THE ASSOCIATION OF AN ENDOGENOUS, PROTEINACEOUS ALPHA-AMYLASE INHIBITOR WITH PRE-HARVEST SPROUTING

BY

DAVID CLARE AUDETTE

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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And finally, all good things must come to an end. However, I would be negligent if I forget my sweety. Thanks for coming home Jess.

Sincerely,

Rusty

TABLE OF CONTENTS

Page

LIST OF TABLES

Table Page

LIST OF FIGURES

ABSTRACT

Audette, David Clare. M.Sc., The University of Manitoba, March, 1990. The Association of an Endogenous, Proteinaceous α-Amylase Inhibitor with Pre-Harvest Sprouting in Wheat.

Major Professor; Dr. R.D. Hill.

An Enzyme-Linked Immunosorbent Assay (ELISA) was developed for the quantitation of an endogenous, proteinaceous cereal α-amylase inhibitor. This technique was used to determine the influence of wheat cultivar and environment during grain development, on the level of the inhibitor. Early and late planting dates of four cultivars with known sprouting character, led to differences in accumulated growing degree days (AGDD). The proportion of inhibitor to total seed protein increased under conditions of greater growing degree days during the period from anthesis to maturity. Sprouting tolerance was examined in a rain simulator apparatus. Varieties from the early planting date were exposed to greater growing degree days during grain development, and generally sprouted more rapidly and to a greater extent than material from the late planting date. These results suggest that the level of the inhibitor may have a role in reducing sprouting damage.

1. INTRODUCTION

The temperate zones are the areas where the majority of wheat production occurs, and particularly in the northern hemisphere, the fall harvest is frequently interrupted by inclement weather (MacKey, 1975). Sprouting damage in wheat is caused by rain-induced events in harvest ripe grain. These events include synthesis and /or activation of several enzymes, such as proteolytic enzymes, starch phosphorylases, hemicellulases, α-amylase and β-amylase (Paleg, 1961; Ching, 1972; Preston and Kruger, 1976). Dormancy in wheat exists when healthy, harvest ripe grains do not germinate when placed under optimum conditions of moisture, temperature and light (Belderok, 1968). Different levels of dormancy (resistance to pre-harvest sprouting) may be expressed in harvest ripe wheat grains, depending on the cultivar and the prevalent environmental conditions during grain development (Belderok, 1968; Grahl and Schrodter, 1975; Olsson and Mattson, 1976; Hagemann and Ciha, 1987).

Two laboratories (Weselake et al., 1983a, 1983b and Mundy et al., 1983) have isolated a protein from barley that specifically inhibits a group of α-amylase isozymes that are synthesized de novo in the aleurone layer during germination. Weselake et al., (1985a) have used antibodies against the barley inhibitor to show partial immunochemical

identity with components in kernel extracts of tetraploid and hexaploid wheat, hexaploid triticale and rye.

The objectives of this study were to determine the influence of various natural environmental conditions during grain filling on the level of the inhibitor protein and sprouting tolerance for four wheat cultivars varying in their level of grain dormancy.

2. LITERATURE REVIEW

2.1 Dormancy and Pre-harvest Sprouting

2.1.1 Introduction

Freshly harvested wheat (*Triticum aestivum* L.) grains might not germinate when placed under optimum moisture, temperature and light conditions. Such grains exhibit a phenomenon known as "post-harvest dormancy" and in order to achieve normal germinative energy, some time must elapse. The length of this dormant period can vary from several days to more than a year, depending on the cultivar and the conditions under which the cultivar is stored (Noll and Czarnecki, 1980; Mares, 1983a). The physiological and biochemical changes occurring within the grains while they are emerging from dormancy are referred to as "after-ripening".

Dormancy ensures the survival of many wild and domesticated plants. In the seed stage, plants are readily able to withstand desiccation and extreme temperatures. However, seedlings and developing plants as well as mature plants may not survive under the same conditions. In many parts of the world it frequently rains at harvest time, which may result in pre-harvest sprouting of cereals such as wheat, barley, corn, rye and oats. Pre-harvest sprouting

can be defined as the germination of either precocious or physiologically mature grains on the parent plant. Whether it be immature or mature grains that are sprouting, the consequence to the farmer and the processing industry is the same - economic losses. Pre-harvest sprouting commonly occurs in Northern and Western Europe (Greer and Hutchinson, 1945), Western Canada (Harrington and Knowles, 1939-40), Australia (Derera et al., 1977), New Zealand (McEwan, 1956-58), and certain regions of the U.S.A. (Hagemann and Ciha, 1987).

2.1.2 Resistance to Pre-harvest Sprouting

Resistance to pre-harvest sprouting in cereals is generally thought to be a result of dormancy (Belderok, 1968). Dormancy in wheat is usually associated with grains possessing a red-pigmented testa (Nilsson-Ehle, 1914; DePauw and McCaig, 1987). However, Derera (1982) has shown that not all red wheats are dormant.

Interestingly, there is evidence to suggest that factors other than dormancy may be influencing the degree of resistance to pre-harvest sprouting, and that these factors are present in varying degrees in different cultivars. Derera et al. (1977) suggested that "any exogenous factor that limits germination and factors either endogenous or exogenous to the caryopsis that limit synthesis of hydrolytic enzymes and reduce paste viscosity when dormancy does not operate, cannot be explained within the frame work

of the traditional definition of dormancy."

There are indications that non-grain tissues of the spike contain germination inhibitors (Derera, 1976), which introduces the concept of spike dormancy. Components of these tissues may be imposing some degree of tolerance to pre-harvest sprouting of otherwise non-dormant kernels (Derera et al., 1977). Water has been shown to penetrate the kernels of different genotypes at varying rates, which may also influence the degree of pre-harvest sprouting (Butcher and Stenvert, 1973; Stenvert and Kingswood, 1976). Also, King and Richards (1984) have demonstrated the effect of spike characteristics on water uptake, with awned cultivars imbibing water more rapidly than awnless ones. These reports clearly indicate that factors other than "dormancy" are also involved in pre-harvest sprouting.

2.2 Physiology of Pre-harvest Sprouting

2.2.1 Cereal Grain Structure and Development

The structure of the wheat grain has been documented extensively by Percival (1921), and Ashford and Gubler (1984). The bulk of the grain is composed of the nonliving starchy endosperm, which contains the carbohydrate and protein reserves. This tissue is surrounded by the living tissues of the aleurone layer, and the scutellum separates it from the embryo. The living tissues, including the embryo, scutellum and aleurone are primarily responsible for reserve mobilization. The entire grain is enclosed within

two maternal tissues, namely the testa (innermost) and pericarp (outer) which at maturity are nonliving. The compounds that determine coat color reside in the testa, as the pericarp is colorless. The maternal tissues account for 50% of grain protein up to about half way through development. The distribution changes so that at maturity 90% of all protein is found within the endosperm with about 80% of this being storage protein (Rijven and Cohen, 1961).

2.2.2 Initial Events of Sprouting

King and Richards (1984), and King and Chadim (1983) demonstrated the importance of spike characteristics in restricting grain wetting and sprouting in the spike. King and Richards (1984) used simulated rainfall to show that spike wetting at 1 hour and 24 hours, and sprouting at 30 hours was cultivar dependent. They note that some of the cultivar variation was associated with the presence or absence of awns, with awned cultivars taking up significantly more water and more likely to sprout. However, even though awned cultivars are more prone to sprout, the awns themselves do not appear to be important as their removal does not affect water absorption. It is evident that some other character of the spike that is associated with the awns, is responsible for both increased water uptake, and faster movement to the grain.

The rates of spike and grain drying must also be influencing sprouting, especially during intermittent

showers (King, 1984). The presence of awns does not affect water loss in the same way as water capture, as spike water loss appears to be solely dependent on evaporation (King and Richards, 1984). As a result, it is possible that all cultivars would dry at the same rate. The kernels must reach a water content of about 45% before germination occurs (Owen, 1952; Lush et al., 1981). Lush et al. (1981) have found that hydration-dehydration effects are additive. Thus it seems logical that each rain storm would have an additive effect when grain moisture exceeds 45%, and as such, sprouting may occur rapidly when grains are rewetted after a cycle of wetting.

It is reasonable to expect water uptake by isolated kernels of different cultivars to be similar, as cereal grains have a permeable grain coat. However, King (1984) has found cultivar differences in water uptake to be as great as two-fold during the first two hours of imbibition. Other authors have also demonstrated that grain wetting is cultivar dependent up to about 24 hours of imbibition (Butcher and Stenvert, 1973; Clarke, 1980; King and Chadim, 1983). The high protein, hard spring wheat, Timgalen, had slower water penetration than Heron, a low protein, soft variety (Butcher and Stenvert, 1973). It is also of interest to note that Timgalen has a thicker cuticle and testa than Heron (Moss, 1973). King (1984) used isogenic lines to demonstrate that grain hardness and seed coat color did not influence grain water uptake or germination. High

protein content and a thick cuticle and testa may have played a role in the cultivars tested. However, King (1984) showed that these factors were not correlated with cultivar differences in water uptake or germination. Small kernels of any given cultivar germinate sooner, but water uptake rates are similar for large and small kernels (King, 1984). Campbell (1958) found that during tempering of Manitoba wheats there was rapid hydration of the dorsal endosperm. This was not found in British wheats. He suggests that mechanical damage of the pericarp in the brush region allows water uptake via capillary action beneath the pericarp. Woodbury and Wiebe (1983b) followed Campbell's procedure and determined that water enters under the pericarp of damaged wheat in the brush region and moves rapidly along the dorsal surface toward the embryo. They also suggest that a crack or tear in the pericarp of the brush region may be the result of the conditions the grain is dried under. The shape of the collar under the brush region varies between cultivars. Some shapes could be more susceptible to cracking (Woodbury, personal communication). Data are also presented by these researchers that indicates water movement along the pericarp to the embryo is transporting an inhibitor, as kernels placed embryo down on moist sand germinated, but those placed brush down did not. However, there is no evidence of cultivar differences for either of these two phenomena.

2.2.3 Hormonal Regulation of Germination

It is clear that the embryo sends a hormonal message to the aleurone layer, and that this message is a gibberellin (GA) (Atzorn and Weiler, 1983). The aleurone layer is thus stimulated to synthesize and secrete germinative enzymes including α-amylase and β-amylase (Groat and Briggs, 1969), whose prime function is to hydrolyse the endosperm reserves. Other tissues are also implicated in the production of hydrolytic enzymes, and there are several reports that the scutellum accounts for the small quantity of α-amylase found early in the germination sequence (Mares, 1987; Okamoto et al., 1980; Cornford et al., 1987). These results appear to support the observation of Palmer (1980) that the endosperm adjacent to the scutellum is the first tissue to be modified. However, only limited production of α-amylase from the scutellum has been demonstrated (Palmer, 1982), and two researchers have estimated that this tissue accounts for 5 to 10% of the total α-amylase produced by the germinating barley grain (Palmer, 1982; Ranki and Sopanen, 1984).

The effects of abscisic acid (ABA) sharply contrast those of GA, as ABA inhibits germination in cereals. This inhibition may be reflected in the ability of ABA to inhibit cell wall loosening in Brassica napus (Schopfer and Plachy, 1985). ABA has been shown to block GA-induced gene transcription and subsequent enzyme production (Jacobsen, 1973), while also stimulating the production of unique proteins (Ho and Varner, 1976), one of which is an inhibitor

of α-amylase (Mundy, 1984). ABA levels increase as the fresh mass of wheat grains increases (King, 1976) with peak levels being attained prior to dehydration. During desiccation at maturity, there is a substantial decrease in the ABA content of the kernels (King, 1982). However, the conditions during grain growth can be expected to contribute to the differences in ABA as water stress, for example, increases ABA levels in wheat (Goldbach and Goldbach, 1977). Also, wheat grown at 25° C has three times as much ABA as wheat grown at 15°C (Radley, 1976). The environment is also very important with respect to the amount of ABA carried over to mature grain. Studies by King (1982) demonstrated that ABA levels were high when mature grains were dried slowly (50% water loss in 79 days). However, ABA levels may drop eight-fold when grains are dried under normal field conditions (50% water loss over 8 days).

Recent studies of an ABA-induced protein, an inhibitor of α-amylase, support a postulated early action of ABA in preventing precocious germination in developing grains (Mundy, 1984). The production of this protein is stimulated by ABA, and the level of this protein increases with ABA accumulation during development.

2.2.4 Cereal Grain Dormancy

Dormancy is a complex control system that prevents a seed from germinating when exposed to optimum conditions of moisture, temperature and light. Explanations of grain

dormancy invoke three distinct concepts, including embryo immaturity, grain coat restrictions, and embryo-specific controls. However, since embryo immaturity is seldom found in wheat (Gordon, 1980 & 1983), the focus of the following discussion will be on the latter two concepts.

The clearest evidence for a grain coat or husk imposed dormancy can be seen in studies of hulled grains. These grains may not germinate in a natural state, but are completely germinable once the hulls have been removed. Germination inhibitors have been found leaching into imbibition solutions and in the hull extracts of Avena fatua (Hsiao, 1987), and barley (Harvey et al., 1983). Miyamoto and Everson (1961) have demonstrated the presence of inhibitory substances in the grain coat of wheat, specifically the testa, with 50% of these substances being catechin- and tannin-like. Of greater significance perhaps, is that the level of catechin-tannin-like compounds in the more dormant red-grained cultivars studied by Miyamoto and Everson (1958 & 1961) were two-fold greater than those found in white cultivars.

Lenoir et al. (1986) have found that the hulls of barley can inhibit germination by restricting the availability of oxygen. Dormancy can be induced at 30°C (Corbineau et al., 1982), and at this temperature the hulls consume up to half the oxygen used by the grain (Lenoir et al., 1986). Furthermore, this temperature induced dormancy was not exhibited by either aged or fresh, naked grains.

Oxygen deficiency would be enhanced during the early hours of germination, if the hulls are indeed imposing dormancy by depriving the grain of oxygen, and this is the case . Oxygen consumption rates of hulls were greatest during the first 10 hours of imbibition, as compared to dehulled grain, where maximum oxygen consumption occurs 20 to 30 hours into germination (Corbineau et al., 1982).

Wheat grains also show a high temperature dormancy (Mares, 1984), but this effect does not appear to be related to oxygen consumption (Durham and Wellington, 1961). Miyamoto and Everson (1961) showed that oxygen consumption for dormant and nondormant cultivars does not differ during the first 10 hours of imbibition.

The embryo-related processes of dormancy include chemical inhibitors, lack of promoters and metabolic regulation. ABA and GA have been implicated in the chemical control of embryo dormancy (Gordon, 1980). When applied to cereal grains ABA will inhibit germination, while GA will promote it. ABA measurements have shown an accumulation in wheat grains at or before the time they enter dormancy (see references in King, 1982). The comments made earlier about the role of ABA in preventing precocious germination and control of enzyme synthesis by the aleurone also apply to this discussion. Measurements of endogenous levels of ABA by King (1976 & 1982) show no relationship between ABA content and wheat grain dormancy. Also, since most of the ABA in the developing grain is degraded during maturation (King,

1976), it is suggested that the reduced level in mature grain is insufficient to impose dormancy unless it is concentrated in the embryo. However, measurements of ABA in the embryo have recently become available (Walker-Simmons, 1987). Interestingly, work with a sprouting-sensitive and a sprouting-resistant wheat cultivar showed that ABA levels during early development were higher in the sprouting-resistant cultivar. However, ABA levels decrease during maturation and at maturity ABA levels were the same for both cultivars. More important though, is that germination studies showed that mature embryos from the sprouting-resistant cultivar were more sensitive to ABA than embryos from the sprouting-susceptible cultivar.

Work by Karssen et al. (1983) has provided evidence for a role for ABA in inducing dormancy. ABA-deficient mutants of Arabidopsis thaliana that were nondormant also had low levels of seed ABA. Furthermore, working with low GA mutants of Arabidopsis thaliana with reduced germinability, Koornneef et al. (1982) were able to cause further mutations for low ABA levels and show that dormancy was removed.

If the role of ABA is to induce dormancy just prior to grain maturation, then it may be irrelevant that ABA levels may or may not be different in dormant and nondormant grain, as embryo sensitivity may be an important factor (Walker-Simmons, 1987). However, the only time a promotive role for GA is likely to occur is on its synthesis and release by the embryo during imbibition. Consequently, GA and ABA balance

would only be important at the time of germination.

Metabolic control over dormancy has been studied by Taylorson and Hendricks (1977), and Gordon (1980). These authors suggest that the more rapid catabolism of glucose-6-phosphate by the pentose phosphate pathway than by the glycolytic pathway may be influencing the germination of dormant grains. The pentose phosphate pathway is an alternate energy producing pathway available under hypoxic conditions. According to Gordon (1980) this condition may exist in embryos because the pericarp impedes oxygen diffusion to the metabolically active tissues embedded beneath it. However, studies with Avena sativa do not indicate a relationship between loss of dormancy and increased activity of the pentose phosphate pathway (Adkins and Ross, 1981; Upadhyaya et al., 1981).

2.2.5 Environmental Influence on Sprouting Resistance

The environment affects sprouting resistance through its effect on the imposition and maintenance of dormancy. The maintenance of dormancy is affected by the temperature and moisture conditions the grains are exposed to following maturity. Belderok and Habekotte (1980) demonstrated a positive correlation between low dormancy and high temperatures. Harvested spikes maintained at 18°C lost their dormancy more rapidly than those left unharvested and held outdoors at lower temperatures. A similar relationship of high temperatures and low dormancy exists for the

imposition of primary dormancy. The duration of the dormancy period has been shown to be inversely correlated with the temperature during the latter 2 weeks of grain development. Belderok (1971) demonstrated that the higher the daily temperature, the shorter the dormant period. However, Hagemann and Ciha (1987) showed that the rate of dormancy loss was accelerated when the grain developed under cooler temperatures. Other authors have also noted that high temperatures during grain ripening reduce the dormancy period of wheat (Olsson and Mattson, 1976; Lalluka, 1976).

The extent of dormancy is also influenced by the environment during development. Sawhney and Naylor (1979) and Peters (1982) reported that low temperatures during development resulted in highly dormant Avena fatua (wild oat) seeds. Similar results have been found in barley (Rauber, 1984) and wheat (Black et al., 1987).

Artificial wetting of wheat heads at various stages of grain development did not produce significant increases in per cent visual sprouting at harvest (Ciha and Goldstein, 1983). Also, this watering treatment did not affect germination promptness or per cent germination, during post-harvest testing. Intermittent showers did not have much influence on α-amylase levels if the humidity was not elevated for long periods (Olered, 1967). However, Gale et al. (1987) did report that cool, wet conditions which led to delayed maturity and harvest resulted in grains containing

increased α-amylase levels.

The application of nitrate fertilizer produces grain of higher protein content, and this grain will have a greater capacity to synthesize α-amylase on germination (Ching and Rynd, 1978). Morris and Paulsen (1985) were able to demonstrate that wheat grains with higher protein levels had greater sprouting. However, Bhatt et al. (1981) showed no significant effect of nitrogen fertilizer on the sprouting activity of a number of hard winter wheats. Protein was reported to be negatively correlated with α-amylase activity by Moss (1967). Huang and Varriano-Marston (1980) reported that grain with a protein content of 15% had a greater capacity to produce α-amylase than grain with 14% protein. These conflicting reports may be the result of environmental influences on grain development.

2.3 Biochemistry of Pre-harvest Sprouting

2.3.1 Introduction

Grain enzyme activity increases rapidly following imbibition and may arise from two possible sources (Cardwell, 1984). Some enzymes are produced prior to maturation, such as β-amylase (Lauriere et al., 1985). The activation of such enzymes is accomplished by rehydration, or via the effects of some other chemical constituent of the grain such as growth substances or proteases.

The other source of enzymic activity is from de novo synthesis (Cardwell, 1984). These enzyme sources can be

further distinguished on the basis of when the mRNA coding for the protein is synthesized. Enzymes coded for by mRNA that is synthesized prior to seed maturation show activity very soon after imbibition. Enzymes coded for by mRNA synthesized following imbibition do not show activity as rapidly as the previously mentioned enzymes (Jacobsen et al., 1982). Alpha-amylase is an example of a kernel enzyme that is coded for by mRNA that is synthesized following imbibition (Jacobsen et al., 1982).

2.3.2 Characteristics of α-amylase

Three groups of α-amylases (1,4- α-D-Glucan Glucanohydrolase, E.C. 3.2.1.1) are present in wheat and barley, and they are referred to as I, II, and III according to their respective isoelectric points (MacGregor, 1980 & 1982). These α-amylases are endohydrolases that cleave the α-1,4 glycosidic linkages of amylose and amylopectin.

The isoelectric point of the α-amylase II group (hi pI) is around 6.2 for triticale and barley (Silvanovich and Hill, 1977; Jacobsen and Higgins, 1982). It has an estimated Molecular range (Mr) of 45 000 (Jones and Jacobsen, 1983), and is the predominant form of amylase synthesized by the aleurone layer of germinating barley kernels (Jacobsen et al., 1970). Alpha-amylase I (low pI) is the only amylase to be found during both development (Olered and Jonsson, 1970) and germination. The α-amylase III enzyme group is a complex formed between α-amylase II

and an endogenous, proteinaceous inhibitor of the α-amylase II group (Weselake et al., 1983a).

2.3.3 Characteristics of the α-amylase Inhibitor

An endogenous protein inhibitor of cereal α-amylase II, and of bacterial subtilisin has been identified by Weselake et al. (1983a) and Mundy et al. (1983). The Mr of the inhibitor is 19 900 (Weselake et al., 1985a), the isoelectric point is nearly neutral at 7.3 (Weselake et al., 1985a), and it has a pH optimum for inhibition of 6.5-7.0 (Weselake et al., 1985a). The action of the inhibitor is to form a complex with α-amylase II. Alpha-amylase extracts from malted barley that were heat treated showed a simultaneous decrease in α-amylase III and increase in α-amylase II (MacGregor and Ballance, 1980). The inhibitor: α-amylase binding ratio has been determined to be 2:1 by Halayko et al. (1986). Weselake et al. (1985a) found the inhibitor protein in kernel extracts of barley, wheat, rye and triticale, but were unable to detect it in oats, rice, millet, sorghum or corn. The inhibitor protein is distributed throughout the endosperm, and to a lesser extent the aleurone layer and embryo. The inhibitor content in mature barley ranges from 180 to 450 mg/kg of grain (Munck et al., 1985), 13 µg/kernel (Lecommandeur et al., 1987) and 20 to 38 µg/kernel (Rassmussen et al., 1988).

2.3.4 Synthesis and Function of the Inhibitor

The regulation of synthesis of the α-amylase inhibitor

is in sharp contrast to the pattern for α-amylase synthesis. ABA promotes and GA abolishes synthesis of the inhibitor (Mundy, 1984; Mundy et al., 1986; Mundy and Rogers, 1986). Inhibitor synthesis in embryo-less half kernels and isolated aleurones is increased upon treatment with ABA, and most of the inhibitor remains in the tissue where it is produced (Mundy, 1984; Mundy and Rogers, 1986).

Various researchers have studied the synthesis of the inhibitor during grain development. A continuous increase in the inhibitor content of barley kernels was shown by Lauriere et al. (1985) between 12 and 37 days post anthesis. For the cultivars studied, this period preceded grain desiccation. Munck et al. (1985) measured inhibitor accumulation in barley using an Enzyme linked Immunoassay (ELISA) and determined that the inhibitor content increases rapidly between 20 and 30 days post anthesis. Mundy and Rogers (1986) employed immunoblotting techniques to detect the inhibitor at 14 days after pollination and also found that accumulation continues up to 44 days following pollination.

The characteristics and the regulation of α-amylase inhibitor synthesis have led to several hypotheses about the physiological role of the inhibitor. Mundy (1984) has speculated that the inhibitor may function in the prevention of precocious germination, based on the observation that ABA induces synthesis of the inhibitor in mature aleurone layers. However, in further studies, Munck et al. (1985)

were unable to show a significant correlation between sprouting and inhibitor levels in several barley cultivars. They concluded that the inhibitor is not likely to be directly responsible for controlling pre-harvest sprouting. Hill et al. (1987) and Weselake et al. (1985) have suggested that a more probable role of the inhibitor is to reduce starch degradation during grain development under conditions where α-amylase II may appear due to precocious germination. This may effectively attenuate germination during this period.

Several workers have studied the use of the inhibitor in the baking industry. The inhibitor has been used successfully as an additive in bread making. Flour containing high α-amylase activity produces poor quality bread loaves (Derera, 1982). However, the addition of the α-amylase inhibitor to this flour during baking counteracts the effects of the α-amylase activity, resulting in the production of normal bread loaves (Zawistowska et al., 1988). Alpha-amylase activity in malt which is used as a dough improver is variable. Different levels of the inhibitor have been shown to have an effect on this α-amylase activity (Henson and Stone, 1988).

2.4 Genetics of Pre-harvest Sprouting

2.4.1 Potential Sources of Resistance

It has been repeatedly recorded in the literature that the duration of dormancy in wheat is largely determined by

genetic factors (Freed et al., 1976; Miyamoto et al., 1961; Derera et al., 1977). This has also been shown for barley (Goldbach and Michael, 1976), triticale (Plett and Larter, 1986) and corn (Fong, 1982).

Czarnecki (1987) has evaluated a diverse population of wheat cultivars as sources of resistance to pre-harvest sprouting. RL4137 has been proven to be an excellent source of resistance, as it possesses a high level of dormancy, low α-amylase levels, and a high falling number. The excellent sprouting resistance of RL4137 has been utilized by Czarnecki (1987), with Neepawa being backcrossed 6 times with RL4137 to produce Columbus. Czarnecki (1987) also used RL4137 to improve the sprouting resistance of Glenlea, Chester and Katepwa. Other potential sources of resistance include Frontana, Chris, Exchange, Kenya 321, Chinese Spring, Park, Spica and Sunelg (Czarnecki, 1987; Mares, 1987). MacKey (1975) suggested that wild diploid wheats with a high level of dormancy may also be incorporated into breeding programs.

2.4.2 Cultivar Variation to Pre-harvest Sprouting

Early studies of graincoat color and its association with post-harvest dormancy have resulted in the generalization that grains with a red graincoat are generally more dormant than white wheats (Wellington and Durham, 1958; Everson and Hart, 1961; Freed et al., 1976). However, more recent research indicates that there are

several white wheats with acceptable levels of sprouting resistance (DePauw et al., 1985; Mares, 1987).

In wheat, red grain color is simply inherited and controlled by 3 independent gene loci. It is not known if this association is due to a pleiotropic effect, or linkage effect between genes responsible for grain color and those conditioning dormancy (Czarnecki, 1987). When red grains are crossed with white grains, at least some of the resistance found in the red grained parent is transferred to the white grained progeny (Depauw and McCaig, 1983), suggesting that some specific factors of dormancy are not pleiotropic or linked with red coat color.

The Australian milling industry traditionally prefers white wheats, and recent efforts to produce a white wheat with acceptable sprouting character have resulted in the discovery of 14 white, resistant cultivars (Mares, 1987). Of these cultivars, AUS 1408, AUS 1490, Saberbeg, and Hellas have sprouting resistance approaching or superior to RL4137 (Mares, 1987). Earlier efforts to find alternative sources of resistance involved the use of GA-insensitivity dwarfing genes (Rht). Five Rht genes are known to exist in wheat (Gale and Gregory, 1977). The Rht3 or 'Tom Thumb' dwarfing gene is the only gene that has a significant effect on the α-amylase response of the aleurone cells to applied or endogenous GA. This gene can limit production of α-amylase by the distal half of the grains to about 1% of tall genotypes (Gale and Marshall, 1973; Gale and Marshall,

1975). The major drawback to this allele is that cultivars exploiting it are generally considered too short for commercial production. Cultivars utilizing this gene include Minister Dwarf (Gale and Marshall, 1973), Tom Thumb (Gale and Marshall, 1973) and Tordo (Derera et al., 1987). Rht1 and Rht2 are the 'Norin 10' semidwarfing genes which have been used extensively in commercial cultivars. However, they have only a minor effect on the α-amylase response of aleurone cells to GA (Gale and Marshall, 1973). As a result, these two genes are not likely to afford an adequate level of sprouting resistance (Gale and Marshall, 1973; Cornford and Black, 1985). The effect of the other two alleles, Rht1S and Rht1O, has not been studied.

2.5 Methods for Assaying Sprouting Resistance

2.5.1 Storage of Biological Material

It is desirable to store intact wheat ears in a state that reduces after-ripening, thereby retaining the level of sprouting resistance found at harvest-maturity. Noll and Czarnecki (1979), and Mares (1983) demonstrated that mature, dry grains (<13% moisture content) stored at -20°C did not lose their dormancy, a fact that has extended the testing period of harvest dormancy in wheat. The freezing and thawing cycle does not appear to cause tissue damage, as long as the grain moisture content is below 15% (Mares, 1983).

2.5.2 Grain Germination Tests

The use of grain germination tests to study the sprouting resistance of wheat and barley at harvest maturity has been described by Noll et al., (1982) and Reitan (1983), respectively. Germination tests are commonly conducted at constant temperature and in the presence of excess water. Woodbury and Wiebe (1983a) have suggested that these are nonphysiological conditions. These researchers have demonstrated that germination of grains exposed to free water differs from germination under conditions where water movement, but not amount, is limited. Also, Mares (1987) has shown that the water content of the non-grain components of the ear following artificial wetting is about 250% of the dry mass, whereas the moisture content of the germination medium is about 800% under standard conditions of 4 ml of water added to a 9-cm diameter filter paper. When the aim of the research is to determine relative sprouting resistance, then the nonphysiological conditions of the standard test should be sufficient.

2.5.3 Wetting of Intact Spikes

Pre-harvest sprouting can be influenced by spike characteristics (King, 1982) and as a result, measurements of grain dormancy are not always adequate for determining resistance to pre-harvest sprouting. A test measuring the sprouting ability of grains within an intact spike would be more beneficial. Intact heads can be studied by utilizing

natural rainfall in the field, or under more controlled conditions in a rain simulator apparatus.

Natural rainfall is generally unpredictable in terms of amount, intensity, and occurrence, however, some useful information is available. Field testing takes into consideration the effects of the crop density, positional relationship of spikes and leaves, and the nodding angle of the spike. However testing under artificial conditions is more likely to produce consistent results as the researcher has more control over environmental parameters and physiological condition of the sample.

Intact spikes and panicles can be induced to artificially sprout by using a rain simulator chamber similar to that described by McMaster and Derera (1976). The apparatus is basically a humidity chamber with a rotating set of trays arranged in a similar fashion to a Ferris wheel. Water is applied in a controlled manner via a series of nozzles located above the rotating assembly. The test is carried out under standardized conditions of temperature, humidity, darkness and duration. Periodic measurements of sprouting are taken, and sprouting damage can be assessed by subjective means, such as a sprouting index or objective methods such as α-amylase activity or falling number. The duration of the dormant period can be determined by repeating the sprouting and germination tests at regular intervals between the stages of physiological maturity and complete after-ripening.

The limitations of this method of testing are the same as the benefits of testing under natural conditions, as the effect of crop micro-environment and canopy architecture on sprouting are not given any consideration. King and Richards (1984) were able to simulate natural wetting rates. However, King (1987) has pointed out that water droplet size, distribution and velocity in rain simulator systems were not comparable with natural rain.

2.5.4 Determination of α-amylase Activity

High α-amylase activity in the flour is the most damaging consequence of sprouting. Since visual sprouting is not necessarily related to the α-amylase activity of the grains, it is beneficial to have a measure of α-amylase activity at various stages of the sprouting test and in mature grains. Numerous methods for detecting α-amylase activity are available, and include the Hagberg Falling Number (Hagberg, 1960), and a technique described by Briggs (1961). The latter assay measures α-amylase activity by determining the rate of substrate degradation over a specific period of time. If the substrate is β-limit dextrin, then only α-amylase activity is measured. The color intensity of the iodinated β-limit dextrin complex is inversely proportional to the α-amylase activity of a given sample.

2.5.5 Relationships between Assay Methods

The relationships between α-amylase activity of non-

weathered and weathered grain samples, visual sprouting in the spike, and grain germination have been studied by DePauw and McCaig (1987). The α-amylase activities of non-weathered grain and artificially weathered grain were not correlated suggesting that the application of simulated rain allowed the genotypes to express their true potential for sprouting when exposed to favourable germination conditions. DePauw and McCaig (1987) were also able to demonstrate a significantly high correlation between α-amylase activity, the number of spikes with visible sprouting and the percentage of grains germinated. Henry and McLean (1987) performed similar experiments, studying the relationship between visual sprouting, α-amylase activity and endosperm modification. They found that all correlations were significant indicating that increased visual sprouting results in elevated α-amylase activity and greater endosperm alterations. However, deviations from this correlation due to environment and cultivars led Henry and McLean (1987) to conclude that sprouting damage due to one factor cannot be based on the levels of other factors.

King (1983) and Mares (1987) have studied the importance of the vegetative components of the spike, by comparing sprouting of intact spikes relative to isolated grains. A linear relationship between the two measurements should exist if the tests are performed under identical conditions, and any deviation from this line would suggest that some characteristic of the spike is influencing

germination. King (1983) found that awned varieties germinated better in the spike, while awnless varieties germinated relatively poorly in the spike as compared to the isolated grains.

3. MATERIALS AND METHODS

3.1 Biological Material

3.1.1 Handling of Wheat Cultivars

Four spring wheat cultivars, Columbus (hard red), RL4555 (soft white), Norquay (soft white) and Tordo (white derivative of Tom Thumb), were grown at the University of Manitoba, Winnipeg, Manitoba, in 1986 and 1987. RL4137 from the 1986 study was replaced by Columbus (Neepawa X RL4137 cross) in the 1987 study. The cultivars were selected to provide a range in sprouting resistance, with Columbus most resistant followed by RL4555, Tordo and Norquay respectively. Two crops were seeded with the intent that each crop would be exposed to different levels of accumulated growing degree days (AGDD) during grain development. To achieve this, seeding dates of each crop were staggered by approximately one month. Growing degree days (GDD) were calculated using the following formula (Belderok, 1965):

GDD = [(Tmax + Tmin.)/2]-12.5°C; where Tmax. and Tmin. were the daily maximum and minimum temperatures. 12.5°C is the base temperature. Temperature above this are considered to have an effect on growth. Accumulated growing degree days (AGDD) were calculated by summing the growing

degree days over the period of grain development.

Individual heads from each cultivar, that flowered 1-2 days apart, were tagged at the beginning of anthesis and subsequently collected at harvest ripeness (approximately 13% grain moisture content) (Hanft and Wych, 1982). In this manner, the length of time from anthesis to maturity is the same for all heads within a cultivar. Plant material was separated into two groups; one group for sprouting tests and the second for inhibitor determination. Intact heads and hand threshed kernels were stored at -20°C as described by Noll and Czarnecki (1980) in order to preserve the dormancy characteristics present at harvest ripeness.

3.2 Determination of Sprouting

A rain simulator chamber similar to that described by McMaster and Derera (1976) was used for sprouting grains on the head. Twenty heads were separated into 4 replicates, and placed on a tray that fits onto the rotating assembly in the chamber. Approximately 50 mm of rainfall was simulated in a two hour period, followed by conditions of 100 % relative humidity, 15°C and darkness , for 7 days. Sprouting was considered to have occurred when coleoptile growth was visually evident. Sprouting was measured qualitatively in 1986 by assigning values to each head based on the degree of sprouting. For example, 0 was no sprouting, 1 was a low level, 2 was moderate, and 3 was high. A quantitative measuring method was adopted for the

1987 studies, and for each head, the number of sprouted kernels and the total number of kernels per head was recorded.

A sprouting index (SI) based on the formula used by Kendrick and Frankland (1969) was calculated in order to determine the level of seed dormancy present. The following formula was used for the determination of SI:

SI= 1/t x P ; where P is the final % germination and t is the time,in hours, at which 1/2 P occurs.

Final % sprouting and the rate at which the sprouting proceeds was taken into consideration with this formula. A larger number indicates greater sprouting.

Germination studies were also performed on the seed samples, and a germination index was calculated in the same manner as the sprouting index (Kendrick and Frankland, 1969). Three replicates of 50 kernels were placed in 9 cm diameter petri plates with two layers of #3 Whatman filter paper. Eight mL of water was added to each plate and the plates were sealed with a strip of parafilm in order to reduce water loss. They were stored under conditions of darkness at 20°C , and exposed to light only when measurements were recorded. Germination was considered to have occurred when radicle length was greater than 2-3 mm. The duration of the germination test was 3 days. Seed surface sterilization following the procedure of Goudy et al.(1987) was adequate in preventing fungal contamination.

3.3 Soluble Protein Extraction

Kernels from each cultivar were ground using a Wiley mill with a #40 mesh screen for 2 minutes, followed by grinding with a #60 mesh screen for another 2 minutes. Kernels from the 1987 study which were analyzed for α-amylase content after 3 days of germination were macerated with a polytron prior to extraction. These samples were kept on ice during the maceration process. The grain protein extraction buffer used was 200 mM acetate buffer, pH 5.5 with 1mM $CaCl_2$. Crude protein extracts were prepared by mixing 1 g of ground material with 10 mLs of extraction buffer in screw cap centrifuge tubes. The samples were kept at 4°C for 24 hours with constant agitation provided by attaching the centrifuge tubes to a multipurpose rotator. The extraction fluids were centrifuged at 12 000 x g for 20 minutes, and the supernatant served as the study extracts. Extracts were kept at - 20°C prior to assaying, at which time they were thawed and diluted as required.

3.4 Protein Determination

Total grain protein (% protein on a dry mass basis) was determined by the Kjeldahl method. This procedure determines the total nitrogen content of the sample, and for wheat, a conversion factor of 5.7 was used to calculate kernel protein.

Buffer extractable proteins were measured using the Biorad protein assay (Bradford, 1976) performed in 96-well

microtitre plates. Using microplates not only speeds up assay and clean-up time, but it also simplifies data recording when using a microplate reader with a paper printout. Two hundred microlitres (uL) of diluted dye reagent (1:4) was added to each well. A standard curve was performed for each plate, and was prepared by adding 10 uL of several dilutions of γ-globulin protein standard, containing from 0.2 to about 1.4 mg/mL of protein. Ten uL of diluted sample was then added to the appropriate well containing the dye reagent. The absorbance at 590 nm was measured using an EIA microplate reader during the period of color stability, which for this assay has been determined to be from 5 minutes to 1 hour after the addition of the sample to the dye reagent. Absorbance at 590 nm was plotted versus the concentration of the standards, a standard curve was calculated, and the concentration of the unknowns was determined.

3.5 Determination of α-amylase Content

For the 1987 study, α-amylase levels were determined in whole, dry kernels, and in kernels germinated for 3 days. This measurement was performed by using a modified method based on Briggs' procedure (1961). The substrate in this assay was β-limit dextrin, as it prevents the expression of β-amylase so only α-amylase is measured. The assay, performed at 35°C, utilized β-limit dextrin at a concentration of 0.05%, and 200 mM acetate buffer, pH 5.5

with 1 mM $CaCl_2$ as the buffering solution. The assay also uses the various solutions at 1/2 the volume suggested by Briggs (1961). Absorbance at 540 nm was measured with a Hewlett-Packard 8452A diode array spectrophotometer. The α-amylase activity in any given sample is inversely proportional to the absorbance of that sample, and it is expressed in iodine-dextrin color units (IDC units). One IDC unit is defined as the amount of α-amylase required to change the absorbance of an iodine-β-limit dextrin complex solution from 0.6 to 0.4 in 100 minutes.

3.6 Determination of α-amylase Inhibitor Content

Alpha-amylase inhibitor contents of whole, dry kernels were determined using the protein extracts prepared in section 3.3. For the 1987 study, inhibitor levels were also measured in the embryo and endosperm fractions of the kernel. Kernels were imbibed for 3 hours, at which time the embryo was removed. This procedure allowed for the removal of the embryo without any endosperm tissue adhering. Embryo and endosperm fractions were macerated prior to extraction.

The assay protocol relies on the formation of an antigen-antibody complex which is fundamental to all immunological assays. The assay for the α-amylase inhibitor makes use of the antigenicity of the inhibitor molecule. The antibody used in this study was prepared according to the protocol of Weselake et al. (1983).

Assay Reagents

Carbonate buffer: 0.2 M, pH 9.6

- sodium carbonate solution added to sodium bicarbonate solution until pH 9.6 was reached.

Phosphate buffered saline (PBS): 0.05 M, pH 7.2

- monobasic sodium phosphate solution added to dibasic sodium phosphate solution until pH 7.2 was reached.
- sodium chloride added at 0.15% w/v.

Blocking solution:

- PBS with 1% bovine serum albumin (BSA).

Phosphate buffered saline with Tween 20 (PBS-Tween):

- as above, plus addition of Tween 20 at 0.05% v/v.

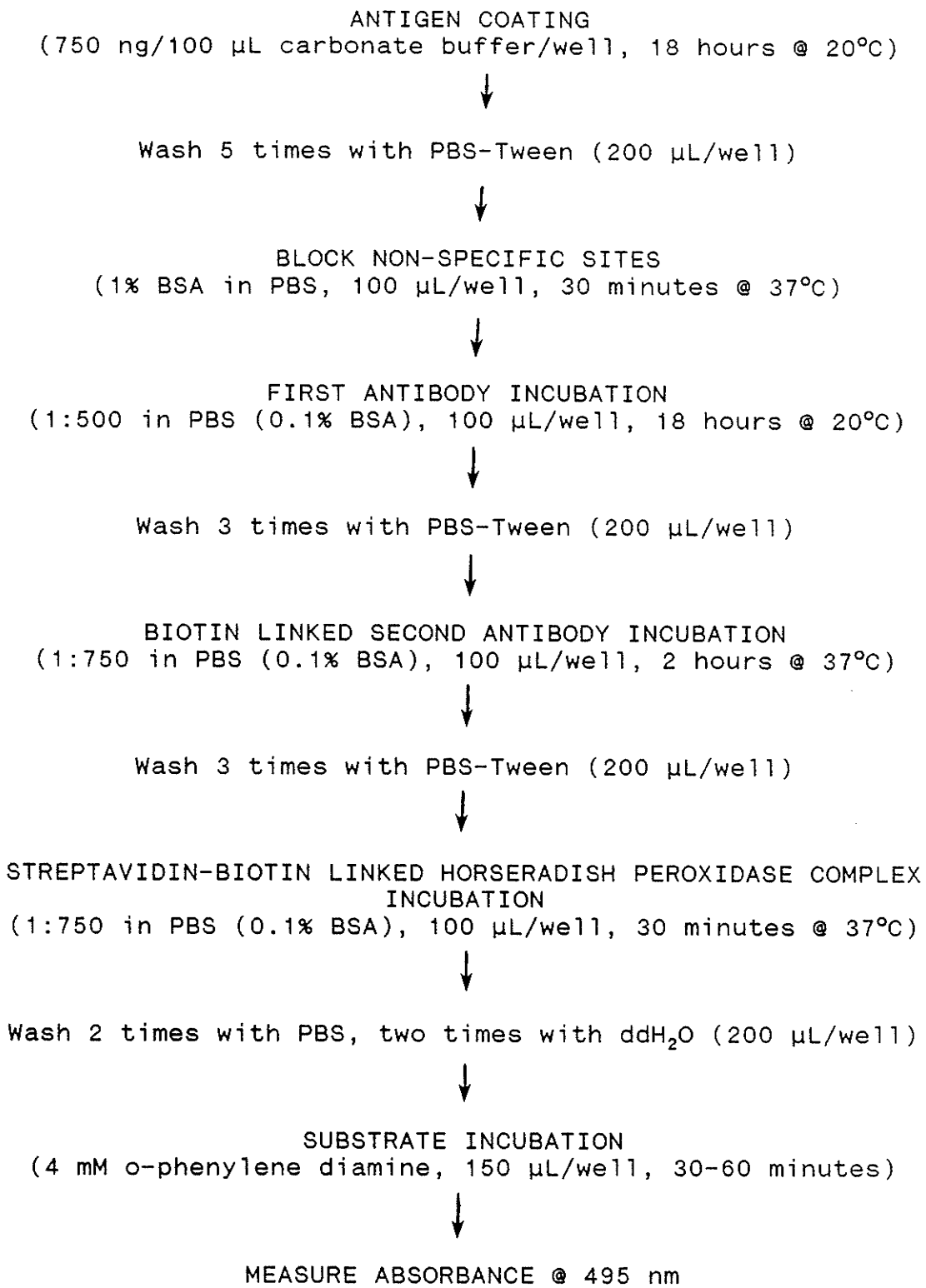
Citric acid (0.02 M) sodium phosphate (0.05 M), pH 5.0:

- citric acid solution added to sodium phosphate solution.

Assay Procedure

A flow chart of the procedure developed for the inhibitor quantitation is illustrated in Figure 1. The assay was performed in new, 96-well polystyrene plates (Titretek), and double distilled water (ddH_2O) was used as the solvent for all reagents. Samples were analyzed in triplicate per plate and each experiment was also conducted in triplicate.

Figure 1. Outline of an ELISA for the α-amylase inhibitor.



1. Antigen coating

A standard curve was created using purified inhibitor protein added to wells at a concentration ranging from 5 ng/100 ul to 50 ng/100 ul. Silanized glassware was used for this step and subsequent steps where antigen and antibody solutions were prepared. Sample protein was added to wells at a concentration of 750 ng/100 uL. Carbonate buffer deprotonates the amino and carboxyl groups of the protein sample, allowing the proteins to undergo ionic binding with the positively charged polystyrene plates. An incubation period of 18 hours was sufficient for antigen binding.

2. Washing

PBS with Tween 20 was used for all washing steps except the last wash, just before the substrate was added. A saline concentration of 0.15% w/v in the PBS with Tween 20 and PBS proved to be adequate in reducing "background noise" from non-specific binding. The last wash was a two step procedure, with 2 PBS washes followed by 2 ddH_2O washes. Five washes, of 5 minute duration each, were sufficient after antigen coating. Washing was not required after the blocking step, and 3 washes of 5 minute duration each, were adequate for subsequent steps. It was critical to ensure that no contamination occurred between wells at all steps of the assay.

3. Blocking

This step was performed to reduce non-specific binding

of antibodies and detection reagents to free binding sites on the wells. These free binding sites exist because of incomplete well coating by the protein of interest. PBS with BSA, added at 100 ul/well for 30 minutes at 37° C was sufficient for blocking these sites. After the blocking solution was evacuated from the wells, it was possible to store the plates, sealed with parafilm, at 2-4°C for extended periods of time. Storage periods of 2 months showed little effect on assay reliability. Freezing of the plates at this step was not performed but may likely yield similar results. This greatly facilitates sample analysis, as a large number of plates can be prepared at one time.

4. First antibody

Affinity purified inhibitor antibody was diluted 1:500 in PBS with 0.1% BSA, and added at 100 uL/well for 18 hours at 20°C.

5. Second antibody

Amershams biotin-linked, anti-rabbit antibody was diluted 1:750 in PBS with 0.1% BSA, and added at 100 uL/well for 3 hours, at 37°C.

6. Detection reagent

This was a biotin-linked peroxidase, streptavidin enzyme complex (Amersham) that bonded to the biotin molecule of the second antibody complex. It was diluted 1:750 in PBS with 0.1% BSA, and added at 100 uL/well for 30 minutes at 37° C.

7. Substrate incubation

O-phenylene diamine (OPD) is a water soluble brown product that absorbs light at 492 nm. OPD (4mM) with hydrogen peroxide (0.004% v/v) was mixed with citric acid, sodium phosphate buffer, and added at 150 uL/well. Color was allowed to develop for 30-60 minutes, or until maximum absorbance was .5 to .6. The reaction was stopped with the addition of 50 uL/well of sulphuric acid (0.1M).

In the assay, the OPD was oxidized and color develops. OPD also oxidizes in the packaging bottle, so color intensity of the assay will decrease over time if precautions are not taken to prevent oxidation in the bottle. Storage in the dark, under an inert atmosphere at low temperature would be the best precaution. A more realistic alternative is to be aware of a potential problem and replace stock periodically.

Assay sensitivity and reproducibility

The formation of an antigen-antibody complex is dependent on the antigen and antibody concentration. Antibody sensitivity and selectivity is determined by the titre of the antibody, and these characteristics are mutually exclusive (Burnette, 1981). A suitable detection range was determined by carrying out a titration of the antibody. This was performed with purified inhibitor and plant protein extracts known to lack inhibitor. In this manner, the selectivity and sensitivity of the antibody was tested. At a dilution of 1500:1, there was poor color

development (lack of sensitivity). At a dilution of 250:1, there was unacceptably intense color development in the wells containing protein extracts lacking inhibitor (lack of selectivity). A dilution of 1000:1 proved to be a suitable balance between selectivity and sensitivity.

Under the conditions of the assay, the lower limits of detection was 5ng, with the standard curve being linear from 5ng to 50ng. The maximum protein level that could be bound to the microplates was 750ng. The addition of 750ng of sample protein to each well yielded absorbance values that corresponded to approximately 15-20ng on the standard curve. Absorbance values for a given amount of inhibitor standard varied between experiments (i.e. the standard curve was not the same each time the experiment was conducted). However, the low coefficients of variation obtained when the replications were combined suggested that the samples and standard curves varied by a proportional amount (see Table 11,12,13 and 14).

3.7 Statistical Analysis

SAS v.5.16 program was used to perform analysis of variance on all data. Treatment means were compared using a least significant difference (LSD) procedure at a significance level of $P<0.05$. In order to estimate a more normal distribution, the following data were transformed prior to analysis:

a) percent germination at time P underwent an arcsine transformation

b) α-amylase activity underwent a log transformation.

Raw data and LSD values are presented in this thesis so that differences can be easily recognized.

4. RESULTS AND DISCUSSION

4.1 Effect of Planting Date on Sprouting Resistance

The staggering of planting dates resulted in a different number of accumulated growing degree days (AGDD) during grain development for each planting date and year (Table 1). For each year of the study, the cultivars from the early planting date were exposed to more AGDD than the cultivars planted later. Cultivar differences in AGDD were also present and were most pronounced when compared on the basis of AGDD per day of development. There were more AGDD for each planting date in 1986 than in 1987. Winnipeg experienced drought conditions in 1987 that may have hastened grain development. Grain development took longer in 1986 than 1987, as evidenced by the number of days from anthesis to harvest maturity (Table 2).

The sprouting index determined for the 1986 studies was useful in showing qualitative differences in the sprouting of the four cultivars tested. The degree of sprouting was greatest for all cultivars grown under more AGDD during grain development and is shown in Table 3. RL4137 showed virtually no sprouting for either planting date. RL4555, Norquay, and Tordo from the early planting date had greater sprouting than the late date. Statistical analysis was not performed on the 1986 sprouting data as the visual

TABLE 1. Accumulated growing degree days (AGDD) from anthesis to maturity for early and late planting dates.

	Planting Date			
	1986		1987	
Cultivar	Early	Late	Early	Late
	(AGDD)			
RL4137	515	426	-	-
Columbus	-	-	630	497
RL4555	621	539	542	495
Norquay	600	514	542	430
Tordo	506	470	428	426

TABLE 2. Time from anthesis to maturity for early and late planting dates.

	Planting Date			
	1986		1987	
Cultivar	Early	Late	Early	Late
	(days)			
RL4137	43	40	--	--
Columbus	--	--	34	38
RL4555	45	49	37	47
Norquay	46	43	37	35
Tordo	34	39	31	42

inspection of the data readily demonstrated that a value of 1, for example, was different from a value of 0.5.

The plant material obtained in the 1987 study was analyzed for sprouting resistance with a quantitative test, and statistical procedures were performed on the data (Table 3). The results were similar to the data obtained in the 1986 study. Cultivars exposed to more AGDD generally had significantly more sprouting than those exposed to fewer AGDD; however, there were two specific differences. First, Columbus from the early planting date showed a significant amount of sprouting and secondly, Tordo from the early planting date showed less sprouting than the late date. Because of the different methods used to determine sprouting indices, the only comparisons that can be made between the 1986 and 1987 data are relative ones. The combined data from both planting dates showed that Columbus and RL4137 were most resistant to sprouting, followed by RL4555, Norquay, and Tordo respectively (Table 4). This order of sprouting resistance was the same for 1986 and 1987. The cultivar Tordo was the exception, where the data showed less sprouting for the early planting date versus the late planting date. For each year of the study, the red wheats Columbus and RL4137, were more resistant to sprouting than the white wheats, Norquay, RL4555 and Tordo.

TABLE 3. Effect of planting date on sprouting index.[1]

	Planting Date			
	1986[2]		1987[3]	
Cultivar	Early	Late	Early	Late
	(sprouting index)			
RL4137	0.00	0.01	--	--
Columbus	--	--	0.12cd	0.01e
RL4555	0.44	0.07	0.28b	0.04de
Norquay	1.50	0.75	0.43a	0.18bc
Tordo	1.00	0.50	0.23b	0.48a

[1]A larger number indicates greater visual sprouting damage.

[2]This data can be compared to 1987 data on a relative scale only.

[3]Treatments with the same group letter are not significantly different ($\alpha = 0.05$) (c.v.= 25.5).

TABLE 4. Combined sprouting indices from early and late planting dates.[1]

Cultivar	1986[2]	1987
	(sprouting index)	
RL4137	0.05	--
Columbus	--	0.07
RL4555	0.26	0.16
Norquay	1.13	0.31
Tordo	0.75	0.36

[1]A larger number indicates greater sprouting.

[2]This data can be compared to 1987 data on a relative scale only.

4.2 Relationship between 1000 Kernel Mass, Planting Date and Sprouting

The kernels from each cultivar were not separated on the basis of density or mass prior to further analysis, as the kernels in the spikes were not sized before undergoing rain simulator tests. It is important to note that wheat spikes will contain grains of differing density and size, and that the smaller grains generally germinate sooner than larger grains (King, 1983; Woodbury and Wiebe, 1983a).

Based on the variable grain size and density, 1000 kernel mass measurements were performed to determine if planting date (and hence differing levels of AGDD) affected kernel mass and if so, to determine if samples of higher average test mass sprouted sooner than samples of lower average test mass.

The association between planting date and 1000 kernel mass for the 1986 crop is demonstrated in Table 5. The early planting date of RL4137, RL4555 and Norquay produced grains of significantly higher 1000 kernel mass than the later planting date, while the situation was reversed for Tordo. Table 6 shows that in 1987, all cultivars from the early planting date yielded samples with a significantly higher 1000 kernel mass than their later planted counterparts.

The relationship between 1000 kernel mass and sprouting observed in this study did not confirm the results of Woodbury and Wiebe (1983a), unless all the grains that

TABLE 5. Effect of planting date on 1000 kernel mass, for the wheat cultivars grown in 1986.

Cultivar	Planting Date[1] Early	Late
	(grams)	
RL4137	33.0a	26.3b
RL4555	33.0a	27.3b
Norquay	31.8a	26.5b
Tordo	18.8c	25.5b

[1]Treatments with the same group letter are not significantly different ($\alpha = 0.05$) (c.v. = 6.1).

TABLE 6. Effect of planting date on 1000 kernel mass, for the wheat cultivars grown in 1987.

Cultivar	Planting Date[1] Early	Late
	(grams)	
Columbus	31.0b	19.0e
RL4555	36.8a	21.8d
Norquay	31.3b	27.0c
Tordo	37.5a	17.0e

[1]Treatments with the same group letter are not significantly different ($\alpha = 0.05$) (c.v. = 5.3).

sprouted were of small size and low density. This may be the case, however, the grains from the sample with the lower average test mass would still be expected to sprout sooner. This was not the case for the cultivars examined, except for the 1986 sample of Tordo. Grain samples of Tordo with a lower 1000 kernel mass did germinate sooner than the samples with a higher mass.

4.3 Effect of Planting Date on Protein Composition

4.3.1 Soluble Protein Content

'Soluble proteins' are proteins that can be extracted with a dilute salt buffer, and are composed of globulins and albumins as well as glycoproteins, nucleoproteins and lipid-protein complexes (Porceddu et al., 1983). The α-amylase inhibitor and α-amylase belong to the globulin group of proteins, and since this study is partially looking at how the levels of these two proteins change under different environmental conditions, it is useful to study how they change relative to the globulins. The data for the proportion of α-amylase inhibitor to soluble protein, and α-amylase to soluble protein is presented in subsequent sections of this study.

The measurement of acetate-buffer soluble protein was primarily performed in order to have a known protein concentration from which to determine inhibitor and α-amylase protein concentration. However, the planting date of a given cultivar did have an effect on the level of

soluble protein. The 1986 studies showed a significantly higher soluble protein content for the early planting date of RL4555, while there was no significant effect of planting date for RL4137, Norquay and Tordo (Table 7). The 1987 data demonstrated a more complete effect of planting date, with all cultivars from the early planting date having a significantly higher soluble protein concentration than the cultivars planted later (Table 8).

4.3.2 Total Protein Content

The total protein content of the wheat cultivars was determined by Kjeldahl analysis and the results for the 1986 and 1987 crops are presented in Table 9 and 10 respectively. Cultivar differences significantly affected protein content, which was in turn influenced by the planting date. The 1986 data showed that total protein content was higher for the early planting date of RL4137, RL4555 and Norquay, but not for Tordo where the later date yielded grains of higher protein content. The growth conditions for the 1987 crop were such that early planting of all the cultivars produced grains of significantly higher protein content than when the cultivars were planted later.

The relationship between protein content and sprouting has been studied by Morris and Paulsen (1985), who were able to show that wheat grains with higher protein content had greater sprouting. The analytical results of studies of the

TABLE 7. Effect of planting date on soluble protein content for the wheat cultivars grown in 1986.

Cultivar	Planting Date[1] Early	Late
	(mg/kernel)	
RL4137	0.76b	0.69bc
RL4555	0.92a	0.74b
Norquay	0.65c	0.62c
Tordo	0.43d	0.50d

[1]Treatments with the same group letter are not significantly different ($\alpha = 0.05$) (c.v. = 8.6).

TABLE 8. Effect of planting date on soluble protein content for the wheat cultivars grown in 1987.

Cultivar	Planting Date[1] Early	Late
	(mg/kernel)	
Columbus	0.97c	0.77d
RL4555	1.31ab	0.97c
Norquay	1.25b	1.01c
Tordo	1.41a	0.65d

[1]Treatments with the same group letter are not significantly different ($\alpha = 0.05$) (c.v. = 8.1).

TABLE 9. Effect of planting date on total protein content for the wheat cultivars grown in 1986.

Cultivar	Planting Date[1] Early	Late
	(mg/kernel)	
RL4137	5.4a	3.4d
RL4555	4.5b	3.4d
Norquay	4.4b	3.8c
Tordo	2.6e	2.9e

[1]Treatments with the same group letter are not significantly different ($\alpha = 0.05$) (c.v. = 2.9).

TABLE 10. Effect of planting date on total protein content for the wheat cultivars grown in 1987.

Cultivar	Planting Date[1] Early	Late
	(mg/kernel)	
Columbus	5.3b	3.6d
RL4555	5.9a	3.8d
Norquay	5.6ab	4.4c
Tordo	6.1a	2.9e

[1]Treatments with the same group letter are not significantly different ($\alpha = 0.05$) (c.v. = 8.0).

cultivars tested in 1986 and 1987 are in general agreement with the findings of Morris and Paulsen (1985). Cultivars from the early planting date of each year, except Tordo, had greater sprouting (Table 3) and higher protein content (Table 9 and 10) compared to grains from the later planting date (Tables 3, 9 and 10). Grain samples of Tordo grown in 1987, from the early planting date, had a higher protein content (Table 10) and less sprouting (Table 3) than the early planting date. In the 1986 study, the early and late planting date of Tordo did not differ in protein content (Table 9), but the late date did give a significantly greater incidence of sprouting (Table 3).

4.3.3 Alpha-Amylase Inhibitor Content

The levels of the α-amylase inhibitor within mature, whole grains of the cultivars from early and late planting dates from the 1986 and 1987 study are presented in Table 11 and 12 respectively. There were no significant differences in inhibitor levels between planting dates for any cultivar grown in 1986. However, there were significant differences between the cultivars for each planting date. The most sprouting resistant cultivar, Columbus, had the highest level of inhibitor followed by the next most resistant cultivar, RL4555. Norquay and Tordo had significantly lower inhibitor levels than either Columbus or RL4555, but did not differ significantly from each other. The levels of inhibitor in the 1987 samples were higher than the 1986

TABLE 11. Effect of planting date on α-amylase inhibitor content for the wheat cultivars grown in 1986.

Cultivar	Planting Date[1] Early	Late
	(ug/kernel)	
RL4137	19.3a	17.3ab
RL4555	15.0b	15.0b
Norquay	11.0c	10.0c
Tordo	10.5c	10.0c

[1]Treatments with the same group letter are not significantly different (α = 0.05) (c.v. = 15.0).

TABLE 12. Effect of planting date on α-amylase inhibitor content for the wheat cultivars grown in 1987.

Cultivar	Planting Date[1] Early	Late
	(ug/kernel)	
Columbus	24.5bc	27.0b
RL4555	24.8b	32.0a
Norquay	15.0d	16.0d
Tordo	21.0c	31.0a

[1]Treatments with the same group letter are not significantly different (α = 0.05) (c.v. = 7.1).

samples, and the late planting dates of RL4555 and Tordo had significantly higher levels than the early dates. For the 1987 study planting date had no significant effect for Columbus or Norquay. For each planting date and year of the study, the most sprouting resistant cultivars generally had the highest levels of α-inhibitor. The exception to this trend was the 1987 sample of Tordo. There was no relationship between α-amylase inhibitor content and the number of genes for red grain coat color, however, the limited number of cultivars tested may have biased this observation.

A more interesting picture is seen when α-amylase inhibitor content was compared on the basis of kernel mass rather than on a per kernel basis. When comparisons were made between planting dates on the basis of α-amylase inhibitor per gram of kernel, planting date showed a significant effect (Tables 13 & 14). For the 1986 studies, planting date showed a significant effect for all cultivars. The late planting date of RL4137, RL4555 and Norquay, and the early planting date of Tordo gave significantly more inhibitor than the early and late dates respectively. The late planting date of all cultivars tested in 1987 gave significantly more α-amylase inhibitor per gram of kernel than the early date.

The α-amylase inhibitor can be extracted with a dilute salt buffer, and therefore falls into the definition of a "soluble" protein. It is desirable to determine if the

TABLE 13. Alpha-amylase inhibitor content expressed on the basis of kernel mass for the wheat cultivars grown in 1986.

Cultivar	Planting Date[1] Early	Late
	(ug inhibitor/g kernel)	
RL4137	584.8b	657.8a
RL4555	454.5c	549.5b
Norquay	345.9e	377.4d
Tordo	558.5b	392.2d

[1]Treatments with the same group letter are not significantly different ($\alpha = 0.05$) (c.v. = 8.6).

TABLE 14. Alpha-amylase inhibitor content expressed on the basis of kernel mass for the wheat cultivars grown in 1987.

Cultivar	Planting Date[1] Early	Late
	(ug inhibitor/g kernel)	
Columbus	790.3c	1421.0b
RL4555	673.9d	1467.9b
Norquay	479.2f	592.6e
Tordo	560.0e	1823.5a

[1]Treatments with the same group letter are not significantly different ($\alpha = 0.05$) (c.v. = 8.8).

environment affects the level of this protein relative to the level of total soluble protein. Data for the proportion of α-amylase inhibitor relative to total soluble protein is presented in Table 15 and 16, for the 1986 and 1987 studies respectively. In the 1986 study, planting date had little effect on the proportion of the inhibitor relative to the soluble protein content. There were large differences between planting dates in the 1987 study. The late planting date of each cultivar had a greater proportion of inhibitor to soluble protein than the early date.

The α-amylase inhibitor levels were also compared to total protein content. The results for the 1986 and 1987 studies are shown in Tables 17 and 18, respectively. Planting date did not have a great effect on the relative proportion of inhibitor to total protein in the 1986 study. In the 1987 study, however, planting date did produce differences in the proportion of inhibitor to total protein. The late planting date resulted in grains having a higher proportion of inhibitor to total protein than the early date, for each cultivar. The proportion of inhibitor to total protein for the late planting date of Columbus, Tordo and RL4555 was nearly double that of the early planting date. There was little difference in the proportion of inhibitor to total protein between either planting date of Norquay.

TABLE 15. Proportion of α-amylase inhibitor to soluble protein for the wheat cultivars grown in 1986.

Cultivar	Planting Date: Early	Planting Date: Late
	Proportion x 100 (%)	
RL4137	2.5	2.5
RL4555	1.6	2.0
Norquay	1.7	1.6
Tordo	2.4	2.0

TABLE 16. Proportion of α-amylase inhibitor to soluble protein for the wheat cultivars grown in 1987.

Cultivar	Planting Date: Early	Planting Date: Late
	Proportion x 100 (%)	
Columbus	2.5	3.5
RL4555	1.9	3.3
Norquay	1.2	1.6
Tordo	1.5	4.8

TABLE 17. Proportion of α-amylase inhibitor to total protein for the wheat cultivars grown in 1986.

Cultivar	Planting Date	
	Early	Late
	Proportion x 100 (%)	
RL4137	0.35	0.47
RL4555	0.33	0.44
Norquay	0.25	0.26
Tordo	0.38	0.33

TABLE 18. Proportion of α-amylase inhibitor to total protein for the wheat cultivars grown in 1987.

Cultivar	Planting Date	
	Early	Late
	Proportion x 100 (%)	
Columbus	0.46	0.75
RL4555	0.42	0.84
Norquay	0.27	0.36
Tordo	0.34	1.10

4.3.4 Distribution of the α-Amylase Inhibitor in the Wheat Kernel

The distribution of the α-amylase inhibitor in the embryo and endosperm fractions was determined from kernels of each cultivar and planting date from the 1987 study. These data may be useful in determining where the inhibitor may be having the greatest effect on α-amylase activity. Table 19 shows that planting date had an effect on the level of the inhibitor found within the embryo of RL4555, but not the other three cultivars tested. The embryo fractions of RL4555 from the late planting date had significantly more inhibitor than the embryo fractions from grains produced in the early planting date. There were no significant differences in the embryo levels of the inhibitor between the early and late planting dates of Columbus, Norquay, and Tordo. The level of the inhibitor within the embryos of the dormant cultivars (Columbus and RL4555) (Table 3) was higher than the non-dormant cultivars (Norquay and Tordo) (Table 3).

Planting date had a significant effect on the concentration of the inhibitor found within the embryo and endosperm fractions. Table 20 shows that the concentration of the inhibitor was significantly higher in the embryo fraction compared to the endosperm fraction for Columbus and RL4555. This observation was found in both the early and late planting dates. There was no significant difference in

TABLE 19. Alpha-amylase inhibitor content of embryo and endosperm fractions of kernels from wheat cultivars grown in 1987.

	Planting Date[1]			
	Early		Late	
Cultivar	Embryo	Endosperm	Embryo	Endosperm
	(ug inhibitor/fraction)			
Columbus	1.4ef	22.5bc	1.5ef	26.0b
RL4555	1.3fg	23.0b	1.7e	30.0a
Norquay	0.8g	14.3d	0.8g	15.1d
Tordo	0.8g	20.2c	1.0g	30.4a

[1]Treatments with the same group letter are not significantly different ($\alpha = 0.05$) (c.v. = 9.3).

concentration between the embryo and endosperm fractions of Norquay for either planting date. There was no significant difference between the two fractions for the early planting date of Tordo. However, for the late planting date, the endosperm fraction had a significantly higher concentration of inhibitor than the embryo portion. Table 21 shows the ratio of the α-amylase inhibitor concentration between the embryo and endosperm fractions. For a given cultivar, planting date does not appear to cause the grains to preferentially accumulate the α-amylase inhibitor in the endosperm versus the embryo fractions.

4.3.5 Alpha-Amylase Activity in Mature Grains

Alpha-amylase activity of the mature grains was determined for the cultivars from the 1987 study. This measurement was undertaken in order to assess the degree of sprouting damage present at maturity, as well as to determine if planting date affected α-amylase activity in mature grains. Bingham and Whitmore (1962) indicated that cultivars differed in their level of α-amylase activity in mature grains.

Table 22 demonstrates that the data from the early planting date did not confirm the results of Bingham and Whitmore (1962). There was no significant difference in α-amylase activity between Columbus, Norquay and Tordo, however, RL4555 was different from Columbus and Norquay.

The results from the late planting date are more in

TABLE 20. Concentration of the α-amylase inhibitor in embryo and endosperm fractions of kernels from wheat cultivars grown in 1987.

	Planting Date[1]			
	Early		Late	
Cultivar	Embryo	Endosperm	Embryo	Endosperm
	(ug inhibitor/gram)			
Columbus	1400.0d	750.0f	2500.0b	1500.0d
RL4555	1182.0e	614.5f	2833.0a	1634.6d
Norquay	571.0f	594.1f	727.0f	653.8f
Tordo	727.0f	657.5f	1667.0d	2000.0c

[1]Treatments with the same group letter are not significantly different (α = 0.05) (c.v. = 7.5).

TABLE 21. Proportion of α-amylase inhibitor concentration in embryo fractions versus endosperm fractions of kernels for the wheat cultivars grown in 1987.

Cultivar	Planting Date: Early	Planting Date: Late
	(embryo:endosperm)	
RL4137	1.9:1	1.7:1
RL4555	1.9:1	1.7:1
Norquay	1.0:1	1.1:1
Tordo	1.1:1	0.8:1

agreement with Bingham and Whitmore (1962) (Table 22). The results from each cultivar differed significantly in α-amylase activity from the other cultivars, except for Columbus and Tordo. The results from these two cultivars did not differ significantly from each other.

Planting date does not appear to have had a significant effect on the level of α-amylase activity. The only cultivar that showed any difference in activity from one planting date to another was Norquay. The late planting date had significantly more α-amylase activity than the early date. This is an interesting observation, as the degree of visible sprouting damage was greater for the early planting date for this cultivar.

4.3.6 Alpha-Amylase Activity in Germinated Kernels

For the 1987 studies, α-amylase activity was determined in kernels germinated for three days. These kernels were stored at -20°C prior to testing in order to maintain the dormancy that was present at harvest. This measurement was performed in order to quantify the sprouting damage that had occurred and see if planting date affected the degree of damage. Hagemann and Ciha (1984) utilized an enzymatic assay to estimate the ability of wheat cultivars to produce α-amylase. These researchers also related the level of α-amylase activity with incidence of precocious germination. The α-amylase activity data for the four cultivars tested in 1987 is presented in Table 23. From the results presented,

it is clear that the 3 day germination period was not long enough to induce significant α-amylase activity in Columbus or RL4555, as compared to Norquay and Tordo. There were no significant differences between Columbus and RL4555; however, these two cultivars differed significantly from Norquay and Tordo. Also, Norquay had significantly higher levels of α-amylase activity than Tordo. There was no significant effect of planting date for Columbus or RL4555, but there was for Norquay and Tordo. The late planting date of Norquay and Tordo had significantly more activity than the early date.

Gordon (1979) and McCrate et al. (1981) demonstrated that α-amylase activity and visible sprouting were not necessarily correlated. The results of this study follow this observation (Table 24). There was no relationship between α-amylase activity and visible sprouting for Columbus or RL4555. The degree of visible sprouting was different for each cultivar, and each planting date, but there was no significant difference in α-amylase activity. This may be due to the germination period not being of sufficient duration. A better estimate of sprouting damage due to enzyme activity may have been obtained if α-amylase activity was determined in the material from the sprouting tests (Table 3). After seven days of sprouting, enzyme activity may have been significantly greater. However, this lack of α-amylase activity may just be an indication of the

TABLE 22. Alpha-amylase activity of whole, mature grains from the wheat cultivars grown in 1987.

Cultivar	Planting Date[1] Early	Late
	(IDC units /20 kernels)	
Columbus	26.5d	30.2cd
RL4555	37.2cb	39.6b
Norquay	26.7d	100.3a
Tordo	30.6cd	23.4d

[1]Treatments with the same group letter are not significantly different (α = 0.05) (c.v. = 13.6).

TABLE 23. Alpha-amylase activity of 3 day germinated grains from the wheat cultivars grown in 1987.

Cultivar	Planting Date[1] Early	Late
	(IDC units /20 kernels)	
Columbus	628.8e	653.8e
RL4555	461.8e	448.3e
Norquay	14808.8b	22965.3a
Tordo	1653.5d	2119.8c

[1]Treatments with the same group letter are not significantly different ($\alpha = 0.05$) (c.v. = 5.1).

TABLE 24. Per cent germination after 3 days germination for the wheat cultivars grown in 1987.

Cultivar	Planting Date[1] Early	Late
	(%)	
Columbus	10e	0ef
RL4555	30c	5e
Norquay	80a	50b
Tordo	20d	75a

[1]Treatments with the same group letter are not significantly different ($\alpha = 0.05$) (c.v. = 7.0).

relative dormancy of these two cultivars as compared to Norquay and Tordo. Alpha-amylase activity was not correlated with sprouting incidence for Norquay. The late planting date of Norquay had significantly more enzyme activity, while the early date had more germination. For Tordo, the kernels with higher α-amylase activity (from the late planting date) did show greater sprouting.

For each cultivar and planting date, the level of activity was greater after three days of germination as compared to the mature, steady state levels (Table 22 and 23).

5. GENERAL DISCUSSION

The first part of this study examined the sprouting of the wheat cultivars RL4137, Columbus, RL4555, Norquay and Tordo. The level of sprouting for each cultivar was determined. The effect of different environmental conditions during grain development on the level of sprouting was also examined. The two planting dates per year were clearly effective in providing substantially different growth conditions. In each year of the study, early planted crops were developing grains under warmer conditions than the crops planted later (Table 1 and 2). RL4137 from the 1986 study was replaced by Columbus (a Neepawa X RL4137 cross) in the 1987 study. RL4137 is the parent cultivar that contributes the dormancy characteristics to Columbus (Campbell and Czarnecki, 1981). This exchange was performed because grain samples of RL4137 were not available at the time of the early planting date. This should not have posed a problem as the sprouting character and falling number values of these two cultivars are very similar (Czarnecki, 1987). The results indicate that RL4137 and Columbus had similar sprouting resistance, although with the early planting date, Columbus did show significant sprouting (Table 3 and 4). Differences in sprouting between RL4137 and Columbus could be genetic or

environmental. Differences in α-amylase inhibitor levels may also be attributed to genetic variation or environmental conditions (Table 11 and 12). All cultivars at both planting dates grown in 1987 had higher inhibitor levels than the cultivars of a given planting date grown in 1986. It would be reasonable to suggest then, that the environment was a source of variation in inhibitor levels between RL4137 and Columbus.

Belderok (1971) showed that the duration of dormancy was decreased if the grains develop under high temperatures, particularly in the last two weeks of development. This study did not examine the length of the dormant period, but did determine the extent of dormancy present at harvest maturity (Table 3 and 4). Black et al. (1987) found a low temperature induction of dormancy for wheat. The results from this study are in general agreement with these researchers. Ears from cultivars exposed to more accumulated growing degree days (AGDD) sprouted more rapidly and to a larger extent than those exposed to fewer AGDD. The exception to this trend was the 1987 crop of Tordo. This cultivar demonstrated significantly higher levels of sprouting when the grains developed under cooler conditions. The reason for this was unclear, but might be related to the drought conditions Winnipeg experienced in 1987. The 1000 kernel mass for kernels from the late planting date of Tordo was significantly lower than the early planting date. This observation by itself may not be of great significance, as

the late planting dates of the other cultivars tested also produced kernels of significantly lower 1000 kernel mass than the early date. Perhaps more important is that these kernels were also shrivelled to a much greater extent than kernels from the late planting date of the other cultivars tested. This suggests that the grains from the late planting date of Tordo were not able to develop normally, and consequently performed poorly in the sprouting test. The known biochemical functions of the inhibitor and α-amylase are used as the basis for discussing the metabolic function of the inhibitor during kernel maturation and sprouting. The α-amylase isozyme detected in this study was not identified; however, the low-pI group is the form of the enzyme that is found in developing wheat kernels (Olered and Jonsson, 1970). Weselake et al. (1983a) showed that the inhibitor binds only to the high-pI group. This group is the form of α-amylase that is found predominantly during germination (Olered and Jonsson, 1970). These findings suggest that the inhibitor is probably not affecting α-amylase activity under conditions of normal development.

A possible function of the inhibitor is to prevent starch degradation during the latter stages of kernel development of some cultivars. Ciha and Goldstein (1984) demonstrated that wetting treatments late in kernel development had a significant effect on α-amylase activity. They also showed that this effect is cultivar dependent. Unfortunately, the isozyme group detected was not specified.

However, Cornford and Black, (1985) and Marchylo et al. (1980) found the high-pI group in mature kernels of high α-amylase containing cultivars. Enzyme increase in these cultivars also occurs late in development, during the period of rapid water loss. A high GA content during desiccation of the kernels may be inducing enzyme synthesis, as GA sensitivity is induced at this time (Armstrong et al., 1982). Another possibility is that a decrease in ABA levels, or sensitivity to ABA allows the kernel to respond to endogenous GA (King et al., 1979). In light of these findings, the inhibitor may be reacting with the high-pI α-amylase late in kernel maturation to prevent starch granule degradation. Furthermore, since the inhibitor is an ABA inducible protein (Mundy, 1984), and ABA is thought to have a role in preventing precocious germination, perhaps the inhibitor is acting in concert with ABA in this preventative manner. That is, ABA is preventing embryo germination, while the inhibitor is preventing starch reserve mobilization.

In addition to attenuating α-amylase activity during the latter part of kernel development, the inhibitor may be modulating the activity of the high-pI group during germination (Weselake et al., 1985b; Hill et al., 1987). This study confirmed the results of Robertson (1989), that the inhibitor was found to a much greater extent in the endosperm than in the embryo (Table 19). The aleurone tissue synthesizes a large amount of the high-pI α-amylase

during germination (Jacobsen et al., 1970; MacGregor and Ballance, 1980), with most of this being secreted into the endosperm (MacGregor et al., 1984) and a small amount present in the embryo. If the role of the inhibitor is to decrease enzyme activity, then it is reasonable to expect that the majority of the inhibitor should be found where the majority of enzyme activity is found. The concentration of the inhibitor was greatest in the embryo (Table 20 and 21). This may reflect on the ability of the kernel to modulate the α-amylase that is synthesized by the scutellum during early germination (Mares, 1987; Cornford et al., 1987).

Robertson (1989) has demonstrated that the inhibitor protein accumulates early in barley kernel development, while other reports indicate that accumulation persists into later stages of development (Lauriere et al., 1985; Munck et al., 1985). These conflicting results could be genetic or environmental. Munck et al. (1985) and Robertson (1989) have shown genetic variations in the inhibitor contents of mature barley kernels. This study demonstrated genetic variation in the final level of the inhibitor of mature wheat kernels (Table 11 and 12). For the cultivars examined, there was no relationship between the number of genes for red grain coat color and inhibitor levels. The more dormant cultivars did, however, have a higher level of inhibitor than the less dormant cultivars.

The relationship between sprouting index and inhibitor levels appears to be in the form of a quadratic regression.

For the 1986 data there was a high correlation (R^2=.77). This relationship was present in the 1987 data, although the second planting date of Tordo did not produce grains that followed the trend of high inhibitor and low sprouting incidence. Consequently, the regression was low when Tordo was included in the test (R^2=.19). However, removal of Tordo from the analysis resulted in a high R^2 value (.80) for the remaining cultivars.

Regression analysis for sprouting index and inhibitor levels between the 1986 and 1987 data was not possible because of the different scales used to determine sprouting index in each year.

Warkentin et al. (1983) suggest that moisture and temperature stress directly influence protein content. Decreased moisture and increased temperature increase protein mainly by reducing yield. Protein content and yield of the cultivars planted early was higher than the later planted crops (Table 5, 6, 9 and 10). This suggests that the cultivars from the early date were not under as much stress as the cultivars from the late planting date. Even though the grains from these cultivars developed under cooler temperatures (Table 1), they probably were exposed to conditions of moisture stress. The later planted cultivars would likely have less available soil moisture, as potential evapotranspiration exceeds rainfall during the growing season in all areas of wheat production in Western Canada (Coligado et al.,1968). The effects of moisture stress were

amplified for the 1987 study, as Winnipeg experienced drought conditions. The effect of the drought conditions was demonstrated as decreased yield and increased protein levels for the 1987 study as compared to the 1986 study.

The effect of the drought conditions of 1987 also served to increase inhibitor levels (Table 11 and 12). Work by Robertson (1989) has demonstrated that the inhibitor is produced in response to stress conditions in barley. The increased inhibitor content under the more stressful conditions of the 1987 season may be following a similar pattern. ABA can be synthesized under stress conditions (Zeevaart and Creelman, 1988) and it has been shown to mediate inhibitor induction during dehydration stress (Robertson, 1989). Under stress conditions, Robertson (1989) has suggested that the inhibitor may be acting against a protease rather than α-amylase.

The environment under which the grains developed also had a significant effect on the final level of the inhibitor within planting dates of each year of the study. This effect was not seen to a great extent when comparisons were made on a per kernel basis (Table 11 and 12), but did become apparent when inhibitor levels per gram of kernel were studied (Table 13 and 14). The kernel mass and size are closely related parameters (Woodbury and Wiebe, 1983a). It may be reasonable to expect that larger kernels have a greater capacity to synthesize α-amylase than smaller kernels of the same cultivar. To guard against this

increased α-amylase activity, the level of the inhibitor may or may not be greater in the larger kernels. The results from this study suggest that the kernel does not synthesize inhibitor to a greater extent in larger kernels (Table 5,6,11 and 12). In fact on a per gram of kernel basis, the smaller kernels accumulated more inhibitor than the large kernels (Table 13 and 14). This was not simply a dilution effect, where the kernel synthesizes a given amount of inhibitor regardless of how the environment affects the level of carbohydrate produced. The results of the 1000 kernel mass experiments clearly indicate that the environment had an effect on the amount of carbohydrate produced (Table 5 and 6). The environment also affected the amount of total protein synthesized, with a lower protein content found in kernels of lower 1000 kernel mass (Table 9 and 10). Under these conditions the kernel was actually synthesizing a greater proportion of inhibitor relative to the total protein content (Table 17 and 18). These same conditions favoured the production of more dormant grains (Table 3).

The total inhibitor content of the kernel does not decrease rapidly. After a five day germination period, degradation by a protease reduces inhibitor levels by less than 50% (Lecommandeur et al., 1987). This suggests that the inhibitor does not likely serve as a reserve protein. This observation combined with the fact that the inhibitor is a 'soluble protein' and that 'soluble proteins' are the

metabolically active proteins (Gottschalk and Muller, 1983) suggests that the inhibitor does have a function in the kernel. As such, the level of the inhibitor should increase relative to the total protein content under conditions where the inhibitor would be expected to have a more prominent role. The early and late planting dates had an effect on the level of sprouting (Table 3) and the concomitant production of α-amylase for the non-dormant cultivar Norquay (Table 23). The inhibitor to total protein ratio was greater where sprouting incidence was higher but not where higher α-amylase activity was observed. This may be an indication that the level of α-amylase activity at the time of measurement was sufficient to overcome the effects of the inhibitor. Tordo did follow the expected path of high sprouting, high α-amylase activity. For RL4555 and Columbus the three day germination period was not long enough to produce high levels of activity compared to Norquay and Tordo (Table 23). Alpha-amylase activity in these two cultivars after three days of germination was, however, higher than in the non-germinated kernels (Table 22). This indicated that there was some synthesis of α-amylase as a consequence of the germination. The inhibitor may have been preventing the full expression of the α-amylase by keeping α-amylase activity levels low compared to Norquay and Tordo. To determine if the inhibitor is modulating α-amylase activity, the enzyme activity should be monitored regularly throughout the germination sequence rather than only once.

6. Conclusion

This study showed that the level of the α-amylase inhibitor in wheat kernels is affected by the environment. The level of the inhibitor is increased under environmental conditions that favour the production of dormant grains. The inhibitor is also induced under stress conditions and this may have been in response to increased ABA content. In some cultivars, the inhibitor may be modulating α-amylase activity even though visible sprouting may or may not occur. This may serve to help prevent precocious germination, or provide some degree of protection from sprouting damage due to enzyme production during the early stage of sprouting. Further investigation into the α-amylase activity present throughout the course of the germination sequence and how this activity relates to the level of the inhibitor would be useful.

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