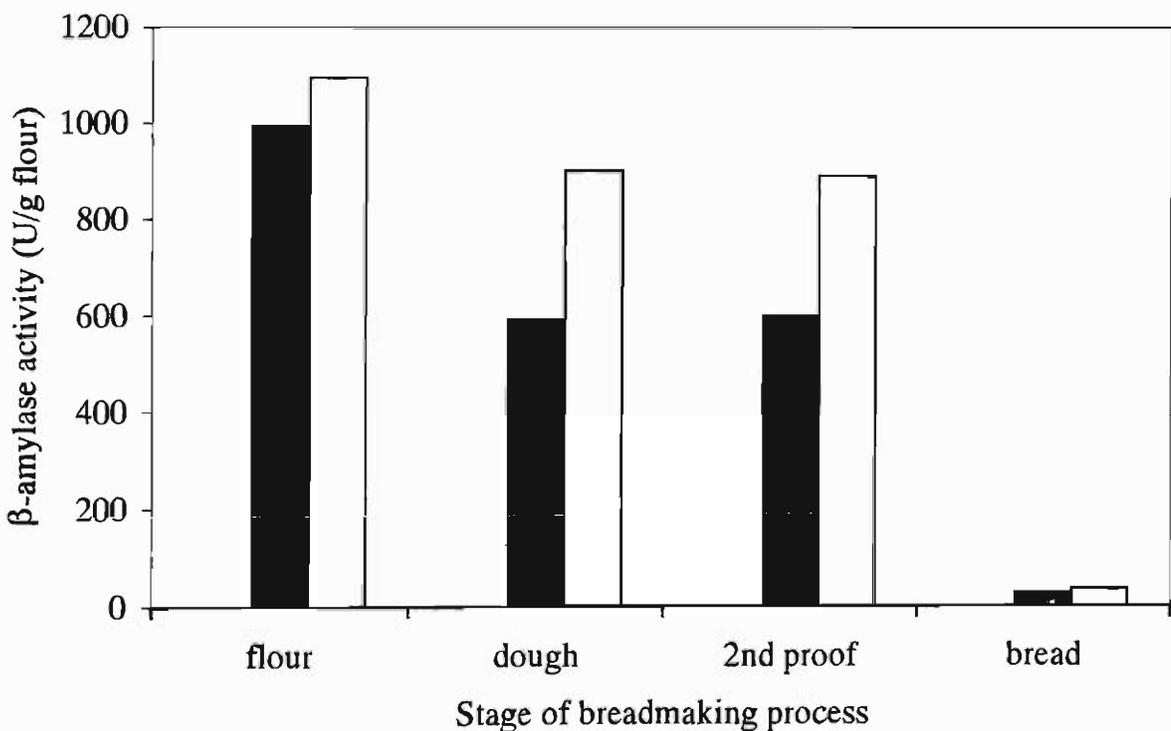
Note: All values are calculated from the results for duplicate assays for each of two separate extractions  
Activities are expressed in Units per g flour

#### **4.4 Results for $\beta$ -amylase activity assayed at various stages of the breadmaking process**

Loaves were baked using the same flour, ingredient formulation and process as previously used in the study of  $\alpha$ -amylase. A further issue investigated was the impact of taking dough and crumb samples from different parts of the sample. Thus measurements were carried out on sub-samples from immediately under the surface of the crust and from the centre of the dough piece or loaf. The results obtained for free and total  $\beta$ -amylase activities are presented in Figures 4.13 and 4.14.



**Figure 4.13** The levels of  $\beta$ -amylase activity found when the enzyme was extracted from samples with (lighter shading) and without added cysteine (darker shading) in the extracting buffer for inner portions of the loaf



**Figure 4.14** The levels of  $\beta$ -amylase activity found when the enzyme was extracted from samples with (lighter shading) and without added cysteine (darker shading) in the extracting buffer for outer portions of the loaf

The patterns found were similar for the two different locations sampled. The results show that the incorporation of cysteine is of greater significance in the measurement of total  $\beta$ -amylase activity. This indicates that a greater proportion of the enzyme is in the bound form in the dough than in the original flour from which the dough was prepared. The enzyme appears to be relatively stable during the proofing of the dough as similar levels were found at the two stages of proofing. However, virtually all of the activity was lost upon the heating of the dough which occurred during baking. It is likely that the amounts remaining after baking were sufficiently low as to have no impact on the storage characteristics of a loaf of bread.

#### **4.5 Summary of results and conclusions**

The enzymes  $\alpha$ - and  $\beta$ -amylase in flour, dough and baked products could be reproducibly measured using Megazyme substrates. The data indicated that the levels of activity remained high during the proofing of a dough, with some apparent increases in the  $\alpha$ -amylase activity. The relative amounts of bound  $\beta$ -amylase were higher in doughs than the corresponding flour. The overall losses in activities of the two amylolytic enzymes were quite different when heating of the dough occurred during baking. Virtually all of the  $\beta$ -amylase was inactivated. On the other hand, the amounts of  $\alpha$ -amylase measured were higher than expected. Further studies are warranted to provide a greater understanding of the factors impacting of the stability of this enzyme in the dough. In particular, the potential significance of the enzyme acting after the completion of baking and the impact on staling are of considerable interest.

## Chapter 5

### Results and discussion—HPLC analysis of sugar contents during the baking process

The purpose of this chapter is to describe the results obtained when samples taken during the breadmaking process were analysed for the sugars present.

#### 5.1 Introduction

In the previous phase of this study, the levels of the amylolytic enzymes were assessed. In order to further investigate the individual sugars present in the dough and bread crumb, an instrumental analysis of sugars using High Performance Liquid Chromatography (HPLC) was carried out. The purpose of this phase was to investigate the levels and types of low molecular weight carbohydrates and their relationship to bread firmness.

Only water extraction samples were analysed due to the incompatibility of the column available to this study with extracts containing ethanol. The retention times for the individual sugars were first identified. The retention of the sugar standards are presented in Table 5.1 and chromatograms of the standards are presented in Figure 5.1.

**Table 5.1** The retention time values obtained when selected standard sugars were analysed by HPLC

| Sugar    | Retention time (min) |
|----------|----------------------|
| Sucrose  | 10.1                 |
| Maltose  | 10.7                 |
| Lactose  | 11.1                 |
| Glucose  | 12.3                 |
| Fructose | 16.8                 |

**Figure 5.1** Typical chromatograms obtained by HPLC for sugar standards  
a) sucrose, b) maltose, c) lactose, d) glucose and e) fructose

## 5.2 HPLC analysis of commercial flour samples

A number of flour samples were analysed and a typical chromatogram is shown in Figure 5.2. The results are presented in Figure 5.3. Sucrose, maltose and glucose were present in all flour samples whereas fructose was not detected. All three flours gave a similar pattern where maltose levels were the highest and glucose the lowest. The presence of maltose was not expected based upon the HPLC data found in food composition tables from USA (Table 5.2). The high levels of maltose may indicate that some sprouting of the grain may have occurred, causing an increase in enzyme activity and elevated levels of maltose (Ponte and others 1962).

## 5.3 HPLC analysis of commercial bread samples

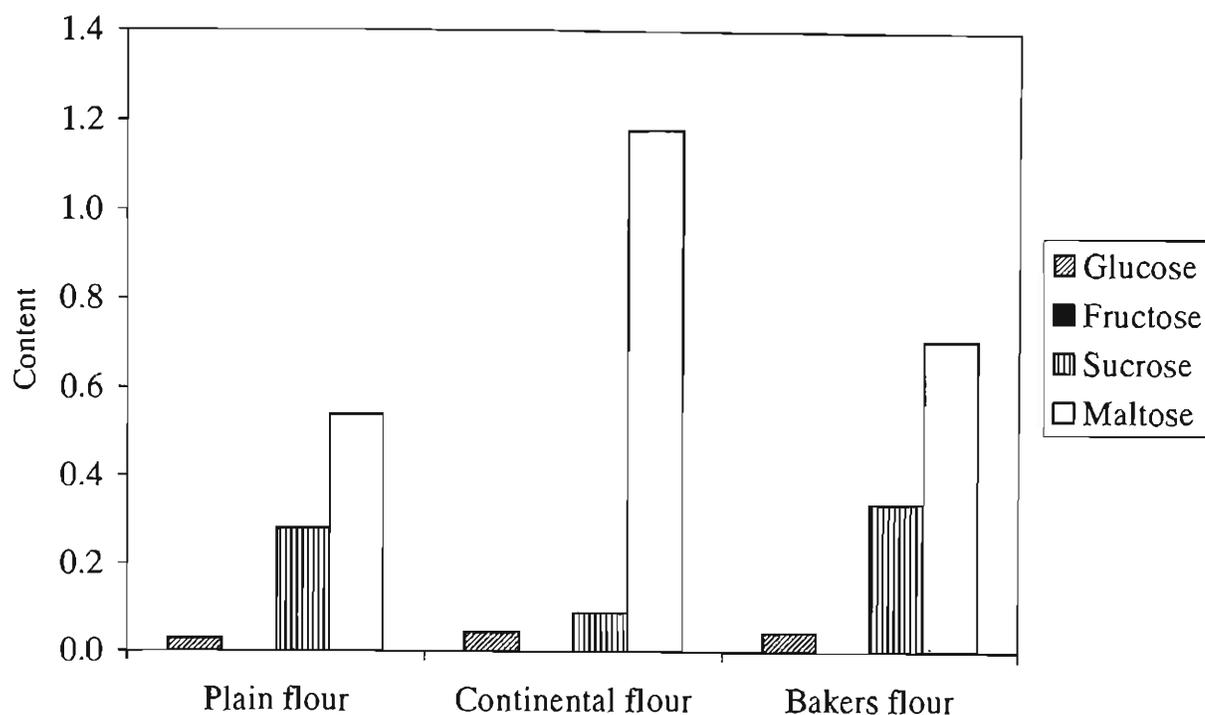
For comparative purposes four commercial white breads were sampled, extracted and analysed for the contents of the individual sugars. The four samples were selected to provide as they were expected to provide a wide range of sugar contents. A typical chromatogram obtained for the breads is shown in Figure 5.4 and the results for all of the samples are presented in Figure 5.5. For each bread product, maltose, glucose, fructose were readily detected whereas sucrose was not. Hamburger rolls were found to contain the most glucose and fructose compared to other bread samples, 1.4% and 1.6% (expressed on an “as is” basis) respectively. High values of glucose and fructose in this product may be due to the incorporation of higher levels of sucrose in the baking formulation while there was no sugar included as an ingredient in the formulation of the other three commercial breads.

Maltose levels found to be present in the three loaf style white breads were higher than the other types of sugars since maltose was the main product of hydrolysis of starch by amylases. In addition, glucose levels found in these products were lower than fructose. This finding confirmed the work of Koch and others (1954) where glucose is fermented at a slightly faster rate than fructose unless very low concentrations of glucose present. As reported by Geddes and others (1930) (Koch and others 1954) sucrose is hydrolysed by yeast at a much faster rate than it is fermented. This explained the absence of sucrose in commercial bread products.

**Figure 5.2 A typical chromatogram obtained for sugars in a commercial flour sample analysed by HPLC**

Note The result shown here is for the bakers flour sample

Peaks were 1) unidentified, 2) sucrose, 3) maltose, 4) glucose, 5) fructose and 6) and 7) unidentified



**Figure 5.3** The contents of sugars in commercial flour samples analysed by HPLC

Notes: The content values are expressed in units of g per 100 g on a 14% adjusted moisture basis

No fructose was detected in any of the flour samples

**Table 5.2** The contents of simple sugars in white flour and reported in literature sources

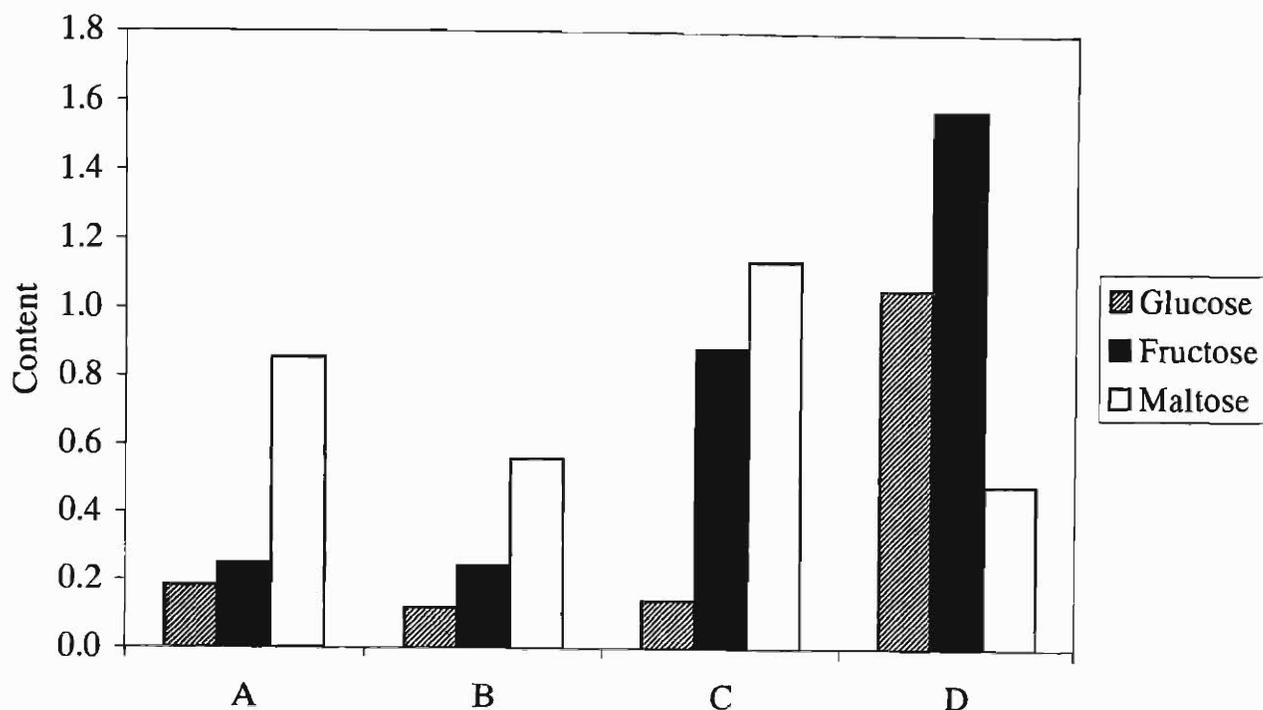
| Amount of sugar (g per 100 g product) |          |         |         |         | Reference                |
|---------------------------------------|----------|---------|---------|---------|--------------------------|
| Glucose                               | Fructose | Lactose | Sucrose | Maltose |                          |
| 0.6                                   | 0.3      | -       | 0.4     | 0.0     | Matthews and others 1987 |

The levels of sugars reported in breads varies widely and selected HPLC data from food composition tables is presented in Table 5.3. These confirm that different results might be expected and some of the likely explanations are the ingredient formulations and the processing method. In order to clarify the situation a series of trials were carried out in which bread loaves were prepared under controlled conditions. For this purpose the formulation and

process variables were controlled. The procedures adopted are those described in Chapter 3 and applied in the earlier investigation of enzyme stabilities during baking (Chapter4).

**Figure 5.4 A typical chromatogram obtained for sugars in a commercial bread sample analysed by HPLC**

Note The result shown here is for the Sunicrust Bakeries Sandwich White bread sample  
Peaks were 1) unidentified, 2) sucrose, 3) maltose, 4) glucose, 5) fructose and 6) and 7) unidentified



**Figure 5.5** The contents of sugars in four different commercial bread samples analysed by HPLC

Note: The content values are expressed in units of g per 100 g on an ‘as is’ moisture basis

The bread samples were:

- A Sunicrust Bakeries Sandwich White
- B Helgas Golden Soughdough
- C Helgas Traditional White
- D Tiptop Hamburger Rolls

**Table 5.3** The contents of simple sugars found in white bread samples and reported in literature sources

| Amount of sugar (g per 100 g product) |          |         |         |         | Reference                |
|---------------------------------------|----------|---------|---------|---------|--------------------------|
| Glucose                               | Fructose | Lactose | Sucrose | Maltose |                          |
| 0.0                                   | 0.3      | 0.0     | 0.0     | 1.7     | English and others 1990  |
| 1.8                                   | 1.5      | np      | 0.1     | 0.5     | Matthews and others 1987 |

Notes: Data are expressed on an “as is” basis

Data for bread from English and others 1990 is for sample 02B1-006

np indicates not presented but probably occurs in the product

## 5.4 HPLC analysis of experimental doughs and breads

A series of loaves were baked and samples taken during the process. These were analysed for the levels of sugars and the changes of individual sugars from dough to end products were observed. Chromatograms obtained for two of the samples are shown in Figure 5.6. The results obtained from the HPLC analyses are presented in Figures 5.7 and 5.8. The data are first presented (Figure 5.7) in terms of an “as is” basis, to facilitate comparisons with the data obtained for the commercial samples reported in Section 5.3. The data in Figure 5.8 has been recalculated to a dry basis. It was observed that no sucrose, lactose or fructose were detected in the dough and loaf samples. However maltose and glucose were detected in each of the dough samples and maltose only in the final products.

Maltose levels decreased slightly during the dough stage and then increased dramatically from 2.7% in dough D to 6.4% in bread that had cooled for 1h. The maltose levels in the bread samples remained in the range of 5.2 – 6.0%. As for the glucose contents, these decreased in the initial dough phase then increased at slow rates from 1.4% in dough B to 1.9% just before baking (dough D). Glucose was not detected in the baked products.

Koch and others (1954) found that inversion of sucrose was completed after 7 minutes of mixing in the straight-dough formula. Similar findings were observed in both control and malt supplemented breads where no sucrose was detected in dough A, that was after 40 minutes of mixing. This was due to the rapid hydrolysis of sucrose to glucose and fructose by an enzyme bound to the cell wall of the yeast rather than through utilisation by yeast for fermentation (Matz 1992).

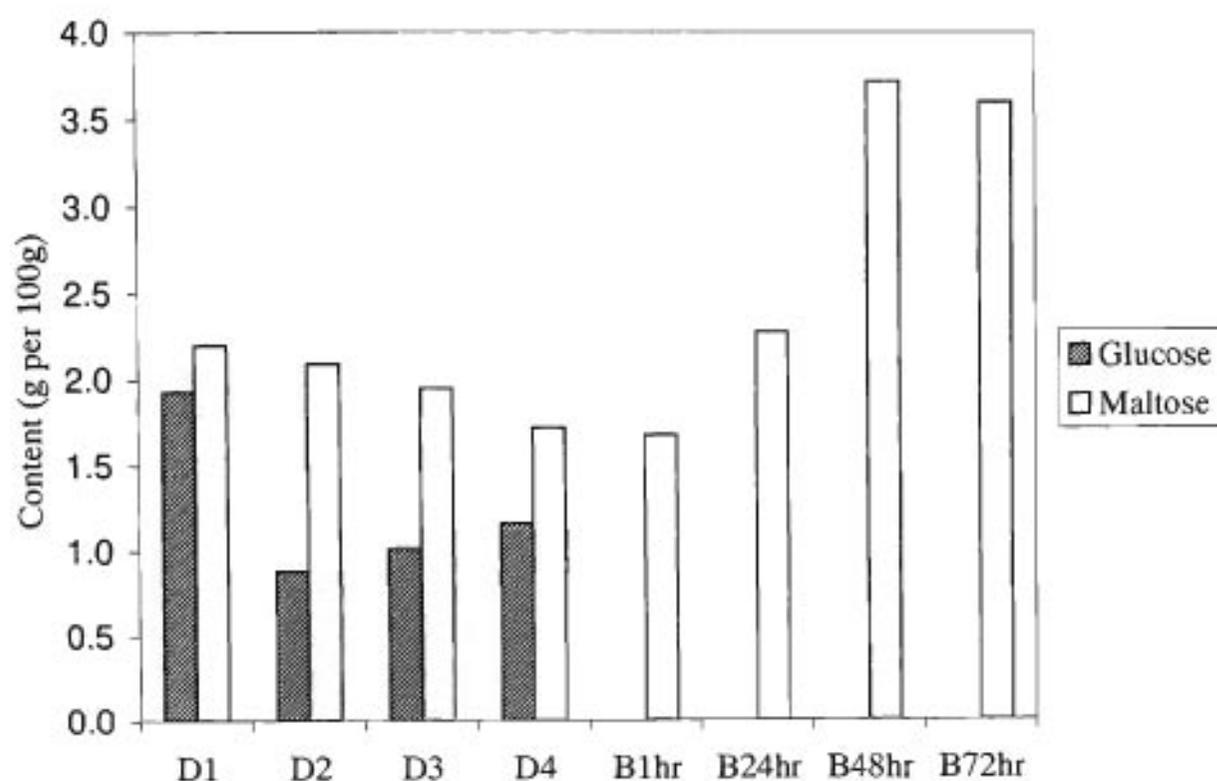
**Figure 5.6** Typical chromatograms obtained for sugars in dough and bread samples prepared in the laboratory and analysed by HPLC

**a) End of mixing stage**

**b) Baked loaf stored for 72 hr**

Note Peaks were 1) unidentified, 2) sucrose, 3) maltose, 4) glucose, 5) fructose and 6) and 7) unidentified

No sucrose or fructose were present



**Figure 5.7** The contents of sugars during the mixing, proofing and storage of experimentally baked loaves analysed by HPLC

Note 1 The content values are expressed in units of g per 100 g

Note 2 No fructose, sucrose or lactose were detected in chromatograms at any stage of processing

The various stages of the process are designated

D1 End of mixing stage

D2 End of first proof

D3 Mid point of second proof

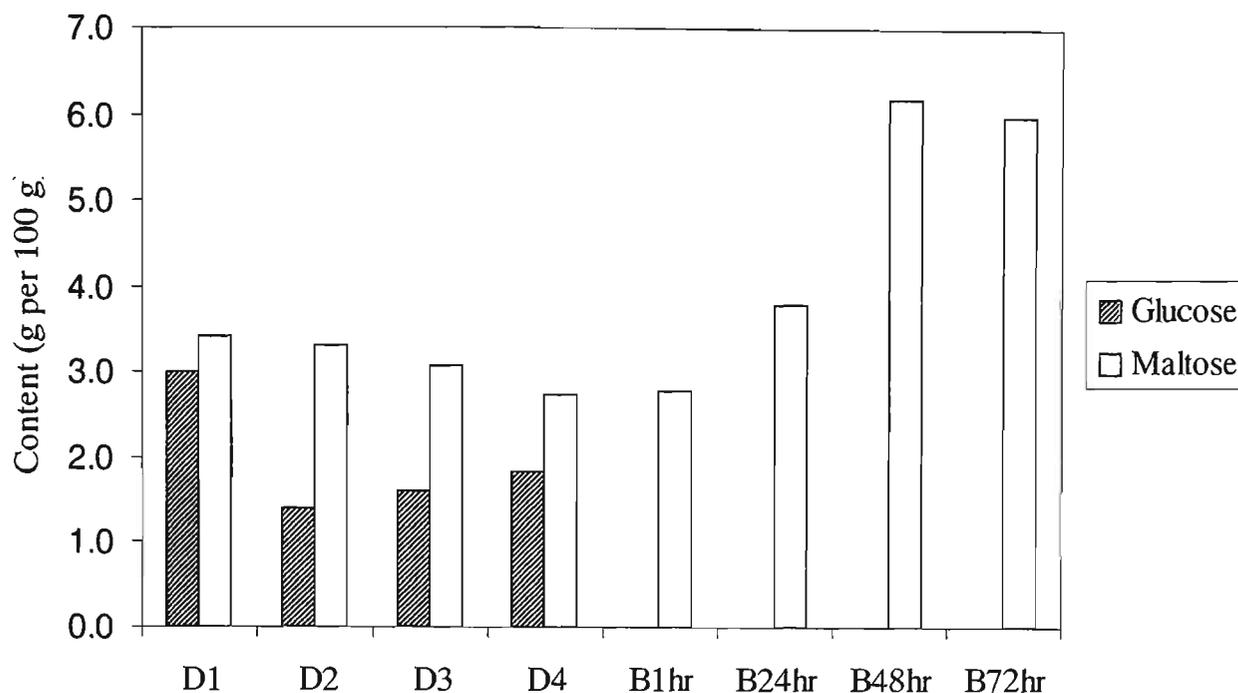
D4 End of second proof (immediately prior to commencement of baking)

B1hr Bread loaf sampled 1hr after completion of baking

B24hr Bread loaf sampled following 24hr of storage

B48hr Bread loaf sampled following 48hr of storage

B72hr Bread loaf sampled following 72h of storage



**Figure 5.8 The contents of sugars during the mixing, proofing and storage of experimentally baked loaves analysed by HPLC and expressed on a dry matter basis**

Note 1 The content values are expressed in units of g per 100 g on an “as is” moisture basis

Note 2 No fructose, sucrose or lactose were detected in chromatograms at any stage of processing

The various stages of the process are designated

D1 End of mixing stage

D2 End of first proof

D3 Mid point of second proof

D4 End of second proof (immediately prior to commencement of baking)

B1hr Bread loaf sampled 1hr after completion of baking

B24hr Bread loaf sampled following 24hr of storage

B48hr Bread loaf sampled following 48hr of storage

B72hr Bread loaf sampled following 72h of storage

In terms of the levels of the individual sugars, results obtained in this study were opposite to those reported by Matthews and others (1987) where glucose levels were slightly higher than fructose. In addition, sucrose was also found present in final baked products. These variations may be due to the differences in bread samples which vary in formulations and ingredients.

For example, the common baking method used in USA is the sponge and dough system where the whole process takes about 7 hours as compared to Chorleywood process

which takes about 4 hours. The fermentation time for these processes is much longer than that used in current study and consequently the types of sugars found in the end products varies.

Due to the hydrolysis of sucrose, higher levels of sucrose and fructose were expected. However no fructose was detected after mixing (dough A). Koch and others (1954) reported that fructose was fermented at a similar rate to glucose only when glucose was absent or present at low concentration. Lee and others (1959) also reported that glucose and fructose were fermented simultaneously, with glucose at a more rapid rate than fructose. Opposite findings were observed in this study where fructose was believed to be fermented first since none of this sugar was detected. However the baking method, sponge and dough used in the published research was different from the method used in the current study.

In the current study, the levels of glucose and maltose decreased, as would be expected since both were utilized by yeast for fermentation with maltose at slower rate. Subsequently the levels increased. The changes in maltose levels could be explained by the production of maltose due to amylase action on starch.

Koch and others (1954) reported that no fructose and glucose were found in bread made from straight dough containing 2.5% of sucrose. The results found here supported this finding where no glucose or fructose were detected in both control and malt supplemented bread samples. During baking, the temperature in the oven increased. Walden (1955) reported that the major activity for malt  $\alpha$ -amylase was in the range of 58 – 78 °C. During this stage the  $\alpha$ -amylase acts on starch and hence increases the levels of malto-oligosaccharides. The maltose in control breads remained relatively constant however in malt supplemented bread this continued to increase during storage. The changes of maltose levels in baked breads was not expected since cereal  $\alpha$ -amylase would be expected to be inactivated during the baking process.

## **5.5 Summary of results for HPLC analyses**

The data presented here demonstrates that using HPLC with refractive index detection allows the analysis of simple carbohydrate molecules in flours, doughs and baked products. Some variation in patterns are found between different flours and also between some commercial

baked goods. When loaves were baked in the laboratory, only glucose and maltose were found in the extracts. The levels of sugars were found to vary during the processing of the dough and the subsequent storage of the loaf for periods of up to 72 hours. The most surprising aspect of the observations made here has been the increases in maltose occurring during storage of the finished baked loaf. It is suggested that this may reflect the presence of  $\alpha$ -amylase. The enzyme would have greater access to starch molecules following baking due to the gelatinization of starch granules. This hypothesis is also consistent with the results described in Chapter 4 where the levels of this enzyme were higher than expected in the baked loaves. The significance of this to an understanding of staling behaviour and the role of partial hydrolysis product of starch are explored further in subsequent chapters.

## Chapter 6

### **Results and discussion—Preparation of microcapsules containing maltodextrin and the incorporation of these into a bakery formulation**

The purpose of this chapter is to firstly describe the preparation of microencapsulated maltodextrin by a spray drying technique. The results obtained when these microcapsules were incorporated into bakery formulations are also presented.

#### **6.1 Introduction to microencapsulation of food ingredients**

The technology of microencapsulation was introduced around 30 years ago and has been particularly applied to pharmaceutical materials (Park and others 1984). The term microencapsulation describes processes by which small particles of solid, liquid or gas are packaged within a secondary material to form a microcapsule (Augustin and others 2001; Zhao and Whistler 1994; Uddin and others 2001; Trindade and Grosso 2000; Gibbs and others 1999; Brazel 1999). Encapsulation has recently started to find application in the food industry and is expected to show rapid growth. There are a variety of reasons for using microencapsulation: these include the protection of the core material, to enhance food product quality and to facilitate the production process (Brazel 1999). In general, three issues are involved: formation of the wall around the material, ensuring that leakage does not occur and also that undesired materials are excluded (Gibbs and others 1999). Microencapsulation technology presents exciting opportunities, particularly for the enhanced stability and shelf life of sensitive ingredients. One of the methods being explored for preparation of microencapsulated ingredients is spray drying (Zhao and Whistler 1994; Trindade and Grosso 2000; Uddin and others 2001). In the context of the results described in earlier chapters a commercial maltodextrin has been used in studies of encapsulation as a means of introducing partial hydrolysis products of starch into a dough during the breadmaking process.

## 6.2 Selection of ingredients and procedures for encapsulation of a commercial maltodextrin

Based upon the published reports of microencapsulation utilising spray drying, consideration was given to this as a potentially useful approach for the current investigation. Spray drying has been used to effectively coat and protect ascorbic acid (Trindade and Grosso 2000; Uddin and others 2001) despite the reported reactivity of this compound. Although heating is integral to the process of spray drying, the exposure of the liquid feed material to high temperatures is typically for a relatively short period of time. The rapid evaporation of water from the atomization of the liquid causes effective cooling.

In spray drying, a variety of carrier materials have been used for microencapsulation (Zhao and Whistler 1994) and are generally based upon either rice starch or gum Arabic. Accordingly these were utilized in a series of preliminary trials. In addition, for the preparations with rice starch, various hydrocolloid gums were used in order to form the microcapsules. Effectively the gums used form an adhesive that binds adjacent starch granules.

Preliminary spray drying trials showed a fine powdered product could be produced with a variety of these materials. However, rice starch appeared to have greater potential. This was based on the observations that the gum Arabic appeared to be relatively soluble when the microcapsules were suspended directly in water. This probably resulted in the rapid loss of integrity and structure of the microcapsules. Accordingly, further spray drying trials therefore utilized rice starch as the carrier material.

Maltodextrins are and commonly used in as food ingredients to act in binding of water and for enhancement of mouthfeel (BeMiller 1993; Chonrakis 1998). The maltodextrin used here was a commercial product, prepared in Australia, and further details of the material are presented in Appendix 2, based upon information provided by the manufacturer.

### 6.3 Preparation of microcapsules by spray drying

In the current study, a Niro pilot scale spray drier was used. The instrument is shown in Figure 6.1. In preparing the liquid for spray drying, guar galactomannan was used as it has been reported to provide effective binding in conjunction with rice starch (Zhao and Whistler 1994). Following dissolution of the guar gum, the rice starch preparation was added slowly with vigorous stirring and gentle heating to ensure thorough distribution without development of unwetted larger particles. Care was also taken to ensure that the mixture did not approach temperatures which might result in gelatinisation of the granules. The maltodextrin was added immediately prior to spray drying of the liquid preparation.

The conditions used in spray drying were chosen to again minimise the likelihood of gelatinisation of the starch granules and so the inlet temperature was set at 120 °C. The feed rate which gave effective drying was 7.0 mL per min. With these conditions, the total time required for drying was approximately seventy min.

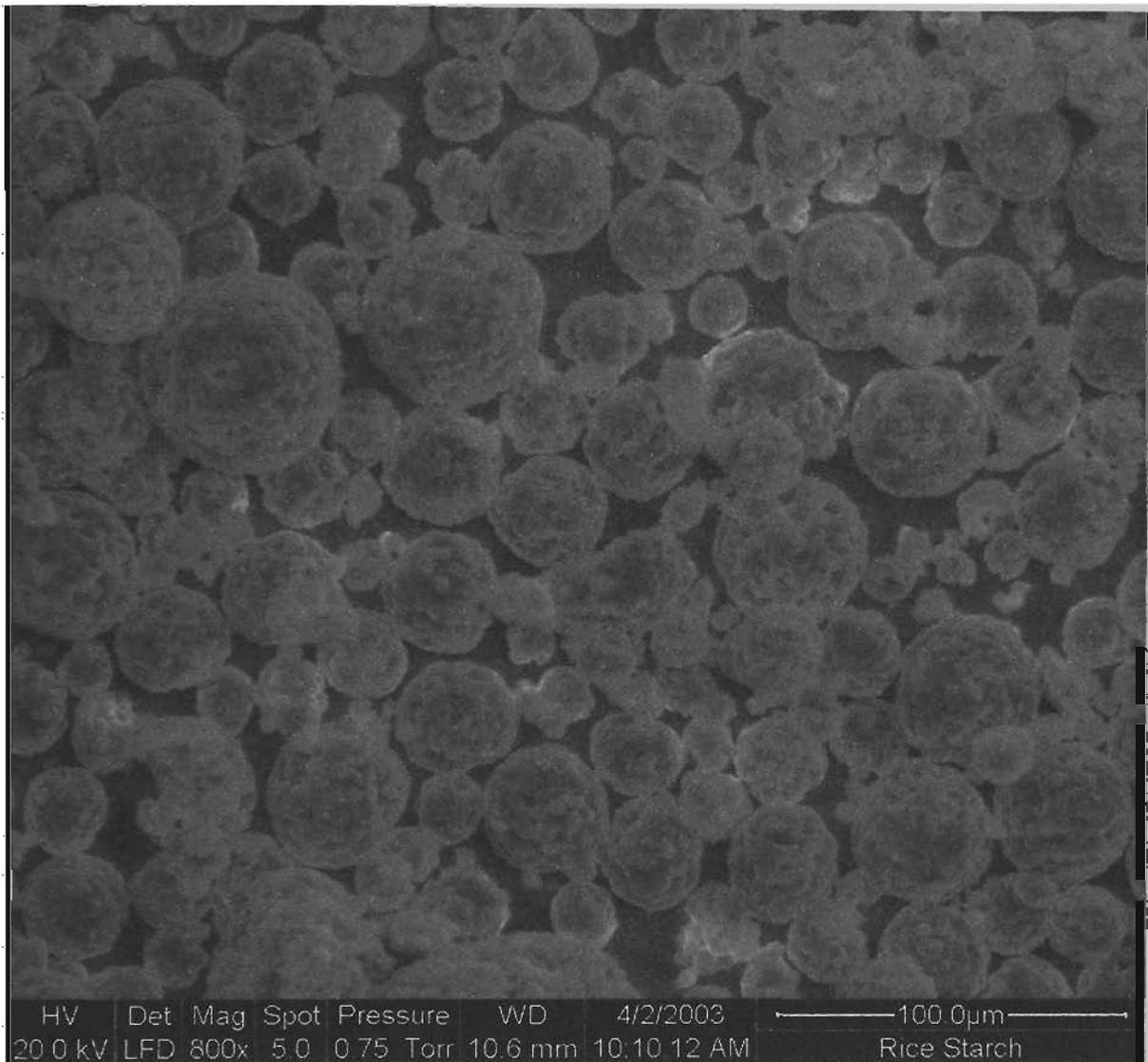
When three separate trials were completed for the preparation of microcapsules based on these ingredients and conditions, the estimated yields of capsules were 78, 74 and 75 percent. The resultant capsules were white in appearance and were indistinguishable in colour from the original rice starch preparation. Samples of the microcapsules were examined by scanning electron microscopy and a typical micrograph is presented in Figure 6.2.

### 6.4 Characteristics of microcapsules

The spray dried capsules containing the maltodextrin preparation were observed to be spherical. The appearance is similar to that of capsules described by other researchers (Zhao and Whistler 1994; Trindade and Grosso 2000; Uddin and others 2001). From the population of particles seen in Figure 6.2 it is estimated that most of the capsules have diameters in the range of 20 to 50  $\mu\text{m}$ . These values can be compared with the diameters of rice starch granules (1.5 to 9  $\mu\text{m}$ ) with rice being a source having smaller granules than most other plant species (BeMiller and Whistler 1996).



**Figure 6.1** The Niro Atomiser spray drying unit used in preparation of microencapsulated materials for incorporation into bakery formulations



**Figure 6.2** The appearance of microencapsulated maltodextrin prepared by spray drying using rice starch and guar gum and viewed by scanning electron microscopy

## **6.5 The incorporation of maltodextrin microcapsules into a breadmaking formulation**

In order to investigate the impact of the microencapsulated material, samples of the capsules were incorporated into a trial using the breadmaking ingredients and processes applied in earlier phases of this project. These are described in more detail in Chapter 3. The level of incorporation selected for the capsules was one percent in relation to the weight of flour used.

For comparative purposes a series of loaves were prepared and included a no-treatment control, as well as others having incorporating unencapsulated maltodextrin at the same effective level as that of the microencapsulated samples, along with capsules prepared in the same way but without any maltodextrin. The purpose of the latter loaves was to assess the impact of the rice starch and the galactomannan used in preparation of the microcapsules.

The baked loaves were assessed for loaf volumes, appearance including symmetry and the crumb characteristics. There was relatively little impact of the various treatments on each of the parameters and characteristics considered here. All loaves were of good volume, appearance and symmetry. However, the crumb characteristics did show differences. Measurements were taken on freshly baked loaves as soon as these had cooled to ambient temperatures. In addition, crumb softness was tested on samples of loaves that had been stored in carefully sealed plastic bags for a period of 48 hours from the completion of baking. The results obtained for measurements of crumb softness are presented in Table 6.1.

The results show that there were differences in the crumb softness values obtained using the standard analysis procedure of the American Association of Cereal Chemists. The differences were apparent in comparing the treatments for the freshly baked loaves and also for the loaves stored for a forty eight hour period. In addition there is evidence of differences in the rates of change in softness differing between the treatments.

**Table 6.1** The crumb texture characteristics of loaves baked with microencapsulated maltodextrin (units of softness are Newtons)

| Loaf formulation                            | Crumb softness of the fresh loaf | Crumb softness of the loaf following storage for 48 hours |
|---|----------------------------------|---|
| Control                                     | 0.92 ± 0.05                      | 1.37 ± 0.06   |
| Control capsules containing no maltodextrin | 0.93 ± 0.06                      | 1.35 ± 0.07   |
| Unencapsulated maltodextrin                 | 0.85 ± 0.04                      | 1.15 ± 0.05   |
| Encapsulated maltodextrin                   | 0.80 ± 0.04                      | 0.95 ± 0.05   |

Note: results are expressed as mean (of triplicate measurements) ± standard deviation

## 6.6 Summary of microencapsulation results

The preliminary conclusions which may be drawn are that the encapsulation procedure trialed here appears to be at least partially effective in protecting the enclosed maltodextrin from release until later in the baking process. In addition, the addition of unencapsulated maltodextrin did have some effect on loaf characteristics. The impact of the encapsulated material had a more pronounced effect. Whilst further studies are recommended, these results provide evidence that at least some of the molecules present in the maltodextrin are having an effect on the softness and the staling of the bread crumb.

## Chapter 7

### General discussion and conclusions

The purpose of this chapter is to summarise and discuss the results obtained in the current study. In addition, recommendations for future research are also made.

#### 7.1 Enzyme stability

The enzymes  $\alpha$ - and  $\beta$ -amylase in flour, dough and baked products could be repeatedly measured using Megazyme substrates. The data indicated that the levels of activity remained high during the proofing of a dough, with some apparent increases in the  $\alpha$ -amylase activity. The relative amounts of bound  $\beta$ -amylase were higher in doughs than the corresponding flour. The overall losses in activity of the two amylolytic enzymes was quite different when heating of the dough occurred during baking. Virtually all of the  $\beta$ -amylase was inactivated. On the other hand, the amounts of  $\alpha$ -amylase measured were higher than expected. Further studies are warranted to provide a greater understanding of the factors impacting of the stability of this enzyme in the dough. In particular, the potential significance of the enzyme acting after the completion of baking and the impact on staling are of considerable interest.

#### 7.2 HPLC analysis of sugars during breadmaking

To investigate the types and levels of sugars present in samples, HPLC analysis was conducted. Five sugar standards (sucrose, maltose, glucose, fructose and lactose) were prepared. Lactose was not expected in the samples since no milk was used in the bread formulation.

In commercial flour samples, sucrose, maltose and glucose were identified from the chromatograms. Maltose level was found to be the highest, followed by sucrose and then glucose. It was not expected that flour would contain high levels of maltose. The presence may indicate that some sprouting had occurred in the grain prior to milling

since the increased enzyme activity of sprouting would lead to high levels of maltose (Ponte and others 1962).

Maltose, glucose and fructose were present in commercial breads. The levels varied and the different profiles of sugars were probably influenced by the raw ingredients used in bread making.

For experimental breads, only maltose was detected in both control and malt supplemented breads. This observation was very different from those found in commercial breads and those reported in the literature. This further confirmed that ingredients and baking process play an important role in determining the characteristics and quality of final products.

### **7.3 Microencapsulation of maltodextrin and the impact in baking**

In the current research, the total sugars determined spectrophotometrically were higher than the sum of individual sugars determined using HPLC. Other sugars were also present in the samples which was indicated by the presence of unidentified peaks shown in the chromatogram. Other standards need to be prepared to further identify those unknown sugars. Results in the current study showed that malt flour did improve loaf volume and bread firmness however maltose was not responsible in reducing crumb firmness. As suggested by Martin and Hosenev (1991) low molecular weight carbohydrates of DP 3 – 9 had an antifirming effect on bread hence more analysis should be conducted to look at the amount of these dextrans present in bread samples and study their relationships to bread staling. This would require a different HPLC column which could separate the low molecular weight carbohydrates according to size.

Considerable amounts of work have been done looking at the impact of fungal and bacterial  $\alpha$ -amylases on bread firmness and many agreed that low molecular weight carbohydrates from hydrolysis by  $\alpha$ -amylases have antistaling effects. However the knowledge on the mechanisms exerted by these molecules remains unclear. Work done by Gerrard and others (1997) added dextrans to standard breads but the research gave inconclusive results. These workers suggested that the added dextrans did not survive the proofing stages of breadmaking due to the hydrolysis by native flour amylases and

yeast metabolism. More research should be directed on the addition of dextrans with varying sizes to bread formulations in different ways. One of the relatively new techniques now becoming available in food science and technology is that of microencapsulation (Augustin and others 2001). In this, an active ingredient is coated with a protective material which limits release until a certain trigger causes changes to occur. The potential of microencapsulation to the current problem is that microencapsulation of dextrans could protect the dextrans from being hydrolysed by native flour amylases and yeast metabolism. The dextrans would then only be released from the capsules just before baking due to the increasing temperature within the loaf. Baked products could then be analysed for their characteristics and the composition of the low molecular weight carbohydrates. It is hoped that further studies using this novel approach might be possible and provide a fresh approach to resolving the questions regarding the role of low molecular weight carbohydrates in baked goods.

#### **7.4 Recommendations for further studies**

The research reported in this thesis provides new evidence on the stability of one enzyme important in baking. In addition the data on changes in carbohydrate levels provides some confirmatory evidence consistent with the findings from the enzyme assay study. The preliminary studies of microencapsulation of a maltodextrin by the spray drying technique indicate that this method may provide a valuable new tool for further investigations of the role of particular maltodextrins or fractions of maltodextrin in determining two important aspects of bread quality. These are

1. product softness; and
2. retardation of staling during storage of baked products.

Some particular areas are recommended for further study. In relation to enzyme stability, this study has focussed on the two enzymes  $\alpha$ - and  $\beta$ -amylase. The study was limited to the enzymes from cereal grain sources. Further reinvestigations of other enzymes and enzymes from other sources, including fungal amylase, are warranted.

The potential role of amylases in determining the carbohydrate composition during the baking process would also be of value and the application of the newer approach of

pulsed-amperometric detection may provide enhanced sensitivity and allow analysis of dextrin components having higher degrees of polymerization.

In relation to the application of microencapsulation, it is strongly recommended that some of the many options for encapsulation be further evaluated. In the current study one particular approach was found to offer potential. Other hydrocolloid materials and encapsulating agents other than rice starch may warrant further investigation. Another possibility is the application of high melting point fats and waxes in combination with melt dispersion techniques. A further extension of the work reported here may eventually be the commercial utilization of encapsulated ingredients not only as investigative tools but also as commercial ingredients providing enhanced product quality and also storage characteristics.

## References

Notes: In this thesis referencing has followed the recommendations found in the Journal of Food Science (see IFT 2002 below). Accordingly journal titles have been abbreviated using the appropriate recommendations (Council of Biology Editors 1994).

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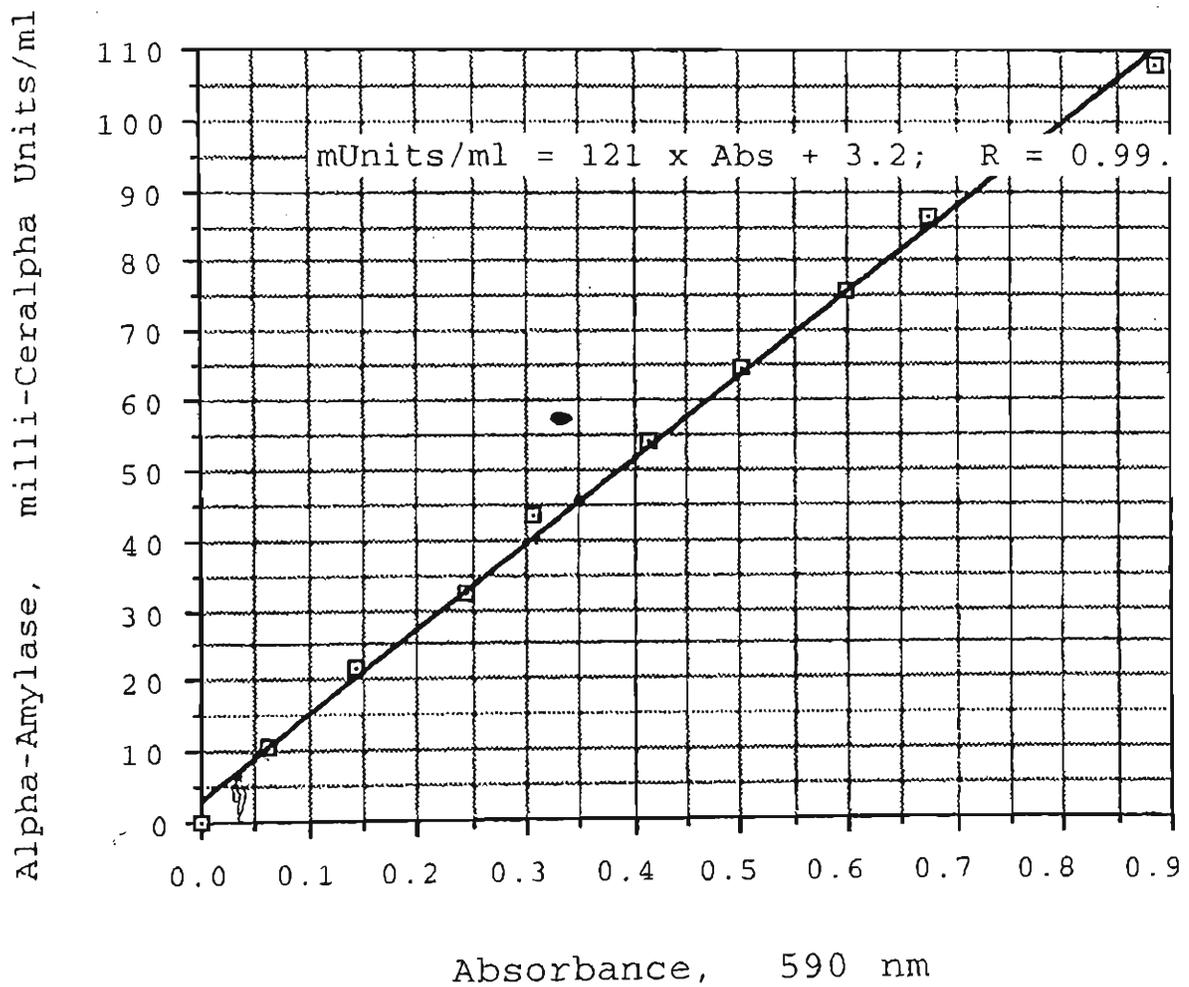
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## Appendix 1

### Calibration curve used in calculating the $\alpha$ -amylase activity values in terms of Ceralpha units

Note: this graph was sourced from Megazyme International 1996a.



## Appendix 2

### Product information and specifications for the commercial maltodextrin (Fieldose 17) supplied by the manufacturer (Starch Australia, Sydney)

#### PRODUCT DESCRIPTION

FIELDOSE 17 is a low DE maltodextrin. It is derived from the controlled hydrolysis of starch and is presented as a spray dried powder.

#### CHARACTERISTICS

FIELDOSE 17 is a white, free flowing powder with low sweetness, hygroscopicity and bland taste. It is cold water soluble and provides a variety of functional properties.

#### APPLICATIONS

FIELDOSE 17 is recommended for use in spray dried products, dry mixes, bakery goods, beverages, frozen foods, non-dairy products and pharmaceuticals.

#### TYPICAL ANALYSIS

Dextrose Equivalent : 17-20

Moisture : 6.0% max

pH : 4.8-5.5

Ash : 0.5% dsb max

Bulk density (compacted) : 0.5-0.7 g/ml (typical)

Particle size Pass 150 micron : 90%

#### Carbohydrate Profile:

Monosaccharides (G<sub>1</sub>) : 1.2

Disaccharides (G<sub>2</sub>) : 5.4

Trisaccharides (G<sub>3</sub>) : 9.8

Tetra & Higher : 83.6

**Solubility:** Disperses readily to produce clear solutions at up to 40% concentrations.

#### Mineral Analysis

Chloride 2500 ppm max

Sodium 2000 ppm max

Phosphorus : 100 ppm max

Calcium : 5 ppm max

Manganese : 5 ppm max

Magnesium : 5 ppm max

Potassium : 5 ppm max

Aluminium : 5 ppm max

Heavy Metals : 5 ppm max

Calorific Value : 16 kj/g

#### Microbiological:

Total Plate count : 5000/g

Yeast : 100/g

Mould : 100/g

## Appendix 2 ...continued

### PACKAGING

Available in 25 kg multiwall paper bags

### LABELLING

Correct labelling is MALTODEXTRIN

### STORAGE & SHELF LIFE

The product should be stored in clean, dry area. Once opened, bags should be used as soon as possible or securely resealed, as product is hygroscopic and will absorb moisture.