THE α-AMYLASE ISOENZYME SYSTEM OF IMMATURE CANADIAN-GROWN WHEAT

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ABSTRACT

Marchylo, Brian Alexander, Ph.D., The University of Manitoba, May 1978. The α-Amylase Isoenzyme System of Immature Canadian-Grown Wheat. Major Professor; Dr. L.J. LaCroix.

The α-amylase isoenzyme system of immature Canadian-grown wheat was studied. An automated fluorometric α-amylase assay was developed for use in the study of the immature wheat α-amylase system as well as for the determination of α-amylase activities in flour. The changes in the α-amylase activity during kernel growth and maturation for 10 cultivars of Canadian-grown wheat then were determined. The 10 cultivars essentially exhibited the same changes in α-amylase activity. The α-amylase isoenzyme composition then was determined at 4 periods during kernel growth and maturation for the 10 wheat cultivars. The α-amylase isoenzymes were separated by the high resolution technique of flat-bed polyacrylamide gel isoelectric focusing. Three groups of α-amylase isoenzymes (i.e. the GI, GII and GIII isoenzyme groups) were detected in the immature wheat. The isoenzyme composition of the immature wheat did change during kernel growth and maturation and

cultivar differences were noted in the isoenzyme composition. The α-amylase isoenzyme distribution in the wheat kernel was ascertained for the 10 wheat cultivars at the 4 developmental stages. Some tissue segregation of the isoenzymes was noted. The GIII isoenzyme group of immature wheat was compared to a similar isoenzyme group in germinating wheat and was found to have comparable isoenzyme patterns and isoelectric points. Further studies indicated that the immature wheat embryo and scutellum and endosperm plus aleurone were able to synthesize GIII-type isoenzymes if these tissues were placed under germination conditions. Similarly, the mature embryo and scutellum had the capacity to synthesize its own α-amylase. Dissection studies indicated that the removal of the seed coat from the immature wheat kernel inhibited α-amylase synthesis and yielded an unique α-amylase isoenzyme composition. Subsequent work confirmed that the seed coat affected the synthesis of α-amylase in both immature and mature germinating wheat. A practical solution to the problem of high α-amylase levels in wheat, at harvest, was suggested from a study involving the treatment of wheat with polyethylene glycol (Carbowax 6000).

INTRODUCTION

In wheat, the enzyme α-amylase is of considerable practical importance as a consequence of the integral role it plays in the breadmaking process. This enzyme is a necessary constituent of flour employed in breadmaking but if present in excess it will produce extensive liquefaction and dextrinization, yielding an inferior loaf of bread with a wet sticky crumb (Bloksma, 1971). Significant α-amylase activity is normally present in the wheat kernel during growth and maturation and germination, while little activity is found in the sound wheat kernel. Unfortunately, elevated α-amylase levels are sometimes found in freshly harvested wheat due to pre-harvest sprouting or reactivation of the immature α-amylase system (Olered and Jonsson, 1970). The presence of the high α-amylase levels in this wheat decreases its quality and market value since it is now less suitable for breadmaking purposes. This problem of high α-amylase levels is widespread and has led to a concentrated study of the wheat α-amylase system. Initially the majority of work was directed towards the α-amylase produced in germinating wheat (Kruger and Tkachuk, 1969; Olered and Jonsson, 1970; Kruger, 1972b; Tkachuk and Kruger, 1974) but recently studies related to the immature wheat α-amylase system have

been carried out (Olered and Jonsson, 1970; Kruger, 1972a; Marchylo et al., 1976).

Subsequent to these studies advances were made in methodology which resulted in the advent of superior separatory techniques such as flat-bed polyacrylamide gel isoelectric focusing. Preliminary studies employing this method indicated that the immature wheat α-amylase system was more complex than previously realized. It was felt that a study of the immature wheat α-amylase system using the isoelectric focusing technique as well as a newly developed fluorometric α-amylase assay would garner further knowledge concerning the wheat α-amylase system. Also, a more comprehensive understanding of this enzyme system eventually could lead to a practical solution to the problem of high α-amylase levels in wheat.

The study initially entailed the determination of the changes in the α-amylase activity and isoenzyme composition in 10 Canadian-grown wheat cultivars during kernel growth and maturation. Subsequently, the isoenzyme distribution in the wheat kernel also was ascertained. The results obtained from these studies led to a number of germination studies designed to give a clearer overall comprehension of the total wheat α-amylase system. Finally,based on the results of a study which involved the treatment of dormant and non-dormant wheat with polyethylene glycol (Carbowax 6000), a possible practical solution to the problem of high α-amylase levels in wheat was suggested.

The main body of this study is divided into 5 major parts. The first part deals with the development of an automated fluorometric α-amylase assay. In the second part, the changes in the α-amylase isoenzymes of 10 Canadian-grown wheat cultivars are determined. This then is followed by a section dealing with the α-amylase isoenzymes of germinating immature wheat. The results of this study subsequently led to preliminary studies concerning the effect of the seed coat on α-amylase synthesis. The concluding section deals with the relationship of seed dormancy to α-amylase levels.

LITERATURE REVIEW

Historically, α-amylase was one of the earliest enzymes to be discovered and studied in cereals. As far back as 1811, Kirchoff noted that wheat extracts possessed a digestive action on starch. In 1883 Payen and Persoz discovered a starch digesting substance in barley malt which they designated diastase. Lintner, in 1887, concluded that although barley malt contained a starch liquefying and starch saccharifying enzyme, the latter component was the only one present in ungerminated barley.

Further studies on these starch digesting substances in cereals definitely showed that more than one type of amylase enzyme was present. The two amylases involved were termed α- and β-amylases by Kuhn (1924, 1925) because the hydrolytic products of the degradation were in the α-form, with downward mutarotation, or in the β-form, with upward mutarotation, respectively. Recently, this has been verified by Semenza *et al*. (1969) using the more sensitive technique of gas-liquid chromatography. An alternate means of referring to the two enzymes was introduced by Ohlsson (1930) who called α-amylase the dextrinizing amylase and β-amylase the the saccharifying amylase.

Since these early studies, extensive work has been

carried out on α-amylase. It is now known that α-amylase (α-1, 4-glucan 4-glucano-hydrolase, EC 3.2.1.1.) specifically catalyses the hydrolysis of α-1, 4 glucosidic linkages of starch, glycogen and their degradation products (Allen and Spradlin, 1974). α-Amylases have been classed as endo-amylases for they can cleave the α-1, 4 glucosidic linkages interior to the substrate chain.

Sources of α-Amylase

α-Amylase may be found throughout the plant and animal kingdom. The sources of α-amylase may be classed into four main groups: a) the higher starch containing plants; b)mammals c) bacteria and d) fungi (Greenwood and Milne, 1968).
It has become apparent that the properties and modes of action of α-amylases are peculiar to their sources (Greenwood and Milne, 1968) although some properties are common to all. The α-amylases from all sources are slightly acidic, water soluble proteins with an approximate molecular weight of 50,000 and contain at least 1 gram atom of calcium, which is essential for their activity (Fischer and Stein, 1960). By contrast, most other properties, such as pH optima, are different between groups. For example, the pH optimum of various plant α-amylases falls between pH 4.7 - 6.0 (Greenwood and Milne, 1968). In comparison, it is found that the mammalian α-amylases have a pH optimum of about 6.9 (Bernfeld et al., 1948: Bernfeld et al., 1950).

In recent years α-amylases have been intensively studied, and as a result, the body of knowledge concerning this enzyme has grown tremendously. α-Amylases have been crystallized or purified from many sources including higher plants. Of particular interest to this discussion are the α-amylases purified from cereals. Schwimmer and Balls (1949) were probably the first to crystallize a cereal α-amylase (barley malt). Since then the enzyme has been purified from other cereal sources such as barley (Greenwood and MacGregor, 1965), Triticale (Lee and Unrau, 1969), malted wheat (Kruger and Tkachuk, 1969), malted rye (Manners and Marshall, 1972), immature barley (MacGregor et al., 1974) and immature wheat (Marchylo et al., 1976).

Because of the immense knowledge now available, even on plant α-amylases, this review will be limited to the discussion of the cereal α-amylases. Some references will be made to α-amylases in other organisms for comparitive purposes.

α-Amylase From Cereal Seeds

Cereals, in common with other plants, synthesize starch as a reserve polysaccharide. The storage tissue for this starch is the endosperm of the seed. Some mechanism must be available for the breakdown of the stored starch to simple sugars which can be subsequently utilized by the growing plant. This is carried out by carbohydrate-metabolizing enzymes located in the seed, with α-amylase being one of the major enzymes responsible.

The level and anatomical location of the α-amylase depends on the natural state of the seed, that is, whether the seed is in the immature, resting or germinating state. The α-amylase present in the resting seed will be discussed first, followed by the α-amylase of the germinating seed and finally, that of the immature seed.

The α- Amylase of the Resting or Dormant Seed

The α-amylase activity of the cereal seed is very low at maturity. Originally it had been felt that the mature seed contained no α-amylase prior to germination (Blish et al., 1937; Grabar and Daussant, 1964). This has since been proven false by Greenwood and MacGregor(1965), MacGregor et al. (1971) and Olered and Jonsson (1970), who have definitely shown that α-amylase activity is present in ungerminated wheat, barley and other cereals.

In wheat and most other cereals, the α-amylase activity

level present in the mature resting seed will vary from cultivar to cultivar and from year to year. In most instances, the activity is not very high and is not significant when compared to the activity present in the germinated seed. However, an elevated level of α-amylase activity is sometimes present due to pre-harvest sprouting or incomplete maturation (Olered and Jonsson,1970; Chojnacki,1976; Olered, 1976; Persson,1976).

Germinated Cereal α-Amylase

Much of our present day knowledge of cereal α-amylase stems from studies of α-amylase present in germinating seeds of cereals. These studies have concentrated on themes such as the synthesis and isoenzymic nature of the enzyme. In the following discussion each of these topics will be considered.

Biosynthesis of α-Amylase in the Germinating Seed. A concerted effort to determine the site of α-amylase synthesis during germination has been carried out in recent years, especially in the barley seed. Originally it was felt that most hydrolytic enzymes were released directly into the endosperm by the scutellar epithelium of the embryo (Brown and Morris, 1890; Brown and Escombe, 1898; Briggs, 1962). This supposition has been discarded and it is now felt that the major portion of the hydrolytic enzymes, including α-amylase, are produced in the aleurone layer in response to gibberellic

acid or a natural hormone of the gibberellin type (MacLeod and Millar, 1962; Varner, 1964; Varner et al., 1965; Briggs, 1963, 1964, 1973). Briggs has found that when barley is germinated for 6 days under malting conditions, around 15% of the α-amylase present in the grain is derived from the embryo, with approximately half of this being located in situ (Briggs, 1964, 1973; Briggs and Clutterbuck, 1973). More pointedly he has indicated that the scutellar epithelium portion of the embryo does synthesize α-amylase although excised embryos require the addition of suitable nutrients (e.g. amino acids) to elicit an appreciable synthesis of this enzyme (Briggs, 1964, 1972, 1973).

In contrast MacLeod and Palmer (1966) contend that the scutellum in fact does not produce any significant amounts of α-amylase. Most recently Palmer (1974) has suggested that the barely perceptible ring of aleurone cells which contaminates the excised embryo or scutellum is responsible for the production of all of the observed α-amylase.

In the case of wheat, all indications are that, as in barley the aleurone is the major α-amylase synthesizing tissue. Dronzek et al., (1972) noted that starch granules in sprouted wheat were more severely damaged near the aleurone layer than those in the interior. This suggests that α-amylase in wheat is synthesized in the aleurone and secreted into the endosperm. Results obtained by Gibson and Paleg (1972, 1975) clearly indicate that wheat aleurones carry out the synthesis of α-amylase.

Another point of contention in the literature related to the synthesis of α-amylase, is concerned with the mode of release of the enzyme in response to the production of gibberellins in the barley embryo.

MacLeod and Palmer (1966, 1967) reported that the axis (i.e. the acrospire, the scutellar node and the roots) was the principal area responsible for the production of gibberellins. They also noted that the scutellar node and the roots were more important than the acrospire in the gibberellin-induced α-amylase synthesis of the aleurone.

Palmer (1969, 1974) proposed that endogenous gibberellins, produced in the axis, were subsequently transported to the apex of the scutellum and released into the aleurone. The gibberellins ultimately enhanced enzyme formation on the dorsal side of the grain. In other words, an asymmetric release of gibberellins and α-amylase is suggested. Palmer (1969) has undertaken a tracer analysis which supposedly verifies this pathway for gibberellin movement.

On the other hand, Radley (1967, 1968, 1969) contends that in the early stages of germination, gibberellins are formed from precursors in the scutellum. If this is the case then a more uniform release of gibberellins would take place and as a consequence a more uniform release of hydrolytic enzymes would be expected (Briggs, 1972).

Briggs (1972) verified that there is an equal distribution of α-amylase between the dorsal and ventral sides of the seed,

which is indicative of a symmetrical release of hormone. At the present time this controversial subject has yet to be resolved to everyone's satisfaction.

The site of synthesis and means of secretion of α-amylase in response to hormones has recently come under close scrutiny. Jacobsen and Knox (1973) using a sensitive immunofluorescence technique appeared to have localized the site of α-amylase synthesis in the aleurone grains and aleurone grain membranes. More recently Jones and Chen (1976), using a similar technique, did not find any indication of α-amylase synthesis related to the aleurone grain membrane or protein body matrix. Their results indicated that the perinuclear region was implicated in α-amylase synthesis. α-Amylase was located in the endomembrane system, i.e. the perinuclear endoplasmic reticulium (E.R.) and nuclear membrane.

Gibson and Paleg (1972, 1975) reported that lysosomal particles derived from wheat aleurone cells may be involved in α-amylase release. On the other hand Jones and Chen (1976) suggested that these lysosomal particles were actually fragments of the α-amylase-containing perinuclear E.R. system.

Jones and Chen (1976) felt that the α-amylase passes to the exterior of the aleurone cell via a mechanism of facilitated diffusion of small molecules.

De Novo Synthesis of α-Amylase. For many years it was not known if the α-amylase was reactivated from a pre-existing protein. It has since been ascertained that the majority of the α-amylase is synthesized de novo. The first proof of de novo synthesis was by Varner and Ram Chandra (1964). Using ^{14}C labelling, they proved that the label was incorporated into the α-amylase of barley during germination. Subsequently, Chrispeels and Varner (1966) found that α-amylase synthesis was halted by metabolic inhibitors. This eliminated the possibility of any part of the α-amylase existing as an enzymatically inactive precursor and being subsequently converted into active enzyme. Finally, Filner and Varner (1967) showed by density labelling techniques that essentially all of the α-amylase arose by de novo synthesis from free amino acids derived from pre-existing aleurone proteins. In the case of germinated wheat α-amylase, Daussant and Renard (1972) used immunochemical methods to show that about 98% of the α-amylase was synthesized de novo. Combined, these results clearly indicate that the major portion of α-amylase in the germinated seed has been synthesized de novo.

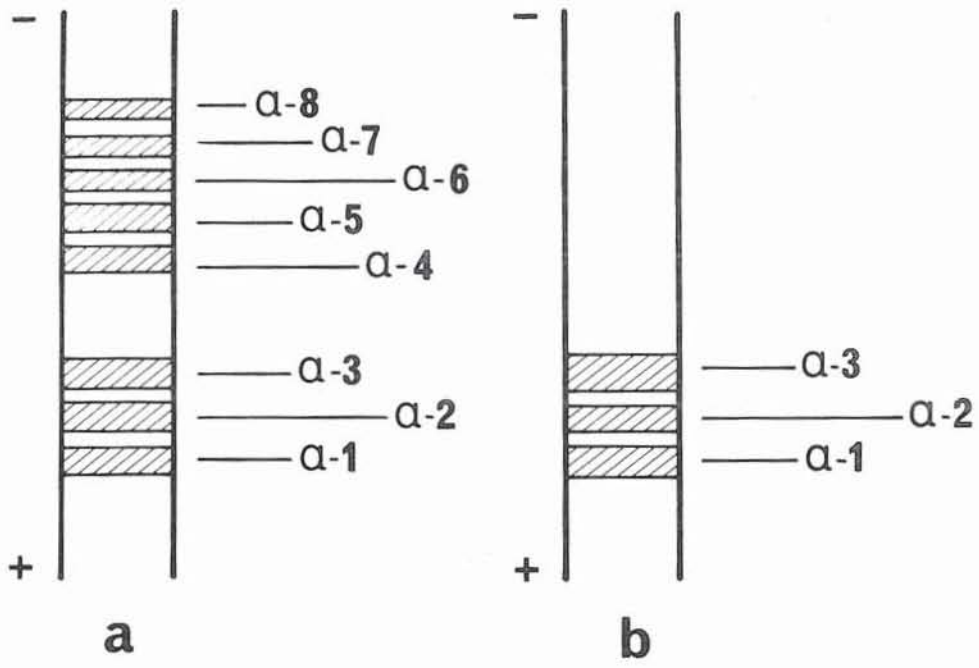
Multiple Forms of Germinated Cereal α-Amylases. As with many other germinated cereal enzymes such as α-amylase, peroxidase, esterase, etc., α-amylase is composed of multiple forms termed isoenzymes. Early research with germinated barley α-amylase indicated that only one molecular species

was present (Grabar and Daussant, 1964). Subsequent research by Frydenberg and Nielson (1965); MacGregor and Meredith (1971), McMasters (1974) and Bilderback (1974) employing electrophoresis, and Daussant et al. (1974) utilizing immunochemical techniques, indicated that at least two molecular species were present. Subsequent research carried out by MacGregor (1976, 1977), on germinated barley, using the high resolution technique of isoelectric focusing has shown that three main groups of isoenzymes may be present with up to fifteen individual isoenzymes being spearable.

Similarly with malted wheat, early studies showed that two electrophoretically separable sets of α-amylases were present (Alexandrescu and Mihailescu, 1970; Olered and Jonnson, 1970; Kruger, 1972b). Kruger (1972b) utilizing the more refined technique of polyacrylamide gel electrophoresis was able to separate the two sets into individual isoenzymes. The isoenzyme pattern he obtained with basic gel electrophoresis is illustrated in Fig. 1.

The more mobile group of α-amylase isoenzymes, on basic gel electrophoresis, was composed of 3 isoenzymes while the less mobile set was composed of 4 and sometimes 5 isoenzymes. Khan et al. (1973) using a similar technique obtained results paralleling those of Kruger. The high resolution technique of isoelectric focusing also has been employed in the study of germinated wheat α-amylase isoenzymes. Various Japanese workers, while carrying out

Fig. 1. Diagramatic illustration of the wheat α-amylase isoenzyme patterns obtained with
a) a germinated heated extract and b) immature extract.
From Kruger (1972 a,b).


−
α-8
α-7
α-6
α-5
α-4
α-3
α-2
α-1
+
a
−
α-3
α-2
α-1
+
b

a number of genetic studies, confirmed the results of Kruger (1972b) and Khan et al. (1973). They found two basic groups of isoenzymes as well as varietal variations in isoenzymes patterns within each group (Nishikawa and Nobuhara, 1971; Nishikawa, 1973; Nagayoshi, 1975).

Other cereals which have been studies include germinated oats (Alexandrescu and Mihailescu, 1973; Smith and Bennett, 1974), malted rye (Manners and Marshall, 1972; Wagennar and Lugtenborg, 1973; Alexandrescu et al., 1975) as well as triticale (Alexandrescu and Mihailescu, 1973). Multiple forms of α-amylase were apparent in all cases.

Most of the studies carried out on germinated cereal α-amylase isoenzymes have involved examination of the activity distribution following some form of electrophoresis. Little work has been directed towards separation of the isoenzymes on a preparative scale, but some preparative work has been carried out on α-amylase isoenzymes of malted wheat (Kruger and Tkachuk, 1969) malted barley (MacGregor et al., 1971, MacGregor, 1977) and malted rye (Manners and Marshall, 1972).

Tkachuk and Kruger (1974) also carried out a physical characterization of the malted wheat isoenzymes. Small differences in isoenzyme properties were evident, such as in energy of activation and pH optima, but the major distinguishing feature related to charge differences between the isoenzymes. These charge differences resulted from basic differences in the amino acid composition of the isoenzymes,

as shown in Table I. It can be seen that the arginine, lysine, glutamic acid and aspartic acid contents are significantly different between the four α-amylase isoenzymes, resulting in differences in charge between the isoenzymes.

It should be noted that the possibility does exist that the isoenzymes are an artifact of the isolation procedures although indications are that this is not so. For example,studies on porcine pancreas α-amylase isoenzymes (Rowe et al., 1968) showed that the α-amylase isoenzymes were definitely not artifacts due to separation procedures or association phenomena. More recently in the study of human α-amylase isoenzymes (Takeuchi et al., 1975) α-amylase isoenzymes were separated by electrofocusing and then re-focused giving an α-amylase of the same isoelectric point. This suggests that isoenzymes are real and not an artifact of the focusing technique. Most probably this is the case with cereal α-amylases.

Immature Cereal α-Amylase

To this date the α-amylase present in immature cereal seeds has not been studied as intensively as the α-amylase found in germinated cereal seeds. Recent work by various researchers, however, has yielded some knowledge as to the level of this enzyme in the immature seed, its morphological distribution and a few of its properties. Immature cereal α-amylases will be discussed in light of this new knowledge.

Table I

Amino Acid Compositions of Malted Wheat α-Amylases
(Moles per g)[a,b]

	Isoenzyme Components				
	I	II	III	IV	Average
Tryptophan[c]	255	297	n.d.	213	255
Lysine	441	415	471	410	434
Histidine	276	292	327	290	296
Ammonia	795	853	824	789	815
Arginine	461	401	396	342	400
Aspartic Acid	939	923	1010	965	959
Threonine	464	451	495	472	471
Serine	469	477	467	475	472
Glutamic Acid	943	970	986	893	948
Proline	589	586	596	642	603
Glycine	972	959	1025	931	972
Alanine	795	714	798	743	763
Cystine[d]	44	68	61	49	51
Valine	582	584	590	535	573
Methionine	117	157	115	182	143
Isoleucine	436	464	492	446	460
Leucine	599	621	694	617	633
Tyrosine	272	280	316	302	293
Phenylalanine	289	309	359	367	331
Cysteine	0	0	0	0	0
Actual Experimental amino acid nitrogen recovery, %	83	89	94	92	

a. Calculated to 100% amino acid residue weight recoveries, e.g., values in column 1 were multiplied by 100/83.

b. Threonine, serine, proline, valine, and isoleucine recoveries multiplied by 105, 109, 105, 108 and 107% to correct for incomplete hydrolysis and decomposition during hydrolysis.

c. Determined by analyzing barium hydroxide hydrolysates.

d. Determined as cysteic acid.

From Tkachuk and Kruger, 1974.

Changes in Activity Levels of Immature Cereal α-Amylase. The developing cereal grain is not a static system, but a dynamic one, undergoing continuous changes in its chemical and physical make up. Chrazaszcz and Janicki (1936) found that α-amylase was a part of this chemical change in wheat, barley, oats and rye. They observed that the α-amylase activity in these immature cereal kernels appeared shortly after anthesis and then gradually disappeared with ripening. Sandstedt (1946) realized that a carbohydrate degrading enzyme was present in the immature wheat kernel, since the pericarp starch, deposited a few days after pollination, was enzymatically digested during kernel development. This enzyme was subsequently shown to be α-amylase and was found to increase in the first week of growth followed by a slow decrease during the ripening of the wheat seed (Sandstedt and Beckford, 1946). Schwimmer (1947), on the other hand, found that on a wheat kernel basis the α-amylase content was relatively constant, although on a dry weight basis the activity decreased rapidly. Recently Olered (1967) and Olered and Jonsson (1970) also found that the α-amylase of immature wheat (which they called "green α-amylase") slowly disappeared with ripening. This confirmed the previous findings of Sandstedt and Beckford (1946). In addition, they found that the decreasing α-amylase activity could be regenerated to an extent by increases in the moisture distribution or equilibrium of the ripening seed (Olered and

Jonsson, 1970; Olered, 1976). The suggested explanation for this was that the "green α-amylase" was inactivated by dehydration of the kernel during the ripening process. More recently Meredith and Jenkins (1973) studied the changes in the pericarp α-amylase for a number of bread wheat cultivars; and obtained results paralleling those of Olered and Jonsson (1970). Subsequently Jenkins and Meredith (1975) also determined the changes in immature α-amylase levels for triticale and rye. Their results indicated that differences in immature α-amylase levels did exist between the species of Gramineae studied.

Similar results have been studied with the developing barley kernel. MacGregor et al. (1971) found that the α-amylase activity increased rapidly from emergence to eleven days past emergence and then declined sharply to one tenth of the maximum level after twenty eight days. This low level was subsequently maintained until maturity. Similar results were found by Duffus (1969) and Duffus and Rosie (1973) with the exception that the maximum activity was reached twenty to thirty days after anthesis. LaBerge et al. (1971) pointed out that there were no great cultivar differences with respect to the changes in α-amylase activity of developing barley.

On the other hand, Riggs and Gothard (1976) in their study of seven developing barley cultivars found different patterns of enzyme development. They found that the timing

of peak activity relative to anthesis agreed with that of LaBerge et al. (1971) as well as Allison et al. (1974); but the peak heights varied considerably between varieties. These workers also deduced a correlation between peak α-amylase activities and germinative energy as measured three weeks after harvest. This suggested to them that in addition to α-amylase synthesized de novo during germination, there may be a reactivation of the "green", i.e. immature-type α-amylases. Riggs and Gothard (1976) also proposed that the peak α-amylase activities obtained between ten and sixteen days after anthesis may be an indication of the degree of dormancy expected in the mature grain during the first few weeks following harvest.

One point of note is that, in contrast to the synthesis of α-amylase in germinating cereal, the synthesis of the α-amylases of immature wheat (Olered and Jonsson, 1970) and barley (Bilderback, 1971; MacGregor, 1972; Radley, 1976) was generally found to be noninducible by gibberellic acid. Conflicting results were reported by Duffus, (1969) who found that chlorcholine chloride, a gibberellic acid synthesis inhibitor, inhibited α-amylase synthesis in immature barley. More recently King (1976), in studying two cultivars of wheat, found that in the case of seed of the cultivar Gabo, gibberellic acid did not induce synthesis until grain drying took place. Conversely, with the cultivar WW15, a more pronounced and earlier induction of α-amylase by gibberellic

acid was evident, although fluctuations in activity were apparent. These results suggest that there are cultivar differences involved, as well as time dependent factors.

Various explanations related to this noninduction have been proposed. MacGregor et al. (1972) suggested that the absence of effect of gibberellic acid may be due to different systems being present for the synthesis of immature and germinated α-amylases, or the presence of an excess of gibberellic acid. On the other hand Radley (1976) discovered a correlation between increasing abscisic acid (ABA) levels in the pericarp and the decrease of α-amylase in this tissue. King (1976) feels that ABA may inhibit premature sprouting as well as possibly preventing the formation of the "green α-amylase". Thus ABA may be one of the factors which results in noninduction by gibberellic acid.

Morphological Distribution. Sandstedt and Beckford (1946) found that α-amylase activity in immature wheat was present in the pericarp and not in the endosperm. These findings have since been verified by Kruger (1972a), who used an electrophoretic technique to show that the major portion of the α-amylase was present in the pericarp. Only small amounts were found in the seed coat and endosperm with none present in the embryo. Banks et al. (1972) obtained a similar result with the α-amylase being present in the endosperm-germ. Meredith and Jenkins (1973)dissected immature wheat grains into white pericarp and green inner

part and found double the activity in the pericarp.

With respect to the α-amylases of immature barley, differences of opinion exist as to the location in the kernel. Duffus (1969) and Bilderback (1971) found that the endosperm as well as the aleurone contained considerable amounts of activity. This conflicts with the results of Stoddart (1971) who found that the α-amylase was almost completely contained in the aleurone. On the other hand, MacGregor et al. (1972) found that the α-amylase activity was confined to the pericarp with very little present in the endosperm. Allison et al. (1974) discovered that the peak activity in the pericarp was twice that of either the endosperm or aleurone. It would appear most likely that the major portion of α-amylase in the immature barley kernel is present in the outer layers of the kernel, as is the case with wheat.

A reasonable assumption for the presence of α-amylase in the outer layers of the immature cereal kernel would be that it serves to hydrolyze the pericarp starch providing some of the energy and sugar requirements of the growing seed (Kruger,1972a; MacGregor et al., 1972). More succinctly, Allison et al. (1974) have suggested that pericarp α-amylase may give rise to short chain primers which would initiate starch synthesis in the endosperm.

Immature Cereal α-Amylase Isoenzymes. Electrophoretic work by many researchers had indicated that α-amylase isoenzymes

are present in immature wheat (Olered and Jonsson, 1970; Kruger 1972a; Iliev, 1974; Daussant and Renard, 1976). Kruger (1972a) found three electrophoretically mobile isoenzymes present in immature wheat. Olered and Jonsson (1970) also found several isoenzymes present but resolution in their electrophoretic technique was not adequate to give an exact number. Kruger (1972a) found that these three isoenzymes were present in all cultivars of Canadian HRS wheat studied. The isoenzymes were not preferentially separated from each other in the various anatomical tissues of the whole seed and were found in the same proportions.

Kruger (1972a) also noted that the 3 α-amylase isoenzymes of immature wheat were electrophoretically identical on basic polyacrylamide gel electrophoresis to a set of three isoenzymes present in germinated wheat and were possibly related. The isoenzyme pattern obtained is illustrated in Fig. 1. It was suggested that the immature α-amylase isoenzymes were regenerated to yield the three isoenzymes found in the germinated seed. Some evidence to support this assumption was the discovery by Daussant and Renard (1972) that 2% of the activity of germinated cereals could be attributed to immature α-amylases. Daussant and Renard (1976) subsequently carried out a closer comparison of the germinated and immature α-amylases. They ascertained that a set of immature α-amylases of the developing period was identical to a set occurring upon germination as noted by

Olered and Jonsson (1970), Kruger (1972b) and Tkachuk and Kruger (1974).

Olered and Jonsson (1970) found that the isoenzymes of immature wheat differed to some degree from the α-amylase of malt. The "green α-amylases" were found to be slightly more heat sensitive, with the sensitivity becoming greater during ripening. More recently Marchylo et al. (1976) purified and partially characterized the three immature isoenzymes present in the developing hard red spring wheat cultivar, Neepawa. These three isoenzymes were found to have similar properties, with the major differences distinguishable on the basis of electrophoretic properties. The immature wheat isoenzymes differed from the malted wheat isoenzymes purified by Tkachuk and Kruger (1974) in a number of respects. The immature wheat α-amylase isoenzymes had a broader pH optimum, a greater heat lability, generally higher activation energies, higher molecular weights and lower isoelectric points.

A simpler isoenzyme system is present in immature barley as indicated by Stoddart (1971) and MacGregor et al. (1974) who found only one isoenzyme of α-amylase to be present in the immature kernel. MacGregor et al. (1974) also found the immature α-amylase isoenzyme to be electrophoretically identical to a distinct isoenzyme of malt barley. On the other hand, Bilderback (1971) found that an immature Himalayan 2-row cultivar apparently contained four α-amylase

isoenzymes. There is the possibility that this was just an isolated case and different from the norm. Stoddart (1971) also found that the α-amylase isoenzymes of the developmental stages differed from those present in the germinating phase.

MacGregor et al. (1974) have continued this work by purifying and partially characterizing the α-amylase isoenzyme present in immature barley.

α-Amylase in Breadmaking

α-Amylase finds a great deal of importance in the breadmaking industry, as it has a large influence on the quality of bread obtained from a flour. To realize the importance of this enzyme in the breadmaking process, it is first necessary to understand the role that it plays.

In breadmaking, α-amylase in dough degrades starch to dextrins and fermentable sugars. In the fermentation stage only the damaged starch granules are available for attack whereas in the baking phase, before the α-amylase is inactivated, both the damaged and undamaged starch granules are attacked (Farrand, 1964). α-Amylase in conjunction with β-amylase can affect dough in three ways, these being: i) formation of fermentable sugars, ii) removal of the damaged starch fraction, and iii) the formation of dextrins (Bloksma, 1971).

In the first case sufficient fermentable sugars must be present so that the yeast will produce enough carbon dioxide

to yield a loaf of proper size, grain and texture (Geddes, 1946). The removal of the damaged starch fraction, in most cases, is not of major importance due to the presence of only a relatively small proportion of damaged starch granules in normal flour (Johnson and Miller, 1953). Some flours, of course, do have a high content of damaged starch especially if they are to be used for a breadmaking process such as the Chorleywood process. In cases such as these, the α-amylase content will become more important. In the gelatinization period, the presence of α-amylase is extremely important since the enzyme is highly active and quickly breaks down the starch to dextrins. Starch is more highly hydrated than dextrins and as a result, excessive liquefaction and dextrinization will occur if too much α-amylase is present. This will yield a loaf of poor quality with a wet and sticky crumb (Geddes, 1946; Bechtel et al., 1964; Bloksma, 1971).

It is evident that α-amylase is extremely important in breadmaking, as it can have a major influence on gassing power, loaf volume, texture, crumb and even crust colour. Minimization of high levels of this enzyme in wheat crops has been, therefore, the concern of cereal technologists around the world.

In most cases, this excess α-amylase results from sprouted wheat. Tipples et al. (1966) carried out baking tests on flour milled from a heavily sprouted wheat and found that it resulted in quality deterioration due to

large holes in the loaf. They also pointed out that many commercial grades of wheat allow a certain amount of sprouted wheat. Thus, since this wheat contains high α-amylase, minimal amounts may cause deleterious effects in the baking quality of the flour.

Olered and Jonsson (1970) suggested that excess α-amylase may actually come from a source other than sprouted wheat. As mentioned in a previous section, they found that "green α-amylase" may be reactivated during the latter stages of maturation. In fact, even when a kernel appears mature "green α-amylase" may be present. In the B-D period of Fig. 2 just before sprouting, the "green α-amylase" may be much more active than the germinated α-amylase. Thus wheat containing reactivated "green α-amylase" or immature kernels could have a detrimental effect on baking quality similar to that obtained with sprouted wheat.

Methods of Analysis for α-Amylase

In the last number of years, there has been a rapid proliferation of methods for the determination of α-amylase activity in mammalian and plant systems. Due to the large numbers of methods which are now available this short review will be limited to consideration of a few methods which may be used to assay for α-amylase in cereal systems.

Two of the standard methods which have been traditionally used as a measure of α-amylase in wheat are the Hagberg falling

Fig. 2. Diagram showing the variations in α-amylase activity (K (α)) in unripe wheat grains during ripening and the appearance of α-amylase from malting.

AB, Kernel development until full ripeness. Variations in α-amylase activity caused by changes in moisture distribution; BC, "primary" α-amylases are reactivated by delayed drying; DE, synthesis of α-amylase during malting.

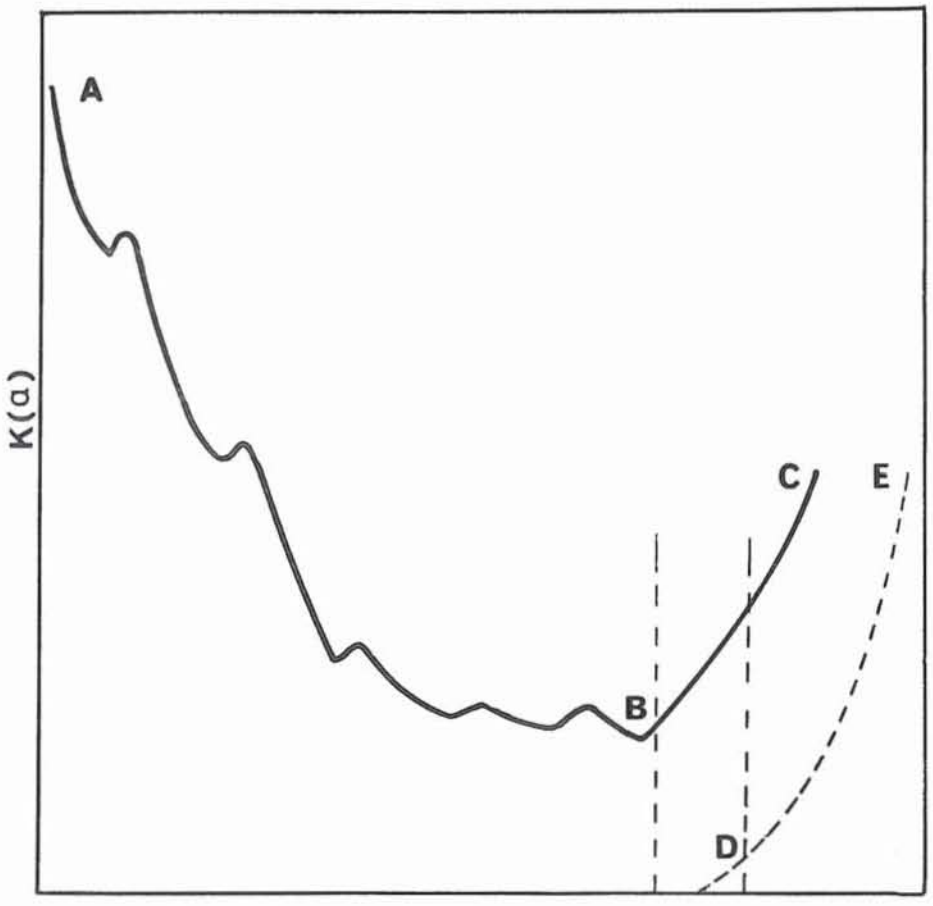
The period between B and D is characterised by a high α-amylase activity and low falling numbers without the appearance of visible sprouts.

From Olered and Jönsson (1970).

number and amylograph methods (American Association of Cereal Chemists, 1975). These methods, which depend upon the principle of a decrease in viscosity of a starch solution, are sensitive and widely used. Unfortunately they suffer from the drawback of only indirectly measuring α-amylase content. Properties of the starch substrate, such as starch damage content and starch susceptibility, may greatly influence the results.

Another group of methods of analysis depend upon the loss in iodine color of a β-limit dextrin solution. Examples of α-amylase assays depending upon this principle are the methods of Briggs (1961), Farrand (1964) and Perten (1966). As with the previous method these also suffer various disadvantages. For example, these methods lack precision, are operator dependent and at high levels of α-amylase will give false results unless special precautions are taken. The presence of β-amylase also will lead to undue loss of iodine color as a result of its synergistic relationship with α-amylase. Since appreciable quantities of β-amylase are present in cereal grains this is a distinct disadvantage with these methods.

A third group of methods depends upon an increase in reducing power of a starch solution. An example of this is the automated procedure of Strumeyer and Romano (1966). Again this suffers disadvantages similar to those of the viscometric methods. Thus, these traditional methods suffer


A
K(a)
C
E
B
D

various disadvantages as mentioned in addition to being generally time consuming.

More recently a new approach involving dye-labelled substrates, developed by Fernley (1963), has been applied by a number of researchers to the study of α-amylase in cereals. For example, Barnes and Blakeney (1974) have employed a commercially available dye-labelled substrate namely Phadebas^{R} tablets.* This substrate was found to be resistant to β-amylase attack and thus did not suffer from its interference during cereal assays. They found that this method was successful with a number of cereals and cereal products. It was also discovered to be a simple, accurate and specific α-amylase technique which was highly reproducible and a great improvement over other methods presently in use. The one possible drawback of such a method could be its unsuitability for an automated analysis system.

In summary, over the years, a large body of knowledge has been collected on the subject of cereal α-amylases. This body of knowledge is expanding continually and is serving as a strong base for further studies pertaining to this enzyme. Many areas of research, related to α-amylase, still remain available for study, and if pursued, this

*Phadebas^{R} tablets contain a substrate made by cross-linking partially hydrolyzed potato starch, using 1,4-butandioldiglycidether as the cross-linking agent. The substrate is labelled with Cibachron Blue by covalent bonds.

research would vastly increase our knowledge and understanding of this enzyme. Research, directed towards the further elucidation of the isoenzymic nature and control of α-amylase, in both the germinating and immature cereal kernel, is essential to obtain a much clearer overall picture of this enzyme system. Fortunately, numerous technological breakthroughs have provided a vast improvement in methodology. In turn, this has furnished the scientist with the means to solve many of the unanswered questions related to the cereal α-amylase system.

PART I

A SENSITIVE AUTOMATED METHOD FOR THE DETERMINATION OF α-AMYLASE

INTRODUCTION

One of the primary steps in this research project was the development of an enzyme assay suitable for the determination of α-amylase activity in wheat. Although numerous α-amylase assays were available, they invariably suffered from various deficiencies which made them unsatisfactory for the assaying of wheat α-amylase (Barnes and Blakeney, 1974). In addition, since it was known that a large number of samples were to be assayed for enzyme activity, it was felt that an automated method would be most suitable. To satisfy these requirements, the automated fluorometric serum α-amylase assay of Rinderknecht and Marback (1970) was adapted to the analysis of cereal α-amylase. The resultant method worked on the principle of α-amylase hydrolyzing the substrate (β-limit dextrin anthranilate) thus producing dialyzable fluorescent products which could be monitored fluorometrically.

MATERIALS AND METHODS

The commercial enzymes used in this study were fungal α-amylase, 5000 SKB units per g; Bacillus subtilus α-amylase, 1187 absorbance units (AU) per mg (Calbiochem, Los Angeles, California); barley α-amylase (Schwartz-Mann, Orangeburg, New York), and sweet potato β-amylase (Sigma Chemical Co., St. Louis, Mo.). These enzymes were dissolved in 0.2M sodium acetate buffer, pH5.5, containing 10^{-3}M $CaCl_2$ prior to use. α-Amylase was also extracted from malted Manitou wheat, heat treated at 70°C and purified by glycogen-complex formation as described by Kruger and Tkachuk (1969).

Amylopectin anthranilate was purchased from Calbiochem, Los Angeles, California.

Extraction of Wheat Flours

Flour (2g) was added to 10 ml of 0.2M sodium acetate buffer, pH 5.5, containing 10^{-3}M $CaCl_2$ and mixed in a VirTis Model 23 Homogenizer for 30 seconds at a speed setting of 60. An aliquot of the suspension was placed in a 10 ml ultracentrifuge tube equipped with a screw cap and rotated on a Labquake rotator (Labindustries, Berkeley, California) for 2 hours at room temperature. The mixture then was

centrifued at 96,000 x g for 10 min and the clear supernatant used for analyses.

α-Amylase Activity Using Reduced β-Limit Dextrin

Fungal α-amylase was used as a reference and was assayed by measuring the reducing sugars liberated from reduced β-limit dextrin at 35°C, as described previously by Kruger and Marchylo (1972).

Reducing sugars were determined by the neocuproin method (Dygert et al., 1965) automated on the Technicon Auto-Analyzer (Marchylo, 1975).

Preparation of β-Limit Dextrin Anthranilate

Amylopectin anthranilate (10g) was added slowly to 400 ml of stirring 0.2M sodium acetate buffer, pH 4.5, and the resultant suspension was brought to a boil. After cooling to room temperature, 0.2 ml of sweet potato β-amylase was added and the mixture was stirred slowly for 1.5 days. It was then reboiled and exhaustively dialyzed against distilled water at 4°C for two days, followed by freeze drying. The freeze dried substrate was ground to a fine powder in a Moulinex electric coffee mill and stored in a dark bottle at 4°C.

Preparation of 0.25% β-Limit Dextrin Anthranilate Working Solution

Sixty ml of 0.2M sodium acetate buffer, pH 5.5, containing 10^{-3}M $CaCl_2$ was combined with 1.2 ml of 30% Brij 35

solution and brought to a boil. After removing from heat, 0.5g β-limit dextrin anthranilate was added with vigorous stirring. The mixture was homogenized for three minutes with a VirTis Model 23 Homogenizer at a speed setting of 15 and added to 100 ml of 0.2M sodium acetate buffer, pH 5.5, containing 10^{-3}M $CaCl_2$, preheated to 90°C. After stirring for a period of 30 minutes at 90°C to ensure a colloidal suspension, the volume was brought to 200 ml with 0.2M sodium acetete buffer, pH 5.5, containing 10^{-3}M $CaCl_2$. The suspension was then quickly cooled to room temperature while being stirred.

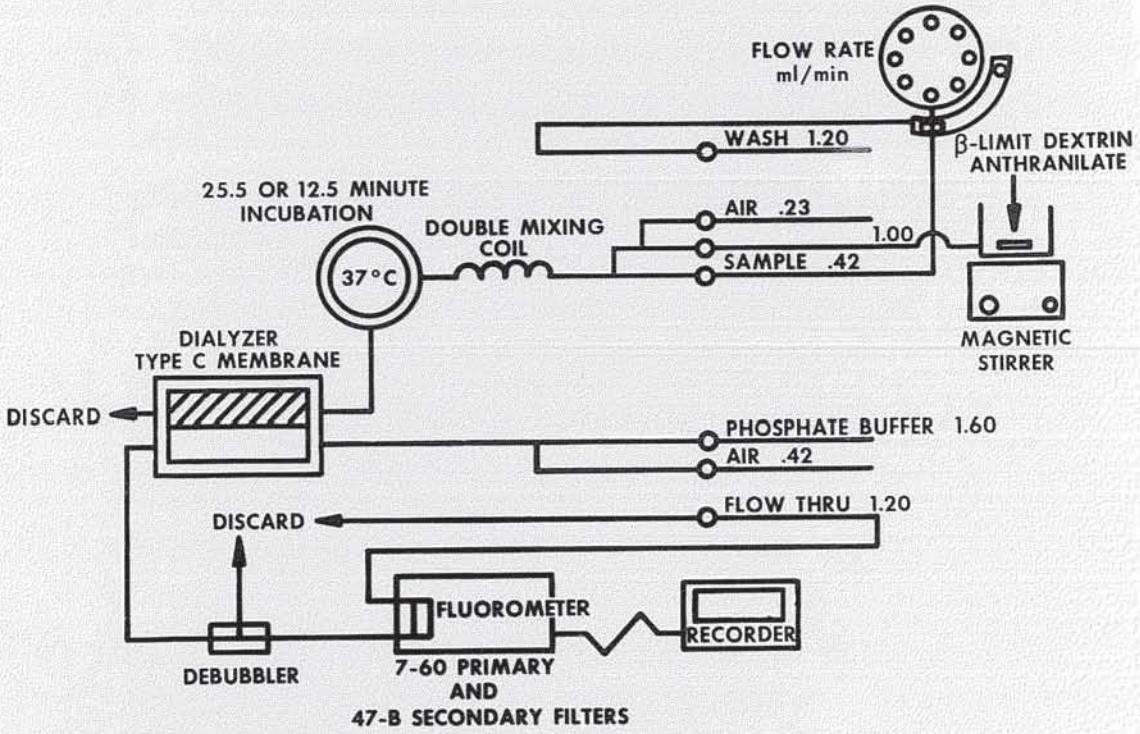
Although initial experiments were carried out with 0.5% substrate, a concentration of 0.25% subsequently was found sufficient to ensure a linear response between fluorescence intensity and enzyme concentration. Triton X-100 in place of Brij 35 proved unsuitable as a coating formed on the walls of the Technicon tubing, coils, etc., and interfered progressively with the analyses.

Operational Procedure

The procedure was adapted from the method of Rinderknecht and Marback (1970) for serum α-amylase, employing amylopectin anthranilate as substrate.

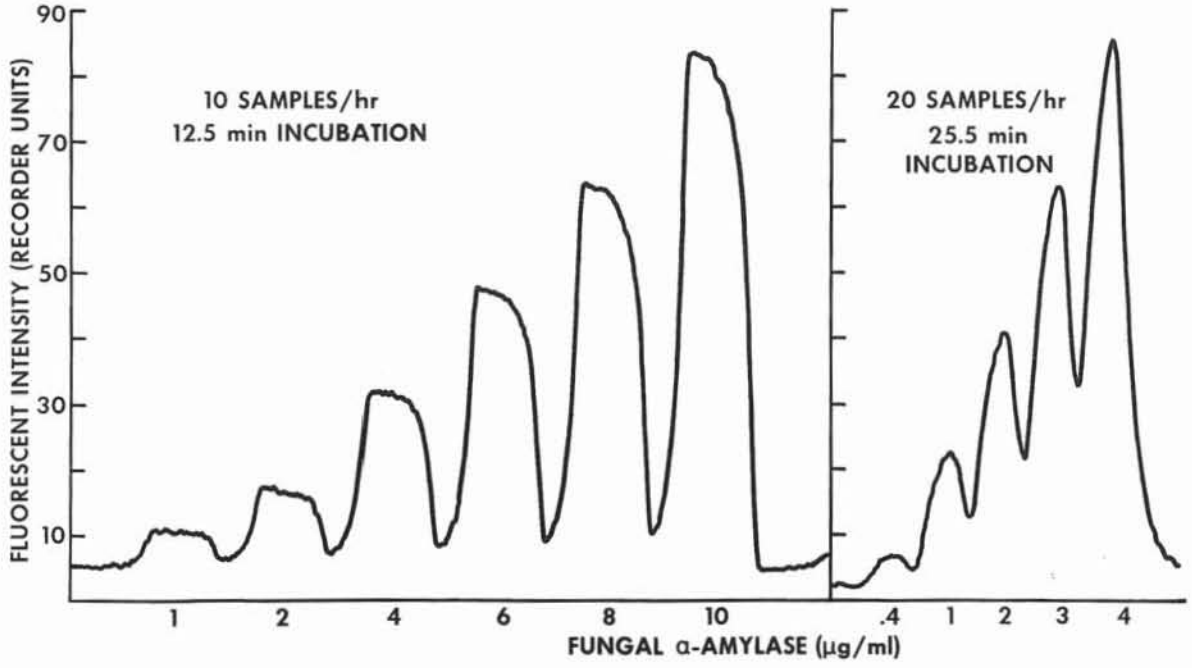
The Technicon AutoAnalyzer (Technicon Corp., Tarrytown, N.Y.) Flow sheet for the assay is shown in Fig. 3. Substrate solution was stirred continuously throughout the assay

Fig. 3. Technicon AutoAnalyzer flow sheet for determination of α-amylase with β-limit dextrin anthranilate as substrate.


FLOW RATE
ml/min
WASH 1.20
β-LIMIT DEXTRIN
ANTHRANILATE
25.5 OR 12.5 MINUTE
INCUBATION
DOUBLE MIXING
COIL
AIR .23
1.00
SAMPLE .42
37°C
MAGNETIC
STIRRER
DIALYZER
TYPE C MEMBRANE
DISCARD
PHOSPHATE BUFFER 1.60
AIR .42
FLOW THRU 1.20
DISCARD
FLUOROMETER
RECORDER
DEBUBBLER
7-60 PRIMARY
AND
47-B SECONDARY FILTERS

in a dark bottle and could be used over a period of 2 - 3 days. Sample, flowing at 0.42 ml per min, met an air segmented stream of substrate flowing at 1 ml per min and then passed through a double mixing coil and was incubated subsequently in a 37°C bath with a 12.5 or 25.5 min coil depending on the sensitivity required. Upon leaving the incubation bath the stream passed through a 24-in dialyzer into an air segmented recipient stream of 0.2M Sorenson's phosphate buffer, pH 7.2, flowing at 1.60 ml per min. The recipient stream was debubbled and passed through a Model III Turner Fluorometer equipped for automated chemistry. The fluorometer contained a 7-60 primary and a No. 49-B secondary filter and the light sensitivity was set at 10x or 30x. The fluorescent intensity which resulted from dialysis of fluorescent hydrolytic products into the recipient stream was measured on the Technicon recorder. Lower and upper limits of fluorescent intensity were set at 5% and 95%, respectively, by running fungal standards of the appropriate concentrations. Typical AutoAnalyzer tracings, with varying concentrations of fungal α-amylase assayed at 10 and 20 samples per hr. are shown in Fig. 4 The pH of the recipient stream was very important and at a pH lower than 7.2 the fluorescent intensity decreased markedly.

Fig. 4. AutoAnalyzer recorder tracings at various concentrations of fungal α-amylase: Left - 10 samples / hour, 12.5 min incubation. Right - 20 samples / hour, 25.5 min incubation.


90
70
50
30
10
FLUORESCENT INTENSITY (RECORDER UNITS)
10 SAMPLES/hr
12.5 min INCUBATION
20 SAMPLES/hr
25.5 min
INCUBATION
1
2
4
6
8
10
.4
1
2
3
4
FUNGAL α-AMYLASE (μg/ml)

RESULTS AND DISCUSSION

α-Amylase from Fungal, Bacterial and Malted Wheat Extracts

α-Amylases from fungal, bacterial and malted wheat extracts were tested for their abilities to break down β-limit dextrin anthranilate into dialyzable fluorescent products. The results obtained with a 12.5 min incubation at 37°C and fluorometer aperture setting of 10x are shown in Figs. 5 - 7. In all cases the formation of dialyzable fluorescent reaction products measured in terms of fluorescent intensity increased linearly with increase in enzyme concentration. Sensitivity of the assay could also be increased considerably. For example, as little as 0.002 SKB units of fungal α-amylase activity could be detected with a 25.5 min incubation and 30x aperture setting of the fluorometer.

As fluorescent intensity is an arbitrary measure of enzyme activity, fungal α-amylase was selected as a reference. In addition to availability in large quantities, its potency decreased extremely slowly with time, if stored in the cold. The potency of the α-amylase was checked from time to time by measuring the amount of reducing sugars in terms of mg maltose liberated from reduced β-limit dextrin substrate

Fig. 5. Hydrolysis of β-limit dextrin anthranilate by different amounts of fungal α-amylase in the presence and absence of β-amylase.

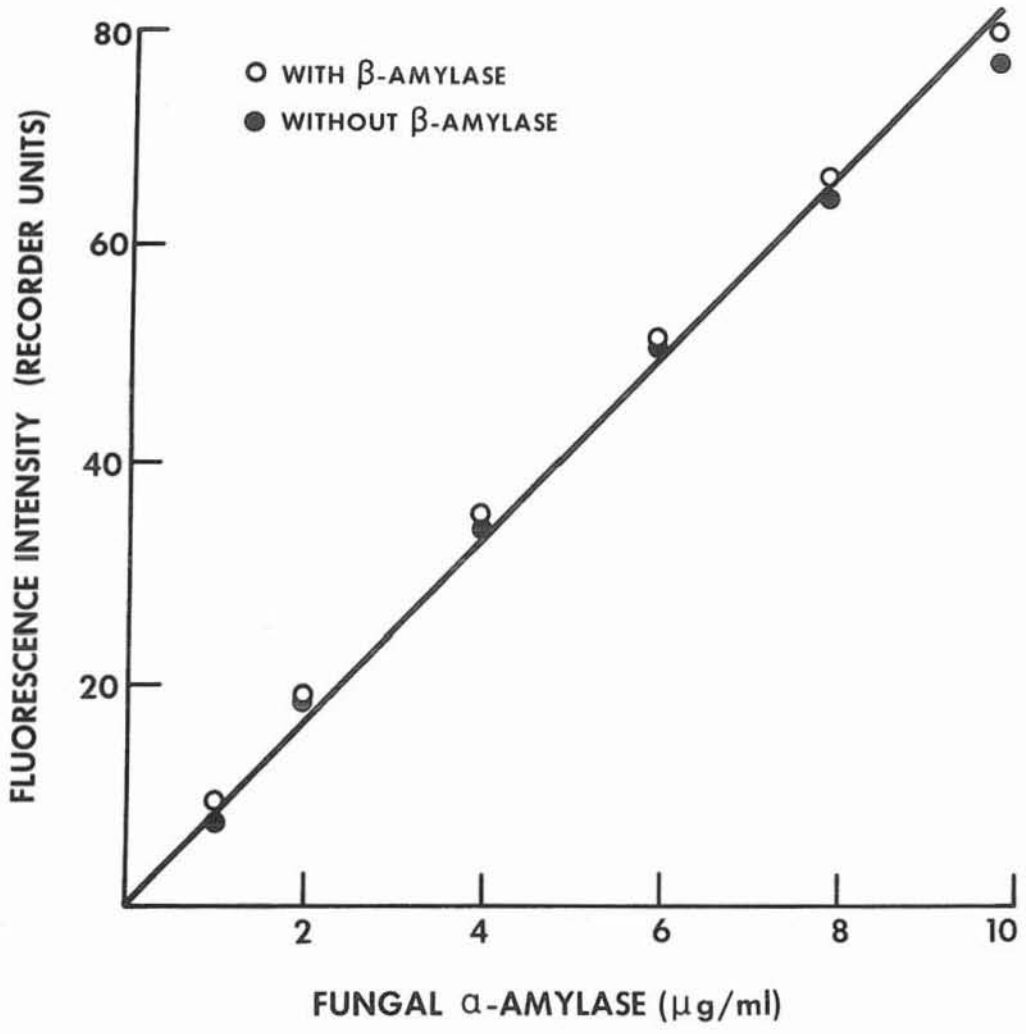

80
60
40
20
O WITH β-AMYLASE
● WITHOUT β-AMYLASE
FLUORESCENCE INTENSITY (RECORDER UNITS)
2
4
6
8
10
FUNGAL α-AMYLASE (μg/ml)

Fig. 6. Hydrolysis of β-limit dextrin anthranilate by different amounts of bacterial α-amylase in the presence and absence of β-amylase.

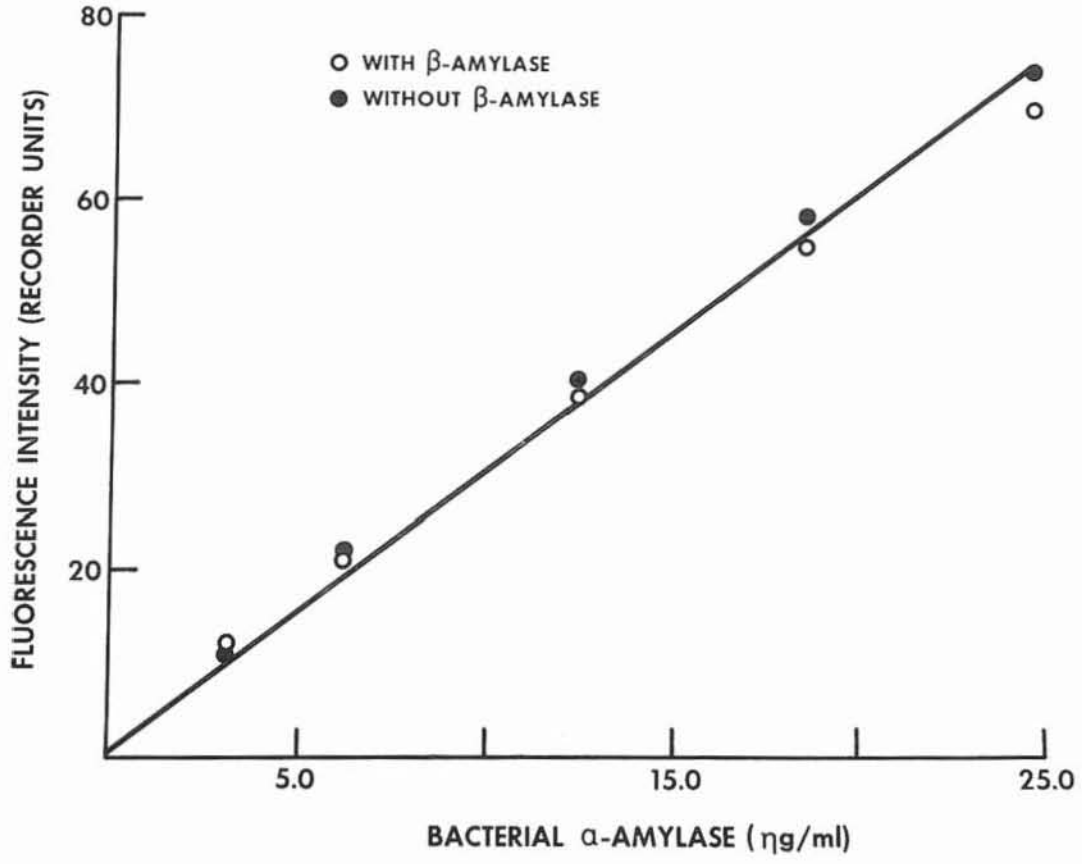
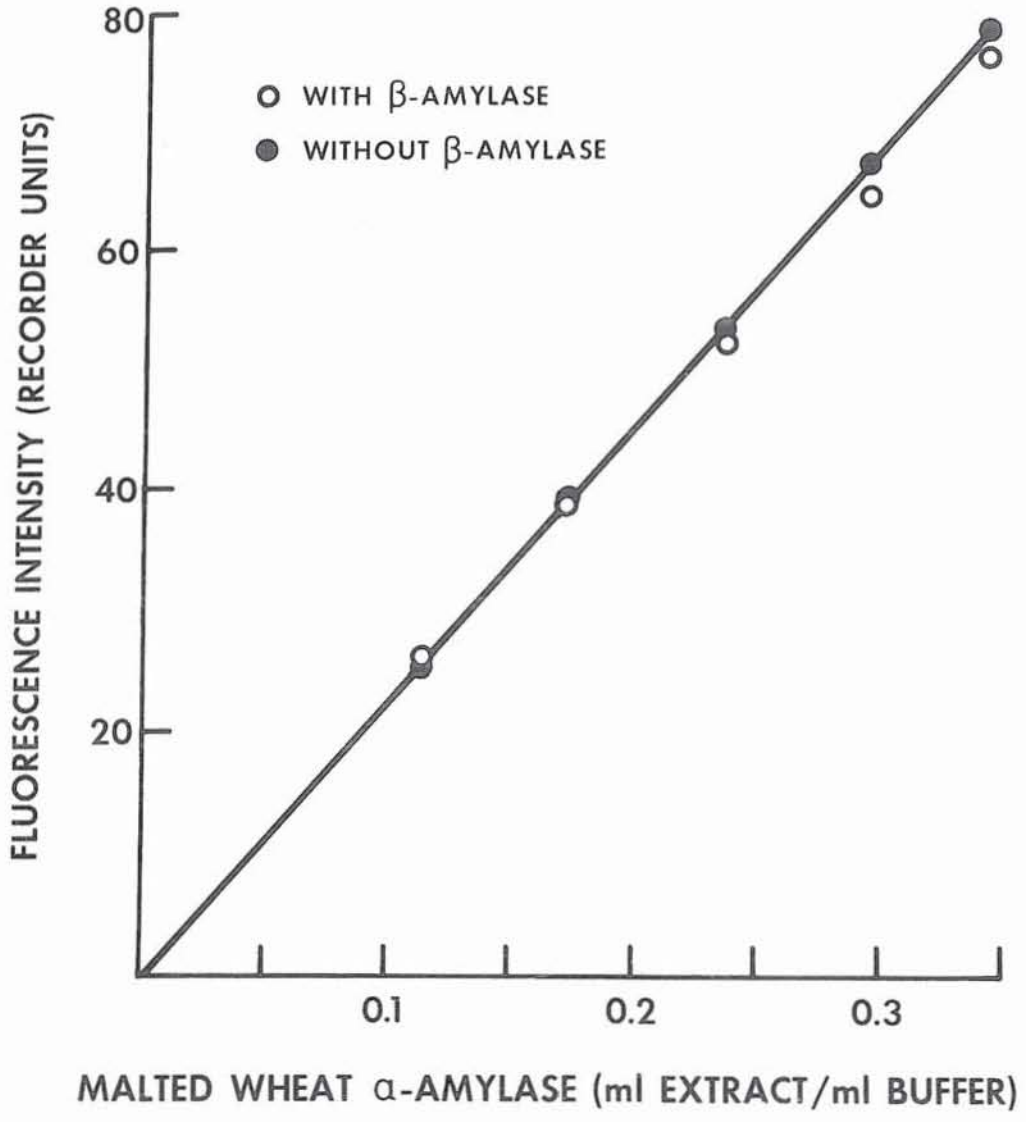

80
60
40
20
FLUORESCENCE INTENSITY (RECORDER UNITS)
WITH β-AMYLASE
WITHOUT β-AMYLASE
5.0
15.0
25.0
BACTERIAL α-AMYLASE (ηg/ml)

Fig. 7. Hydrolysis of β-limit dextrin anthranilate by different amounts of malted wheat α-amylase in the presence and absence of β-amylase.


80
60
40
20
FLUORESCENCE INTENSITY (RECORDER UNITS)
WITH β-AMYLASE
WITHOUT β-AMYLASE
0.1
0.2
0.3
MALTED WHEAT α-AMYLASE (ml EXTRACT/ml BUFFER)

at 37°C per min per mg of enzyme. Appropriate dilutions of fungal α-amylase were run at least twice during a normal working day, and fluorescent intensity values could be converted into the amount of maltose liberated from reduced β-limit dextrin.

Recently some controvery arose concerning the kinetic aspects of a fluorometric α-amylase assay. Eikenberry (1977) suggested that a fluorometric α-amylase assay is in actuality a kinetic assay for the glucose released by the action of α-amylase after a specified time. Guilbaut (1977) countered this point with the following argument. In the assay the cleavage of starch by α-amylase is the rate determining step, and since the rate of such conversion is still proceeding in a linear first order manner at the time of assay, then this method is a kinetic assay of α-amylase as well as of glucose.

The fluorometric α-amylase assay described in this study, although using β-limit dextrin anthranilate as substrate, does proceed in a linear first order manner during the time of assay (Figs. 5 - 7) and, as noted by Guilbaut (1977), is then a kinetic assay of α-amylase.

The most useful feature of using β-limit dextrin anthranilate for the determination of α-amylase was that excess β-amylase (barley, 20 ug/ml) had no effect on the assay. Similar behavior has been reported with β-limit dextrin azure (Bilderback, 1973). The resistance of these

substrates to β-amylase is probably due to the dye-label interfering with the exo mode of attack characteristic of β-amylase.

The resistance of β-limit dextrin anthranilate to β-amylase indicated its particular suitability for the determination of cereal α-amylase where β-amylases are often in excess.

α-Amylase from Wheat Flour

To analyze for the α-amylase present in wheat flours, a 25.5-min coil in the 37°C incubation bath of the Auto-Analyzer and the 30x aperture setting on the fluorometer were chosen. This provided the required sensitivity to detect the low levels of α-amylase activity in flours with very high amylograph viscosities. As flour extracts contained some dialyzable fluorescent compounds a blank was run with each sample. These were prepared by heating an aliquot of the flour extract at 90°C for 10 minutes to inactivate the α-amylase, cooling rapidly to room temperature and filtering through glass wool.

Twenty-four flour samples with varying amylograph viscosities selected from Canadian HRS wheats (either plant breeders' cultivars or Canadian export cargoes) were examined by the present method. The results are shown in Table II. Actual fluorescent intensities in terms of recorder units have been included to show typical values

Table II

Determination of α-amylase by the automated fluorometric method of 24 Hard Red Spring wheat flours of varying amylograph viscosity.

Amylograph[a] Viscosity B.U.	Fluorescent Intensity of Blank Recorder Units	Fluorescent Intensity of Sample-blank Recorder Units	α-amylase Activity[b] mg maltose/min/gm x 10^{-3}
200	11.0	48.5	9.8
205	22.5	48.0	10.6
260	13.0	52.5	10.0
265	15.5	38.5	8.2
350	12.0	35.0	7.6
355	12.5	35.0	7.2
400	13.0	36.5	7.0
430	14.0	29.0	6.4
430	13.0	34.5	6.6
460	11.5	29.5	6.4
470	13.0	33.0	7.3
490	12.0	30.0	6.7
530	17.0	31.0	6.4
580	13.0	28.0	5.3
585	15.5	28.5	5.4
595	11.5	25.5	5.6
615	14.5	21.5	4.8
620	13.5	33.0	6.2
635	22	25.5	4.8
645	16	28.5	5.4
650	11.0	21.5	4.6
660	12.0	28.5	6.2
725	11.0	24.5	5.4
860	12.5	21.0	4.6

(a) 65 g flour, 450 ml water

(b) Converted from fluorescent intensity recorder units with a fungal α-amylase reference.

for flours and blanks. Thus blank values due to inherent dialyzable fluorescent substances in the flours accounted for 18 - 46% of the total fluorescent intensity although the average contribution was around 30%. Fluorescent intensity values for all the flours fell within the scale of the recorder and dilutions were not necessary. The fluorescent intensity values of the sample minus blank were converted to α-amylase activity with a fungal α-amylase reference and a two-fold difference in activity was found between the high and low amylograph viscosity flours. The α-amylase activity decreased curvilinearly with increase in amylograph viscosity as shown in Fig. 8. Similar behavior has been observed when the α-amylase was determined by other methods such as the ICC (Carr and Spillane, 1969), viscometric (Tipples, 1969), or Phaedebas tablet method (Barnes and Blakeney, 1974). Deviations from the curve, which were observed for some samples, could be expected since the α-amylase activity of a particular wheat cultivar may be affected by additional factors, such as the nature of the starch (Meredith, 1970). Falling number values were determined by the standard ICC method (International Association for Cereal Chemistry, No. 107) for a number of the whole wheats shown in Table II. When plotted against the α-amylase activity a curvilinear plot was obtained (Fig. 9) similar to that shown in Fig. 8. It is advantageous to plot the α-amylase activity versus the reciprocals of the

Fig. 8. Wheat α-amylase activity determined by the automated fluorometric method versus amylograph viscosity.

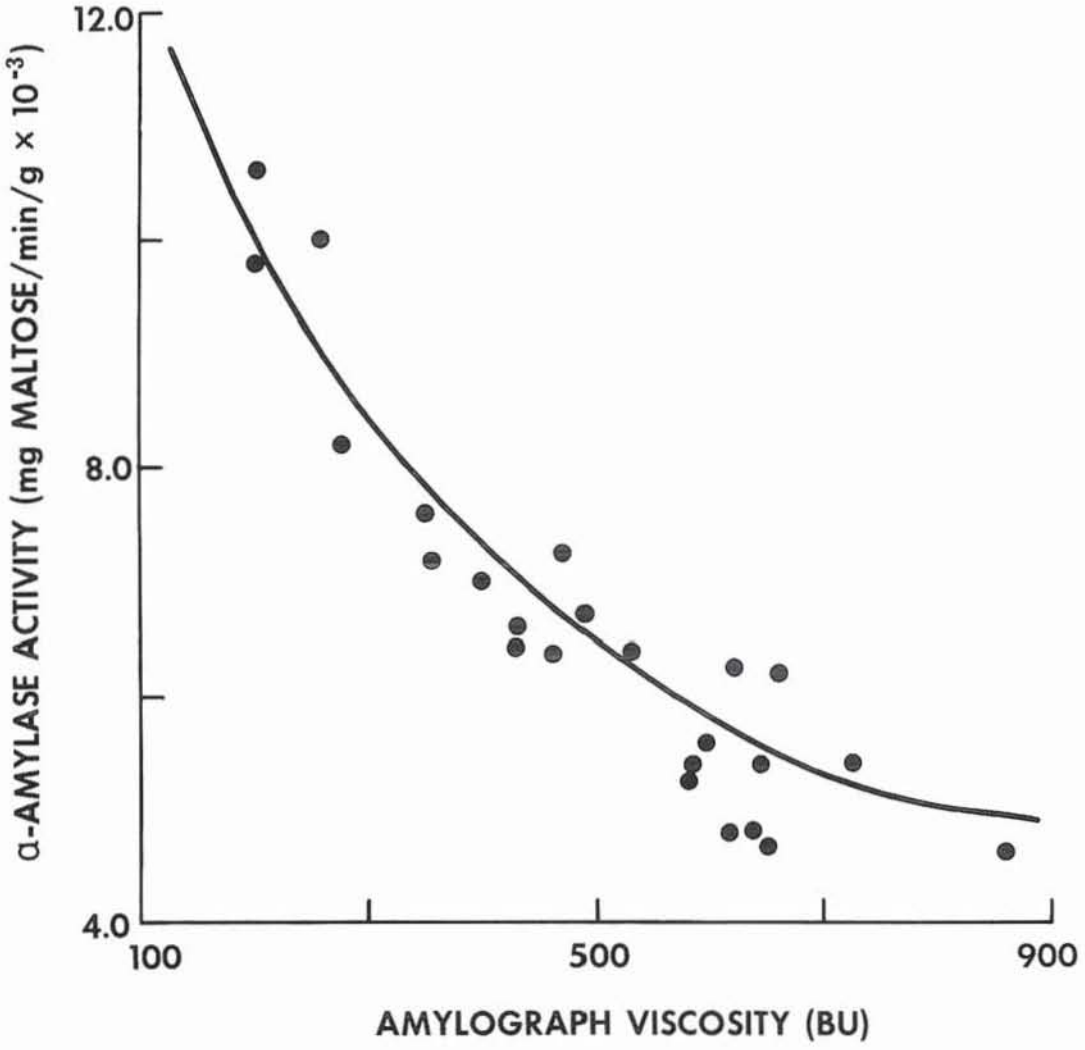
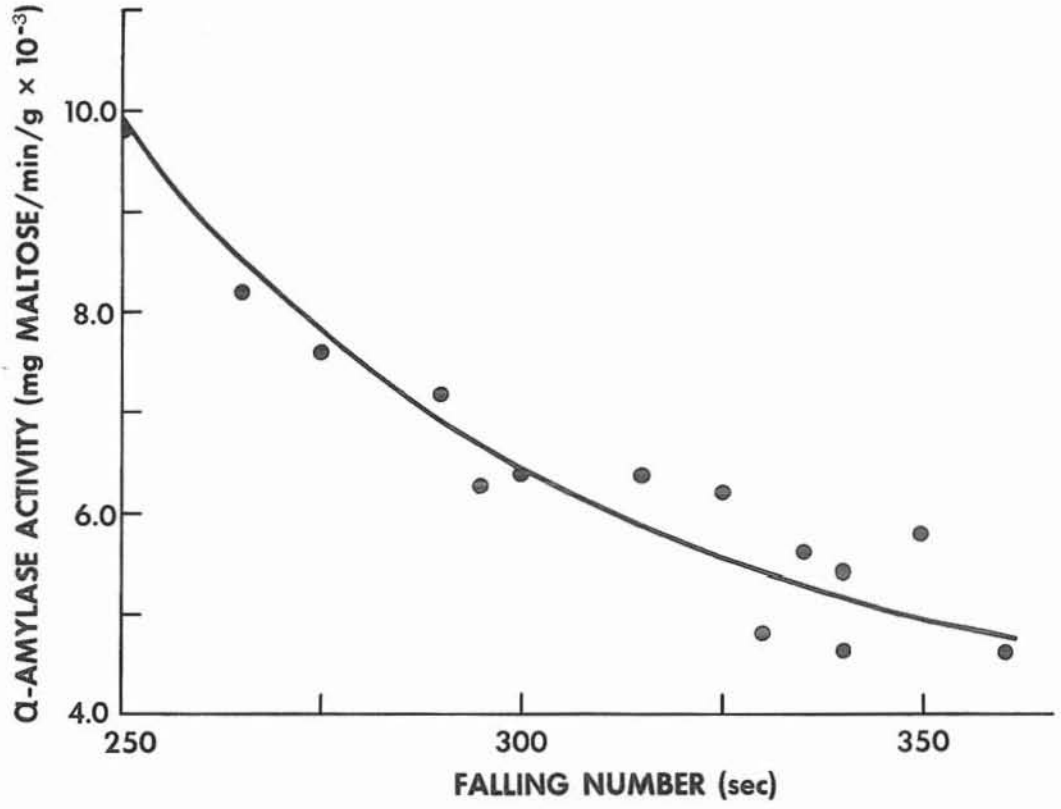
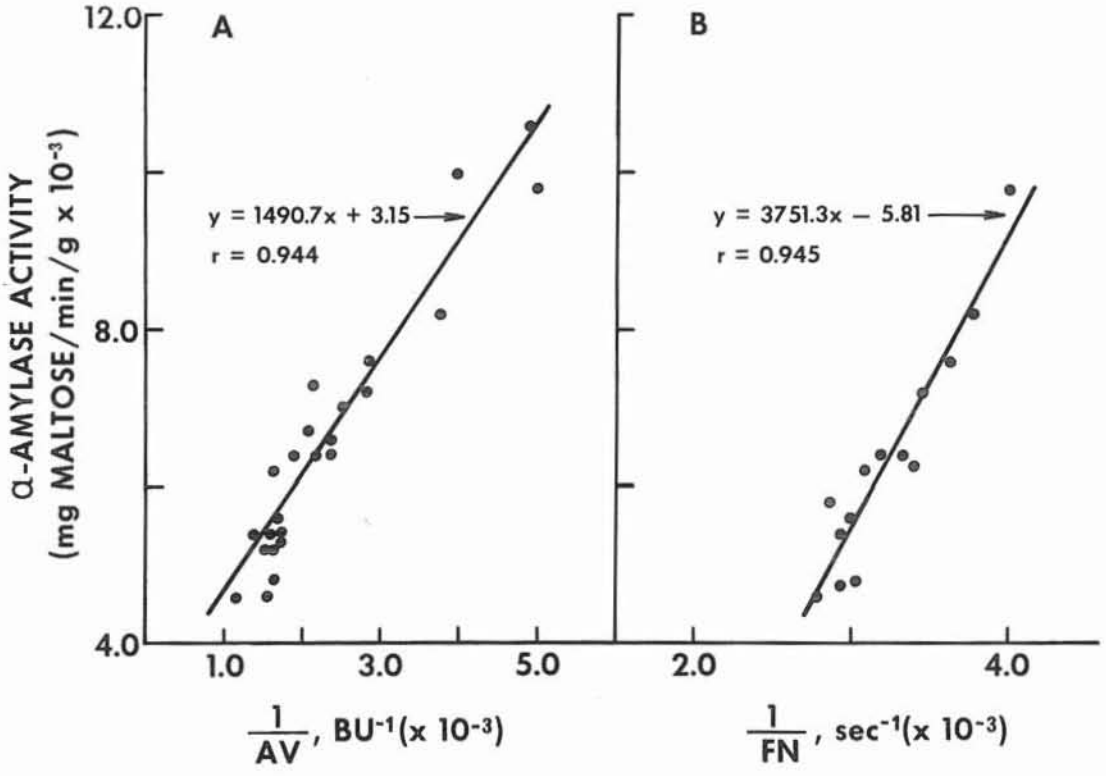

12.0
8.0
4.0
100
500
900
α-AMYLASE ACTIVITY (mg MALTOSE/min/g × 10⁻³)
AMYLOGRAPH VISCOSITY (BU)

Fig. 9. Wheat α-amylase activity determined by the automated fluorometric method versus falling number.


α-AMYLASE ACTIVITY (mg MALTOSE/min/g × 10⁻³)
10.0
8.0
6.0
4.0
250
300
350
FALLING NUMBER (sec)

amylograph or falling number, as suggested by Hlynka (1968). This results in a straight line relationship between the methods as illustrated in Fig. 10. Excellent correlations can be seen between α-amylase determined by the present automated method and the reciprocals of both the amylograph and falling number values.

Fig. 10. Wheat α-amylase activity determined by the automated fluorometric method versus the reciprocals of the amylograph viscosity (AV) and falling number (FN).


12.0
8.0
4.0
α-AMYLASE ACTIVITY
(mg MALTOSE/min/g x 10⁻³)
A
y = 1490.7x + 3.15
r = 0.944
1.0
3.0
5.0
1/AV, BU⁻¹(x 10⁻³)
B
y = 3751.3x − 5.81
r = 0.945
2.0
4.0
1/FN, sec⁻¹(x 10⁻³)

GENERAL DISCUSSION

The fluorometric method described has overcome the difficulties normally associated with many of the standard α-amylase assays presently in use. For example, the fluorometric method employs a synthetic substrate which precludes any interference resulting from either variations in starch properties or excess β-amylase. These are pitfalls frequently associated with the viscometric and reducing power methods as well as the iodine color methods (Barnes and Blakeney, 1974). The fluorometric method also requires a very small sample size with as little as 0.84 ml needed for one analysis. A further asset of this method is incurred as a consequence of its complete automation. Many methods such as the amylograph or falling number are very time consuming whereas up to 20 samples per hour can be analyzed by the fluorometric method. Finally, this assay can be used with confidence for the determination of the low α-amylase levels associated with sound wheat. This is indicative of its high sensitivity.

Recently the Model III Turner fluorometer has been replaced by a Turner Model 430 spectrofluorometer equipped

for automated chemistry. This substitution has further improved the method by introducing a larger peak separation, a larger range for blank suppression, a more controlled wavelength selection, as well as a higher sensitivity.

PART II

CHANGES IN THE α-AMYLASE ISOENZYMES OF CANADIAN-GROWN WHEAT CULTIVARS DURING GROWTH AND MATURATION

INTRODUCTION

In recent years several researchers have studied the α-amylase present in germinating and immature wheat. These studies have been valuable in obtaining a great deal of basic understanding relating to the physical and chemical properties of this enzyme. Unfortunately, there are many facets of the wheat α-amylase system which are not yet fully understood. For example, a more complete understanding of wheat α-amylase is required with respect to: 1) its isoenzymic nature, 2) the relationship between the immature and germinating wheat enzyme systems, 3) its control in immature and germinating wheat.

In this part of the study, the changes in the α-amylase isoenzymes of Canadian-grown wheat cultivars were determined during kernel growth and maturation. As well as providing information related to cultivar differences, it was hoped that this would also provide a better understanding of the isoenzymic nature of this enzyme which would aid in reaching a more complete and integrated picture of the wheat α-amylase system.

MATERIALS AND METHODS

Wheat Samples

Ten cultivars of Canadian-grown wheat were grown in the Canada Department of Agriculture experimental plots at Glenlea, Manitoba, during the summer of 1975. The cultivars grown were: the hard red spring wheats, Manitou; Neepawa; Cypress; Park; Glenlea; and, the unlicensed cultivar RL4137; the amber durum wheats, Wakooma; and Wascana; a soft spring wheat, Pitic 62; and, the soft white spring wheat; Idaed. After flowering, excised spikes of wheat were collected at regular intervals during the period of kernel growth and maturation. The intact spikes were harvested in the morning and brought directly into the laboratory where kernels were selected for moisture and α-amylase determinations. The remaining spikes were placed in plastic bags and stored in a deep freeze for subsequent use in the α-amylase iso-enzyme study.

Enzymes

Sweet potato β-amylase (750-1000 Sigma units/mg protein) was obtained from the Sigma Chemical Co., St. Louis, Mo.

Moisture

The air oven method (AACC, 1975) was modified to use with a test sample of 25 whole kernels selected from 2 freshly harvested spikes.

α-Amylase Activity

Extraction of Whole Kernels. Immediately following a harvest, 50 kernels of each wheat cultivar were selected from at least 2 spikes and homogenized in a mortar with sand and 2 ml portions of 0.2M sodium acetate buffer, pH 5.5, containing 10^{-3}M $CaCl_2$. The extracts were centrifuged at 96,600 x g for 10 min and the clear extracts were used for activity analysis.

Determination of Activity. The fluorometric method, as previously described in Part I was employed. The 25.5-min coil in the 37°C incubation bath of the AutoAnalyzer and the 30x aperture setting on the fluorometer were utilized. In some cases it was necessary to use appropriate dilutions to bring the fluorescent intensity values within the scale of the recorder.

Blanks, prepared as described in Part I initially were determined for each sample. It was found that the blank readings were negligible and thereafter they were determined at spaced intervals to ensure that they remained negligible throughout the study.

α-Amylase Isoenzymes

Dissection Technique. Kernels used for dissection purposes were obtained from spikes harvested on 4 separate days during the period of kernel growth and maturation. The spikes which had been stored in the deep freeze following harvest were thawed first before use. For each of the 10 cultivars, 20 kernels were selected from a minimum of 2 spikes, at each of the four harvest dates, with 10 kernels being utilized for dissection purposes and 10 for a whole kernel control.

The wheat kernel was dissected into 5 separate tissues: pericarp (outer pericarp); seed coat (inner pericarp, testa, hyaline layer); endosperm and aleurone; scutellum; and, embryo (embryonic axis).

Great care was taken in these dissections to obtain only pure tissue sections and as a result it was not always possible to effect quantitative dissections. The tissue sections, during the dissection process were placed in separate containers, with 0.5 ml of 10^{-3}M $CaCl_2$ and were maintained near freezing with the aid of a cooling plate. After the kernel dissections were completed the tissue sections were frozen until required for extraction.

Extraction of Dissected Tissue Fractions. The tissue fractions were homogenized in a mortar with sand and 0.5 ml of 10^{-3}M $CaCl_2$. The extracts then were placed in centrifuge tubes and allowed to sit overnight at 8^{o}C.

The following morning the extracts were centrifuged at 96,600 x g for 10 min with the clear extracts being used for isoelectric focusing.

The whole kernel controls were extracted with 1.0 ml of 10^{-3}M $CaCl_2$ in the same manner as the tissue fractions.

Isoelectric Focusing. The LKB multiphor system was used for all the isoelectric focusing experiments. The gel mixture was prepared by mixing acrylamide (10 ml, 39.1%), N, N^1-methylene - bisacrylamide (Bis) (10 ml, 0.9%), sucrose (7.5gm in 36.6 ml of water), LKB ampholine (3.0 ml, pH 3.5-10; 0.5 ml pH 4-6; 0.5 ml pH9-11), degassing for 5 min and then mixing with ammonium persulfate (0.8 ml, 1.0%). The gel mixture then was polymerized overnight, sandwiched between 2 glass plates (125 x 260 mm). The resultant polyacrylamide gel had a monomer concentration (T) of 6.5% and a cross linking concentration (C) of 2.3%.

Enzyme samples were applied to the cathode side of the gel on small double pieces (ca. 12 x 6 mm) of Whatman No. 1 filter paper saturated with extract. A current of 35 mA was maintained through the gel until the voltage reached 600V. This voltage then was maintained while the current decreased to a constant value of ca. 10mA., after which the power remained connected for an additional 1 hr. This procedure required a running time of about 4 hr.

Detection of α-Amylase Isoenzymes. The α-amylase isoenzymes were detected using the dextrin plate technique first described by Doane (1967) and modified by MacGregor et al. (1974). This method was further modified with the use of a higher β-limit dextrin content of 0.3gm. The gels were incubated at room temperature, in a closed moist box, for ca. 1.5 hrs. Following the development of the isoenzyme bands the plates were dried and stored in plastic bags for future reference.

RESULTS

Ten cultivars of Canadian-grown wheat were planted on May 20, 1975 and were sampled at regular intervals ranging from the early stages of kernel growth to the final stages of maturation. These cultivars were chosen with the intention of representing a variety of Canadian-grown wheats with a range of quality parameters. The hard red spring (HRS) wheat cultivars Manitou, Neepawa, Cypress and Park were chosen to represent cultivars associated with excellent milling and baking quality. The high yielding HRS wheat Glenlea and Pitic 62, a soft spring wheat, represented poorer quality wheats used for feed purposes. Wakooma was chosen as an amber durum wheat with good spaghetti making quality as contrasted to Wascana which is decidedly inferior. The amber durums were also chosen on the basis of their different genetic makeup. Finally, 2 wheats were chosen for their contrasting dormancy characteristics. Idaed, a soft white spring wheat, was very susceptible to premature sprouting and had little or no dormancy while the unlicensed HRS wheat cultivar RL4137 displayed a high degree of sprout resistance and dormancy.

Changes in α-Amylase Activity Levels During Growth and Maturation

The changes in α-amylase activity levels and moisture content of ten Canadian-grown wheat cultivars are illustrated in Figs. 11 and 12. The α-amylase activity on a per kernel basis generally increased very soon after flowering. Thereafter the level of α-amylase activity declined with increasing maturity and concomitant with decreasing moisture. The HRS wheat cultivar Park appeared to be the exception to this general trend since it maintained a relatively constant activity during the first 15 - 17 days after flowering. Some variations were noted in the decline of α-amylase activity. Most notably a particularly strong resurgence in activity was observed between 23 and 28 days after flowering for all cultivars with the exception of Pitic 62.

The α-amylase activity on a per gram basis declined sharply from the level present in the early stages of growth and reached very low levels between 20 and 24 days after flowering for the majority of cultivars. The cultivars Wakooma and Idaed deviated from this trend slightly by first showing a sharp increase to a peak activity before decreasing rapidly in activity. The low activity levels which were between 1% and 10% of the initial levels were

Fig. 11. Changes in α-amylase activity levels and moisture content for 5 Canadian wheat cultivars during kernel growth and maturation. The α-amylase activity was calculated on a 14% moisture basis.

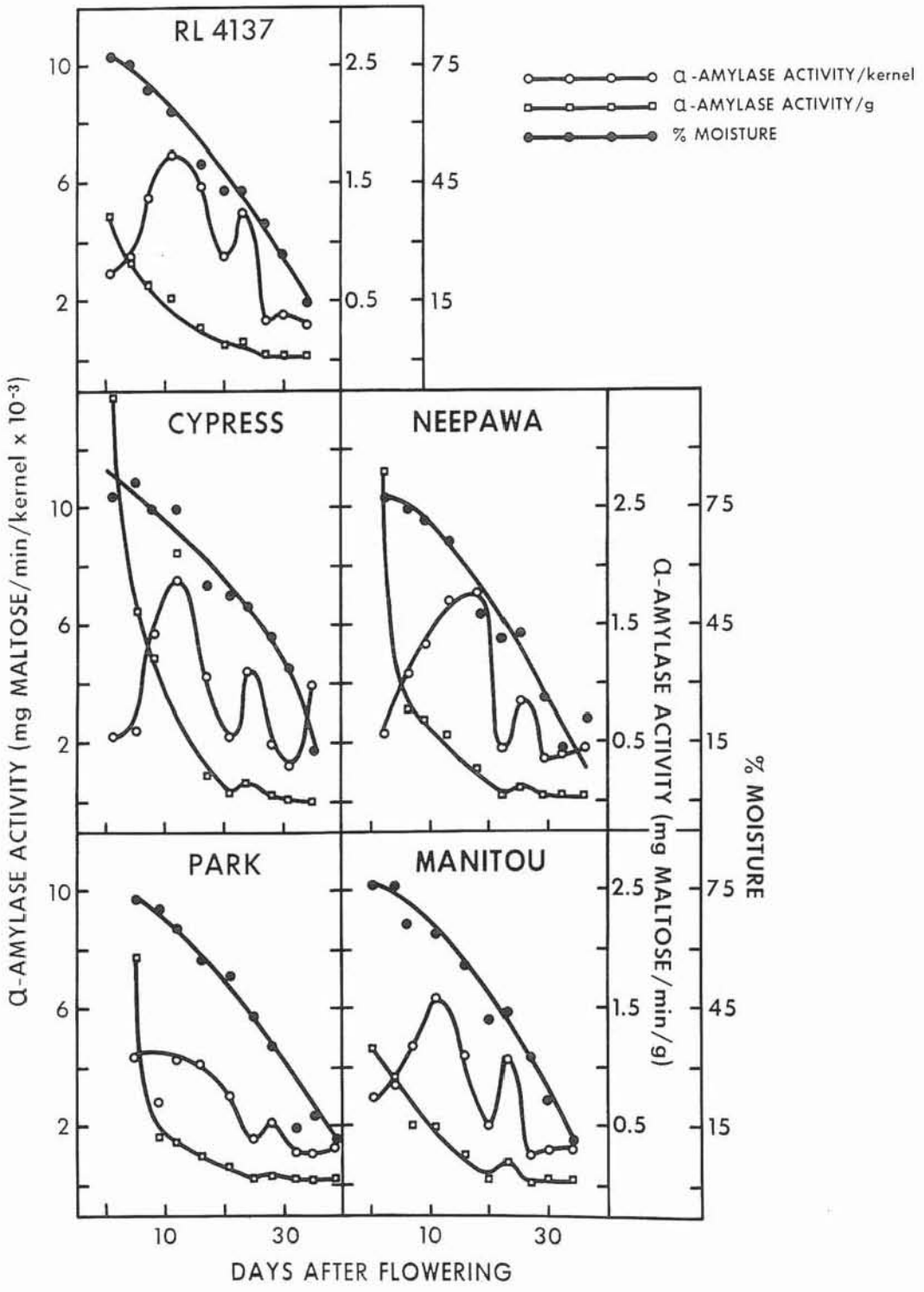
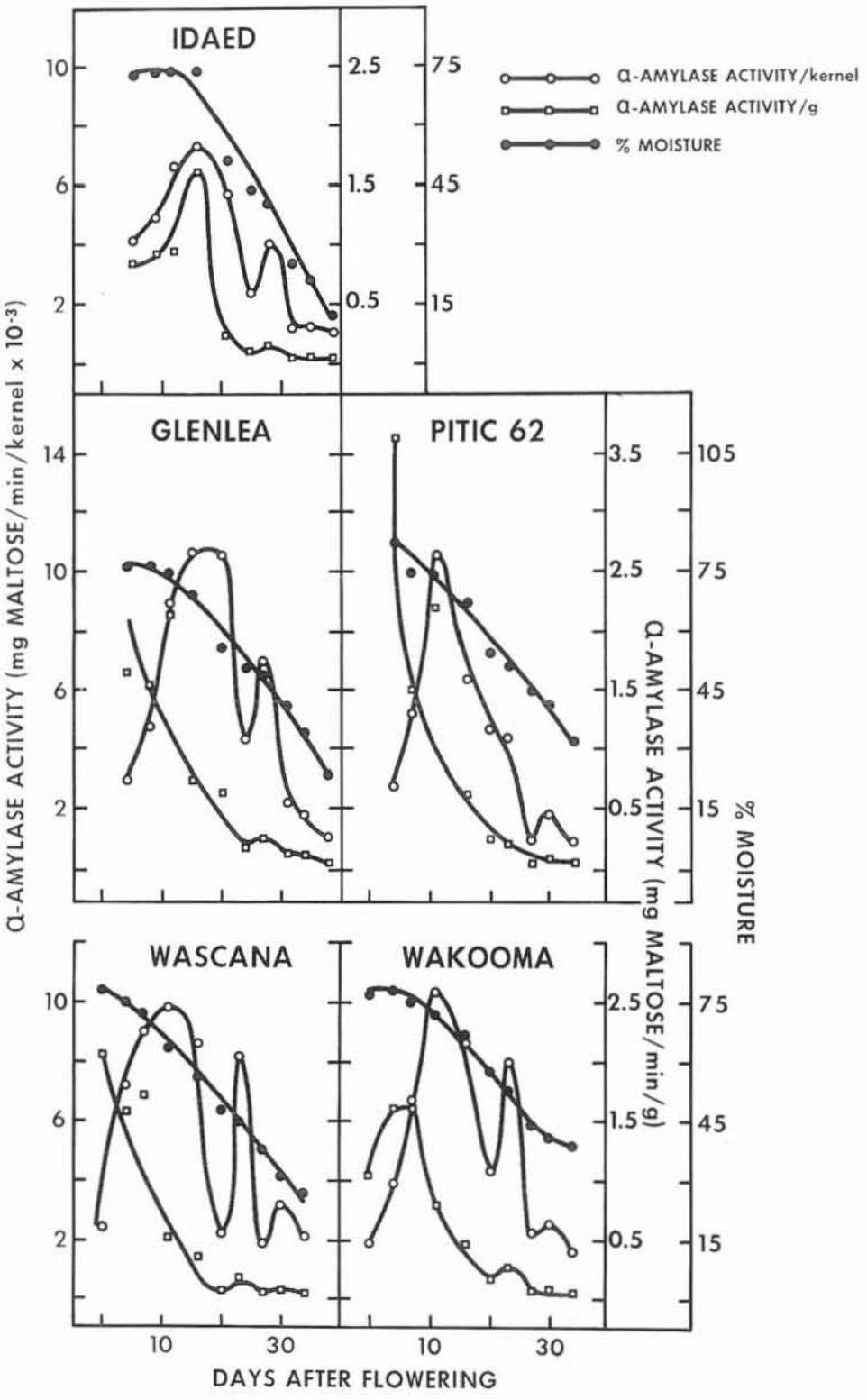

RL 4137
CYPRESS
NEEPAWA
PARK
MANITOU
α-AMYLASE ACTIVITY/kernel
α-AMYLASE ACTIVITY/g
% MOISTURE
α-AMYLASE ACTIVITY (mg MALTOSE/min/kernel x 10⁻³)
α-AMYLASE ACTIVITY (mg MALTOSE/min/g)
% MOISTURE
DAYS AFTER FLOWERING
10
6
2
2.5
1.5
0.5
75
45
15
10
30

Fig. 12. Changes in α-amylase levels and moisture content for 5 Canadian wheat cultivars during kernel growth and maturation. The α-amylase activity was calculated on a 14% moisture basis.


IDAED
GLENLEA
PITIC 62
WASCANA
WAKOOMA
α-AMYLASE ACTIVITY/kernel
α-AMYLASE ACTIVITY/g
% MOISTURE
α-AMYLASE ACTIVITY (mg MALTOSE/min/kernel x 10⁻³)
α-AMYLASE ACTIVITY (mg MALTOSE/min/g)
% MOISTURE
DAYS AFTER FLOWERING

maintained subsequently in the latter stages of maturation. Some resurgence in activity was noted also in the region between 23 and 28 days after flowering.

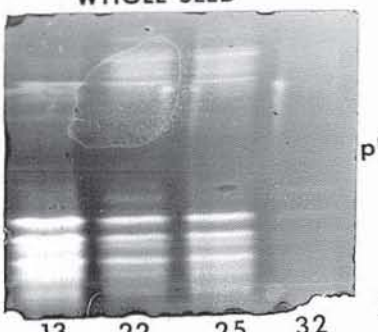
α-Amylase Isoenzymes During Growth and Maturation

The high resolution separational technique of isoelectric focusing was used to study the multiple forms of α-amylase present in 10 cultivars of Canadian-grown wheat, at 4 stages during the period of kernel growth and maturation. The first stage was selected to coincide with the maximum activity per kernel as determined in Figs. 11 and 12, while the remaining stages were chosen at intervals up to and including the final harvest date. The morphological distribution of the α-amylase isoenzymes was obtained also at each of the 4 stages studied. The resulting zymograms for the whole kernel and tissue dissections are shown in Figs. 13 - 22. As an aid for visualizing the isoenzyme patterns and to facilitate discussion and comparison of results, line drawings, as shown in Figs. 23 - 27, were prepared for 5 of the 10 cultivars studied. Line drawings were not prepared for the remaining cultivars since they would not have contributed any additional information.

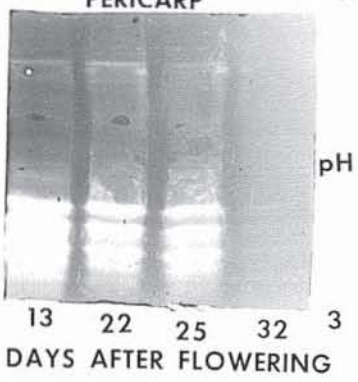
At this point a further simplification of the isoenzyme patterns would be advantageous for subsequent

Fig. 13. Zymograms on β-limit dextrin slabs of the α-amylase isoenzymes from the whole seed and anatomical parts of the HRS wheat cultivar Neepawa, at 4 stages during kernel growth and maturation.

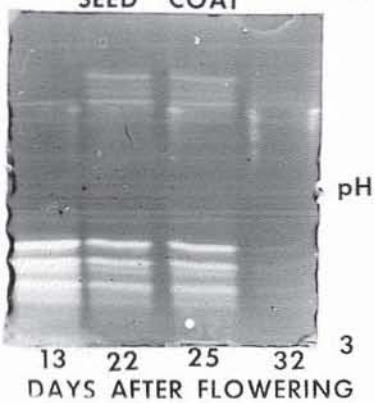
IMMATURE NEEPAWA
WHOLE SEED



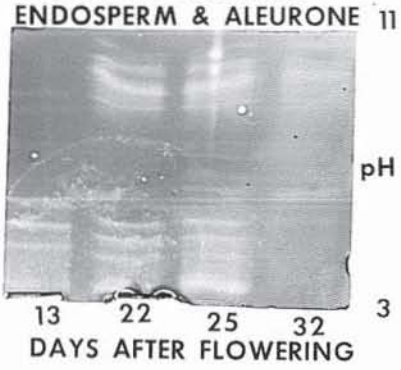
IMMATURE NEEPAWA
PERICARP



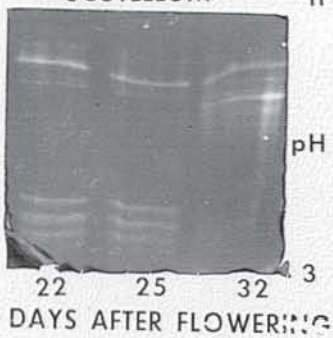
IMMATURE NEEPAWA
SEED COAT



IMMATURE NEEPAWA
ENDOSPERM & ALEURONE

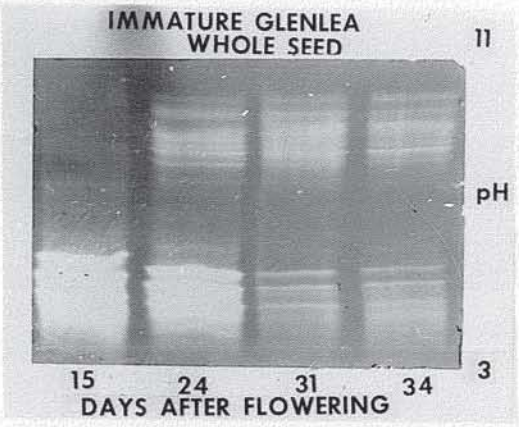


IMMATURE NEEPAWA
SCUTELLUM

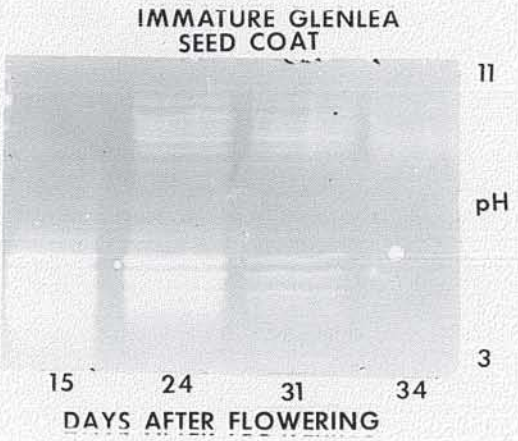


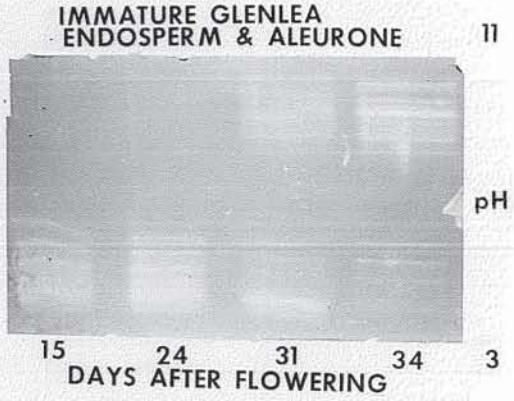
IMMATURE NEEPAWA
EMBRYO

Fig. 14. Zymograms on β-limit dextrin slabs of the α-amylase isoenzymes from the whole seed and anatomical parts of the HRS wheat cultivar Glenlea, at 4 stages during kernel growth and maturation. The embryo is not shown as α-amylase isoenzymes were not detected in this tissue.


IMMATURE GLENLEA
WHOLE SEED
11
pH
3
15
24
31
34
DAYS AFTER FLOWERING


IMMATURE GLENLEA
PERICARP
11
pH
3
15
24
31
34
DAYS AFTER FLOWERING


IMMATURE GLENLEA
SEED COAT
11
pH
3
15
24
31
34
DAYS AFTER FLOWERING


IMMATURE GLENLEA
ENDOSPERM & ALEURONE
11
pH
3
15
24
31
34
DAYS AFTER FLOWERING


IMMATURE GLENLEA
SCUTELLUM
11
pH
3
24
31
34
DAYS AFTER FLOWERING

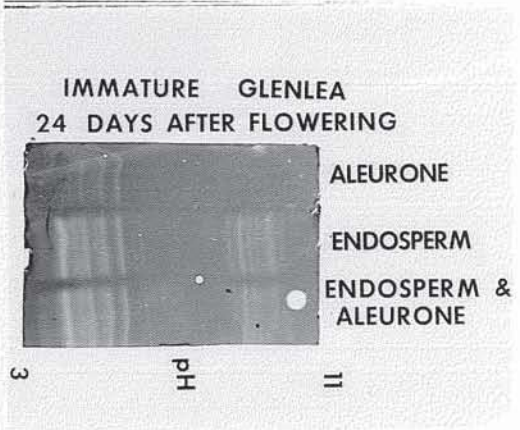
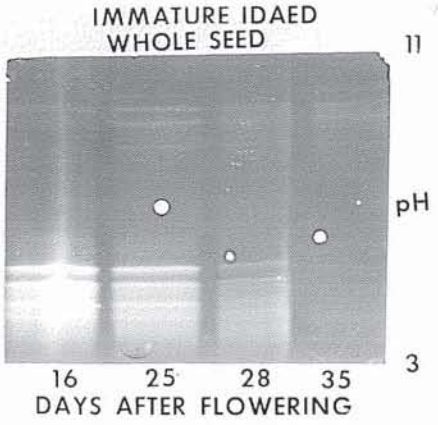
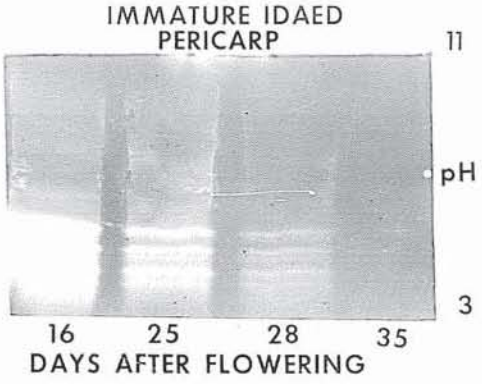
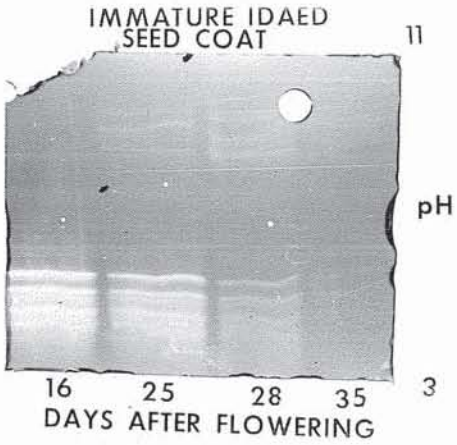
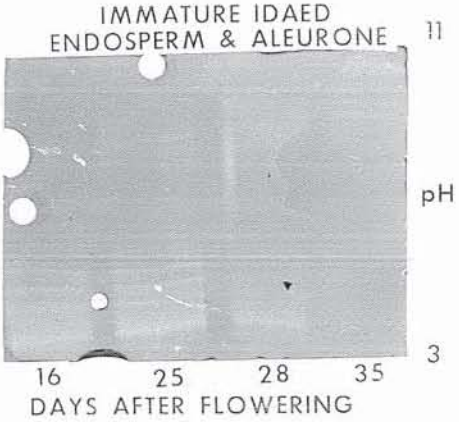

IMMATURE GLENLEA
24 DAYS AFTER FLOWERING
ALEURONE
ENDOSPERM
ENDOSPERM &
ALEURONE
3
pH
11

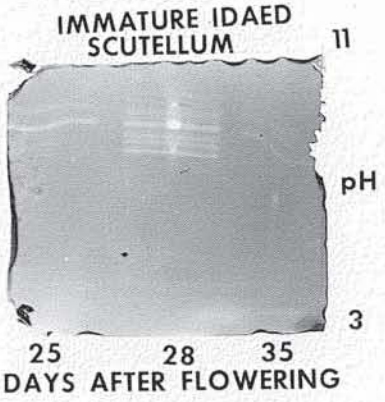
Fig. 15. Zymograms on β-limit dextrin slabs of the α-amylase isoenzymes from the whole seed and anatomical parts of the soft white spring wheat cultivar Idaed, at 4 stages during kernel growth and maturation.


IMMATURE IDAED
WHOLE SEED
11
pH
3
16 25 28 35
DAYS AFTER FLOWERING


IMMATURE IDAED
PERICARP
11
pH
3
16 25 28 35
DAYS AFTER FLOWERING


IMMATURE IDAED
SEED COAT
11
pH
3
16 25 28 35
DAYS AFTER FLOWERING


IMMATURE IDAED
ENDOSPERM & ALEURONE
11
pH
3
16 25 28 35
DAYS AFTER FLOWERING


IMMATURE IDAED
SCUTELLUM
11
pH
3
25 28 35
DAYS AFTER FLOWERING

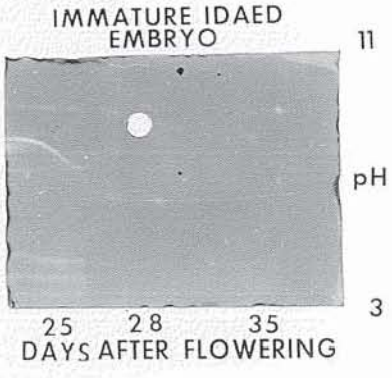
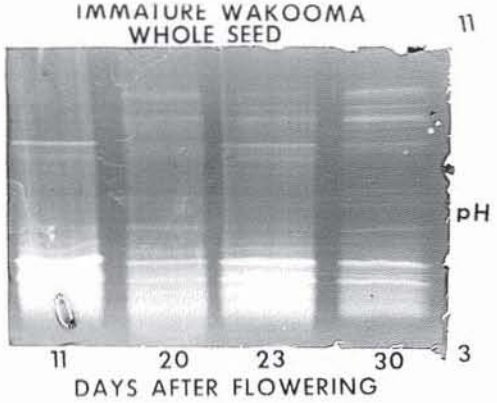
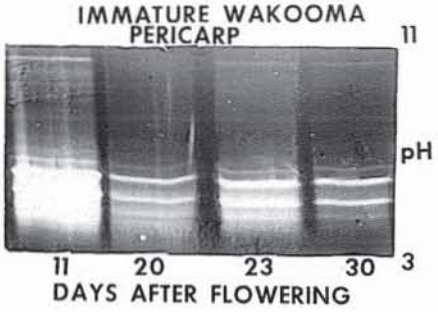
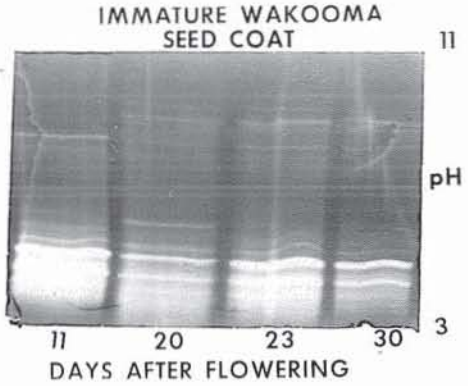
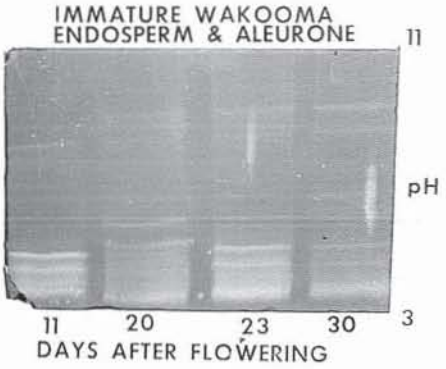

IMMATURE IDAED
EMBRYO
11
pH
3
25 28 35
DAYS AFTER FLOWERING

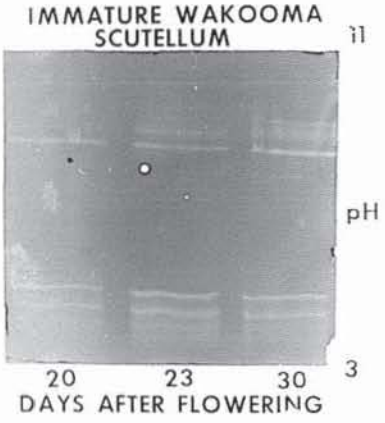
Fig. 16. Zymograms on β-limit dextrin slabs of the α-amylase isoenzymes from the whole seed and anatomical parts of the amber durum wheat cultivar Wakooma at 4 stages during kernel growth and maturation.


IMMATURE WAKOOMA
WHOLE SEED
11
pH
3
11 20 23 30
DAYS AFTER FLOWERING


IMMATURE WAKOOMA
PERICARP
11
pH
3
11 20 23 30
DAYS AFTER FLOWERING


IMMATURE WAKOOMA
SEED COAT
11
pH
3
11 20 23 30
DAYS AFTER FLOWERING


IMMATURE WAKOOMA
ENDOSPERM & ALEURONE
11
pH
3
11 20 23 30
DAYS AFTER FLOWERING


IMMATURE WAKOOMA
SCUTELLUM
11
pH
3
20 23 30
DAYS AFTER FLOWERING

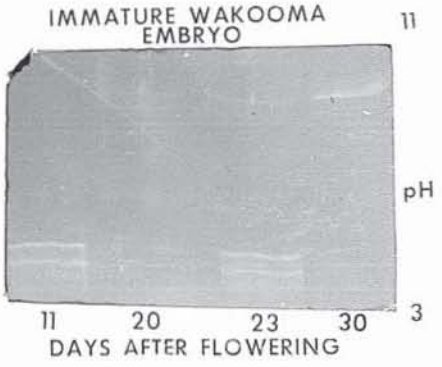
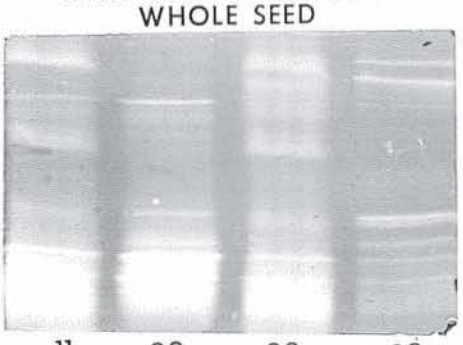
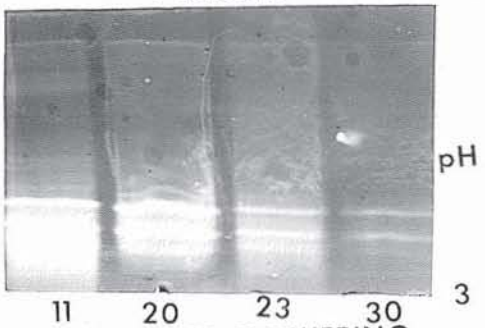

IMMATURE WAKOOMA
EMBRYO
11
pH
3
11 20 23 30
DAYS AFTER FLOWERING

Fig. 17. Zymograms on β-limit dextrin slabs of the α-amylase isoenzymes from the whole seed and anatomical parts of the amber durum wheat cultivar Wascana, at 4 stages during kernel growth and maturation.

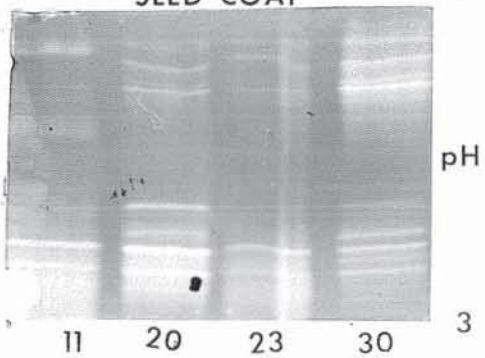
IMMATURE WASCANA
WHOLE SEED



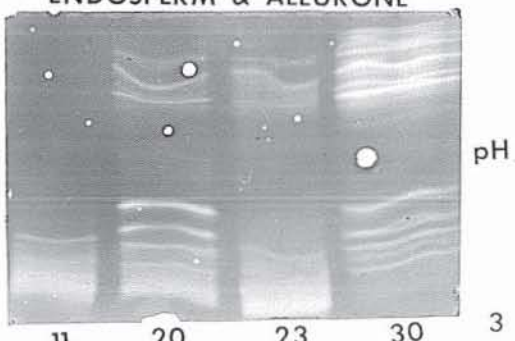
IMMATURE WASCANA
PERICARP



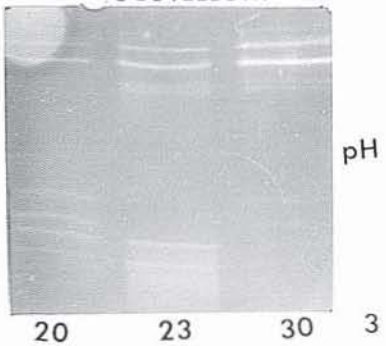
IMMATURE WASCANA
SEED COAT



IMMATURE WASCANA
ENDOSPERM & ALEURONE



IMMATURE WASCANA
SCUTELLUM



IMMATURE WASCANA
EMBRYO

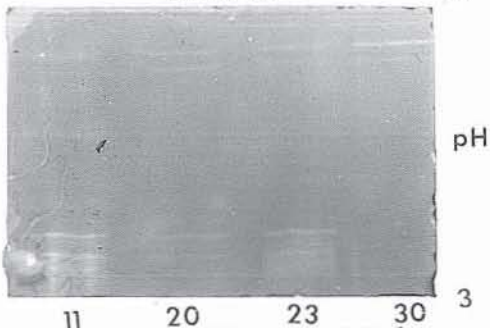
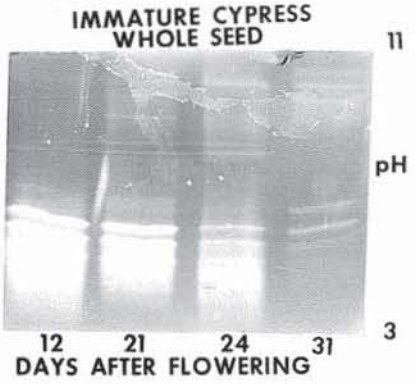
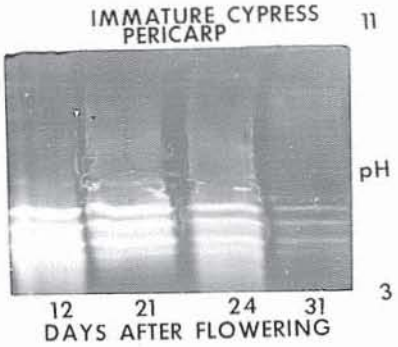
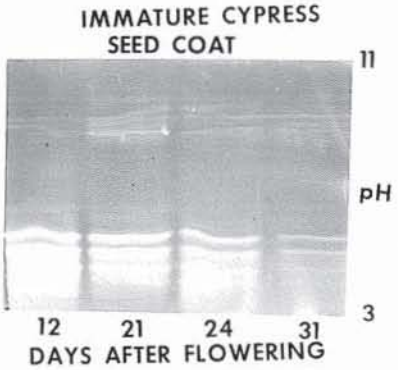
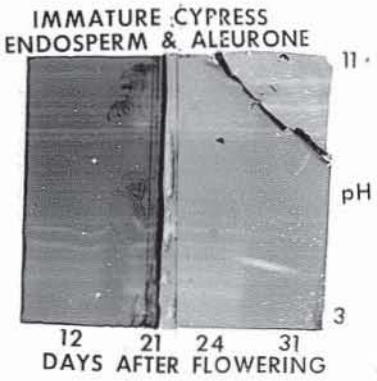


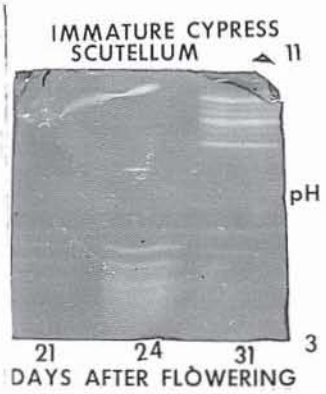
Fig. 18. Zymograms on β-limit dextrin slabs of the α-amylase isoenzymes from the whole seed and anatomical parts of the HRS wheat cultivar Cypress, at 4 stages during kernel growth and maturation.


IMMATURE CYPRESS
WHOLE SEED
11
pH
3
12 21 24 31
DAYS AFTER FLOWERING


IMMATURE CYPRESS
PERICARP
11
pH
3
12 21 24 31
DAYS AFTER FLOWERING


IMMATURE CYPRESS
SEED COAT
11
pH
3
12 21 24 31
DAYS AFTER FLOWERING


IMMATURE CYPRESS
ENDOSPERM & ALEURONE
11
pH
3
12 21 24 31
DAYS AFTER FLOWERING


IMMATURE CYPRESS
SCUTELLUM
11
pH
3
21 24 31
DAYS AFTER FLOWERING

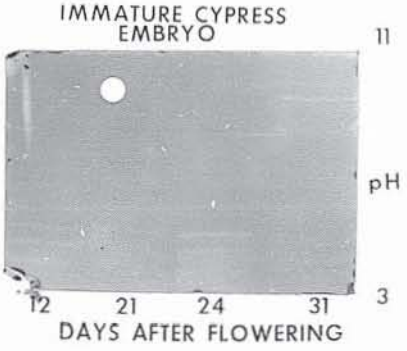
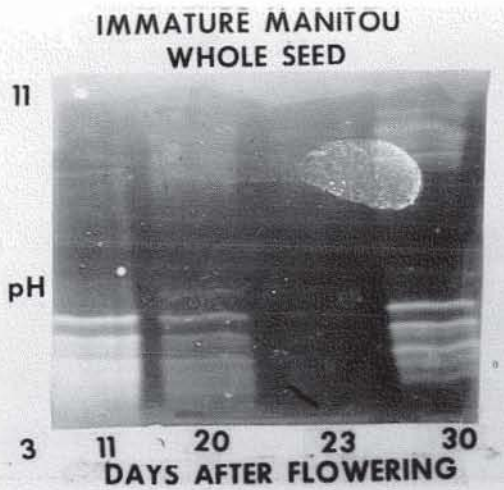
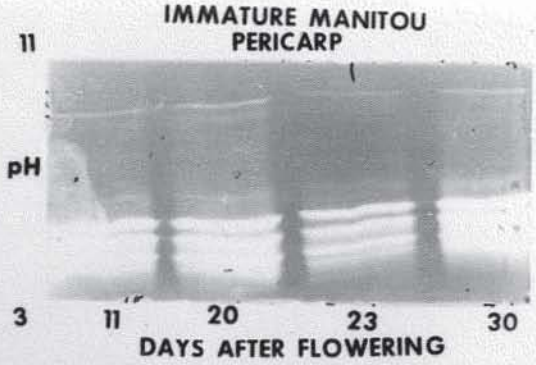
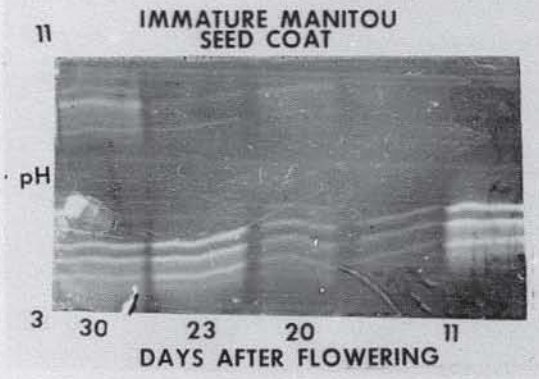

IMMATURE CYPRESS
EMBRYO
11
pH
3
12 21 24 31
DAYS AFTER FLOWERING

Fig. 19. Zymograms on β-limit dextrin slabs of the α-amylase isoenzymes from the whole seed and anatomical parts of the HRS wheat cultivar Manitou, at 4 stages during kernel growth and maturation. The embryo is not shown as α-amylase isoenzymes were not detected in this tissue.


IMMATURE MANITOU
WHOLE SEED
11
pH
3
11
20
23
30
DAYS AFTER FLOWERING


IMMATURE MANITOU
PERICARP
11
pH
3
11
20
23
30
DAYS AFTER FLOWERING


IMMATURE MANITOU
SEED COAT
11
pH
3
30
23
20
11
DAYS AFTER FLOWERING


IMMATURE MANITOU
11 ENDOSPERM & ALEURONE
pH
3
11
20
23
30
DAYS AFTER FLOWERING

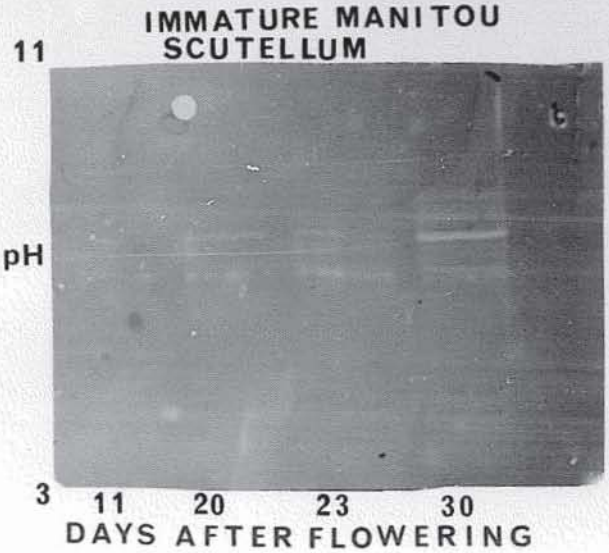
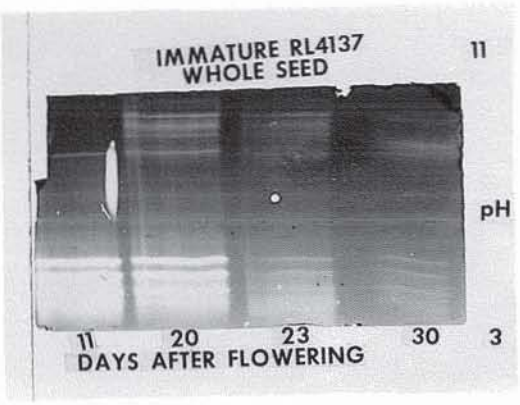
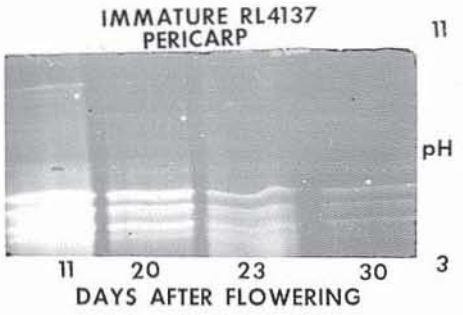
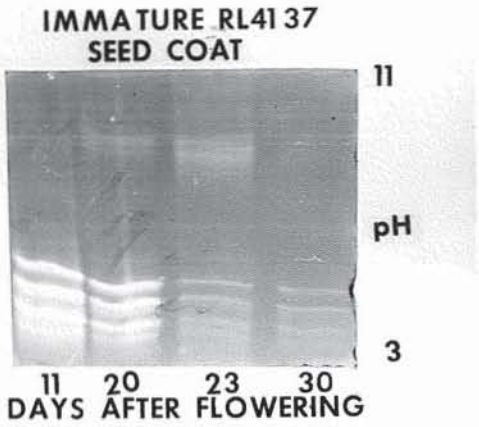
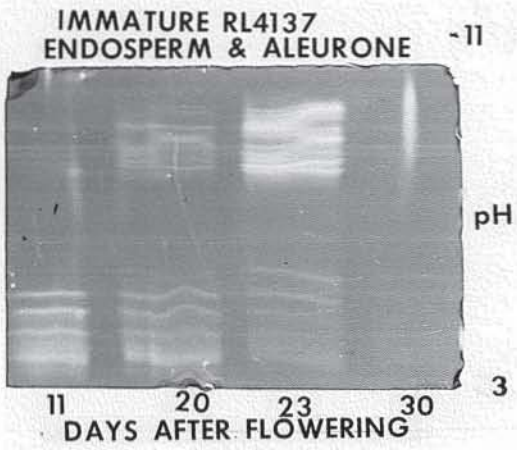

IMMATURE MANITOU
SCUTELLUM
11
pH
3
11
20
23
30
DAYS AFTER FLOWERING

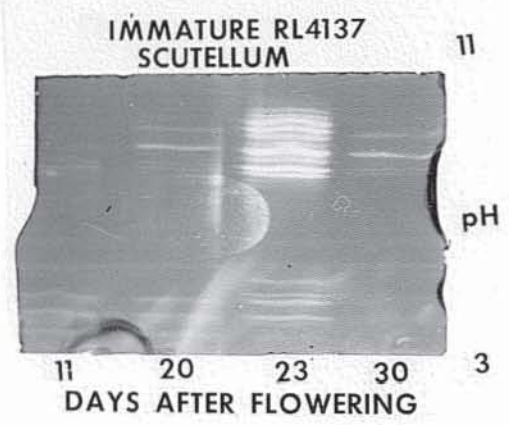
Fig. 20. Zymograms on β-limit dextrin slabs of the α-amylase isoenzymes from the whole seed and anatomical parts of the unlicensed HRS wheat cultivar RL4137, at 4 stages during kernel growth and maturation.


IMMATURE RL4137
WHOLE SEED
11
pH
3
11 20 23 30
DAYS AFTER FLOWERING


IMMATURE RL4137
PERICARP
11
pH
3
11 20 23 30
DAYS AFTER FLOWERING


IMMATURE RL4137
SEED COAT
11
pH
3
11 20 23 30
DAYS AFTER FLOWERING


IMMATURE RL4137
ENDOSPERM & ALEURONE
11
pH
3
11 20 23 30
DAYS AFTER FLOWERING


IMMATURE RL4137
SCUTELLUM
11
pH
3
11 20 23 30
DAYS AFTER FLOWERING

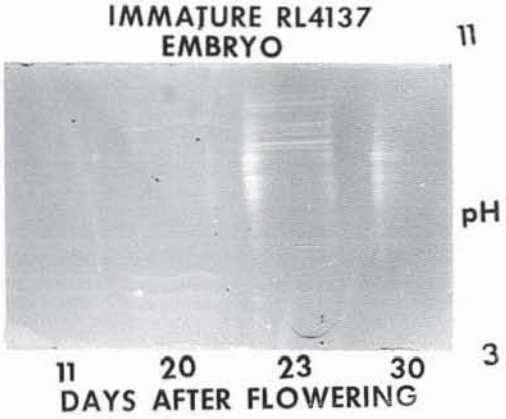
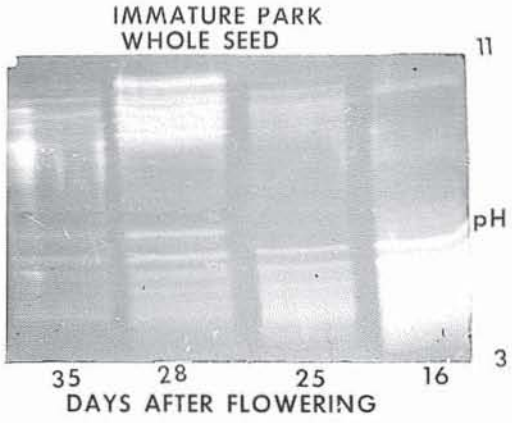
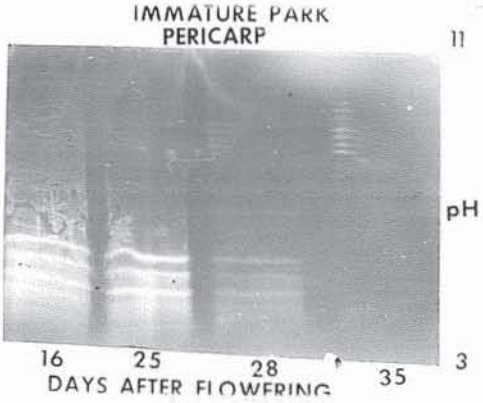
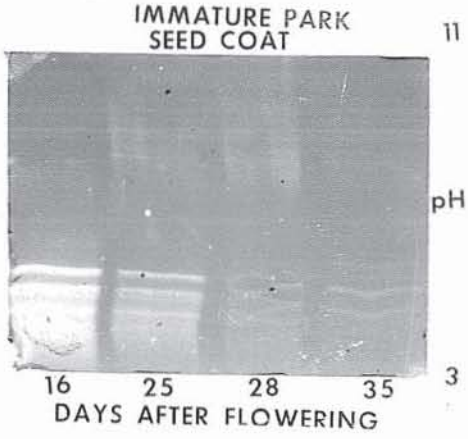
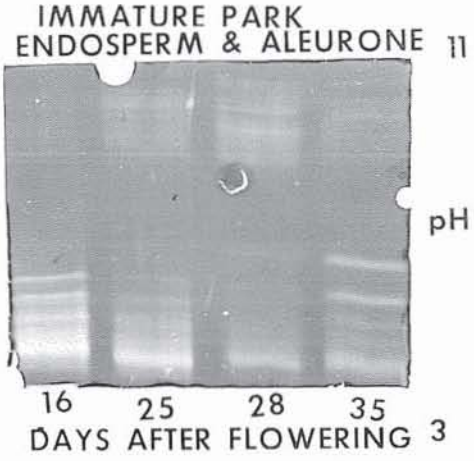

IMMATURE RL4137
EMBRYO
11
pH
3
11 20 23 30
DAYS AFTER FLOWERING

Fig. 21. Zymograms on β-limit dextrin slabs of the α-amylase isoenzymes from the whole seed and anatomical parts of the HRS wheat cultivar Park, at 4 stages during kernel growth and maturation.


IMMATURE PARK
WHOLE SEED
11
pH
3
35 28 25 16
DAYS AFTER FLOWERING


IMMATURE PARK
PERICARP
11
pH
3
16 25 28 35
DAYS AFTER FLOWERING


IMMATURE PARK
SEED COAT
11
pH
3
16 25 28 35
DAYS AFTER FLOWERING


IMMATURE PARK
ENDOSPERM & ALEURONE
11
pH
3
16 25 28 35
DAYS AFTER FLOWERING


IMMATURE PARK
SCUTELLUM
11
pH
3
25 28 35
DAYS AFTER FLOWERING

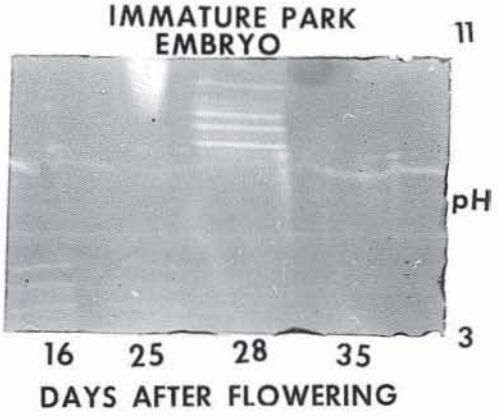

IMMATURE PARK
EMBRYO
11
pH
3
16 25 28 35
DAYS AFTER FLOWERING

Fig. 22 Zymograms on β-limit dextrin slabs of the α-amylase isoenzymes from the whole seed and anatomical parts of the soft spring wheat cultivar Pitic 62, at 4 stages during kernel growth and maturation.

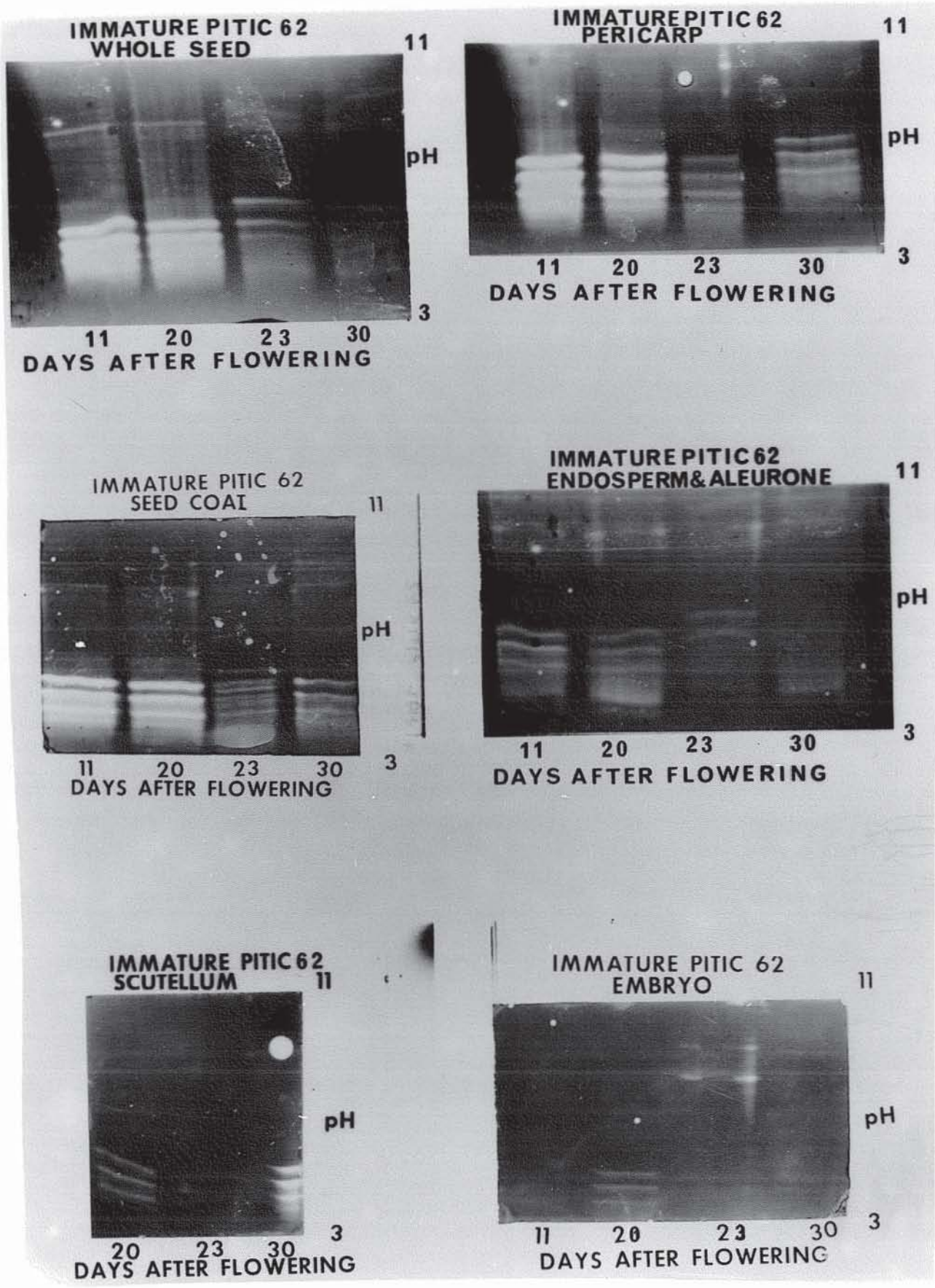
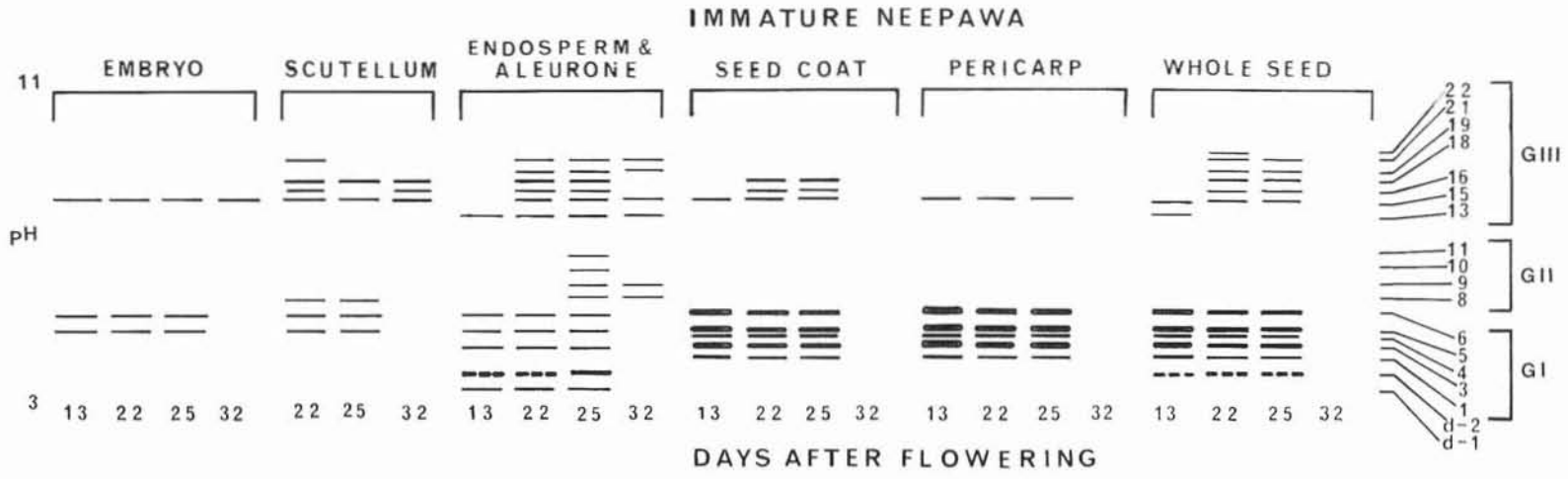

IMMATURE PITIC 62
WHOLE SEED
11
pH
3
11 20 23 30
DAYS AFTER FLOWERING
IMMATURE PITIC 62
PERICARP
11
pH
3
11 20 23 30
DAYS AFTER FLOWERING
IMMATURE PITIC 62
SEED COAI
11
pH
3
11 20 23 30
DAYS AFTER FLOWERING
IMMATURE PITIC 62
ENDOSPERM & ALEURONE
11
pH
3
11 20 23 30
DAYS AFTER FLOWERING
IMMATURE PITIC 62
SCUTELLUM
11
pH
3
20 23 30
DAYS AFTER FLOWERING
IMMATURE PITIC 62
EMBRYO
11
pH
3
11 20 23 30
DAYS AFTER FLOWERING

Fig. 23. Line drawing of the α-amylase isoenzymes from the whole seed and anatomical parts of the HRS wheat cultivar Neepawa, at 4 stages during kernel growth and maturation.
D-1 and d-2 denote debranching enzymes.

Fig. 24. Line drawing of the α-amylase isoenzymes from the whole seed and anatomical parts of the HRS wheat cultivar Glenlea, at 4 stages during kernel growth and maturation.
GI isoenzymes, 1-6; GII isoenzymes, 7 and 8; GIII isoenzymes 13-22.
D-1 and d-2 denote debranching enzymes.


IMMATURE NEEPAWA
EMBRYO
SCUTELLUM
ENDOSPERM & ALEURONE
SEED COAT
PERICARP
WHOLE SEED
11
pH
3
13 22 25 32 22 25 32 13 22 25 32 13 22 25 32 13 22 25 32 13 22 25 32
DAYS AFTER FLOWERING
22 21 19 18 16 15 13
11 10 9 8
6 5 4 3 1 d-2 d-1
GIII
GII
GI

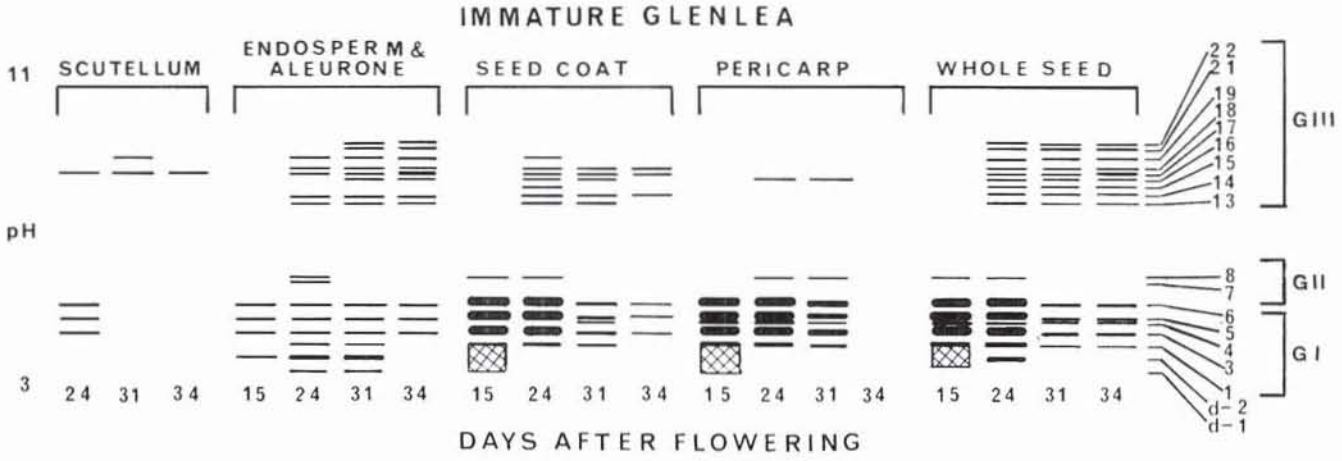

IMMATURE GLENLEA
SCUTELLUM
ENDOSPERM & ALEURONE
SEED COAT
PERICARP
WHOLE SEED
11
pH
3
24 31 34 15 24 31 34 15 24 31 34 15 24 31 34 15 24 31 34
DAYS AFTER FLOWERING
22 21 19 18 17 16 15 14 13
8 7 6 5 4 3 1 d-2 d-1
GIII
GII
GI

Fig. 25. Line drawing of the α-amylase isoenzymes from the whole seed and anatomical parts of the soft white spring wheat cultivar Idaed, at 4 stages during kernel growth and maturation.

D-1 and d-2 denote debranching enzymes.

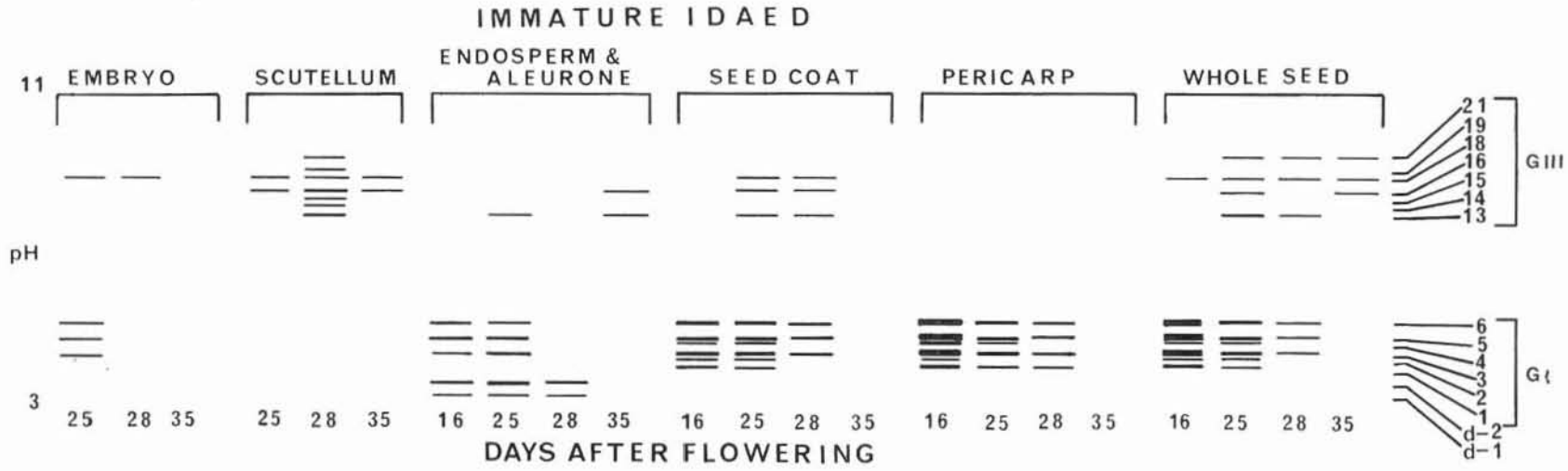
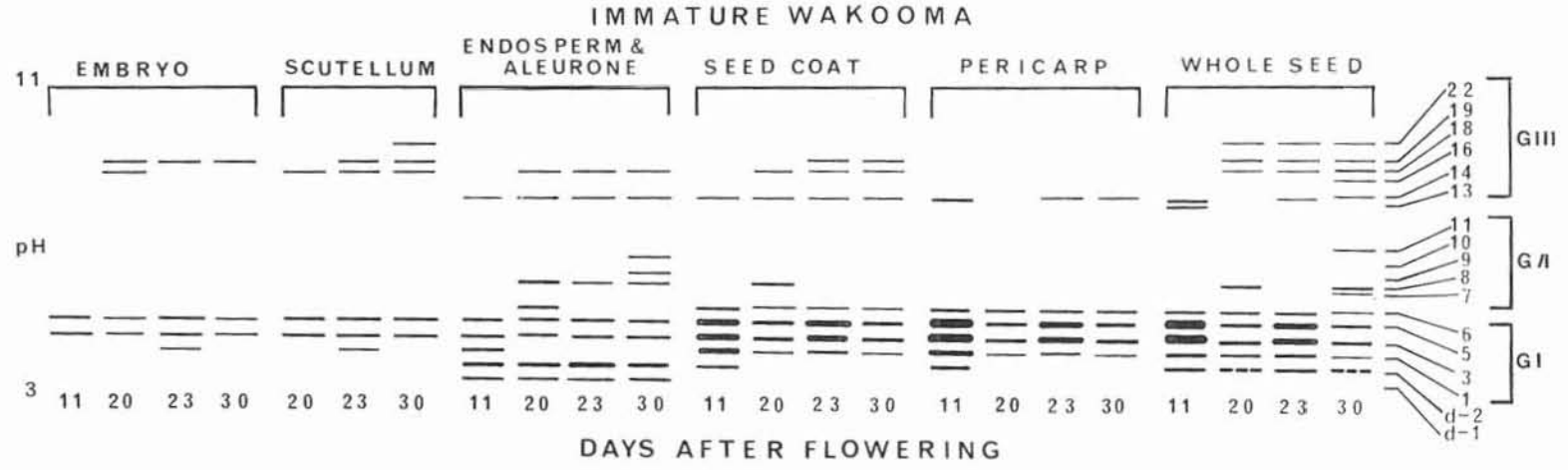
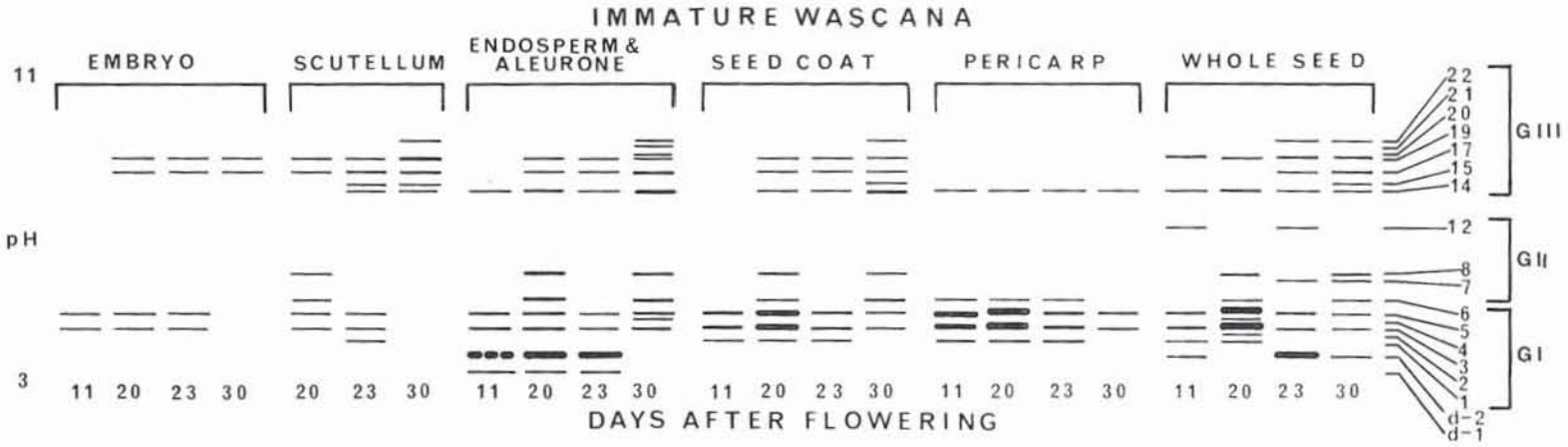

IMMATURE IDAED
11
pH
3
EMBRYO
SCUTELLUM
ENDOSPERM & ALEURONE
SEED COAT
PERICARP
WHOLE SEED
25 28 35
25 28 35
16 25 28 35
16 25 28 35
16 25 28 35
16 25 28 35
DAYS AFTER FLOWERING
21
19
18
16
15
14
13
GIII
6
5
4
3
2
1
d-2
d-1
G

Fig. 26. Line drawing of the α-amylase isoenzymes from the whole seed and anatomical parts of the amber durum wheat cultivar Wakooma, at 4 stages during kernel growth and maturation.
D-1 and d-2 denote debranching enzymes.

Fig. 27. Line drawing of the α-amylase isoenzymes from the whole seed and anatomical parts of the amber durum wheat cultivar Wascana, at 4 stages during kernel growth and maturation.
D-1 and d-2 denote debranching enzymes.


IMMATURE WAKOOMA
11
EMBRYO
SCUTELLUM
ENDOSPERM & ALEURONE
SEED COAT
PERICARP
WHOLE SEED
pH
3
11 20 23 30 20 23 30 11 20 23 30 11 20 23 30 11 20 23 30 11 20 23 30
DAYS AFTER FLOWERING
22 19 18 16 14 13
GIII
11 10 9 8 7
GII
6 5 3 1 d-2 d-1
GI


IMMATURE WASCANA
11
EMBRYO
SCUTELLUM
ENDOSPERM & ALEURONE
SEED COAT
PERICARP
WHOLE SEED
pH
3
11 20 23 30 20 23 30 11 20 23 30 11 20 23 30 11 20 23 30 11 20 23 30
DAYS AFTER FLOWERING
22 21 20 19 17 15 14
GIII
12 8 7
GII
6 5 4 3 2 1 d-2 d-1
GI

discussion. An inspection of the zymograms (Figs. 13-22) revealed the presence of 2 discrete groups of α-amylase isoenzymes in the immature wheat kernel. As a consequence, those isoenzymes clustered in the low pH region of an isofocusing gel were labelled as Group I (GI) isoenzymes, while those clustered in the higher pH regions were labelled Group III (GIII) isoenzymes. The isoenzymes situated between GI and GIII were labelled Group II (GII) isoenzymes. These 3 isoenzyme groups shown on the line drawings include isoenzymes 1-6 in GI, isoenzymes 7-12 in GII and isoenzymes 13-22 in GIII.

Before beginning an analysis of the results it should be realized also that some anomalies in isoenzyme activities or patterns, as indicated on the zymograms, may be due to variations in the flowering date within a cultivar. One flowering date was assessed for the general population of each wheat cultivar. Consequently, some error in the flowering date was incurred unavoidably for those spikes ahead or behind the average growth rate. This would lead to a spread in the maturity of excised spikes of wheat harvested on the same day. Ultimately this sampling error could result in the anomalous variations in isoenzyme activities and patterns.

The α-Amylase Isoenzymes of the Whole Seed

A detailed analysis of the whole seed zymograms indicated that in all cultivars α-amylase activity was present

early in kernel growth primarily in the form of GI isoenzymes. The durum wheat cultivars Wakooma and Wascana had 2 principal GI isoenzymes (Figs. 26 and 27) numbered 3 and 5, while the remaining cultivars had 3 principal isoenzymes 3, 5 and 6 as illustrated for the cultivars Neepawa, Glenlea and Idaed in Figs. 23-25. These major isoenzymes contained the main portion of the α-amylase activity in the whole kernel during the early stages of kernel growth and maturation. It should be noted that although isoenzyme 6 was present in the durum wheats Wakooma and Wascana (Figs. 26 and 27) it contributed little activity as compared to isoenzymes 3 and 5 or to the isoenzyme 6 present in the other 8 cultivars.

The intensity of the major GI isoenzymes relative to each other remained the same during each growth stage and for the majority of cultivars their activity decreased considerably in the later stages of maturation. The cultivars Glenlea, Wascana and Wakooma retained higher GI isoenzyme levels in the final harvest stage but this could be attributed to their elevated moisture still remaining at this stage.

Three minor GI isoenzymes 1, 2 and 4 were present in addition to the major 3, 5 and 6 isoenzymes. Isoenzyme 1 was present in all of the wheat cultivars studied as contrasted to isoenzymes 2 or 4 which were absent in a number

of cultivars as illustrated in Figs. 23-27. In the early stages of kernel growth these minor isoenzymes contributed substantial activity to the whole kernel but this activity was quickly lost during maturation.

A total of 6 GII isoenzymes were present in the 10 wheat cultivars studied. The complete complement of 6 GII isoenzymes was not present together in any of the 10 cultivars. Those which were detected contributed little activity to the whole kernel at any stage during kernel growth and maturation.

A surprising feature of the whole seed α-amylase isoenzyme composition was the presence of GIII isoenzymes in all 10 wheat cultivars. A total of 10 GIII isoenzymes were detectable in the 10 cultivars with up to a maximum of 9 being present in a single cultivar, as was the case with the HRS wheat Glenlea (Fig. 24). There was little or no indication of GIII isoenzyme activity in the early stages of growth. However, as maturation progressed these isoenzymes increased in activity until the later stages of maturation. At this stage there was a general decrease in activity for all of the α-amylase isoenzymes coincident with a decrease in moisture to very low levels. As was the case with the GI isoenzymes, the cultivars Glenlea, Wakooma and Wascana retained elevated GIII activity levels in the final stages of maturation, which was probably a direct result of the retention of high moisture levels.

An inspection of the zymograms (Figs. 13-22) revealed apparent cultivar differences in the GIII activity levels. Unfortunately, due to the qualitative nature of the zymograms it was not feasible to procure an accurate assessment of those differences.

It is interesting to note that the GIII isoenzyme levels were increasing during maturation even though the total kernel activity (Figs. 11 and 12) exhibited a general decline during this period. This was a reflection of the overshadowing GI isoenzyme activity levels. Although the GIII isoenzyme activity was low and never reached the GI activity levels found early in kernel growth, in most cultivars (Figs. 13-22) the GIII activity in the final stages of maturation was comparable to the GI activity in the same stage.

α-Amylase Isoenzyme Distribution in the Immature Whole Seed

In all cultivars the bulk of the α-amylase activity in the early stages of growth and maturation was present in the outer pericarp. The pericarp activity was attributed basically to the major GI isoenzymes 3, 5 and 6, although the minor isoenzymes 1, 2 and 4 were present to a lesser extent. The GII isoenzymes were either completely absent or present with little activity. Only 1 GIII isoenzyme was present in the pericarp in the form of one of the 3 isoenzymes 14, 15 or 16.

The GI activity decreased rapidly as maturation continued and little or no activity remained in the final stages. The GIII isoenzyme activity, although slight, remained basically constant until the final stages of maturation where it was lost.

In the early stages of kernel growth and maturation the seed coat also contained appreciable GI α-amylase activity. As in the pericarp, the major isoenzymes 3, 5 and 6 contained most of the activity with the minor isoenzymes 1, 2 and 4 contributing lesser amounts. With the exception of the durum wheat cultivars (Figs. 26 and 27) there was little evidence of GII isoenzymes. Surprisingly, the seed coat also contained a considerable number of GIII isoenzymes. The activity of the GI isoenzymes rapidly decreased with maturation whereas the GIII isoenzymes generally increased in number and retained a reasonably constant activity until the final stages of maturation, when much of this was lost. The number of GIII isoenzymes present in the seed coat varied with the cultivar.

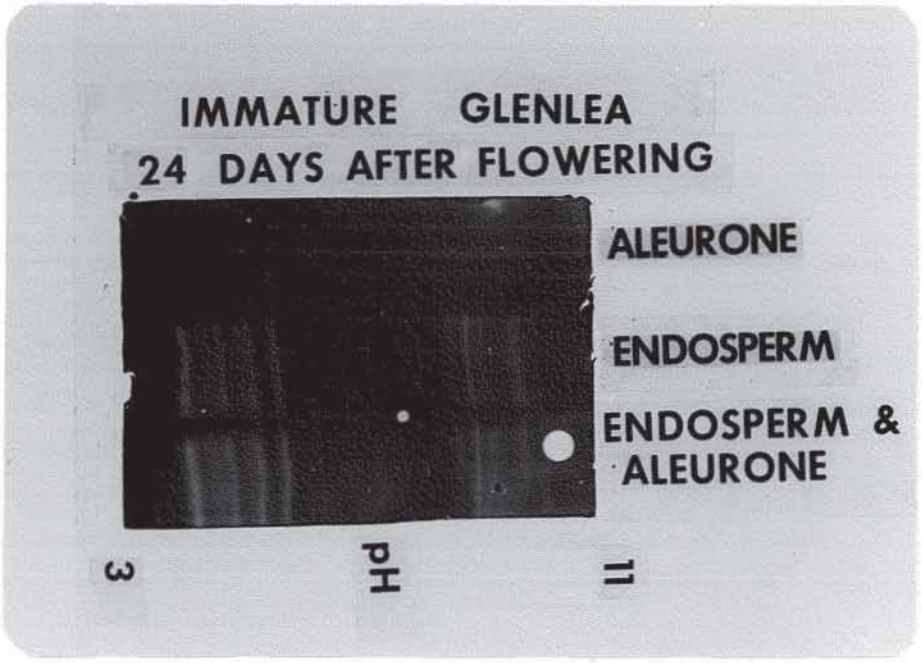
The combined endosperm and aleurone also contained detectable α-amylase activity represented by GI, GII and GIII isoenzymes. The major GI isoenzymes 3, 5 and 6, although much weaker than in the pericarp or seed coat, were present in all cultivars while the minor GI iso-

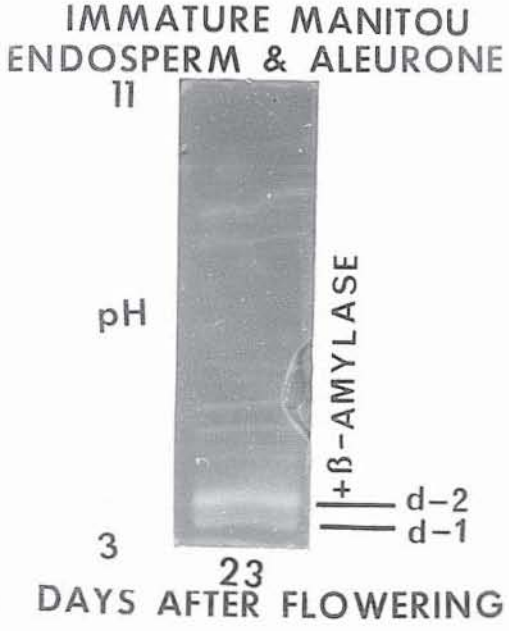
enzymes 1, 2 and 4 generally were absent. As in the other tissues the GI isoenzyme activity decreased to low or undetectable levels with maturation. The GII isoenzymes basically were found in the combined endosperm and aleurone although these isoenzymes contributed little activity. Similarly, the majority of the GIII isoenzyme activity resided in this tissue. As in the seed coat the GIII isoenzymes generally increased in number and activity with maturation until the final stages of maturation when the activity decreased. As noted previously, the cultivars Glenlea, Wakooma and Wascana (Figs. 24, 26, 27) retained higher GIII isoenzyme activity in the last stages of maturation. The cultivar Idaed also showed some deviation from this trend since, as noted in Fig. 25, the highest levels of GIII isoenzyme activity appeared in the scutellum.

Two diffuse bands of activity also were observed below the GI isoenzymes in the zymograms (Figs. 13-22). These 2 bands of activity designated d-2 and d-1, as noted in Figs. 23-27 , were present in all cultivars. The appearance of these bands was not typical of α-amylase isoenzymes which appeared as clear sharp bands. D-2 appeared as a diffuse blue tinged clear band while d-1 was a diffuse completely blue band (Fig. 28). It was very difficult to see d-1 since it showed blue on a purple background. These 2 bands increased in intensity as maturation proceeded until the final stages whereupon the activity was lost. In an effort

Fig. 28. Zymogram on β-limit dextrin slab of the α-amylase isoenzymes and debranching enzymes from: the endosperm and aleurone; endosperm; aleurone.

Fig. 29. Zymogram on β-limit dextrin slab illustrating the debranching enzymes after the addition of β-amylase.


IMMATURE GLENLEA
24 DAYS AFTER FLOWERING
ALEURONE
ENDOSPERM
ENDOSPERM &
ALEURONE
3
pH
11


IMMATURE MANITOU
ENDOSPERM & ALEURONE
11
pH
3
+ß-AMYLASE
d-2
d-1
23
DAYS AFTER FLOWERING

to more readily visualize these bands β-amylase (5mg) was incorporated into a dextrin plate. This transformed d-2 and d-1 into clear bands (Fig. 29) which were easily visible. Subsequent studies by Kruger and Marchylo (unpublished results) showed that these bands of activity were indicative of debranching enzyme activity.

The endosperm and aleurone were not separated in the dissection study so in order to more specifically locate the GI, GIII and debranching activity these tissues were separated for 1 wheat cultivar. The endosperm and aleurone of the immature HRS wheat cultivar Glenlea (harvested 24 days after flowering) were separated carefully and the α-amylase isoenzyme patterns were obtained. The α-amylase isoenzyme patterns shown on the zymogram in Fig. 28 clearly indicated that the endosperm contained the major portion of the GIII isoenzyme as well as the debranching enzymes and GI isoenzymes. The aleurone contained only small amounts of GI isoenzyme activity.

The scutellum generally contained only small amounts of α-amylase isoenzyme activity during growth and maturation. In the early stages of growth it was impossible to separate the scutellum and embryo and as a result only the last 3 stages were studied with the separated scutellum. GI isoenzyme activity was very weak or nonexistent as with Idaed (Fig. 25), even in the early stages of growth. Generally in the final stages of maturation this activity had disappeared.

GII isoenzymes were completely absent but all the cultivars contained discernable GIII isoenzyme activity. This activity generally increased slightly as growth and maturation proceeded although the cultivars RL4137 (Fig. 20) and Idaed (Fig. 25) showed very intense GIII isoenzyme activity in the intermediate stage during maturation.

The embryo contained either traces of α-amylase activity or no activity as was the case with Glenlea (Fig. 24). Those cultivars which did display some activity contained GI and GIII isoenzymes but in such trace amounts that it was all but impossible to follow any changes during growth and maturation.

DISCUSSION

Ten cultivars of Canadian-grown wheat essentially exhibited the same changes in α-amylase activity levels during kernel growth and maturation. Some deviation was noted for the HRS wheat cultivar Park, which showed a fairly constant activity level in the early stages of kernel growth, instead of the typical rapid increase to a maximum. Minor cultivar differences were evident in the maximum activities achieved on a per kernel basis. Glenlea, Pitic 62, Wascana and Wakooma attained slightly higher maximum levels which could be attributed to the large kernel size of these cultivars. Some differences also were observed in the date at which the maximum activity was reached and in the incidence and levels of activity variations. In the early stages of kernel growth the cultivars Idaed and Wakooma displayed minor differences in their activity profiles on a per gram basis. The activity of the 2 cultivars initially was at a low level and subsequently increased to a maximum, whereas the other eight cultivars exhibited a rapid decline in activity from an initial maximum level. This would imply that in the case of Idaed and Wakooma the synthesis of α-amylase in the early stages of growth was progressing at a rate greater

than the increase in kernel weight. Minor differences in initial activities per gram were present for the cultivar Pitic 62, Cypress and Neepawa which had appreciably higher initial activities. These elevated activities very quickly declined to lower levels comparable to the other cultivars.

These results were in general agreement with similar studies reported in the literature. Sandstedt and Beckford (1946) showed that the α-amylase in wheat increased during the first week of growth followed by a slow decrease during the ripening of the wheat seed. Schwimmer (1947), on the other hand, found that on a wheat kernel basis the α-amylase activity was relatively constant while on a dry weight basis the activity decreased sharply. More recently, similar α-amylase activity profiles have been obtained with a number of Swedish (Olered, 1967) and Australian (Meredith and Jenkins, 1973; Jenkins et al., 1974; Jenkins and Meredith, 1975) wheat cultivars.

Olered (1967) also found a great deal of variation in the α-amylase activity during growth and maturation, as did Meredith, Jenkins and co-workers. Olered and Jonsson (1970) have suggested that these variations in α-amylase activity (Fig. 2) may be due to the regeneration of the "green" α-amylase as a result of changes in the moisture distribution and equilibrium of the wheat kernel. This hypothesis may be applicable to the variations encountered in the α-amylase activity profiles of the Canadian-grown wheat cultivars (Figs. 11 and 12).

It is of interest to note that the variations in α-amylase activity profiles of the Canadian-grown wheat cultivars were less frequent than those found in the Swedish and Australian cultivars. This difference may be a result of inherent cultivar characteristics or more reasonably, different climactic conditions in the wheat growing regions.

Other cereal grains also have been found to follow similar α-amylase changes during growth and maturation. For example, MacGregor et al. (1971) found that with the barley cultivar Conquest, α-amylase activity on a per kernel basis increased very rapidly from ear emergence to 11 days and then declined sharply to one tenth of its maximum level after 28 days. This level then remained almost constant until maturity. Similar findings for barley have been reported by LaBerge et al. (1971), Allison et al. (1974) and Riggs and Gothard (1976), as well as for triticale (Hill et al. (1974)). It is also of interest that another immature wheat enzyme system, the proteolytic system (Kruger, 1973) behaves in a manner similar to α-amylase.

Of some possible significance, was the observation that the cereals noted above, along with the 10 Canadian-grown wheat cultivars, exhibited a primary activity peak at the same period during growth and maturation i.e. around 14 days after flowering. The secondary activity peak found

at approximately 25 days after flowering in the 10 Canadian-grown wheat cultivars also can be observed in the α-amylase activity profiles of Swedish-grown wheat cultivars (Olered, 1967), some barley cultivars (Riggs and Gothard, 1976), and in the proteolytic activity profiles of HRS wheat cultivars (Kruger, 1973, Preston and Kruger, 1976).

Similarly, the seed pigment system in durum wheat (LaCroix and Lier, 1975) also exhibited primary and secondary peaks at these 2 periods. Surprisingly, the primary and secondary peaks also closely coincided with the final stages of embryo (Merry, 1941) and endosperm (LaCroix and Lier, 1975) growth, respectively. Possibly the appearance of these peaks at comparable times during cereal development may be indicative of a common control mechanism integrated with specific growth stages during kernel development.

The high resolution separational technique of flat bed polyacrylamide isoelectric focusing in combination with the dextrin slab detection system was found ideally suited to the study of immature wheat α-amylase isoenzymes. This technique could resolve isoenzymes with closely spaced isoelectricpoints while the dextrin slab detection system allowed for a long incubation period without appreciable diffusion of the isoenzyme activity. In contrast previous studies of immature wheat α-amylase isoenzymes (Olered and Jonsson, 1970, Kruger, 1972a) involved the use

of electrophoretic techniques and agar gel detection systems which did not have the resolving power and sensitivity of the isoelectric focusing system. As a result these authors observed a much simpler immature α-amylase isoenzyme system. For example, Kruger (1972a) detected 3 strong α-amylase isoenzymes in the immature wheat kernel (Fig. 1) whereas in this study a more complex system consisting of up to 22 α-amylase isoenzymes (Figs. 23-27) was observed. The 3 immature wheat α-amylase isoenzymes, α-1, α-2, α-3, detected by Kruger (1972a) undoubtedly correspond to the major GI isoenzymes 3, 5 and 6, although the α-1, α-2, α-3 components probably masked the minor isoenzymes 1, 2 and 4 which were not separable by electrophoretic systems. Similarly, the immature wheat α-1, α-2, α-3 isoenzymes purified and characterized by Marchylo et al. (1976) could have been partially contaminated with small amounts of the minor α-amylase isoenzymes.

A number of cultivar variations were evident in the whole seed isoenzyme patterns. The most obvious cultivar difference was related to the major GI isoenzyme. The durum wheat cultivars Wakooma and Wascana contained two highly active GI isoenzymes 3 and 5 (Figs. 26 and 27) while the remaining 8 cultivars possessed 3 major isoenzymes 3, 5 and 6 (Figs. 23-25). This variation probably is indicative of the different genetic background associated with the durum wheat cultivars. An identical cultivar

variation between germinated T. durum and T. aestivum wheat also has been found by Nagayoshi (1975). Some variation among cultivars was evident with the minor GI isoenzymes 1, 2 and 4, as isoenzyme 1 was the only component present in every cultivar.

The GII isoenzymes, 7 - 12 also displayed a degree of cultivar variability since each cultivar possessed only a portion of the total complement of 6 isoenzymes.

Cultivar variations also were exhibited in the GIII isoenzyme compositions. In the 10 cultivars studied a total of 10 GIII isoenzymes could be detected. The largest complement of GIII isoenzymes was displayed by the HRS wheat cultivar Glenlea which had 9. Distinctly different whole seed isoenzyme compositions were displayed by the 2 durum wheat cultivars Wakooma and Wascana (Figs. 26 and 27) and 2 HRS wheat cultivars Neepawa and Glenlea (Figs. 23 and 24). Wakooma and Neepawa represented good quality durum and bread wheat cultivars respectively in contrast with Wascana and Glenlea. Thus, α-amylase isoenzyme composition could be linked to some quality aspect of these cultivars. Furthermore, cultivar variations in the α-amylase isoenzyme composition also could be utilized for cultivar identification. This would provide a means for the separation of wheat cultivars of differing quality but identical appearance.

In the early stages of kernel growth and maturation

the major portion of the whole seed α-amylase activity was located in the outer pericarp in the form of GI isoenzyme activity. The seed coat also contained appreciable activity while the combined endosperm and aleurone contained only minimal amounts of GI activity. Little activity was attributable to the embryo or scutellum. In the early stages of growth and maturation the same morphological distribution was exhibited with all 10 cultivars. In the latter stages of maturation some cultivars exhibited a shift in the distribution primarily of the major GI isoenzymes. The cultivars Glenlea, Wakooma, Wascana and Park exhibited an expected large decrease in the pericarp activity but the GI activity in the seed coat and endosperm did not decrease in such a marked manner. This could have resulted from the moisture levels being lower in the outer pericarp than the inner tissues.

These results generally agreed with those of Kruger (1972a) although in this study the seed coat contributed appreciable activity in the early growth stages. As noted by Meredith and Jenkins (1973) the outer pericarp contained the largest portion of the total activity but there was no clear cut distribution between this tissue and those immediately below. Kruger (1972a) reported that the α-1, α-2, α-3 isoenzymes of immature wheat were not preferentially separated from each other and were present in the same relative proportions in the particular anatomical portion

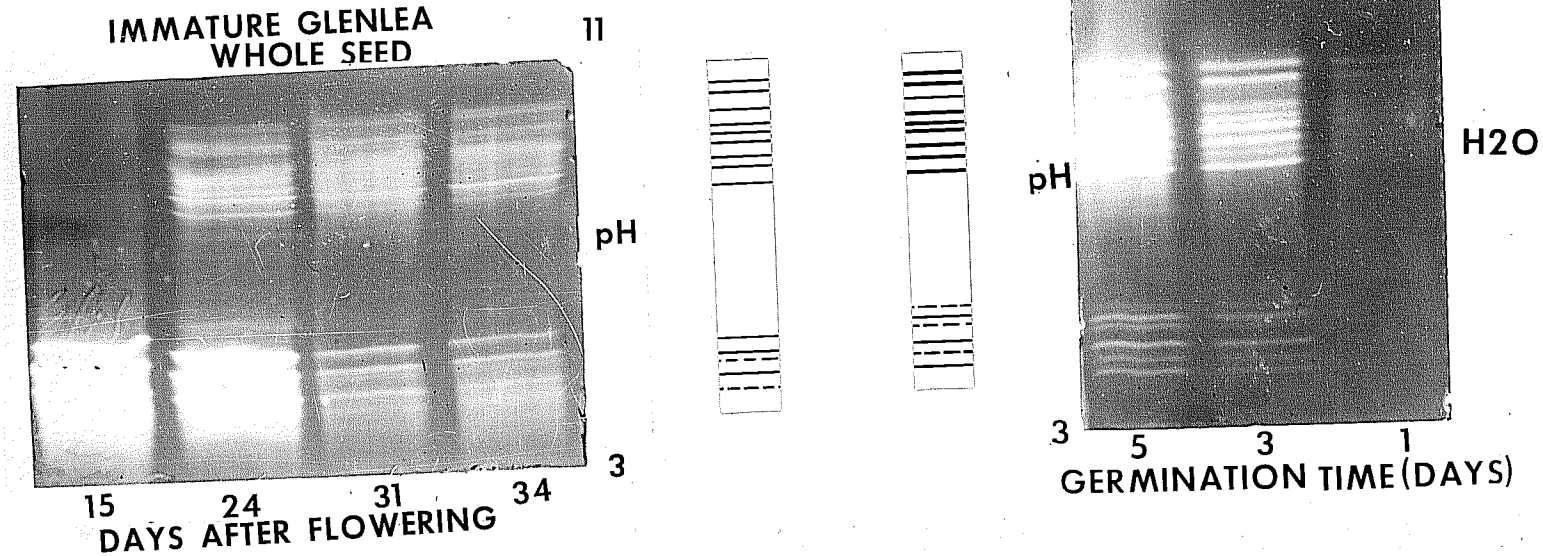
of the kernel in which they were found. This was the case for the major GI isoenzymes 3 and 5 of the durum wheat cultivars and 3, 5 and 6 of the remaining cultivars. On the other hand the minor GI isoenzymes basically were present in the pericarp and seed coat and were seldom found in the endosperm and aleurone, scutellum or embryo. Unlike the major GI isoenzymeshe minor GI isoenzymes were separable from each other. As suggested by a number of workers (MacGregor et al., 1971; Kruger, 1972a; Meredith and Jenkins, 1973) the α-amylase activity in the early stages of growth and maturation probably is associated with the metabolism of pericarp starch. It is a mystery why the endosperm contained detectable GI activity in view of the starch synthesis which is taking place in this tissue. It would seem logical to assume that the α-amylase would interfere with the starch synthesis.

The GII isoenzymes were present primarily in the endosperm although in the cultivar Glenlea (Fig. 24) traces were found in the pericarp and seed coat. The cultivar Wascana (Fig. 27) had traces of the GII isoenzymes in the seed coat.

A surprising feature of the isoenzyme composition of immature wheat was the presence of GIII isoenzymes in the 10 cultivars. These were found essentially in the endosperm with less activity in the seed coat and scutellum and only traces in the pericarp and embryo. The morphological distribution remained the same for all cultivars

and it generally did not change during growth and maturation. The surprising aspect of the GIII isoenzymes was their close resemblance to a similar group of isoenzymes in germinating wheat. This may best be illustrated in Fig. 30 where the isoenzyme patterns displayed by an immature and germinated wheat are compared. It is immediately apparent that germinated wheat contains 2 groups of α-amylase isoenzymes comparable to the GIII as well as GI isoenzymes. This suggests that in both the immature and germinating phases of seed life one common isoenzyme system is present but in each stage different isoenzyme groups are emphasized. Results published by Varner and Ram Chandra (1964); Chrispeels and Varner (1966); Filner and Varner (1967) have shown that the aleurone is the site of the de novo synthesis of α-amylase in the germinating seed. Therefore it was surprising to find the GIII isoenzymes present in the endosperm and not the aleurone as illustrated in Fig. 28. This may be accounted for if GIII α-amylase isoenzymes were secreted immediately into the endosperm upon synthesis. GIII isoenzymes could be present in the aleurone but in such low levels that they were not detectable during the 1.5 hr. incubation period. The reason for the presence of GIII isoenzymes in the seed coat is obscure but their presence in the embryo and scutellum may indicate that these tissues may also have the apparatus for synthesizing α-amylase during germination.

Fig. 30. Zymograms on β-limit dextrin slab illustrating the similarity between the immature and germinating wheat α-amylase isoenzymes.


IMMATURE GLENLEA
WHOLE SEED
11
pH
3
15
24
31
34
DAYS AFTER FLOWERING
MATURE CYPRESS
WHOLE SEED
11
pH
3
H2O
3
5
3
1
GERMINATION TIME (DAYS)

Previously the α-amylase activity in immature wheat has only been found in the form of pericarp type α-amylase isoenzymes (i.e. GI isoenzymes) (Olered and Jonsson, 1970; Kruger,1972a) but recently Daussant using immunological techniques (unpublished results) has confirmed that germinated wheat α-amylase (i.e. GIII isoenzymes) is present in immature wheat. He also has shown previously (Daussant and Renard, 1976) using similar techniques, that the immature seed isoenzymes (i.e. GI isoenzymes) are found in germinated wheat which appears to be the case in Fig. 30. It should be realized that in this study there was no indication of sprouting with any of the 10 cultivars studied. Unfortunately, to illustrate beyond a doubt that the GIII and GI isoenzymes are in fact identical to those in germinating wheat, it would be necessary to purify and characterize each isoenzyme.

The reason for the presence of the GIII isoenzymes in the immature seed is obscure but results recently published by King (1976) could assist in a better understanding. King found that abscisic acid (ABA), an inhibitor of GA_3-induced α-amylase synthesis, increased in concentration in the early stages of growth and then decreased as the seed dehydrated and reached full maturity. King suggested that ABA may be important in the control of grain dormancy and prevention of premature sprouting in immature wheat. This could signify

that ABA may be important in the control of grain dormancy and prevention of premature sprouting in immature wheat. This could signify that ABA may be involved directly or indirectly in controlling the synthesis of germinated wheat α-amylase isoenzymes. The appearance of GIII isoenzymes during maturation possibly coincides with a decrease in ABA. Perhaps a decrease in ABA levels would remove the total inhibition of α-amylase synthesis enabling a small amount of GIII isoenzyme activity, typical of germination to accumulate. If this were the case then the total activity i.e. intensity of the GIII isoenzyme bands could be related to the dormancy characteristics of a cultivar at full maturity.

PART III

THE α-AMYLASE ISOENZYMES OF GERMINATING IMMATURE WHEAT

INTRODUCTION

In Part II it was shown that immature wheat contained two major groups of α-amylase isoenzymes, i.e. the GI and GIII isoenzyme groups (Fig. 23-27). Germinating wheat had two groups of isoenzymes comparable in pI and isoenzyme pattern, to the GI and GIII isoenzyme groups of immature wheat (Fig. 30). Daussant and Renard (1976) had shown previously that immature seed α-amylases (i.e. GI isoenzymes) were present in germinating wheat. But no reports were found in the literature to indicate that immature wheat contained α-amylase isoenzymes (GIII isoenzymes) comparable to the major α-amylase isoenzymes group of germinating wheat. Studies then were initiated to determine the sites and levels of production of the GIII α-amylase isoenzymes during the development of the seed.

MATERIALS AND METHODS

Immature hard red spring (HRS) wheat, cultivar Cypress, was used in all the germination studies. This cultivar was grown in the Canada Department of Agriculture experimental plots at Glenlea, Manitoba, during the summer of 1976. Excised spikes of wheat were collected at 3 stages of kernel development i.e. 25, 32 and 39 days after flowering. The intact spikes of wheat were placed in plastic bags after harvest and stored at -19°C for subsequent use.

Dissection and Germination Procedure

Two spikes of wheat, harvested at one of the 3 developmental stages were selected and allowed to thaw. A total of 10 kernels was chosen from the 2 spikes and divided into 2 groups of 5 kernels. These kernels were then dissected into: pericarp (outer pericarp); seed coat (inner pericarp, testa, hyaline layer); endosperm and aleurone, embryo (embryonic axis) and scutellum. The tissues were placed into separate petri dishes containing sterilized sand saturated with 10^{-3}M $CaCl_2$, with or without 10^{-4}M GA_3 (ICN Pharmaceuticals, Cleveland). The tissues were then placed in a germination cabinet for

5 days at 18.5°C and 99% humidity. To facilitate discussion and for comparative purposes this process will be referred to as germination. Further germinations, employing the above procedure also were carried out for periods of 4, 3, 2 and 1 day. After the samples were removed from the germination cabinet they were stored at -19°C for subsequent analysis. This procedure also was employed for the 2 remaining developmental stages. To facilitate the dissection of the last developmental stage it was necessary to soak the kernels for about 2 hours prior to dissection.

α-Amylase Activity

The tissues were extracted into calcium chloride solution (0.5 ml, 10^{-3}M) as described previously. The α-amylase isoenzymes were separated and detected with the flat-bed polyacrylamide gel isoelectric focusing system and dextrin plate technique respectively, basically as described in Part II. However, in this study the incubation time of the detection varied depending upon the sample activity.

RESULTS

The HRS wheat cultivar Cypress was planted on May 11, 1976 and sampled at intervals of 25, 32 and 39 days after flowering. The moisture content of the wheat at each developmental stage was 46.8%, 40.9% and 17.1%, respectively. This cultivar was selected for study on the basis of its milling and baking quality and its tendency to exhibit, in some crop years, higher levels of α-amylase at harvest.

Changes in α-Amylase Levels during Germination

In preliminary germination studies, pericarp and seed coat, at each developmental stage, did not exhibit any increase in α-amylase activity within the 5-day germination period, either in the presence or absence of GA_3. Consequently, in subsequent studies these tissues were discarded following their dissection. Conversely, the remaining tissues i.e. the embryo and scutellum and the endosperm and aleurone exhibited an increase in α-amylase activity within the 5-day germination period. Therefore, at each developmental stage these tissues were germinated and analyzed in triplicate. The resultant changes in α-amylase activity are illustrated in Figs. 31 and 32.

Fig. 31. Changes in the α-amylase activity levels during germination of the immature embryo and scutellum at 3 developmental stages and the mature embryo and scutellum.

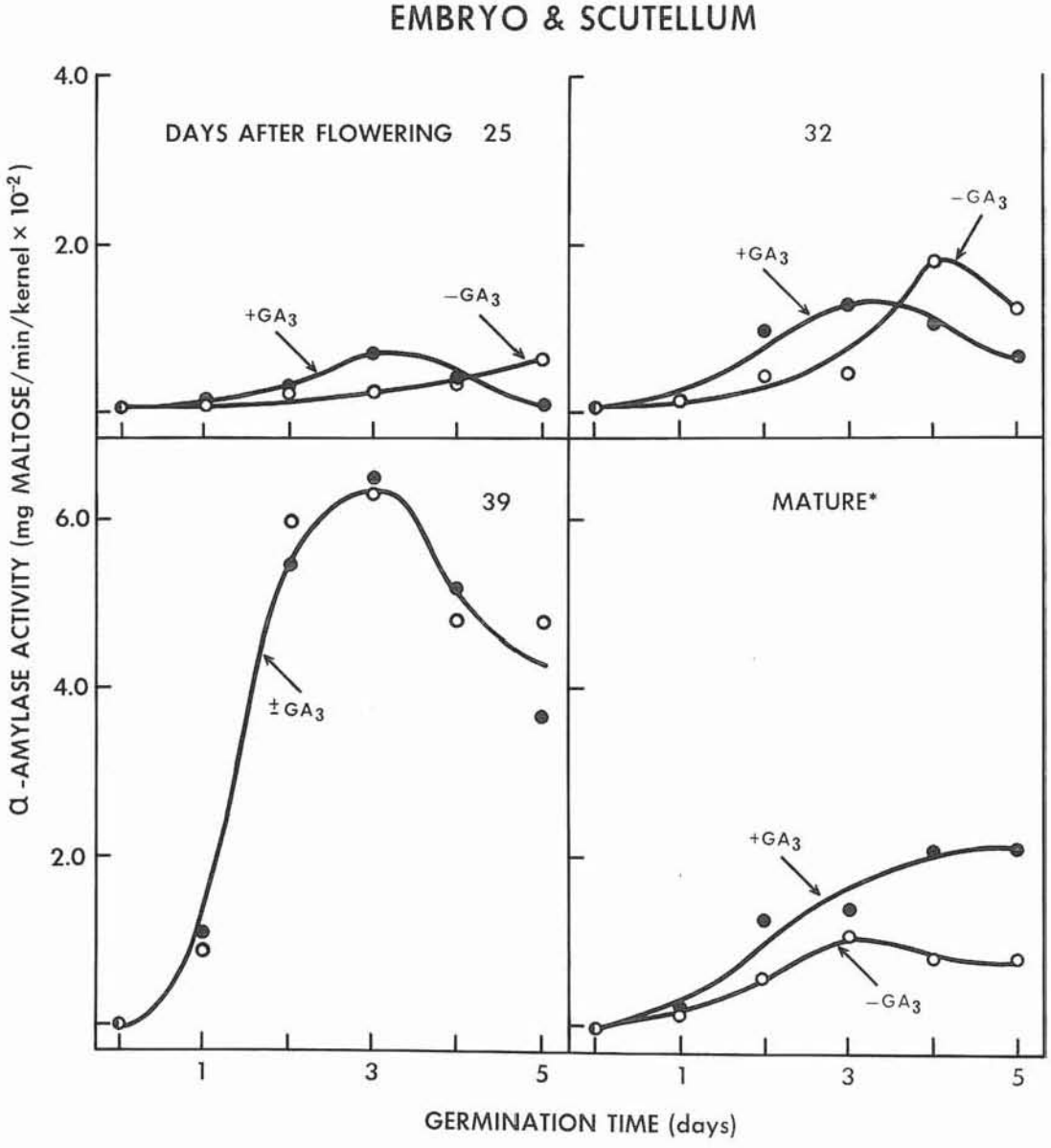
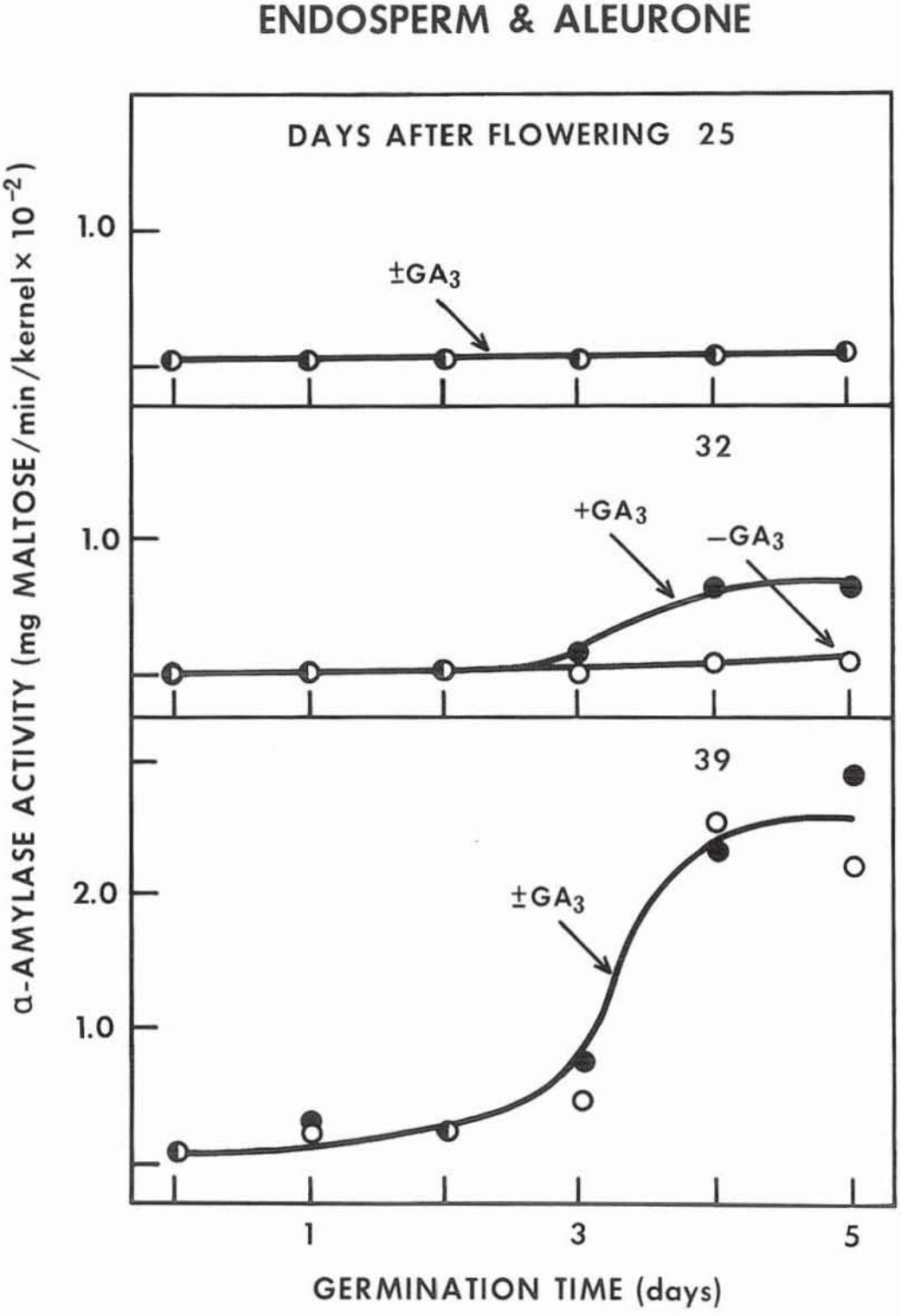

EMBRYO & SCUTELLUM
DAYS AFTER FLOWERING 25
32
39
MATURE*
+GA3
−GA3
±GA3
α-AMYLASE ACTIVITY (mg MALTOSE/min/kernel × 10⁻²)
4.0
2.0
6.0
4.0
2.0
1
3
5
GERMINATION TIME (days)

Fig. 32. Changes in the α-amylase activity levels during germination of the immature endosperm and aleurone at 3 developmental stages.


ENDOSPERM & ALEURONE
DAYS AFTER FLOWERING 25
±GA3
32
+GA3
−GA3
39
±GA3
1.0
1.0
2.0
1.0
α-AMYLASE ACTIVITY (mg MALTOSE/min/kernel × 10−2)
1
3
5
GERMINATION TIME (days)

The change in α-amylase activity in the embryo and scutellum at the first developmental stage differed during germination relative to the presence or absence of GA_3 (Fig. 31). A maximum α-amylase activity was attained after 3 days germination in the presence of GA_3 whereas in the absence of GA_3 the activity gradually increased during the 5-day germination period. The maximum activities reached after 3 and 5 days germination, in the presence and absence of GA_3 respectively were approximately equal.

The embryo and scutellum at the second developmental stage also exhibited different changes in α-amylase activity during germination, in the presence and absence of GA_3 (Fig. 31). The α-amylase activity increased to a maximum after 3 days of germination with GA_3, while a maximum was attained after 4 days germination without GA_3. The maximal activity without GA_3 was slightly higher than with GA_3.

In the presence and absence of GA_3 the third developmental stage embryo and scutellum exhibited the same changes in α-amylase activity during germination. The α-amylase activity increased very rapidly during the early stages of germination until a maximum activity was reached after 3 days. Subsequently, the activity declined between 3 and 5 days of germination. The maximal α-amylase activity attained at this stage was over 3 times greater than the preceeding stage and over 6

times higher than the first developmental stage.

The mature embryo and scutellum was germinated under the same conditions as the immature samples, for comparative purposes. The mature Cypress, employed in this study, was harvested 45 days after flowering, with a moisture of approximately 7%, from the same crop as the immature samples. The α-amylase activity of the mature embryo and scutellum increased to a maximum after 4 days germination in the presence of GA_3. This activity them remained constant up to 5 days germination. The activity, in the absence of GA_3, reached a maximum after 3 days germination then showed a subsequent decrease to a constant level which was maintained between 4 and 5 days of germination. The maximum activity with GA_3 was approximately double the maximal activity without GA_3 but was only one third of the maximum activity attained in the third developmental stage.

The embryo and scutellum at each developmental stage displayed evidence of root and shoot elongation, as did the mature tissue. The first developmental stage exhibited the least elongation whereas the third developmental stage and the mature tissue showed the most. This is consistent with the results of King (1976) who found that embryos could germinate at about 20 days after anthesis.

The germination of the first developmental stage endosperm and aleurone did not induce any increase in α-amylase activity within the 5-day germination period, either in the presence or absence of GA_3 (Fig. 32).

The endosperm and aleurone at the second developmental stage displayed only a slight increase in α-amylase activity in the absence of GA_3, after 5 days of germination (Fig. 32). In contrast, a sharp increase in activity occurred between 3 and 4 days in the presence of GA_3. The α-amylase activity then remained constant between 4 and 5 days germination.

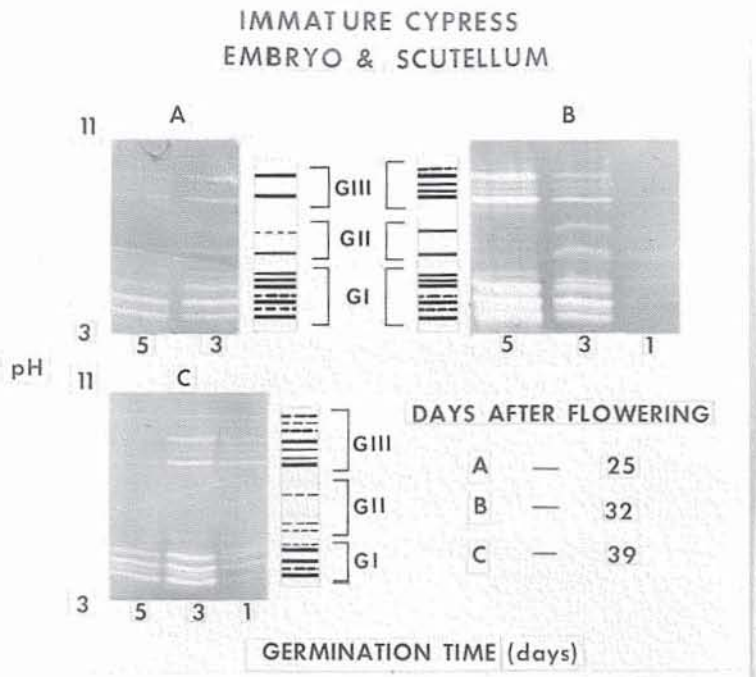
The endosperm and aleurone at the third developmental stage exhibited a gradual increase in activity during the first 3 days of germination (Fig. 32). Subsequently, there was a sharp increase in activity, between 3 and 4 days germination to a maximum level which was maintained up to 5 days germination. The change in activity was found to be the same either in the presence or absence of GA_3. The maximum activity reached in the third developmental stage was about 3 times greater than that reached in the second stage with GA_3. The endosperm and aleurone from the mature wheat could not be used for comparative purposes since it was not feasible to separate it from the surrounding tissues.

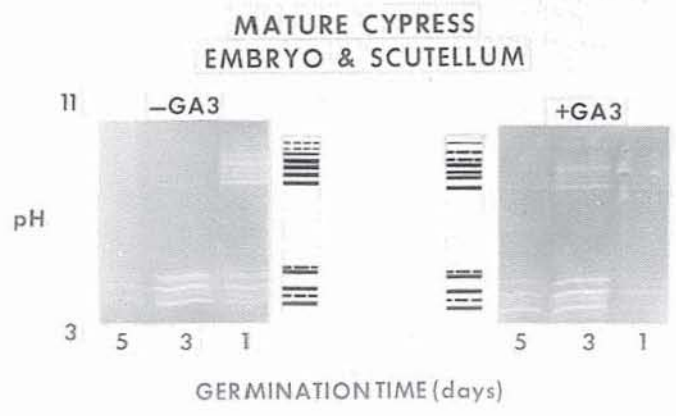
α-Amylase Isoenzymes of Germinated Immature Wheat

The α-amylase isoenzyme composition of the embryo and scutellum and endosperm and aleurone from each developmental stage was determined after 0, 1, 3 and 5 days of germination, in the presence and absence of GA_3. In some instances the α-amylase isoenzyme activity was not sufficient for detection via the dextrin plate technique in the incubation time chosen. For this reason, the α-amylase isoenzyme composition is not shown for a number of germination trials. The α-amylase isoenzyme composition of the embryo and scutellum is illustrated in Fig. 33 for each developmental stage. The presence or absence of GA_3 did not alter the isoenzyme composition for any developmental stage but it did affect their intensity. The α-amylase isoenzymes illustrated in Fig. 33 were separated into three groups to conform with the terminology initiated in Part II.

The bulk of the α-amylase activity in the germinated first developmental stage embryo and scutellum was contributed by three major GI isoenzymes. Four additional minor GI isoenzymes were also detected. Two GII isoenzymes were evident but they contributed little activity. In contrast, two GIII isoenzymes were present with substantial activity.

The isoenzyme composition of the germinated embryo and scutellum at the second developmental stage was basically


IMMATURE CYPRESS
EMBRYO & SCUTELLUM
A
B
C
11
3
pH
5 3
5 3 1
GIII
GII
GI
DAYS AFTER FLOWERING
A — 25
B — 32
C — 39
GERMINATION TIME (days)


MATURE CYPRESS
EMBRYO & SCUTELLUM
–GA3
+GA3
11
3
pH
5 3 1
5 3 1
GERMINATION TIME (days)

the same as the first growth stage although three additional minor GIII isoenzymes were detected. The intensity of the major GIII isoenzymes also appear to have increased relative to the major GI isoenzymes. Unfortunately, a quantitative assessment of this relationship could not be made with the dextrin plate technique.

The germinated third developmental stage embryo and scutellum isoenzyme composition was much the same as the previous two developmental stages but some differences were readily apparent. The majority of the α-amylase activity again was associated with three major GI isoenzymes but in this case only two minor GI isoenzymes were detectable. Three GII isoenzymes were present but they contributed an insubstantial amount of activity. A total of seven GIII isoenzymes were detectable in this stage with the two major isoenzymes being the most active in addition to five minor isoenzymes.

The α-amylase isoenzyme composition of the germinated mature embryo and scutellum is illustrated in Fig. 34 in the presence and absence of GA_3. Two main groups of isoenzymes were detectable in the germinated tissue. The same isoenzymes were present with and without GA_3 but their activities varied in a different manner during germination in each case. In both instances the group of isoenzymes in the low pI region was composed of three major and two minor isoenzymes. The pI group of isoenzymes

was composed of two major and five minor isoenzymes, whereas without GA_3 there were five major and only two minor isoenzymes.

The endosperm and aleurone at the first developmental stage did not show an increase in α-amylase activity within the 5-day germination period (Fig. 32) so the isoenzyme composition was not studied. The second developmental stage endosperm and aleurone did show an increase in α-amylase activity after germination (Fig. 32) so the isoenzyme composition was determined as illustrated in Fig. 35. The tissues germinated without GA_3 basically contained three major GI isoenzymes and two debranching enzymes d-1 and d-2. In contrast, the tissues germinated with the GA_3 contained three GIII isoenzymes in addition to the GI and debranching enzymes. The GIII isoenzymes appeared to be increasing in intensity with increasing germination time. The control sample, i.e. 0 day germination, had the same isoenzyme composition as the tissue germinated for one day without GA_3, so it was not shown.

The endosperm and aleurone at the third developmental stage exhibited a radically different α-amylase isoenzyme composition following germination (Fig. 35) in both the presence and absence of GA_3. After three days of germination GI and GII isoenzymes as well as debranching enzymes were detected. The activity of the GI and GII isoenzymes

Fig. 35. The α-amylase isoenzyme composition of the endosperm and aleurone from 2 developmental stages, following germination in the presence and absence of GA_3. The symbols GI, GII and GIII designate groups of α-amylase isoenzymes and d-1 and d-2 refers debranching enzymes.

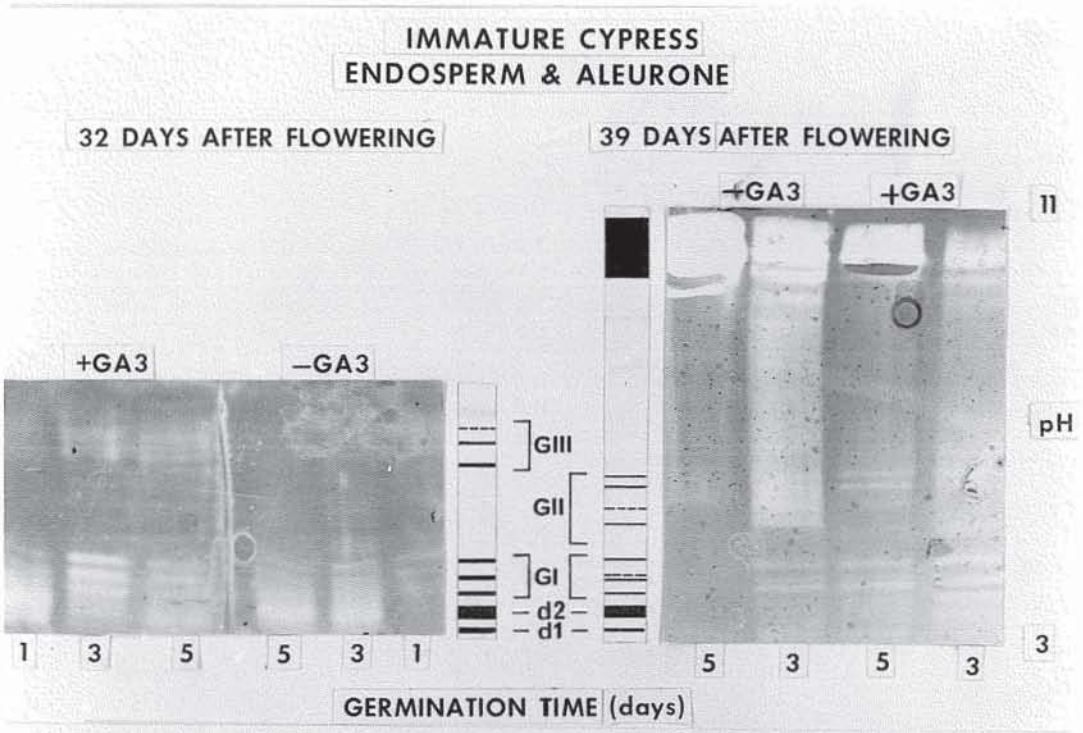
Fig. 36. The GA_3 induced α-amylase isoenzyme composition of:

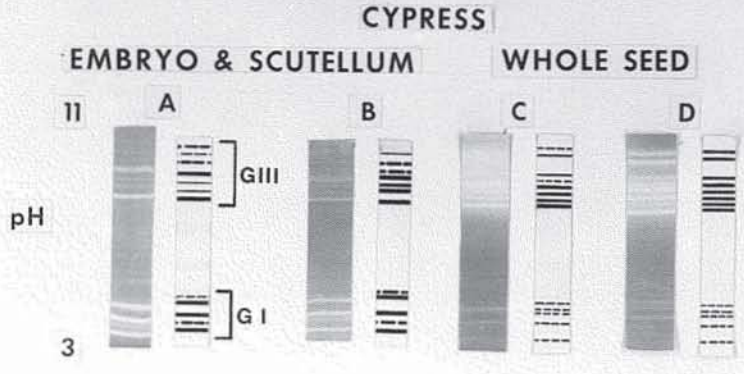
A. 3 Day germinated immature embryo and scutellum (harvested 39 days after flowering),

B. 3 Day germinated mature embryo and scutellum,

C. 5 Day germinated immature whole seed (harvested 39 days after flowering),

D. 3 Day germinated mature whole seed.


IMMATURE CYPRESS
ENDOSPERM & ALEURONE
32 DAYS AFTER FLOWERING
39 DAYS AFTER FLOWERING
+GA3
−GA3
+GA3
+GA3
11
pH
3
GIII
GII
GI
d2
d1
1 3 5 5 3 1
5 3 5 3
GERMINATION TIME (days)


CYPRESS
EMBRYO & SCUTELLUM
WHOLE SEED
11
A
B
C
D
pH
3
GIII
G I

and debranching enzymes showed a slight decrease after five days germination with GA_3 while without GA_3 these isoenzymes virtually lost all of their activity. GIII isoenzymes were not detected within the 5-day germination period either in the presence or absence of GA_3. The majority of the α-amylase activity appeared in the high pH region of the isoelectric focusing gel (Fig. 35) and increased in activity during germination both in the presence and absence of GA_3. This activity was not sharply defined into distinct isoenzyme bands but was present as a very broad region of activity. A number of trials were carried out at this developmental stage and in each case the same results were invariably obtained. Unfortunately, the mature endosperm and aleurone could not be studied since the separation of this tissue from the surrounding tissues was not possible.

DISCUSSION

It is evident that germination of the embryo (embryonic axis) and scutellum,at 3 developmental stages (i.e. 25, 32 and 39 days after flowering), elicited an increase in α-amylase activity in the presence and absence of GA_3 (Fig. 31). This would appear to be an indication of α-amylase synthesis in the embryo and scutellum well in advance of full maturity. As the tissue matured, it was able to produce higher levels of α-amylase upon germination. Evidence of this may be seen in Fig. 31 where higher enzyme levels were obtained with each successive growth stage, both in the presence and absence of GA_3. This behavior simply could be the result of an increase in the size of the embryo and scutellum during maturation concomitant with an increase in the amount of enzyme synthetic apparatus in the tissue. On the other hand, this behavior also could stem from a change in the balance of the gibberellins (activators of α-amylase synthesis) and abscisic acid (ABA, an inhibitor of gibberellin-induced α-amylase synthesis) levels in the tissue. These 2 plant hormones have both been implicated

in the control of α-amylase synthesis (King, 1976; Radley, 1976). Surprisingly, the germinated mature embryo and scutellum did not produce α-amylase levels as high as those found in the last developmental stage. The only major difference between these two samples was in their moisture content with the immature and mature tissues containing moisture levels of approximately 17% and 7%, respectively. Thus it would appear that the final desiccation of the embryo and scutellum is the cause of the decrease in the synthesis of α-amylase in the mature tissue. Perhaps the desiccation of the tissue decreased the availability of the nutrients required for α-amylase synthesis or again it may have changed the balance of gibberellin and ABA levels, as previously discussed.

The immature embryo and scutellum at each developmental stage exhibited the same pattern of change in α-amylase activity when germinated in the presence of GA_3 (Fig. 31). In each case, the activity reached a maximum after 3 days germination and subsequently decreased between 3 and 5 days germination. The decrease in activity after 3 days germination could be indicative of degradation via proteolytic enzymes. In the absence of GA_3 the pattern of change in α-amylase activity during germination changed with each succeeding stage of development until in the third growth stage the pattern of activity change was identical to that

shown in the presence of GA_3. This behavior again could be indicative of an increase in GA_3 tissue levels or of a change in balance of ABA and GA_3 levels. The mature embryo and scutellum showed a pattern of activity change different from that of the last developmental stage. This again could be the result of the final desiccation of the tissue.

The α-amylase isoenzyme composition of the germinated embryo and scutellum was similar for each developmental stage as illustrated in Fig. 33. In each stage, 3 GI and 2 GIII isoenzymes contributed the bulk of the activity to this tissue. The number of minor GIII isoenzymes increased in each successive developmental stage whereas there was a decrease in the number of minor GI isoenzymes in the final developmental stage. The number of GII isoenzymes also increased from 2 to 3 into the final stage but they contributed negligible activity at that stage. The overall result of these changes was that the isoenzyme pattern of the germinated immature embryo and scutellum more closely resembled the isoenzyme pattern of the germinated mature tissue in each succeeding developmental stage. In fact, barring the presence of the basically negligible GII isoenzyme activity, the embryo and scutellum at the final developmental stage had an isoenzyme pattern identical to that of the germinated mature tissue. This similarity in α-amylase isoenzyme

pattern is evidence that the immature embryo and scutellum can synthesize α-amylase isoenzymes traditionally associated with germinated mature wheat. The α-amylase isoenzyme composition of the immature or mature embryo and scutellum was not affected by germination in the presence or absence of GA_3. However, the presence of GA_3 during germination of the mature embryo and scutellum, did appear to have some bearing upon the timing of the appearance of the GIII-type α-amylase isoenzymes as well as on their relative activities (Fig. 34). The mature tissue again exhibited different behavior as compared to the final developmental stage. As noted previously, this difference could be an indication of the physical and chemical affects of the final desiccation of this tissue.

The germination of the immature endosperm and aleurone also resulted in an increase in α-amylase activity in the last two developmental stages, as illustrated in Fig. 32. This is one indication that the endosperm and aleurone also can synthesize α-amylase in advance of full maturity. In the latter stages of maturity the endosperm and aleurone also were able to produce higher α-amylase levels during germination with or without GA_3. The increased production of α-amylase with maturity is probably due to actual growth and maturation of the aleurone, which is known to be the site of α-amylase synthesis during germination (Rowsell and Goad, 1963; Filner and Varner, 1967). Thus in the first developmental stage, where no

increase in activity was noted, the aleurone probably was not sufficiently developed to carry out the synthesis of the enzyme. However, in the following 2 developmental stages the tissue was probably sufficiently developed to carry out the synthesis of α-amylase. This is consistent with the findings of Morrison et al. (1975) who found that the aleurone, as assessed by its ultrastructure, does not mature until 6 - 10 days before cessation of grain dry weight accumulation for wheat grown in the Canberra Phytotron.

A change in the response of this tissue in the presence and absence of GA_3 also was observed. In accordance with the above hypothesis the first growth stage showed no response during germination in the presence of GA_3. In contrast the next developmental stage exhibited a definite increase in activity during germination with GA_3 while in the last developmental stage an identical increase in activity was obaained in the presence or absence of GA_3. As with the embryo and scutellum this behavior may be attributed to changes in the balance of gibberellin and ABA levels in this tissue. This is consistent with the results of King (1976) and Radley (1976) which have shown that the GA_3 and ABA levels vary during the course of kernel development.

The α-amylase isoenzymes of the second developmental stage endosperm and aleurone, germinated without GA_3,

were primarily the major GI isoenzymes (Fig. 35) traditionally associated with the immature seed. In this case, the slight increase in activity noted in Fig. 32 could be the result of a reactivation of these isoenzymes as suggested by Olered and Jonsson (1970). In contrast, in the presence of GA_3, GIII isoenzymes were formed in increasing amounts during the 5-day germination period (Fig. 35). The formation of these isoenzymes presumably is responsible for the majority of the α-amylase activity increase noted in Fig. 32, and is probably due to synthesis via the aleurone. Also, as was shown for the embryo and scutellum (Fig. 36), these GIII isoenzymes are comparable to α-amylase isoenzymes found in germinating mature wheat.

The α-amylase isoenzyme composition of the germinated third developmental stage endosperm and aleurone was quite different than that of the previous stage or other immature and germinated wheat samples previously studied. Although GI isoenzymes were present, their activities relative to each other were different than those of the GI isoenzymes of the second developmental stage (Fig. 35). The appearance of these isoenzymes, plus two debranching enzymes again could have resulted from the reactivation of the immature wheat isoenzyme system as suggested by Olered and Jonsson (1970), or

they may have been synthesized during the germination period. Whatever the case, this α-amylase isoenzyme activity did give rise to a portion of the activity increase as noted in Fig. 32.

Surprisingly, GIII isoenzymes were not detected even though they had been formed during germination of the endosperm and aleurone at the second developmental stage. But unlike the second stage a large amount of α-amylase activity with a very high pI was detected in the germinated third developmental stage endosperm and aleurone. This activity increased during germination and contributed the bulk of the α-amylase activity present in the tissue. α-Amylase isoenzymes with such a high pI previously have not been detected and thus it is difficult to rationalize their appearance in this case. One possible explanation for the existence of the α-amylase activity is that under these germination conditions the GI, GII and GIII isoenzymes in the endosperm and aleurone may become bound to certain highly charged particles which would give the α-amylase isoenzymes a very high pI. If this binding changed during germination and was slightly different for each isoenzyme it also could explain the absence of GIII isoenzymes, the differences in the relative GI and GII isoenzyme activities and it also could account for the apparent decrease in the GI and GII activities during the later stages of germination concomitant with

an increase in the high pI α-amylase activity.

Another possible explanation is that the high pI α-amylase activity may be the same as the D2 α-amylase activity detected by Daussant and Renard (1976) in immature wheat. These workers found that some of the immature wheat α-amylase activity migrated towards the cathode during agarose gel electrophoresis, at pH 8.6, whereas the D1 α-amylase typical of immature wheat moved towards the anode. This indicates that the D2 α-amylase has a high pI which in turn suggests that it may be equivalent to the high pI α-amylase activity shown in Fig. 35. Perhaps under the germination conditions employed in this study the synthetic machinery responsible for the synthesis of the high pI α-amylase was stimulated to produce excessive amounts of this enzyme.

The presence of GA_3 did not appear to have any marked effects on the isoenzyme composition. However, in the absence of GA_3 more high pI α-amylase was formed and there also was a larger decrease in the GI and GII isoenzyme activities in the later stage of germination. This study has confirmed that both the immature embryo and scutellum and endosperm and aleurone can synthesize α-amylase isoenzymes comparable to α-amylase isoenzymes found in germinating mature wheat. Thus, as was suggested in Part II, these tissues must contain the necessary

synthetic appartus used for the synyhesis of α-amylase during germination. Although the third stage endosperm and aleurone showed anomalous behavior, this was probably the result of other factors and should not detract from the fact that the second growth stage endosperm and aleurone synthesized GIII α-amylase isoenzymes comparable to those present in germinating mature wheat. In light of these findings, the presence of GIII isoenzymes in the immature wheat, as described in Part II, can be more easily understood. As suggested in Part II, the control mechanisms which prevent the synthesis of the germinated wheat α-amylase isoenzymes (i.e. GIII-type isoenzymes) in the immature kernel may not be 100% efficient. This would then enable a small amount of GIII isoenzymes to be synthesized in the immature wheat.

The increase in α-amylase activity in the endosperm and aleurone in the presence and absence of GA_3 is relatively consistent with the findings of other workers. Although Bilderback (1971) found that immature barley aleurone layers would not respond to GA_3, he did observe that if immature seeds 20 days or older were dried then large amounts of α-amylase were produced when the aleurone layers were subsequently incubated with GA_3. King's (1976) results agreed with this since he discovered that isolated wheat aleurone was unable

to produce α-amylase until the grain has dried. King (1976) also found that one immature wheat cultivar showed an increase in α-amylase activity in the absence of GA_3. In this study the endosperm and aleurone of the final 2 developmental stages were taken from seeds with moisture contents of 41% and 17%. As noted by King these moistures were low enough to allow for the α-amylase synthesis which was found.

Although this study has shown that the immature embryo and scutellum and endosperm and aleurone can synthesize GIII isoenzymes, this was not shown to be the case with the seed coat (inner pericarp, testa, hyaline layer). Unfortunately, this does not explain the presence of the GIII isoenzymes which were detected in this tissue, as was shown in Part II. This anomalous behavior could simply stem from the seed coat's direct attachment to the aleurone layer. In all probability, some α-amylase was synthesized and excreted into the seed coat from the aleurone resulting in the presence of the GIII isoenzymes.

Another conclusion which may be drawn from the results of this study is that the embryo and scutellum can synthesize α-amylase during germination. It previously has been established that the aleurone is tha major site of α-amylase synthesis in the germinating seed (Filner and Varner, 1967; Briggs, 1973; Gibson and Paleg, 1972,

1975) but some controversy has arisen over α-amylase synthesis by the embryo (Briggs, 1973; Palmer,1974). The results of this study agree with the conclusions of Briggs (1964, 1972, 1973) which state that the embryo does synthesize α-amylase and excludes the contention of Macleod and Palmer (1966) that the scutellum portion of the embryo does not produce any α-amylase. This is shown firstly in Fig. 31 where increases in α-amylase activity are exhibted by the mature and immature embryo and scutellum. Secondly, the α-amylase isoenzyme composition of the germinated immature and mature embryo and scutellum is quite distinct from that of the germinated whole seeds as illustrated in Fig. 36. Palmer (1974) has suggested further that a barely perceptible ring of aleurone cells, which contaminate the excised embryo, is responsible for the production of the observed α-amylase activity. According to the results of this study this is not a valid argument. As shown in Fig. 31 the first developmental stage embryo and scutellum can synthesize α-amylase with or without GA_3. In contrast, the endosperm and aleurone at the same stage of development could not synthesize α-amylase under the same conditions (Fig. 32). Thus any aleurone cells present on the first developmental stage embryo and scutellum did not contribute any additional α-amylase through synthesis. This then indicates that the embryo and scutellum can synthesize α-amylase.

Briggs (1973) also pointed out that excised embryos required the addition of suitable nutrients (e.g., amino acids) to elicit any appreciable synthesis of α-amylase. According to this study excised embryos could synthesize α-amylase without the addition of these nutrients but it is not unreasonable to assume that the addition of nutrients could yield a larger enzyme production.

The synthesis of α-amylase by the embryo and scutellum is reasonable in light of its nutritional requirements during germination. During normal germination the embryo rapidly begins to grow and thus requires a source of nutrient sugars very early in the germination process. The synthesis of α-amylase by this tissue would ensure an immediate supply of nutrients from the endosperm in advance of the main supply produced by the action of the aleurone α-amylase on the endosperm.

PART IV

PRELIMINARY STUDIES ON THE EFFECT OF THE SEED COAT ON α-AMYLASE SYNTHESIS

INTRODUCTION

In Part III, it was found that the dissected endosperm and aleurone tissues of the HRS wheat, cultivar Cypress (harvested 39 days after flowering), displayed an unique α-amylase isoenzyme composition after being placed for 3-5 days under typical germination conditions (Fig. 35). It was found that the majority of the α-amylase activity was present in the form of a wide band of activity found in the very high pH regions of the isoelectric focusing gel. There was some indication that the removal of the seed coat from the endosperm and aleurone may have had some relationship to this unique isoenzyme composition. Therefore, preliminary studies were performed to determine if the seed coat has some effect on the synthesis of α-amylase.

MATERIALS AND METHODS

Immature and mature hard red spring(HRS) wheat, cultivar Cypress, was used in all the germination studies. This cultivar was grown in the Canada Department of Agriculture experimental plots at Glenlea, Manitoba, during the summer of 1976. The immature wheat was harvested 39 days after flowering with a moisture content of 17.1% and was stored at -19^{o}C. The mature wheat was harvested from the same crop 45 days after flowering at a moisture level of 7.0%.

Dissection and Germination Procedure

The endosperm and aleurone was dissected from the immature kernel and was germinated by the same procedure described in Part III. In order to de-embryonate the immature and mature wheat it was necessary to soak the kernels for about 2 hours prior to dissection. The embryo was carefully removed to ensure that none of the scutellar tissue remained attached to the endosperm. The whole and de-embryonated kernels were germinated by the same procedure and under the same conditions employed in Part III.

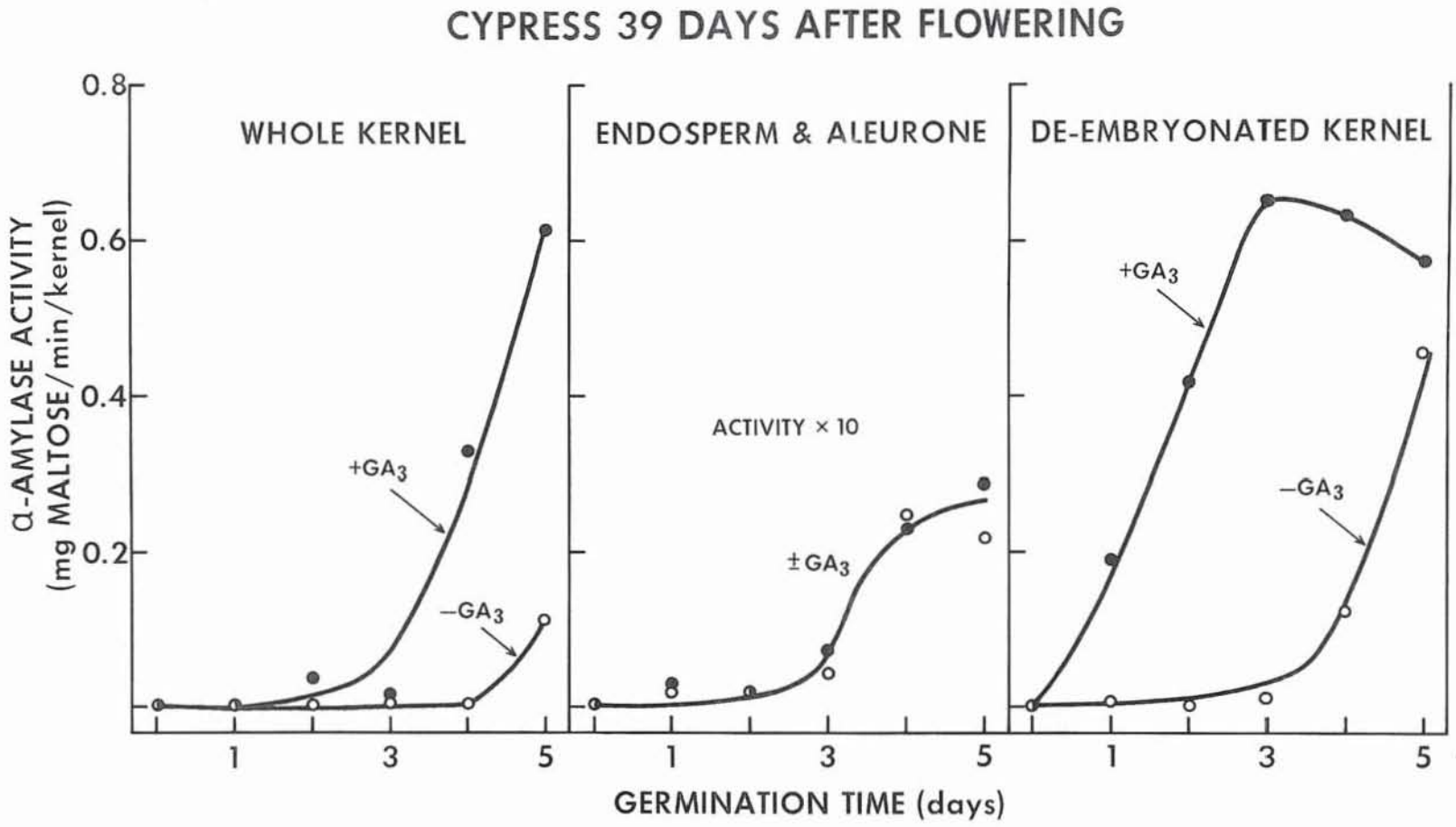
Determination of α-Amylase Activity and Isoenzyme Composition

The α-amylase activity was determined by the fluorometric procedure employed in Part III. Similarly, the isoenzyme composition was obtained as described in Part III. In both cases the sample preparation also was carried out in accordance with the method described in Part III.

RESULTS

The α-amylase activity in the immature whole kernel, in the presence of GA_3, slowly increased between 1 and 3 days of germination and then exhibited a rapid increase in activity between 3 and 5 days of germination. In the absence of GA_3 the α-amylase activity showed no increase until between 4 days and 5 days of germination (Fig. 37). The change in the α-amylase activity during the germination of the immature endosperm and aleurone was the same in the presence or absence of GA_3 (Fig. 37). A slight increase in activity which took place during the first 3 days of germination was followed by a sharp increase in activity between 3 and 4 days germination. Subsequently, the rate of α-amylase activity increase levelled off. The maximum activity produced in this tissue was approximately one twentieth of the activity produced in the whole kernel. The immature de-embryonated whole kernel synthesized α-amylase rapidly during the first 3 days of germination in the presence of GA_3. Thereafter the activity showed a slight decline. In contrast the de-embryonated kernel, in the absence of GA_3, exhibited a

Fig. 37. Changes in the α-amylase activity during germination of the whole kernel, endosperm and aleurone and de-embryonated kernel in the presence and absence of GA_3. The immature HRS wheat cultivar Cypress (harvested 39 days after flowering) was employed.


CYPRESS 39 DAYS AFTER FLOWERING
WHOLE KERNEL
ENDOSPERM & ALEURONE
DE-EMBRYONATED KERNEL
α-AMYLASE ACTIVITY (mg MALTOSE/min/kernel)
0.8
0.6
0.4
0.2
+GA3
−GA3
ACTIVITY × 10
±GA3
1
3
5
GERMINATION TIME (days)

minimal increase in activity which continued up to 5 days germination (Fig. 37).

For comparitive purposed mature wheat also was germinated in the presence and absence of GA_3. In the presence of GA_3, the α-amylase activity increased rapidly after 1 day germination (Fig. 38), but showed a decline in the rate of activity increase after 3 days germination. The α-amylase activity also increased rapidly after 1 day germination in the absence of GA_3 but the rate of increase and the activity levels reached during the 5-day germination period were lower (Fig. 38). The de-embryonated mature wheat exhibited a rapid increase in activity to a maximum level which was reached after 4 days germination in the presence of GA_3 (Fig. 38), thereafter a decrease in activity was exhibited. In the absence of GA_3, the de-embryonated kernel showed a slight increase in activity after 2 days of germination to a relatively constant level which was maintained between 3 and 5 days of germination.

The α-amylase isoenzymes of the germinated immature whole kernel were divided into 2 distinct groups (Fig. 39 A). The bottom group of isoenzymes located in the low pH regions of as isoelectric focusing gel was composed of 5 isoenzymes where the top group was composed of 9 isoenzymes. The top group contributed the majority of the activity to the whole kernel with the bottom 4 isoenzymes in the top group being the most active.

Fig. 38. Changes in the α-amylase activity during germination of the whole and de-embryonated kernel in the presence and absence of GA_3. The mature HRS wheat cultivar Cypress was employed.

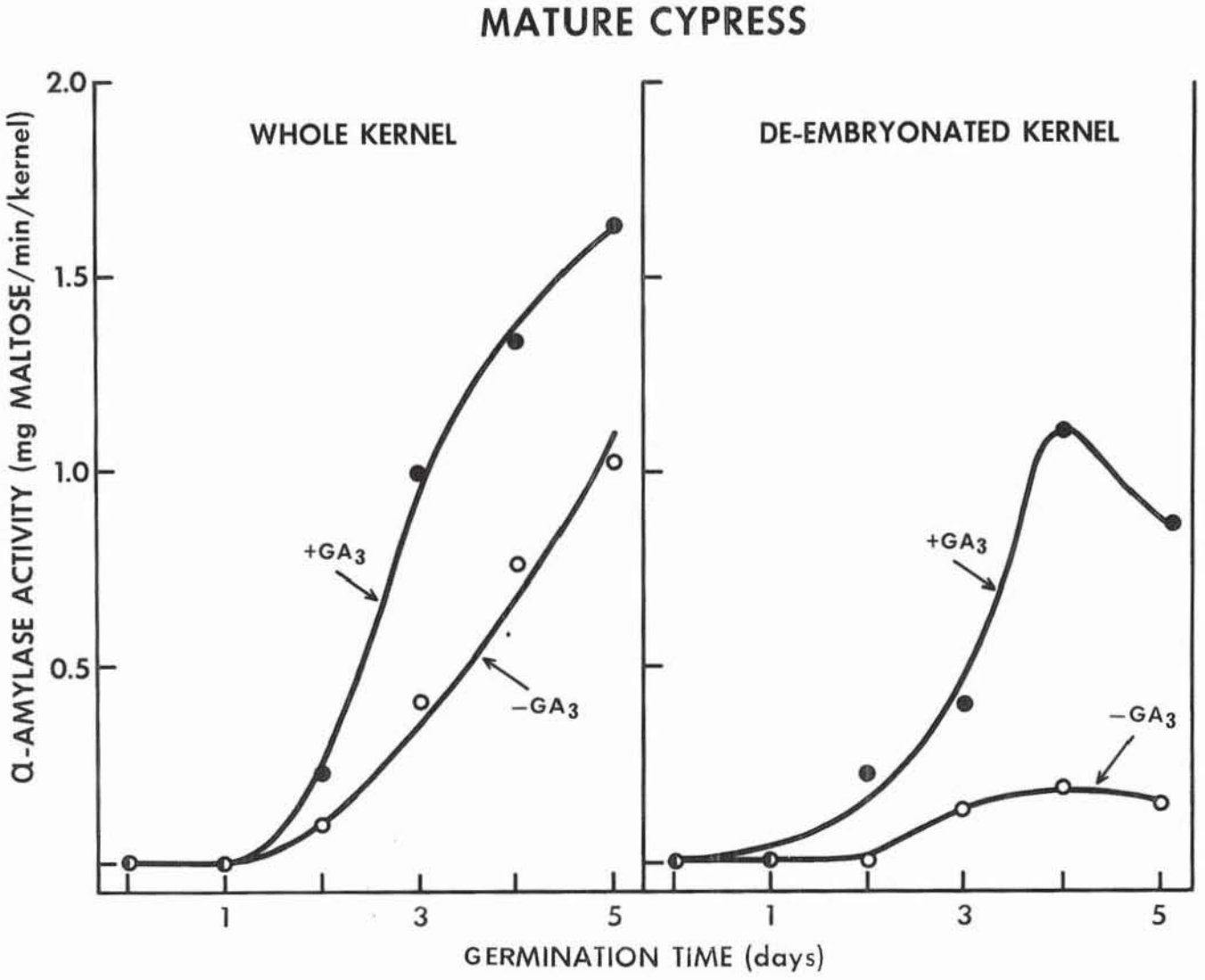

MATURE CYPRESS
WHOLE KERNEL
DE-EMBRYONATED KERNEL
α-AMYLASE ACTIVITY (mg MALTOSE/min/kernel)
2.0
1.5
1.0
0.5
+GA3
−GA3
+GA3
−GA3
1
3
5
1
3
5
GERMINATION TIME (days)

Fig. 39 The α-amylase isoenzyme composition of germinated wheat:

A. immature whole kernel

B. immature endosperm and aleurone

C. immature de-embryonated kernel

D. mature whole kernel

E. mature de-embryonated kernel

The wheat used was the HRS wheat cultivar Cypress and the immature wheat was harvested 39 days after flowering. All the germinations were carried out in the presence of GA_3.

Fig. 40 The α-amylase isoenzyme composition of germinated wheat:

A. immature de-embryonated wheat in the absence of the seed coat, as noted in operation 2, Table III

B. immature de-embryonated wheat in the presence of the seed coat, as noted in operation 1, Table III

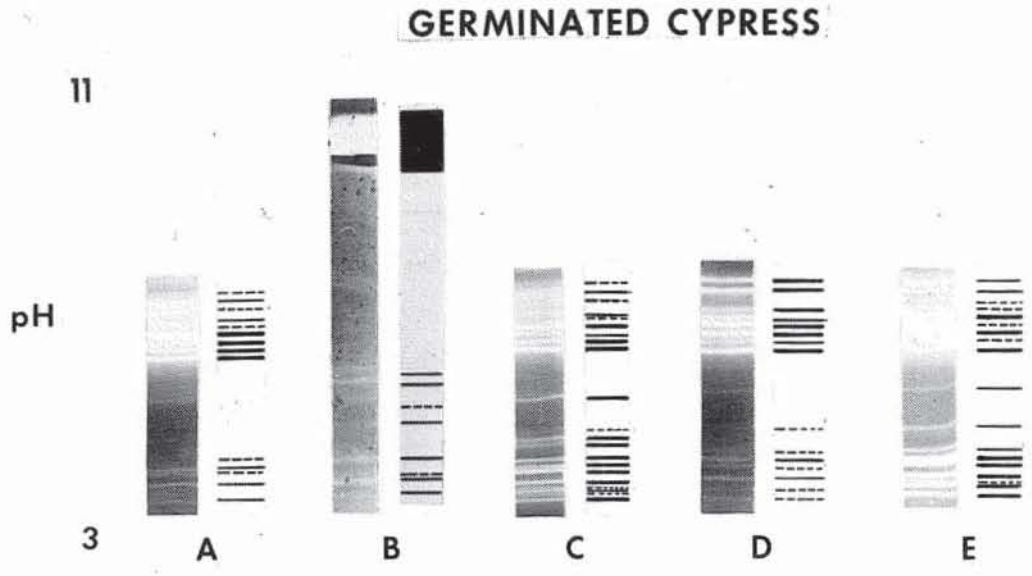
C. mature de-embryonated wheat in the absence of the seed coat, as noted in operation 2, Table III

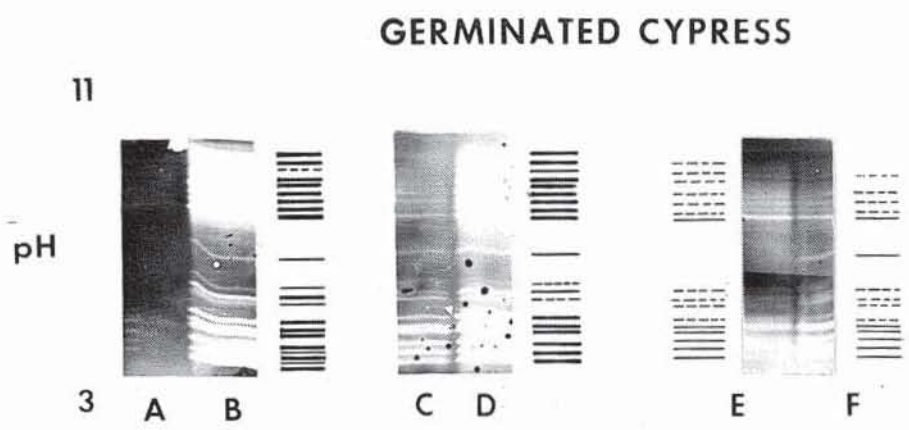
D. mature de-embryonated wheat in the presence of the seed coat as noted in operation 1, Table III

E mature de-embryonated wheat in the partial absence of the seed coat as noted in operation 3, Table III

F. immature de-embryonated wheat in the partial absence of the seed coat as noted in operation 3, Table III

The wheat used was the HRS wheat cultivar Cypress and the immature wheat was harvested 39 days after flowering. All germinations were carried out in the presence of GA_3.


GERMINATED CYPRESS
11
pH
3
A
B
C
D
E


GERMINATED CYPRESS
11
pH
3
A
B
C
D
E
F

The isoenzyme composition of the germinated mature whole kernel Fig. 39 D) was basically the same as that of the immature whole seed although minor differences were apparent. For example, the bottom isoenzyme group contained one more isoenzyme although this was barely detectable. Also, 1 isoenzyme was detectable in the intermediate region between the 2 main isoenzyme groups. This isoenzyme as shown in Fig. 39 D was barely detectable but in the later stages of germination it was more apparent. Similarly, the top isoenzyme group in the mature kernel appeared to have 1 less isoenzyme but again with further germination this isoenzyme was detectable.

The germinated immature and mature de-embryonated kernels also exhibited similar isoenzyme compositions although again minor differences were apparent as shown in Figs. 39 C and E, respectively. In both cases the bottom isoenzyme group contained the same 6 major isoenzymes but the immature de-embryonated wheat contained 1 more weakly active minor isoenzyme. The immature de-embryonated wheat also appeared to have a larger number of intermediate isoenzymes but in the later germination stages the mature de-embryonated wheat acquired these missing isoenzymes. The top isoenzyme group contained 9 isoenzymes in both cases but there were differences between the activity of equivalent isoenzymes from each source. With further germination

these differences disappeared. In both instances the isoenzymes present in the bottom groups of the germinated de-embryonated kernels were much more active relative to the upper isoenzyme group than their counterparts present in the germinated kernels.

The α-amylase isoenzyme composition of the germinated immature endosperm and aleurone was totally different from the germinated immature or mature samples discussed above. A complete description of the isoenzyme composition may be found in the Results of Part III.

In each of the above cases the isoenzyme compositions were the same in the presence or the absence of GA_3. The only variation conferred by the presence of GA_3 was higher activity of the individual isoenzymes.

The above results plainly pointed out that the α-amylase activity changes and isoenzyme composition of the germinated immature endosperm and aleurone were vastly different from the germinated immature and mature de-embryonated kernels and whole kernels. The immature de-embryonated kernel and the immature endosperm and aleurone differed only in the presence of a seed coat on the de-embryonated kernel. This suggested that the seed coat was in some way involved with the synthesis of α-amylase and prompted a number of further studies.

To ascertain if some factor required for synthesis was

present in the seed coat, this tissue after dissection from the immature kernel was homogenized and added back to the endosperm and aleurone. The seed coat was homogenized in a mortar with sand in 0.5 ml of 10^{-3}M $CaCl_2$ with or without 10^{-4}M GA_3 as required. The homogenate was then poured over the endosperm and aleurone and germinated as described in Part III. The endosperm and aleurone was then assayed for α-amylase activity and isoenzyme composition. The activity curves obtained with the germinated endosperm and aleurone, in the presence or absence of GA_3 were not changed from the results shown in Fig. 37. The α-amylase isosenzyme composition in the absence of GA_3also remained the same as that shown in Fig. 39 B, following germination. In contrast, in the presence of GA_3 a number of the uppermost isoenzymes which were found in the germinated immature whole seed (Fig. 39 A) appeared after 3 days germination but these were only weakly active. The high pI α-amylase shown in Fig. 39 B in this case also lost some of its activity.

To further ascertain the effect of the seed coat upon the synthesis of α-amylase a number of germination studies were carried out with de-embryonated mature and immature wheat in the presence and absence of the seed coat. The germination studies were carried out on samples of 5 kernels under the germination conditions

described in Part III. The germinated samples then were assayed for activity and isoenzyme composition as described in Part III. In each case the dissections were carried out as carefully as possible to ensure the least possible damage to the aleurone layer.

The α -amylase activities obtained in the presence, absence and partial absence of the seed coat are illustrated in Table III. The α-amylase isoenzyme composition of the germinated samples in Table III are illustrated in Fig. 40. The immature de-embryonated wheat had the expected isoenzyme composition after 4 days of germination in the presence of GA_3 (Fig. 40 B). In comparison, the de-embryonated immature wheat which had its seed coat removed after 1 day germination exhibited little activity (Fig. 40 A) but the isoenzyme composition was basically the same as the control (Fig. 40 B). The isoenzyme composition exhibited by the germinated immature endosperm and aleurone (Fig. 39 B) was not found in this case. The mature Cypress, which was used for comparative purposes, displayed the same behavior as the immature samples after the same treatment (40 C, D). Finally, when only part of the seed coat was removed from the immature and mature wheat samples (as described in Table III) their isoenzyme compositions (Fig. 40 E, F) were basically the same as the control (Fig. 40 B, D).

TABLE III

GERMINATION OF DE-EMBRYONATED CYPRESS IN THE PRESENCE AND ABSENCE OF THE SEED COAT

Operation (In the presence of GA_3)	α-AMYLASE ACTIVITY (Mg Maltose/min/K)	
	Immature Cypress (39 days after flowering)	Mature Cypress
1 Presence Germinated for 4 days.	5.22	11.21
2 Absence The seed coat was removed after 1 day germination. The remaining endosperm and aleurone then was germinated for 3 additional days.	0.025	0.26
3 Partial Absence One half of the seed coat was removed after 1 day germination. The endosperm and aleurone and the remaining attached seed coat then were germinated for 3 additional days.	0.26	0.58

DISCUSSION

Both the immature whole kernel and de-embryonated whole kernels appear capable of synthesizing α-amylase during germination (Fig. 37). The synthesis of α-amylase by the immature whole kernel proceeded at a much faster rate and to higher activity levels in the presence of GA_3. The change in α-amylase activity in the presence of GA_3 was relatively the same as the change in activity shown by the mature whole kernel germinated with GA_3 (Fig. 38), although the activity level reached by the germinated mature kernel was more than double that of the immature whole kernel. In the absence of GA_3, the immature kernel did not shown any synthesis until after 4 days of germination which was unlike the mature whole kernel which showed substantial α-amylase synthesis beginning after 1 day germination. In both cases, in the absence of GA_3 the activity levels were substantially lower (Figs. 37 and 38) for the mature and immature whole kernels. The change in α-amylase activity during germination of the mature and immature whole kernels, in the presence of GA_3 was similar to that obtained by Khan *et al.* (1973) in the early stages of germination of the wheat

cultivar, Yorkstar. On the other hand, in later stages of germination Khan et al. (1973) found a decrease in activity which was not shown by either the mature or immature whole kernel. The results of Khan et al. (1973) for germination in the absence of GA_3 was similar to those observed for the mature Cypress whole kernel in the early stages of germination but again in the later stages of germination Khan et al. (1973) observed a decrease in activity which was not found with the mature Cypress (Fig. 38). This difference between the two cultivars simply could be indicative of a cultivar dependent characteristic. The lower activity levels reached by the immature whole kernel during germination both in the presence and absence of GA_3, as compared to the mature whole kernel may be a result of the dormancy phenomenon. This is most evident during germination without GA_3, where the immature kernel didn't show any activity increase or visible growth until after 4 days germination. Results reported by Miyamoto et al. (1961) may explain these differences between the activity results found for the mature compared to the immature wheat kernel. These authors studied the factors controlling dormancy in wheat and discovered that catechins and tannins, as well as other growth inhibitors, were involved in conferring dormancy to a wheat kernel. They also found that upon drying most of the inhibitors were lost concomitant with a loss in dormancy. The mature Cypress,

used in this study had a moisture content of 7% which was 10% lower than that of the immature kernel. Thus, this loss in moisture in going from the immature to completely mature kernel could have resulted in the loss of these inhibitors thus allowing the mature kernel to subsequently carry out normal α-amylase synthesis.

The de-embryonated immature wheat kernel showed much the same change in activity during germination, in the presence of GA_3 (Fig. 37) as the mature de-embryonated kernel (Fig. 38). The activity levels again were higher in the mature kernel and the mature kernel also showed its peak activity after a slightly longer germination period. The immature de-embryonated wheat kernel exhibited a more rapid increase in activity which could in part be due to the higher initial moisture level of the immature kernel. More specifically, although the embryo was removed from both the immature and mature kernel, which probably allowed for faster moisture uptake, the immature kernel may have been able to reach higher levels due to its higher initial level. This in turn may have led to a more rapid commencement of α-amylase synthesis. The activity changes of the germinating de-embryonated immature and mature wheat, in the presence of GA_3 are in agreement with the results of Khan et al. (1973) who studied embryoless wheat, as well as MacGregor (1976) for

barley distal half seeds and Goldstein and Jennings (1975) for de-embryonated maize kernels.

In the absence of GA_3, the de-embryonated immature and mature wheat did not show the same activity changes during germination. Surprisingly, the de-embryonated immature kernel even reached higher activity levels in the later stages of germination (Figs. 37 and 38). The reason for this result again may be an indication of the effects of the final desiccation of the immature wheat during maturation. As previously mentioned, immature wheat does contain GA_3 (Radley, 1976) but the GA_3 content of the grain decreases towards full maturity. Thus, the fully mature de-embryonated grain would contain very little endogenous GA_3, and one might expect a strong response to added GA_3 during germination. Conversely, the immature de-embryonated wheat has not completely dried and thus may still contain enough endogenous GA_3 to promote the synthesis of α-amylase and show a strong response in the absence of externally added GA_3. The reason that this behavior was not displayed in the whole seed germinations could be due to other intervening factors, such as the dormancy phenomenon. The results obtained for the de-embryonated mature kernel agreed with those of Khan et al. (1973) for wheat, and MacGregor (1976) for barley.

The immature endosperm and aleurone when germinated under the same conditions as the immature and mature whole

and de-embryonated kernels did not produce comparable levels of α-amylase activity. The activity level produced by the immature endosperm and aleurone during germination was significantly lower even though it has been shown that the aleurone is the source of α -amylase during germination (Filner and Varner, 1967; Gibson and Paleg, 1972, 1975). The immature endosperm and aleurone also did not exhibit activity changes comparable to the immature de-embryonated kernel even though these two samples were basically equivalent. In both instances the embryo was removed and each sample was at the same stage in maturity. The major difference between these two samples was the presence of the seed coat on the de-embryonated kernel. This would appear to implicate the seed coat as being involved in some capacity in the synthesis of α-amylase.

The α-amylase isoenzyme composition of the germinated mature and immature whole kernels were basically the same except for a number of very minor variations (Fig. 39 A, D). The two groups of isoenzymes found in germinated wheat also have been found by other workers (Kruger, 1972; Daussant and Renard, 1976). As noted in Part II, the isoenzyme composition as obtained by the isoelectric focusing technique had much greater resolution than previously used separational techniques. Thus, whereas Kruger (1972) found up to 5 isoenzymes in the top group; a total of up to 9 isoenzymes

were detected in this study (Fig. 39 A, B). Similarly, the bottom group contained from 5 - 6 isoenzymes in this study, whereas Kruger previously found 3. These results basically also agree with results obtained by Nagayoshi (1975) who used a disc-isoelectric focusing technique.

The α-amylase isoenzyme compositions of the germinated de-embryonated immature and mature whole seeds also were very similar to each other (Fig. 39 C, E). Some variation in the intensity of equivalent isoenzymes was visible and again these differences may have been due to the effects of the final drying of the wheat to full maturity. The germinated de-embryonated kernels, as with the whole kernels also exhibited a more complicated isoenzyme pattern due to the high resolution isoelectric focusing technique. More specifically, Khan et al. (1973) found 4 - 5 isoenzymes in the upper and 4 in the lower isoenzyme group as compared to 9 in the upper, up to 4 in the intermediate and up to 8 in the lower group of α-amylase isoenzymes found in this study.

Although the germinated immature and mature de-embryonated kernels basically had the same isoenzyme composition they exhibited a decidedly different isoenzyme composition from the germinated whole mature and immature kernels. The isoenzyme composition of the upper groups were comparable in both instances but the intermediate and lower groups showed major differences. The germinated

de-embryonated kernels showed a larger number of strong intermediate group of isoenzymes many of which were not present in the germinated whole kernels. Similarly, the bottom group of isoenzymes in the germinated de-embryonated kernel contained a number of additional isoenzymes besides those found in the germinated whole seed. In addition, the lower group of isoenzymes in the germinated de-embryonated kernel were much more active relative to the top isoenzyme group than was the case in the germinated whole seed. Since the embryo was the missing tissue in the de-embryonated seed, it would appear that it is involved in the control of synthesis of the lower isoenzyme group. Normally the embryo is considered to be the source of the gibberellins, such as GA_3 which promote the synthesis of α-amylase (Briggs, 1973) but these results suggest that the embryo also contains other factors responsible for the inhibition of synthesis. Conclusions similar to this also have been suggested by Khan et al (1973).

The isoenzyme composition of the germinated immature endosperm and aleurone (Fig. 39 B) bore little resemblance to the isoenzyme composition of the immature or mature whole or de-embryonated kernels. Again, some resemblance would have been expected especially between the germinated immature endosperm and aleurone and the germinated de-embryonated immature kernel. This resemblance was not displayed to a large extent and confirms the suggestion

made previously that the seed coat is in some manner involved with the synthesis of α-amylase.

In all the above cases, the α-amylase isoenzyme composition did not change in the presence or absence of GA_3. This is in agreement with the results of Khan et al. (1973) for the whole wheat kernel but disagrees with his observed results for embryoless wheat which indicated that GA_3 was required to synthesize any α-amylase isoenzymes. In contrast, MacGregor (1976) found that the presence of GA_3 preferentially enhanced the formation of two α-amylase isoenzymes during the germination of barley.

The germination study which entailed the addition of a seed coat homogenate to the germinating immature endosperm and aleurone was designed to determine if a seed coat factor was involved in the synthesis of α-amylase. The results were inconclusive in that the activity change in this study was comparable to that shown in Fig. 37 whereas in the presence of GA_3, there appeared to be some synthesis of the top isoenzyme group which was not found previously in the germinated immature endosperm and aleurone (Fig. 39 B). A more definitive study would be required to settle the apparent contradiction in the above results. The germination of de-embryonated mature and immature wheat in the presence and absence of the seed coat further illustrated the involvement of the seed coat in the synthesis of α-amylase.

In the presence of the seed coat both the mature and immature de-embryonated wheat synthesized large quantities of α-amylase as shown in Table III. In contrast, in the absence of the seed coat the α-amylase activity was drastically reduced in both the mature and immature samples as illustrated by operation 2 in Table III. The partial removal of the seed coat (operation 3, Table III) also decreased the α-amylase synthesis drastically but not to the extent shown by complete removal of the seed coat.

The α-amylase isoenzyme composition of the germinating mature and immature de-embryonated wheat in the absence and partial absence of the seed coat (Fig. 40 A,C,E,F) was comparable to the isoenzyme composition of the germinated control samples (Fig. 40 B, D). Although the isoenzyme activity was much lower in the absence and partial absence of the seed coat, the anomalous isoenzyme composition of the germinated immature endosperm and aleurone (Fig. 39 B) was not found in either case. It must be realized that the seed coat was not removed or partially removed until after one day germination of the de-embryonated kernel, as noted in operations 2 and 3, Table III. Thus, if the seed coat remained attached for the first day of germination it was sufficient to give the normal isoenzyme composition (Fig. 40) not the anomalous isoenzyme composition (Fig. 39 B) but was not sufficient to yield normal activity levels (Table III). This could further indicate that some factor or factors in

the seed coat are required for normal α-amylase synthesis and/or the removal of the seed coat may in some way disrupt the aleurone such that normal synthesis and secretion of the enzyme cannot take place. To delineate the seed coat effect further more comprehensive and detailed studies will have to be carried out.

PART V

THE RELATIONSHIP OF SEED DORMANCY TO α -AMYLASE LEVELS

INTRODUCTION

Up to this point, the studies performed have produced a much better understanding of the wheat α-amylase system. Unfortunately, they have not led to a practical solution to the problem of high α-amylase levels in wheat. As a possible means of rectifying this situation a study was undertaken, in an attempt to learn more about the control of α-amylase in the wheat kernel.

This study was prompted by the recent work of Heydecker et al. (1973) on the pretreatment of onion seeds with the osmotic agent polyethylene glycol (P.E.G. (Carbowax 6000)). It was discovered that during treatment with this agent, the seed reaches elevated moisture levels with out the appearance of any visible sprouting. Thus, by observing the effects of osmotic pretreatment on the α-amylase system, in dormant and non-dormant wheat, it was hoped that a better understanding of the control processes in the wheat kernel, would be obtained. In turn it was felt that a clearer insight into these control processes could lead to a practical solution to the problem of high α-amylase levels in wheat.

MATERIALS AND METHODS

The Canadian hard red spring wheat, unlicensed cultivar RL4137, was used in all the studies due to the high levels of dormancy associated with this cultivar. This cultivar was grown in a growth cabinet and harvested at full ripeness i.e. at a moisture content of 7%. The dormancy of the wheat was checked immediately following harvest by placing 100 kernels on filter paper moistened with 4 ml of water and determining the percentage of newly germinated wheat kernels each day at 18.5^{o}C and 99% humidity. Following the dormancy check 10 kernels which had not sprouted during the test were selected and dried for future use.

Immediately following harvest wheat also was osmotically pretreated with P.E.G. (Carbowax 6000) (25.7%). This P.E.G. (Carbowax 6000) solution, which had an osmotic potential of -15 bar, as determined from the results of Williams and Shaykewich (1969), was the most suitable since it did not allow any germination to take place even when used in the pretreatment of non-dormant wheat. Pretreatment was carried out in 100 kernel batches of dormant and non-dormant (control) wheat. The kernels were placed

on filter paper moistened with 6 ml of P.E.G. (Carbowax 6000) solution at 18.5°C and 99% humidity for a period of 8 days. Following pretreatment the wheat was thoroughly washed with distilled water and then air dried.

One hundred pretreated dormant kernels were then tested for their dormancy levels as previously described. Pretreated and untreated samples were analyzed in 10-kernel batches for their isoenzyme composition as described in Part III.

RESULTS AND DISCUSSION

The results of this study showed that osmotic pretreatment with P.E.G. (Carbowax 6000) did increase dormancy levels of the HRS wheat RL4137. The percentage of newly germinated kernels showed a decided increase in the pretreated kernels as shown in Fig. 41. The pretreatment of the wheat did not appear to accelerate germination to the extent found by Heydecker et al. (1973) for onion seeds.

The effect that this treatment had upon the α-amylase isoenzyme activity, as shown in Fig. 42, has several interesting implications. Firstly, the non-dormant pretreated wheat showed sizeable isoenzyme activity in the upper group of isoenzymes yet the lower group of isoenzymes basically was undetected (Fig. 42). This result suggests that even though the pretreated non-dormant kernel showed no growth, the aleurone still could synthesize α-amylase. This would be relevant in the visual grading of wheat which relies on the appearance of chiting as an indicator of sprouting and an increase in α-amylase activity. These results suggest that the levels of α-amylase may increase even though there is no visible sprout damage. The

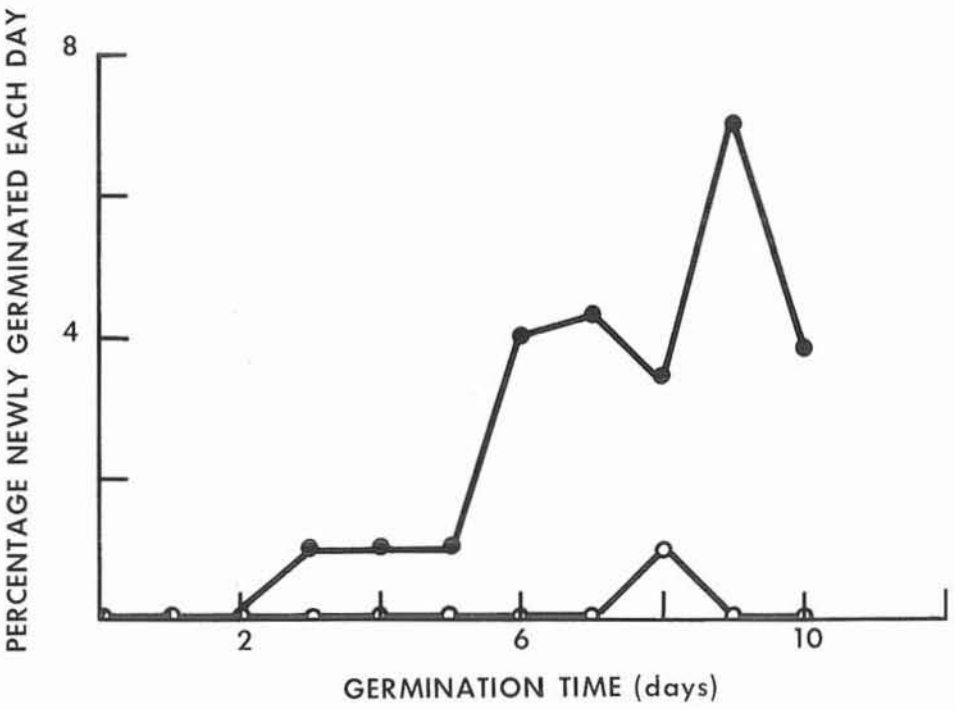
Fig. 41. Germination of dormant HRS wheat, cultivar RL4137, before osmotic pretreatment 0-0-0 and after osmotic pretreatment in P.E.G. (Carbowax 6000) of -15 bar potential **0-0-0**.

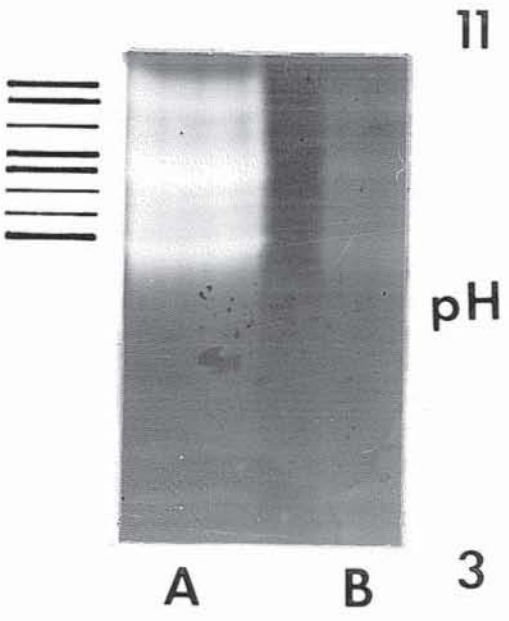
Fig. 42. α-Amylase isoenzyme composition of:

A. Non-dormant wheat, cultivar RL4137, osmotically pretreated in P.E.G. (Carbowax 6000) of -15 bar potential,

B. Dormant wheat, cultivar RL4137, which remained unsprouted after 10 days under germination conditions at 18.5°C and 99% humidity.

The α-amylase isoenzyme composition of osmotically pretreated and untreated dormant RL4137 was also studied but isoenzyme activity was not detectable.


PERCENTAGE NEWLY GERMINATED EACH DAY
8
4
2
6
10
GERMINATION TIME (days)


11
pH
3
A
B

fact that the lower isoenzyme group was basically undetectable could be a further indicator of control over these isoenzymes by the embryo, as was suggested in Part IV. It has been shown previously (Part III) that concomitant with embryo growth there is a synthesis of α-amylase and in Part IV it also was observed that removal of the embryo caused an oversynthesis of the enzyme. Thus in this study, pretreatment effectively stopped any embryo growth and, as a consequence, this tissue could not synthesize α-amylase. But since the embryo still remained attached it could still exert control over the aleurone and not allow for the oversynthesis of the bottom isoenzyme group. This also may explain the differences in the activity of the upper group isoenzymes relative to each other in this case (Fig. 42) compared to that found in normal germinated wheat (Fig. 39 D).

In comparison, the pretreated dormant wheat showed no α-amylase isoenzyme activity. This then shows that the dormancy phenomenon also has control over enzyme synthesis by the aleurone. As a control, the dormant wheat which had shown no growth during the dormancy test also was checked for isoenzyme activity. Slight activity was noted in the upper isoenzyme group (Fig. 42) but this activity was much lower than in the pretreated non-dormant wheat (Fig. 42). This is a further illustration that α-amylase synthesis is controlled while the kernel remains dormant. This is a good

indication that a dormant wheat cultivar will retain low enzyme levels prior to harvest, even it is subjected to harsh environmental conditions conducive to germination and the synthesis of high enzyme levels.

GENERAL DISCUSSION AND CONCLUSIONS

Wheat α-amylase is now recognized for its important role in the breadmaking process. This enzyme can have a major influence on gassing power, loaf volume, texture, crumb and crust color. High α-amylase levels in a flour also result in excessive liquefaction and dextrinization which yield an inferior loaf with a wet sticky crumb (Geddes, 1946; Bechtel et al., 1964; Bloksma, 1971). The high α-amylase levels found in wheat mainly arise via pre-harvest sprouting but alternately, the reactivation of the immature wheat α-amylase system may also produce high enzyme levels (Olered and Jonsson, 1970). The production of high α-amylase levels concomitant with pre-harvest sprouting or reactivation substantially decreases the quality and consequently the market value of wheat. This problem, which is encountered in many countries including Canada has provided the incentive for an intense study of α-amylase. The main objective of these studies is eventually to achieve a practical solution to the problem of high α-amylase levels in wheat. One of the primary steps on the road to attaining this objective is the acquisition of detailed knowledge concerning the wheat α-amylase system.

To this end a detailed study of the α-amylase isoenzyme system of immature Canadian-grown wheat cultivars was performed.

The initial phase in the study of the immature wheat α-amylase system was the development of a suitable α-amylase assay which did not suffer from the drawbacks encountered in the numerous conventional or traditional methods. This was accomplished by adapting the fluorometric assay of Rinderknecht and Marback (1970)for the study of wheat α-amylase. The resultant method enjoyed many advantages such as high sensitivity, small sample size, short analysis time, and no interference by variations in starch properties or excess β-amylase. This method was ideally suited for use in the study of the immature α-amylase system and for the determination of α-amylase levels in flour.

The fluorometric method then was employed to determine the changes in the α-amylase levels during kernel growth and maturation for 10 Canadian-grown wheat cultivars. The 10 wheat cultivars essentially exhibited similar α-amylase activity changes (Figs. 11 and 12). Minor cultivar variations were detected but they could not be related to quality or the incidence of high α-amylase levels at maturity. Possibly a prolonged study over a number of years and under various environmental conditions would provide such information.

The α-amylase isoenzyme composition of the 10 wheat

cultivars changed during kernal growth and maturation. In the initial stages of kernel growth the α-amylase activity was derived from the GI isoenzymes (Figs. 23 - 27). As growth and maturation continued the GI isoenzymes decreased in activity concomitant with an increase in GIII isoenzyme activity (Figs. 23 - 27). α-Amylase isoenzymes (GII isoenzymes) also were detected between the GI and GIII groups of isoenzymes but they contributed little activity to the whole kernel. Cultivar variations were exhibited in each of the GI, GII and GIII isoenzyme groups. There was some indication that these variations could be linked to quality as well as being used for cultivar identification. To determine if such a link does exist, further studies would be required with a large number of samples grown under various environmental conditions.

The pericarp contained the majority of the GI α-amylase isoenzyme activity in the early stages of kernel growth although the seed coat also contained substantial GI isoenzyme activity. The endosperm and aleurone, embryo and scutellum only contained low levels of GI isoenzyme activity. The most striking feature of the α-amylase isoenzyme composition of immature wheat was the appearance of GIII isoenzyme activity during kernel growth and maturation.

The GIII isoenzymes were located mainly in the endosperm although they were detectable in the seed coat and scutellum and to a slight degree in the embryo. The GIII group of isoenzymes bore a close resemblance, with respect to pI and isoenzyme pattern, to an α-amylase enzyme group present in germinating wheat (Fig. 30). This similarity strongly suggests that the two groups of isoenzymes are equivalent. In turn, this would indicate that immature wheat can synthesize α-amylase isoenzymes previously thought to be synthesized only in germinating wheat. This implies that immature and germinating wheat have the same α-amylase synthetic machinery but in each case the majority of synthesis is directed either towards the GI isoenzymes in the immature kernel or the GIII-type isoenzymes in the germinating mature kernel.

All of the cultivars studied contained GIII isoenzyme activity but the activity of this isoenzyme group varied among the cultivars. Since GIII isoenzyme activity is mainly associated with the germinating kernel, the intensity of the isoenzymes in the immature kernel may be related to susceptibility of cultivars to the problem of high α-amylase levels. Such a relationship could be ascertained by quantitatively determining the GIII isoenzyme activity in a number of immature wheat cultivars grown under various environmental conditions. This activity

then would have to be related to the incidence of high α-amylase levels in the mature cultivars at harvest.

These results also indicated that the endosperm contained detectable levels of GI, GII and GIII α-amylase isoenzymes. This is surprising in view of the starch synthesis taking place in this tissue during kernel growth and maturation. One would reasonably assume that the α-amylase would interfere with the process of starch synthesis by degrading the starch as it was being formed. Possibly, as has been suggested by Jenkins et al. (1974) the α-amylase isoenzymes play some role in starch synthesis. Debranching enzymes also were located in this tissue and presumably these enzymes were involved in starch synthesis.

The studies employed to determine the validity of the theory that immature wheat can synthesize germinating wheat α-amylase isoenzymes i.e. GIII-type isoenzymes proved this theory correct. The embryo and scutellum and endosperm and aleurone were able to synthesize GIII isoenzymes under typical germination conditions. Since these isoenzymes are mainly produced in the germinating wheat kernel and not the immature wheat kernel, it illustrates the large degree of control placed on α-amylase synthesis in the immature kernel. The presence of low activity levels of GIII isoenzymes in the immature kernel probably is evidence that the controls are not 100% efficient, enabling a small amount of GIII isoenzyme synthesis to take place in the immature kernel.

This study also provided further evidence that the embryo and scutellum do synthesize α-amylase during germination as suggested by Briggs (1964, 1972, 1973). Later studies also indicated that the embryo exerted a great deal of control over the GI-type isoenzyme group in the germinating wheat kernel. This control appeared to be directed towards α-amylase synthesis in the aleurone. More specifically if the embryo was present in the germinating mature wheat kernel synthesis of the GI- and GII-type isoenzymes was considerably lower than the GIII-type isoenzymes. However, if the embryo was removed, synthesis of the GI- and GII-type isoenzymes increased to a level comparable with the GIII-type isoenzymes (Fig. 39). The conclusion from these results is that in the germinating wheat kernel the embryo must exert a control over the synthesis of the GI- as well as the GII-type isoenzymes by the aleurone. This control of course is in conjunction with promotional effects exerted by the gibberellins produced in the embryo.

The changes which took place in α-amylase activity (Figs. 11 and 12) and isoenzyme composition (Figs. 23 -27) of immature wheat during kernel growth and maturation, also intimate that the synthesis and possibly loss of this enzyme may be under some system of control. It has been suggested that this control involves endogenous growth substances such as the gibberellins and ABA

(Duffus, 1969; King, 1976; Radley,1976). In view of the overlapping changes in concentration of these substances (King, 1976; Radley, 1976) during kernel growth and maturation, perhaps the balance between their concentration levels is another factor which should be considered as part of the control system.

Throughout this study various results indicated the importance of the final desiccation of the wheat kernel. Although the moisture loss during this period was minimal in going from about 20% to around 10%, it affected α-amylase synthesis significantly, as was illustrated by comparison of the α-amylase synthesis by the immature and mature; embryo and scutellum; whole; de-embryonated kernels. These results imply that even though a wheat kernel is physiologically mature (i.e. all the tissues have completed their growth and development) it cannot be considered mature until the final desiccation takes place.

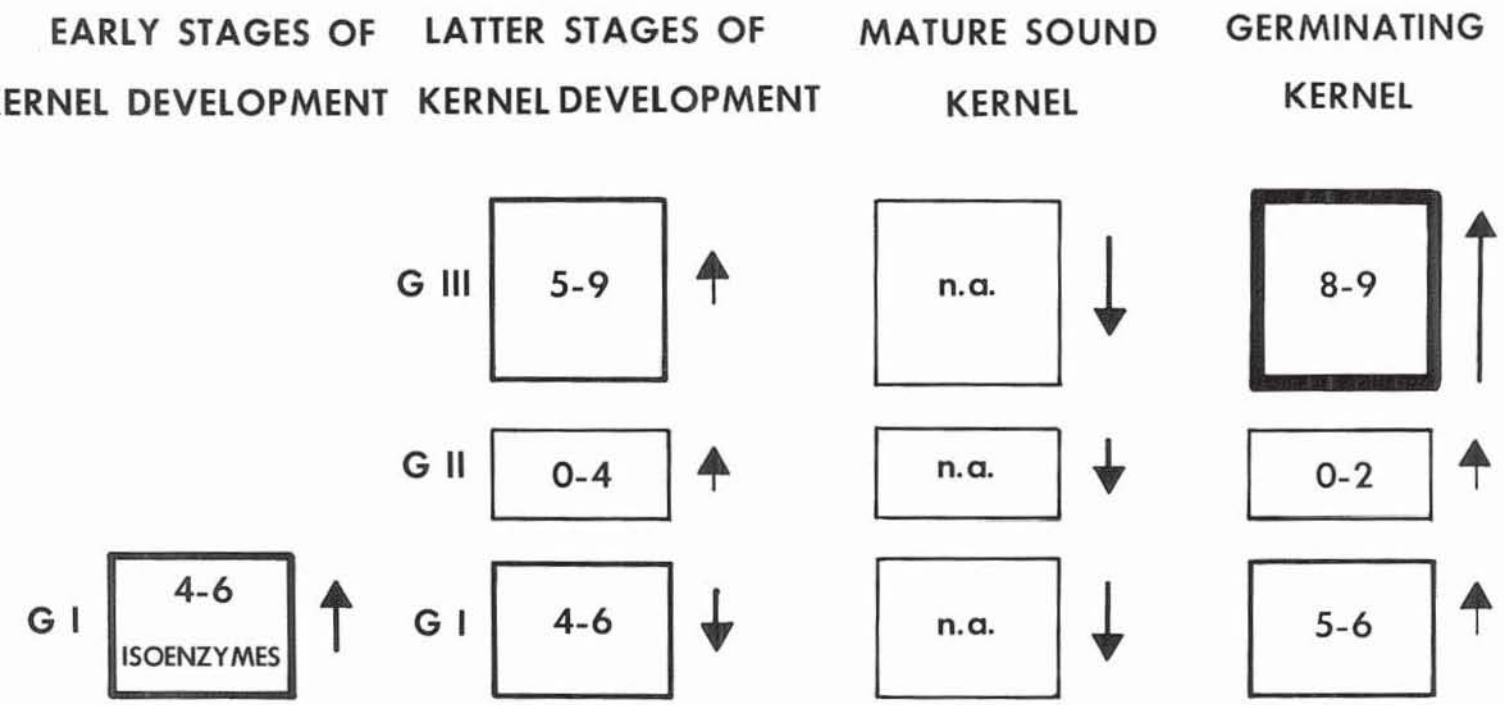
Germination of the final developmental stage endosperm and aleurone produced anomalous results which suggested that the seed coat in some capacity is involved with the synthesis of α-amylase. Further studies with immature and mature wheat provided additional evidence that the removal of the seed coat inhibited the normal synthesis of α-amylase. Possibly some factor (s) present in the seed coat are required for normal synthesis of the enzyme. These factors could be involved with secretion

of the enzymes as well as the actual synthesis. Alternately, the removal of the seed coat could physically affect the aleurone so that α-amylase synthesis is inhibited.

This study of the wheat α-amylase system has indicated that it is more complicated that previously thought. The results obtained have clarified the changes which take place in the α-amylase isoenzyme system during the life cycle of the wheat kernel. These changes are most easily represented diagramatically as shown in Fig. 43. From a more practical point of view, based on the results of this study, one may suggest a possible solution to the problem of high α-amylase levels in mature wheat. More specifically, from studies of germination of the immature whole kernel, (Fig. 37) it would appear that the synthesis of α-amylase could be controlled by the presence of dormancy. This was confirmed in the polyethylene glycol study Part V when it was found that a dormant wheat under ideal conditions would not produce high α-amylase levels. In contrast, non-dormant wheat, which was treated with P.E.G. (Carbowax 6000) to prevent sprouting still produced high α-amylase levels under identical germination conditions. This implies that the practical solution to the problem of high α-amylase levels in wheat at harvest is to breed for wheat with a high degree of dormancy. Unfortunately, other studies (Boyd et al., 1971) have indicated that dormancy

Fig. 43. Generalized schematic representation of the changes in the α-amylase isoenzyme composition at various stages during the life cycle of a wheat kernel. The density of the boxes represents the relative activities of the isoenzymes. ↑ and ↓ designates increasing or decreasing activity, respectively . Isoenzyme composition is variable due to cultivar variations or length of germination. N.A. is equivalent to negligible activity.

CHANGES IN THE α-AMYLASE ISOENZYME SYSTEM DURING THE LIFE CYCLE OF A WHEAT KERNEL



also plays a significant role in seedling vigor. Therefore caution would have to be demonstrated if wheat was bred for higher dormancy. Consideration also must be given to the work of Gale (1976), who has shown that the dwarf varieties Tom Thumb and Minister dwarf carry a GA-insensitive gene. These varieties exhibit very small increases in α-amylase activity in response to added GA and they also display lower residual α-amylase activities at maturity. Thus, the use of this gene to control α-amylase synthesis also is very attractive.

In conclusion, ths wheat α-amylase system, which is now more clearly understood, could serve as an ideal enzyme system to utilize as an aid in the study of the dormancy phenomenon.

BIBLIOGRAPHY

Alexandrescu, V., and Mihailescu, F. 1970. Electrophoretic study of wheat α-amylases during the first growing stages. Rev. Roum. Biochim. 7:3-8.

Alexandrescu, V., and Mihailescu, F. 1973. Immunochemical investigations of germinated seeds endosperms α-amylase of some cereals. Rev. Roum. Biochim. 10:89-94.

Alexandrescu, V., Mihailescu, F., and Paun, L. 1975. Amylases in the endosperms of wheat, rye and triticale germinated seeds. 1. Electrophoretic and immunoelectrophoretic investigations. Rev. Roum. Biochim. 12:3-6.

Allen, W.G., and Spradlin, J.E. 1974. Amylases and their properties. Bakers Digest. 48(3):14-57.

Allison, M.J., Ellis, R.P., and Swanston, J.S. 1974. Tissue distribution of α-amylase and phosphorylase in developing barley grain. J. Inst. Brew. 80:488-491.

American Association of Cereal Chemists. 1975. "AACC Approved Methods". St. Paul, Minn.

Banks, W., Evers, A.D., and Muir, D.D. 1972. The location of α-amylase in developing cereal grain. Chem. Ind. 14:573-574.

Barnes, W.C., and Blakeney, A.B. 1974. Determination of cereal alpha amylase using a commercially available dye-labelled substrate. Staerke. 26:193-197.

Bechtel, W.G., Geddes, W.G., and Gilles, K.A. 1964. Carbohydrates. In: Wheat Chemistry and Technology., I. Hlynka, ed. American Association of Cereal Chemists, St. Paul, Minn. 277-352.

Bernfeld, P., Duchert, F., and Fischer, E.H. 1950. Propriétés de l'α-amylas de pancreas humain. Comparison avec les autres α-amylases cristallisées. Sur les enzymes amylolytiques XV. Helv. Chim. Acta. 33:1064-1070.

Bernfeld, P., Staub, A., and Fischer, E.H. 1948. Sur les enzymes amylolytiques XI. Propriétés de l'α-amylase de salvic humaine cristallisée. Helv. Chim. Acta. 31:2165-3172.

Bilderback, D.E. 1971. Amylases in developing barley seeds. Plant Physiol. 48:331-334.

Bilderback, D.E. 1973. A simple method to differentiate between α- and β-amylase. Plant Physiol. 51:594-595.

Bilderback, D.E. 1974. Amylases from aleurone layers and starchy endosperm of barley seeds. Plant Physiol. 53: 480-484.

Blish, M.J., Sandstedt, R.M., and Mecham, D.K. 1937. Action of wheat amylases on raw wheat starch. Cereal Chem. 14:605-628.

Bloksma, A.H. Rheology and Chemistry of Dough. 1971. In: Wheat Chemistry and Technology., Y. Pomeranz, ed. American Association of Cereal Chemists, St. Paul, Minn. 523-584.

Boyd, W.J.R., Gordon, A.G., and LaCroix, L.J. 1971. Seed size, germination resistance and seedling vigor in barley. Can. J. Plant Sci. 51:93-99.

Briggs, D.E. 1961. A modification of the Sandstedt, Kneen and Blish assay of α-amylase. J. Inst. Brew. 67:427-431.

Briggs, D.E. 1962. Development of enzymes by barley embryos in vitro. J. Inst. Brew. 68:470-475.

Briggs, D.E. 1963. Effects of gibberellic acid on barley germination and its use in malting: A Review. J. Inst. Brew. 69:244-248.

Briggs, D.E. 1964. Origin and distribution of α-amylase in malt. J. Inst. Brew. 70:14-24.

Briggs, D.E. 1972. Enzyme formation, cellular breakdown and the distribution of gibberellins in the endosperm of barley. Planta (Berl.). 108:351-358.

Briggs, D.E. 1973. Hormones and carbohydrate metabolism in germinating cereal grains. In: Biosynthesis and its Control in Plants, B.V. Millborrow, ed. Academic Press, New York and London. 219-277.

Briggs. D.E., and Clutterbuck, V.J. 1973. Generation of α-amylase in germinating Hordeum Distichon. Phytochemistry. 12:1047-1050.

Brown, H.T., and Escombe, F. Cited In Briggs, D.E. 1963. Effects of gibberellic acid on barley germination and its use in malting: A Review. J. Inst. Brew. 69:244-248.

Brown, H.T., and Morris, G.H. Cited In Briggs, D.E. 1963. Effects of gibberellic acid on barley germination and its use in malting: A Review. J. Inst. Brew. 69:244-248.

Carr, J.G., and Spillane, P.A. 1969. Some factors affecting amylograph behavior in Irish flours. J. Sci. Fd. Agric. 20:638-641.

Chojnacki, G., Brykczyinski, J., and Tymieniecka, E. 1976. Preliminary information on sprouting Triticale. Cereal Res. Commun. 4:111-114.

Chrazaszcz, T., and Janicki, J. 1936. Amylase during the growth and ripening of grains. Biochem. J. 30:1298-1302.

Chrispeels, M.J., and Varner, J.E. 1967. Gibberellic acid-enhanced synthesis and release of α-amylase and ribonuclease by isolated barley aleurone layers. Plant Physiol. 42: 398-406.

Daussant, J., and Renard, M. 1972. Immunochemical comparisons of α-amylases in developing and germinating wheat seeds. FEBS. Letters. 22:301-304.

Daussant, J., and Renard, M. 1976. Immunochemical identification of α-amylases in developing and germinating wheat seeds. Cereal Res. Commun. 4:201-212.

Daussant, J., Skakoun, A., and Niku-Paavola, M.L. 1974. Immunochemical study on barley α-amylases. J. Inst. Brew. 80:55-58.

Doane, W.W. 1967. Quantitation of amylases in Drosphilia separated by acrylamide gel electrophoresis. J. Exp. Zool. 164:363-378.

Dronzek, B.L., Hwang, Paulina, and Bushuk, W. 1972. Scanning electron microscopy of starch from sprouted wheat. Cereal Chem. 49:232-239.

Duffus, C.M. 1969. α-Amylase activity in the developing barley grain and its dependance on gibberellic acid. Phytochem. 8:1205-1209.

Duffus, C., and Rosie, R. 1973. Starch hydrolyzing enzymes in the developing barley grain. Planta (Berl.). 109:153-160.

Dygert, S., Li, L.H., Florida, D., and Thoma, J.A. 1965. Determination of reducing sugar with improved precision. Anal. Biochem. 13:367-374.

Eikenberry, J.W. 1977. Fluorometry of α-Amylase. Clin. Chem. 23:766.

Farrand, E.A. 1964. Flour properties in relation to the modern bread process in the United Kingdom, with special reference to alpha-amylase and starch damage. Cereal Chem. 41:98-111.

Fernley, H.N. 1963. The use of reactive dyestuffs in enzymology: New substrates for cellulolytic enzymes. Biochem. J. 87:90-95.

Filner, P., and Varner, J.E. 1967. A test for de novo synthesis of enzymes: Density labelling with H_2O^{18} of barley α-amylase induced by gibberellic acid. Proc. Nat. Acad. Sci. U.S.A. 58:1520-1526.

Fischer, E.H., and Stein. E.A. α-Amylases. 1960. In: The Enzymes, P.D. Boyer, H. Lardy and K. Myrback, ed. Academic Press, New York. 4:313-343.

Frydenberg, D., and Nielsen, G. 1966. Amylase isoenzymes in germinating barley seeds. Heredatis. 54:123-139.

Gale, M.D. 1976. High α-amylase - Breeding and genetical aspects of the problem. Cereal Res. Commun. 4:201-212.

Geddes, W.F. 1946. The amylases of wheat and their significance in milling and baking technology. Advanc. Enzymol. 6:415-468.

Gibson, R.A., and Paleg, L.G. 1972. Lysosomal nature of hormonally induced enzymes in wheat aleurone cells. Biochem. J. 128:367-375.

Gibson, R.A., and Paleg, L.G. 1975. Further experiments on the α-amylase-containing lysosomes of wheat aleurone cells. Aust. J. Plant Physiol. 2:41-49.

Goldstein, L.D., and Jennings, P.H. 1975. The occurence and development of amylase enzymes in incubated de-embryonated maize kernels. Plant Physiol. 55:893-898.

Grabar, P., and Daussant, J. 1964. Study of barley and malt amylases by immunochemical methods. Cereal Chem. 41:523-532.

Greenwood, D.T., and MacGregor, A.W. 1965. The isolation of α -amylase from barley and malted barley, and a study of the properties and action patterns of the enzymes. J. Inst. Brew. 71:405-417.

Greenwood, C.T., and Milne, E.A. 1968. Studies on starch degrading enzymes. Part VIII. A comparison of α-amylases from different sources: their properties and action patterns. Staerke. 20:139-150.

Guilbaut, G.G. 1977. Fluorometry of α-amylase. Clin. Chem. 23:767.

Heydecker, W., Higgins, J., and Gulliver, R.L. 1973. Accelerated germination by osmotic seed germination. Nature. 246:42-44.

Hill, R.D., Klassen, A.J., and Dedio. W. 1974. Metabolic factors influencing kernel development in Triticale. In: Triticale: proceedings of an international symposium El Batan, Mexico. Ed. R. MacIntyre and M. Campbell.

Hlynka, I. 1968. Amylograph mobility and liquefaction number. Cereal Sci. Today. 13:245-254.

Iliev, S.V. 1974. Amylase isoenzymes in developing wheat seeds. Comptes rendus de l'Academie Bulgare des Sciences. 27:113-116.

International Association for Cereal Chemistry. Determination of "falling number" (according to Hagberg-Perten) as a measure of the degree of alpha-amylase activity in grain and flour. ICC Standard No. 107.

Jacobsen, J.V., and Knox, R.B. 1973. Cytochemical localization and antigenicity of α-amylase in barley aleurone tissue. Planta. 112:213-224.

Jenkins, L.D., Loney, D.P., and Meredith, P. 1974. Periodicity of growth and starch deposition in the developing wheat grain. Cereal Chem. 51:718-733.

Jenkins, L.D., and Meredith, P. 1975. Grain development and amylase activities in rye and triticale compared with wheat. N.Z. Journal of Science. 18:189-194.

Johnson, J.A., and Miller, B.S. 1953. Enzyme systems important in breadmaking. Wallerstein Lab. Comm. 16: 105-115.

Jones. R.L., and Chen, R. 1976. Immunohistochemical localization of α-amylase in barley aleurone cells. J. Cell. Sci. 20:183-198.

Khan, A.A., Verbeek, R., Waters, E.C., Jr., and Van Onchelen, H.A. 1973. Embryoless wheat grain. Plant Physiol. 51:641-645.

King, R.W. 1976. Abscisic acid in developing wheat grains and its relationship to grain growth and maturation. Planta (Berl.). 132:43-51.

Kirchoff, G.S.C. Cited In Fischer, E.H., and Stein, E.A. α-Amylases. In: The Enzymes. 1960. P.D. Boyer, H. Lardy and K. Myrback, ed. Academic Press, New York. 4:314.

Kruger, J.E. 1972a. Changes in the amylases of hard red spring wheat during growth and maturation. Cereal Chem. 49:379-390.

Kruger, J.E. 1972b. Changes in the amylases of hard red spring wheat during germination. Cereal Chem. 49:391-398.

Kruger, J.E. 1973. Changes in the levels of proteolytic enzymes from hard red spring wheat during growth and maturation. Cereal Chem. 50:122-131.

Kruger, J.E., and Marchylo, B. 1972. The use of reduced β-limit dextrin as substrate in an automated amylase assay. Cereal Chem. 49:453-458.

Kruger, J.E., and Tkachuk, R. 1969. Wheat alpha-amylases. 1. Isolation. Cereal Chem. 46:219-225.

Kuhn, R. 1924. Ueber die konstitution der starke and die verschiedenen wirkungsivusen der amylasen. Ber. 57: 1965-1968.

Kuhn, R. 1925. Mechanism of the action of amylases. Constitution of starch. Ann. 443:1-71. Chem. Abstracts. 19: 3098-3099.

LaBerge, D.E., MacGregor, A.W., and Meredith, W.O.S. 1971. Changes in alpha and beta-amylase activities during the maturation of different barley cultivars. Can. J. Plant Sci. 51:469-477.

LaCroix, L.J., and Lier, J.B. 1975. Carotenoids in durum wheat: Developmental patterns during two growing seasons. Can. J. Plant Sci. 55:679-684.

Lee, W.Y., and Unrau, A.M. 1969. Alpha-Amylase of a synthetic cereal species. J. Agr. Food Chem. 17:1306-1311.

Lintner, C.J. 1886. Studien ueber diastase. J. Prakt. Chem. 34:378-394.

MacGregor, A.W. 1976. A note on the formation of -amylase in de-embryonated barley kernels. Cereal Chem. 53:792-796.

MacGregor, A.W. 1977. Isolation, purification and electrophoretic properties of an α-amylase from malted barley. J. Inst. Brew. 83:100-103.

MacGregor, A.W., Gordon, A.G., Meredith. W.O.S., and LaCroix, L. 1972. Site of α-amylase in developing barley kernels. J. Inst. Brew. 78:174-179.

MacGregor, A.W., LaBerge, D.E., and Meredith, W.O.S. 1971. Changes in barley kernels during growth and maturation. Cereal Chem. 48:255-269.

MacGregor, A.W., and Meredith, W.O.S. 1971. A low pH disc electrophoresis system for studying -amylase enzymes. J. Inst. Brew. 77:510-512.

MacGregor, A.W., Thompson, R.G., and Meredith, W.O.S. 1974. α-Amylase from immature barley. Purification and properties. J. Inst. Brew. 80:181-187.

MacLeod, A.M., and Millar, A.S. 1962. Effects of gibberellic acid on barley endosperm. J. Inst. Brew. 68:322-332.

MacLeod, A.M., and Palmer, G.H. 1966. The embryo of barley in relation to modification of the endosperm. J. Inst. Brew. 72:580-589.

MacLeod, A.M., and Palmer, G.H. 1967. Gibberellin from barley embryos. Nature. 216:1342-1343.

Manners, D.J., and Marshall, J.J. 1972. Studies on carbohydrate metabolising enzymes. Part XXVIII. Preparation and some properties of α-amylase isoenzymes from malted rye. Staerke. 24:3-8.

Marchylo, B. 1975. The α-amylase from immature hard red spring wheat; purification and characterization. M.Sc. thesis. University of Manitoba.

Marchylo, B., Kruger, J.E., and Irvine, G.N. 1976. α-Amylase from immature hard red spring wheat. I. Purification and some chemical and physical properties. Cereal Chem. 53:157-173.

Meredith, P. 1970. Inactivation of cereal α-amylase by brief acidification: the pasting strength of flour. Cereal Chem. 47:492-500.

Meredith, P., and Jenkins, L.D. 1973. Amylases of developing wheat, barley and oat grains. Cereal Chem. 50:243-254.

Merry, J. 1941. Studies on the embryo of Hordeum Sativum-I. The development of the embryo. Bulletin of the Torrey Club. 68:585-599.

Miyamoto, T., Tolbert, N.E., and Everson, E.H. 1961. Germination ingibitors related to dormancy in wheat seeds. Plant Physiol. 36:739-746.

Morrison, I.N., Kuo, J., and O'Brien, T.P. 1975. Histochemistry and fine structure of developing wheat aleurone cells. Planta. (Berl.). 123:105-116.

McMasters, D. 1974. Barley α-amylase isozymes induced by different gibberellins. Ph.D. thesis. University of Arkansas.

Nagayoshi, T. 1975. Studies on Amylase isozymes of Triticeae I. Variation of α -amylase isozymes in genus Triticum. Sci. Rep. Fac. Agr. Kobe Univ. 11:249-256.

Nishikawa, K. 1973. Alpha-Amylase isozymes and phylogeny of hexaploid wheat. In Proceedings of the Fourth International Wheat Genetus Symposium. E.R. Sears, and L.M.S. Sears, ed. Agricultural Experiment Station, College of Agriculture, University of Missouri. 851-855.

Nishikawa, K., and Nobuhara, M. 1971. Genetic studies of α-amylase isozymes in wheat. 1. Location of genes and variation in tetra and hexaploid wheat. Japan. J. Genetics. 46:345-353.

Ohlsson, E. 1930. The two components of malt diastase, especially with reference to the mutarotation of the products formed in the hydrolysis of starch. Z. Physiol. Chem. 189:17-63; Chem. Abstracts. 24:3521-3522.

Olered, R. 1967. Development of α-amylase and falling number in wheat and rye during ripening. In Vaxtodling. Plant Husbandry No. 23, Uppsala: Almquirst and Wiksells Boktryckeri AB.

Olered, R. 1976. α-Amylase isozymes in cereals and their influence on starch properties. Cereal Res. Commun. 4:195-199.

Olered, R. and Jonsson, G. 1970. Electrophoretic studies of α-amylase in wheat. II. J. Sci. Fd. Agric. 21:385-392.

Payen, A., and Persoz, J. 1833. Memoire sur la diastase, les principaux produits de ses reactions et leur applications aux arts industriels. Ann. Chem. Phys. 53:73-92.

Palmer. G.H. 1969. Increased endosperm modification of abraded barley grain after gibberellic acid treatment. J. Inst. Brew. 75:536-541.

Palmer, G.H. 1974. The industrial use of gibberellic acid and its scientific basis: A Review. J. Inst. Brew. 80:13-30.

Persson, E. 1976. Otello - a result of amylase selection for sprouting resistance. Cereal Res. Commun. 4:107-110.

Perten, H. 1966. A colorimetric method for the determination of alpha-amylase activity (ICC.method). Cereal Chem. 43:336-342.

Preston, K. and Kruger, J.E. 1976. Location and activity of proteolytic enzymes in developing wheat kernels. Can. J. Plant Sci. 56:217-223.

Radley, M. 1967. Site of production of gibberellin-like substances in germinating barley embryos. Planta (Berl.). 75:164-171.

Radley, M. 1968. Production of gibberellin-like substances in barley seed and seedlings. In: Plant Growth Regulators, S.C.I. Monograph. 31:53-69.

Radley, M. 1969. The effect of the endosperm on the formation of gibberellin by barley embryos. Planta (Berl.). 86:218-223.

Radley, M. 1976. The development of wheat grain in relation to endogenous growth substances. J. Exp. Bot. 27:1009-1021.

Riggs, T.J., and Gothard, P.G. 1976. The development of barley grain: comparisons between cultivars for growth rate and α-amylase activity. J. Agric. Sci. Camb. 86:603-608.

Rinderknecht, H. and Marback, E.P. 1970. A new automated method for the determination of serum α-amylase. Clin. Chim. Acta. 29:107-110.

Rowe, J.J.M., Wakim, J., and Thoma, J.A. 1968. Multiple forms of porcine pancreatic α-amylase. Anal. Biochem. 25: 206-220.

Rowsell, E.V., and Goad L.J. 1964. Some effects of gibberellic acid on wheat endosperm. Biochem. J. 90:11p.

Sandstedt, R.M. 1946. Photomicrographic studies of wheat starch. I. Development of the starch granules. Cereal Chem. 23:337-359.

Sandstedt, R.M., and Beckford, O.C. 1946. Photomicrographic studies of wheat starch. II. Amylolytic enzymes and the amylase inhibitor of the developing wheat kernel. Cereal Chem. 23:548-559.

Schwimmer, S. 1947. Development and solubility of amylase in wheat kernels throughout growth and ripening. Cereal Chem. 24:167-178.

Schwimmer, S., and Balls, A.K. 1949. Isolation and properties of crystalline α-amylase from germinated barley. J. Biol. Chem. 179:1063-1074.

Semenza, G., Curtius, H.-Ch., Raunhardt, O., Hore, P., and Mueller, M. 1969. The configurations of the anomeric carbon of the reaction products of some digestive carbohydrates. Carbohyd. Res. 10:417-428.

Smith, J.B., and Bennett, M.D. 1974. Amylase isozymes of oats (Avena sativa L.). J. Sci. Fd. Agric. 25:67-71.

Stoddart, J.L. 1971. Sequential changes in amylase isozymes during grain maturation in barley. Planta (Berl.) 97:70-82.

Strumeyer, D.H., and Romano, A.T. 1966. Automated assay of submicrogram levels of amylase by a reducing sugar procedure In: Automation in Analytical Chemistry, Technicon Symposia, Mediad: New York. I:469-473.

Takeuchi, T., Matsushima, T., and Sugimura, T. 1975. Separation of human α-amylase isozymes by electrofocusing and their immunological properties. Clin. Chim. Acta. 60:207-213.

Tipples, K.H. 1969. A viscometric method for measuring alpha-amylase activity in small samples of wheat and flour. Cereal Chem. 46:589-598.

Tipples, K.H., Kilborn, R.H., and Bushuk, W. 1966. Effect of malt and sprouted wheat. Cereal Sci. Today. 11:362-375, 416-418.

Tkachuk, R., and Kruger, J.E. 1974. Wheat α-amylases. II. Physical characterization. Cereal Chem. 51:508-529.

Varner, J.E. 1964. Gibberellic acid-controlled synthesis of α-amylase in barley endosperm. Plant Physiol. 39:413-415.

Varner, J.E., and Ram Chandra, G. 1964. Hormonal control of enzyme synthesis in barley endosperm. Proc. Nat. Acad. Sci. 52:100-106.

Varner, J.E., Ram Chandra, G., and Chrispeels, M.J. 1965. Gibberellic acid-controlled synthesis of α-amylase in barley endosperm. J. Cell. Comp. Physiol. 66:55-68.

Wagennar, S., and Lugtenborg, T.F. 1973. α-Isoamylases of rye seeds. Phytochem. 12:1243-1247.

Williams, J., and Shaykewich, C.F. 1969. An evaluation of polyethylene glycol (P.E.G.) 6000 and P.E.G. 20,000 in the osmotic control of soil water matric potential. Can. J. Soil. Sci. 49:397-401.