

EFFECT OF ENVIRONMENT AND ABA ON α -AMYLASE/SUBTILISIN
INHIBITOR ACCUMULATION DURING KERNEL DEVELOPMENT AND
EARLY SEEDLING GROWTH IN HORDEUM VULGARE L.

BY

MASUMI ROBERTSON

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Submitted to the Faculty

of

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ABSTRACT

Robertson, Masumi. Ph. D., The University of Manitoba, July 1989. Effect of Environment and ABA on α -Amylase/Subtilisin Inhibitor Accumulation During Kernel Development and Early Seedling Growth in *Hordeum vulgare* L. Major Professor; Dr. R.D. Hill.

Regulation of α -amylase/subtilisin inhibitor synthesis was studied in the kernel and embryo during barley development. Effects of ABA and dehydration stress were also examined to elucidate the physiological function of the inhibitor.

In the developing barley kernel, inhibitor synthesis was initiated shortly after fertilization and most active protein accumulation occurred early in kernel development parallel to an increased level in inhibitor mRNA. Inhibitor mRNA content after 19 DPA was higher than expected from a small amount of protein accumulation, suggesting the presence of translational control. The α -amylase content was very low throughout kernel development, thus the inhibitor is unlikely to attenuate the enzyme activity in immature kernels.

In mature kernels, the inhibitor was mostly in the endosperm, although a small amount was present in the embryo. Inhibitor expression is confined to kernel tissue since neither the protein nor its mRNA was detected in vegetative tissue, indicating that its function is limited to the kernel.

The α -amylase inhibitor was induced by dehydration stress in barley. The inhibitor accumulated following increases in ABA content in isolated immature embryos and young seedlings under dehydration. Exogenous application of ABA induced inhibitor synthesis in isolated immature embryos and young seedlings. In addition, during normal embryo development, the inhibitor accumulated as embryonic ABA increased up to grain desiccation. The evidence suggests that the induction of the inhibitor is mediated by ABA under dehydration stress. Of the two known biochemical functions of the inhibitor, the protein may function as a protease inhibitor rather than an α -amylase inhibitor under stress.

Exogenous ABA arrested germination in isolated immature embryos, while in its absence, the immature embryos germinated. As the embryos aged, responsiveness to ABA decreased as more embryos germinated at a given ABA concentration. Responsiveness to ABA, however, was different for inhibitor synthesis and germination. More

inhibitor was accumulated by ABA application in isolated immature embryos at stage IV than those at stage III. Both germinated and non-germinated embryos responded to ABA treatment by increasing the inhibitor. In contrast to the embryos, exogenous application of ABA did not show any change in inhibitor synthesis in the kernel.

A modified RNA isolation procedure was developed to obtain RNA from starch-rich barley kernels. Kernel starch was removed by centrifugation of tissue homogenate, followed by an addition of guanidinium to inactivate RNase completely, then RNA was isolated by ultracentrifugation through CsCl/EDTA. Using the RNA thus isolated, an immunoprecipitation of total in vitro translation products showed a single polypeptide having Mr of 21,500. This protein was processed by canine pancreatic microsomal membranes to a smaller protein of about 20,000, demonstrating the presence of a signal peptide. Northern analysis of kernel poly(A)-RNA for the inhibitor mRNA showed a single transcript of about 900 nucleotides.

OBJECTIVES

During germination of cereal grains, many hydrolytic enzymes are produced by the aleurone layers. Alpha-amylase is the dominant protein synthesized and secreted by the aleurones. This enzyme has been extensively studied due to its importance in the hydrolysis of kernel reserves during germination. The enzyme is important because of its effect on sprout-damaged cereal grains and its role in the modification of endosperm starch during the malting process. Alpha-amylase is composed of two isozyme groups, the high-pI isozyme group is germination-specific whereas the low-pI group is not. Synthesis of the high-pI isozyme group is regulated by plant growth substances in the mature aleurone layers. Alpha-amylase synthesis is promoted by gibberellic acid (GA_3) and inhibited by abscisic acid (ABA).

An endogenous α -amylase inhibitor has been isolated and characterized from barley and wheat. It inhibits the high-pI isozyme group of barley α -amylase and a bacterial protease, subtilisin. ABA induction of the protein in the aleurone, together with its inhibitory action against germination specific α -amylase, suggests that its physiological function is to attenuate germination

processes by retarding starch hydrolysis upon forming a complex with α -amylase. In order to gain better understanding of inhibitor function for the benefit of crop improvement, it would be beneficial to understand factors that may affect inhibitor synthesis during kernel development.

Although activity assays provide meaningful information for inhibitor action, investigation at the molecular level was desirable to further characterize inhibitor synthesis. To detect and quantify the α -amylase inhibitor protein and its message, analytical methods were developed. Tissue specificity of the inhibitor will be used to illustrate these techniques. Isolation of RNA from the kernel tissue was difficult using the established extraction procedure, due to the high starch content in the kernel. To reliably isolate undegraded RNA, a modified procedure was developed combining two separate procedures.

It is essential to understand the pattern of inhibitor synthesis in immature kernels, thus inhibitor synthesis was studied in developing kernels and embryos. This would help to postulate metabolic function of the inhibitor during development and to examine the possible presence of regulating factors. Its temporal pattern was also compared with those of other kernel proteins.

ABA is widely considered to regulate embryo development and to inhibit germination. A set of proteins is induced by ABA in germination-arrested embryos. Since both inhibitor synthesis and germination can be regulated by ABA, the role of the α -amylase inhibitor in controlling germination was further investigated in isolated immature embryos. ABA is also involved in plant stress response. Plants exposed to water stress undergo adaptation to stress by means of physiological and biochemical changes. Synthesis of a unique set of dehydration-induced proteins has been shown to be mediated by ABA and these proteins may be involved in plant adaptation to imposed stress. A condition known to increase endogenous ABA in plants, dehydration stress, was applied to the embryos and young seedlings to investigate a possible physiological function for the inhibitor under stress.

LITERATURE REVIEW

2.1. Cereal α -amylase Inhibitor

Cereal kernels contain proteins with inhibitor activity against α -amylase. Various homogeneous protein preparations from different extraction fractions exhibit inhibitory activity. The most extensively studied proteinaceous inhibitors are the albumin components of wheat (Buonocore et al., 1977; Deponte et al., 1976). A small fraction of inhibitory activity is also found in wheat gliadin fraction (Pace et al., 1978) and in barley globulin (Barber et al., 1986). These inhibitors are effective against α -amylases of bacterial, insect and mammalian origin. Inhibitors that are effective against endogenous plant α -amylases are more recent in their discovery. Heat-stable inhibitors have been isolated from wheat (Warchalewski, 1976, 1977a, 1977b and 1983) and from maize (Blanco-Labra and Iturbe-Chinas, 1981). Heat-labile inhibitors of barley malt α -amylase in cereals have been isolated and characterized from two independent workers (Mundy et al., 1983 and 1984; Weselake et al., 1983a). Further attention will be focused on these inhibitors.

2.1.1. Isolation and Characterization of the α -Amylase Inhibitor

Weselake and Hill (1982) hypothesized the presence of an endogenous inhibitory factor in triticale as a result of abnormalities observed during the isolation of α -amylase. Earlier, using isoelectric focusing to separate α -amylase isozyme groups according to their isoelectric points (pIs), MacGregor and Ballance (1980) noted the disappearance of a group of α -amylase isozymes termed α -amylase III and observed increased activity of the α -amylase II group after heat treatment. Subsequent investigations led to the isolation of an endogenous α -amylase inhibitor from barley (Mundy et al., 1983; Weselake et al., 1983a and 1983b) and wheat (Mundy et al., 1984). The purified material yielded a single band on isoelectric focusing and SDS-PAGE, indicating that the inhibitor consisted of a single protein.

This proteinaceous inhibitor has a molecular weight, based on the amino acid sequences, of 19,865 for barley (Svendsen et al., 1986) and 19,641 for wheat (Maeda, 1986). These figures are consistent with values obtained for the inhibitor from mature barley kernels (Hordeum distichum cv. Klages) (Weselake et al., 1983b), green barley malt (H. vulgare cv. Gula) (Mundy et al., 1983) and wheat (Triticum aestivum cv. Solid) (Mundy et al., 1984), which have estimated relative molecular weights between 20,000 and 21,000 using SDS-PAGE or gel filtration. The protein has a

pI of 7.3 (Weselake et al., 1983b) or 7.2 (Mundy et al., 1983 and 1984). The amino acid sequences (Maeda, 1986; Svendsen et al., 1986) show a homology of greater than 90% between barley and wheat inhibitors. The inhibitor is 59% homologous to Kunitz-type trypsin inhibitors in soybean (Koide and Ikenaka, 1973), and 58% homologous to that in winged bean (Yamamoto et al., 1983). The amino acid compositions of these inhibitors also showed similarities to the previously reported subtilisin inhibitor from barley (Yoshikawa et al., 1976) and band-2 protein (Rodaway, 1978).

2.1.2. Inhibition Characteristics

The inhibitory activity of the protein is specific towards high-pI α -amylases from germinated barley and wheat, whereas low-pI α -amylase activities are not affected by the inhibitor (Weselake et al., 1983a). Upon binding with the high-pI isozymes, the inhibitor converts the enzyme to a previously reported (MacGregor and Ballance, 1980) higher pI amylase group (Weselake et al., 1983a). Two moles of the inhibitor bind one mole of the enzyme during the inhibition process (Halayko et al., 1986). The binding affinity between the inhibitor and high-pI amylase is pH dependent; it is 7 fold greater at pH 7.0 than at pH 5.5 (Halayko et al., 1986). The difference in binding affinity corresponds to the observed difference in inhibitory ability, suggesting that complex formation is the mechanism of inhibition

(Weselake et al., 1983b). Salt concentration affects complex dissociation; a concentration of 0.3M NaCl reduces the association by 50-fold (Halayko et al., 1986). Alteration of a specific tryptophan residue on high-pI α -amylase reduces inhibitor binding, suggesting that this region is the site of action of the inhibitor (Halayko et al., 1986). In order to elucidate the structure-function relationship of the inhibitor, a crystallographic study is reported to be underway (Maeda et al., 1987). Examination of hydrolysis products by α -amylase in the presence of the inhibitor shows no significant differences when amylose is used as a substrate, however the composition of the low molecular weight products is slightly altered when starch granules are used (MacGregor et al., 1986).

The protein also inhibits the bacterial serine protease, subtilisin, at 1000-fold greater binding affinity than its binding to α -amylase (Mundy et al., 1983; Yoshikawa et al., 1976). Inhibitors of microbial proteases of similar sizes and pIs have also been isolated from wheat, rye, and triticale (Mosolov and Shul'gin, 1986) and these are probably the same inhibitor, since proteins having immunochemical similarity have been identified from wheat, rye, and triticale but not from sorghum, oats, millet, rice, and maize (Weselake et al., 1985a).

2.1.3. Regulation of Inhibitor Synthesis and its Function

Regulation of α -amylase inhibitor synthesis has been studied during grain development and in aleurone layers, with particular reference to its hormonal control by GA₃ and ABA. In contrast to α -amylase, α -amylase inhibitor synthesis is promoted by ABA and abolished by GA₃ (Mundy, 1984; Mundy et al., 1986; Mundy and Rogers, 1986). ABA increases de novo synthesis of the inhibitor in embryo-less half seeds or isolated aleurone layers with most of the protein synthesized being retained in the tissue (Mundy, 1984; Mundy and Rogers, 1986). Enhanced synthesis results from an increased level of translatable message for the inhibitor (Mundy et al., 1986; Mundy and Rogers, 1986).

Inhibitor synthesis during grain development has been studied by various methods. Single radial immuno-diffusion techniques used by Lauriere et al. (1985) show a steady increase of the inhibitor content from 12 to 37 days post anthesis (DPA), a period prior to kernel desiccation. Inhibitor accumulation in three barley cultivars, as measured by ELISA, increases logarithmically at 20 to 30 days after pollination (Munck et al., 1985). The inhibitor is detectable at 14 DPA by immunoblotting and its relative abundance increases up to 44 DPA (Mundy and Rogers, 1986). Translatable message for the inhibitor, is slightly higher relative to total poly(A)-RNA at 30 DPA than at 20 DPA (Mundy et al., 1986; Mundy and Rogers, 1986). The relative

inhibitor translation capacity per kernel, however, is about double for 20 DPA endosperm than 30 DPA (Mundy et al., 1986). In addition, during the 10 and 30 DPA period examined, the relative amount immunoabsorbed increases only slightly after 22 DPA (Rasmussen et al., 1988). In mature barley kernels, inhibitor contents ranged from 20 to 38 $\mu\text{g}/\text{kernel}$ (Rasmussen et al., 1988), 13 $\mu\text{g}/\text{grain}$ (Lecommandeur et al., 1987), 180 to 450 mg/kg kernel (Munck et al., 1985). This range in value probably represents the variation of a number of factors including the crop and cultivar of barley used, and the assay procedure.

Although inhibitor synthesis is induced by ABA in mature aleurone layers, no translatable inhibitor message was detected in immature aleurone layers at 20 and 30 DPA or in mature, dry aleurone layers, and synthesis appears to be localized in the starchy endosperm during kernel development (Mundy et al., 1986; Mundy and Rogers, 1986). Tissue localization shown by a immunohistochemical method indicates the association of the inhibitor around starch granules within the starchy endosperm (Lecommandeur et al., 1987). No positive presence of the inhibitor has been detected in aleurone layer cells, using the same method.

During the germination of barley kernel, protease degradation of the inhibitor appears to be slow; more than a half of the protein can be detected after a 5 day germination period (Lecommandeur et al., 1987).

Several workers have speculated on the physiological significance of the α -amylase inhibitor based on the analyses of inhibitor characteristics and the regulation of its synthesis. In mature aleurone layers, inhibitor synthesis can be induced by ABA leading to a suggestion that the inhibitor may function in the prevention of precocious germination (Mundy, 1984). However there was no significant correlation between pre-germination incidence and levels of the inhibitor in many barley cultivars, thus Munck et al. (1985) concluded that the inhibitor did not seem to be a directly limiting factor in the control of preharvest sprouting. Rather, the protein is more likely to attenuate germination during kernel maturation or early germination by reducing starch degradation (Hill et al., 1987; Weselake et al., 1985b). There have been suggestions that the significant quantities of the protein synthesized specifically in endosperm tissue during grain development may protect the kernel proteins against microbial attack due to its anti-protease activity and limit degradation of starch granules by endogenous α -amylase (Mundy and Rogers, 1986).

The role of the inhibitor in the end use of cereal grains has also been investigated. α -amylase activity, a critical factor in determining malt quality, is modulated by different levels of inhibitor activities in barley cultivars (Henson and Stone, 1988). The inhibitor is also shown to be

an effective additive in bread making. The addition of purified α -amylase inhibitor to flour containing high α -amylase activity, a serious problem encountered in pre-harvest sprout-damaged grains, during the baking process neutralizes the detrimental effect of excess α -amylase activity and normal bread loaves are produced (Zawistowska et al., 1988). The nutritional quality of barley grain, in particular the lysine content, is affected by the level of high lysine proteins. The inhibitor, which contains 7 lysine residues, may contribute to this effect. However, the inhibitor content was lower in a high lysine cultivar than in a low lysine cultivar, an opposite pattern to that found for other lysine-rich proteins (Rasmussen et al., 1988).

2.2. Protein Synthesis During Barley Kernel Development

Examination of the developmental and hormonal regulation of inhibitor synthesis in the immature barley system, and its comparison to other kernel proteins, will give a better understanding of the possible physiological significance of the protein in cereal kernels. This section outlines a general developmental pattern of barley kernels with an emphasis on the patterns of various kernel protein syntheses.

Several studies of barley kernel development have been conducted. A comparison of these studies is often

difficult, however, due to the variable growth conditions used. Temperature, for example, plays an important role in grain development as higher temperature hastens the ripening process (Goldbach and Michael, 1976). A set of growth parameters devised for wheat caryopses, that includes growth and size measurements as well as morphological characteristics, provides a convenient basis for a comparison of the different stages of development (Rogers and Quatrano, 1983). These parameters will be used to correlate the results of the various studies, where possible. The development stages are divided into 5 groups, stages I through V. Among the characteristics, grain colour and endosperm characteristics are most easily identified. Stage I caryopses are white in colour with watery endosperm, those in stage II are white to mint green in colour with milky to early dough endosperm, at stage III, grain colour remains green having soft-dough endosperm, stage IV grain colour is still green and the endosperm is hard-dough, and finally at stage V, caryopses are tan coloured and endosperm is hard.

In general, there is a rapid increase in fresh weight until the start of desiccation at stage IV (Goldbach and Michael, 1976; MacGregor et al., 1971; Rogers and Quatrano, 1983; Rahman et al., 1982). Dry weight increase occurs slightly after the fresh weight increase and the dry weight increase continues through stage V. The kernel protein

content increase is parallel to the dry weight increase (Kirsi, 1973; Macgregor et al., 1971; Rahman et al., 1982). Kernel maturation generally occurs between 40 and 60 DPA (Goldbach and Michael, 1976; MacGregor et al., 1971; Rahman et al., 1982; Rogers and Quatrano, 1983).

2.2.1. Temporal Control of Protein Synthesis

Coordination of protein synthesis during kernel development is one of many significant biochemical processes required for survival of the kernel. Kernel proteins are classified into four main groups according to their solubility characteristics (Osborne, 1895). Albumins are soluble in water and low ionic strength solutions; solutions with a higher concentration of salt will extract globulins. Prolamins are extracted with aqueous alcohols and glutelins in acidic or basic solutions. In cereals, the major storage proteins are prolamins (Higgins, 1984; Kreis et al., 1987) which are termed hordeins in barley. Proteins in other fractions are mostly metabolic, although some proteins exhibit storage protein-like characteristics (Kreis et al., 1987). The general sequence of deposition for these proteins has been studied by differential extraction and nitrogen determination of each fraction (Brandt, 1976; Rahman et al., 1982). Most of the albumins are accumulated during the early stages of development while the globulins,

glutelins and hordeins account for kernel protein increases later in development.

The largest fraction at maturity is hordein, accounting for 30 to 50% of kernel protein (Brandt, 1976; Kreis et al., 1987; Rahman et al., 1982). This fraction consists of proteins divided into three groups termed B, C and D hordeins that are present in decreasing relative abundance. B and C hordeins are present in 10 DPA kernels at very low levels and increase very rapidly later in development (Giese and Hopp, 1984; Giese et al., 1983; Rahman et al., 1982). The increase in rate of accumulation for hordein proteins corresponds with an increase in the proportion of hordein mRNA (Dailey et al., 1988; Rahman et al., 1984). There appears to be a coordination of hordein genes, but, subsequent analyses show a slight differential expression of B and C hordeins and their subgroups (Giese et al., 1983; Rahman et al., 1982 and 1984). The expression of hordeins is influenced by the nutritional status of plants during development, in particular nitrogen (Bottacin et al., 1985; Giese et al., 1983; Giese and Hopp, 1984), and sulfur (Bottacin et al., 1985; Rahman et al., 1983).

Control elements for hordein genes have been isolated from genomic clones and studied in homologous and heterologous expression systems. By use of a chimeric gene system, the 5' flanking region of a B hordein gene was shown to contain control elements for temporal expression of the

protein in transgenic tobacco plants (Marris et al., 1988). The reporter gene, chloramphenicol acetyl transferase (CAT), is expressed in tobacco kernels at the appropriate developmental interval, demonstrating that the flanking region contains controlling elements directing the temporal expression of the gene in the seed. Within this region, strongly conserved sequences are identified that are homologous to similar regions from storage protein genes of wheat and corn (Forde et al., 1985). Regulatory elements of low molecular weight glutenin-CAT and Z4 zein-GUS (beta-glucuronidase) fusion genes are capable of imposing temporal control on the synthesis of these reporter enzymes in transgenic tobacco seeds (Colot et al., 1987; Scherthaner et al., 1988).

Among the globulins, small amounts of particular storage-type proteins provide a significant lysine contribution to cereal kernel proteins. These are of interest in improving the nutritional quality of cereal kernels. For example, β -amylase, chymotrypsin inhibitors (CI) 1 and 2, and protein Z, which contain high proportions of lysine, are present in larger quantities in high lysine barley mutants (Jonassen, 1980) at the expense of hordeins (Kreis et al., 1984). Globulin accumulation starts early in kernel development with synthesis occurring at 10 DPA, before the period of hordein synthesis (Giese and Hejgaard, 1984). Beta-amylase and protein Z, however, are synthesized

later than these minor globulin species, at about the same time as hordeins (Giese and Hejgaard, 1984; LaBerge and Marchylo, 1986). The inhibitory activity of CI, also increased most rapidly later in development (Kirsi, 1973). In vitro translation and Northern analyses demonstrated that the temporal control of these globulin genes is similar to that for hordeins (Giese and Hopp, 1984; Mundy et al., 1986; Rasmussen et al., 1988; Williamson et al., 1988).

In addition to the temporal control applied to the expression of these kernel proteins, storage proteins are found exclusively in the kernel (Kreis et al., 1987). Hordein polypeptides are present only in endosperm and not in embryo tissue (Goldberg et al., 1989). Regulatory elements for this tissue specificity have been located within the same region as that for temporal control (Forde et al., 1985; Marris et al., 1988). Storage-like protein, CI-1, mRNA is also detectable only in endosperm and not in vegetative tissues, which include shoots, leaves and roots (Williamson et al., 1988). CI-1 and 2 messages are also present in immature aleurones (Mundy et al., 1986). A probable amylase-protease inhibitor is present primarily in aleurones (Mundy and Rogers, 1986). A kernel protein with a known enzymatic activity, β -amylase, is not restricted to kernel tissue and is found in various tissues of the barley plant, and corresponding abundance of β -amylase mRNA is found in these tissues (Kreis et al., 1987).

2.2.2. Characterization of Immature Kernel α -Amylase and Temporal Control of its Synthesis

Alpha-amylase, one of the major hydrolytic enzymes produced by cereal kernels during germination, is also present in immature cereal kernels. It appears in small amounts early in development and declines by the second week after anthesis in barley (MacGregor et al., 1971). This isozyme has similar characteristics to the low-pI α -amylase that appears during germination (MacGregor et al., 1974). In wheat, this isozyme is limited to the outer pericarp of the developing kernel and has a similar temporal relationship to the barley enzyme (Radley, 1976). For a cultivar with higher dormancy, however, the activity remains very low throughout development. In a cultivar with lower post-harvest grain dormancy, α -amylase activity increases following grain drying coinciding with a decline in ABA (King, 1976). Even greater activity is observed for a triticale cultivar which lacks dormancy (King et al., 1979). Further study revealed that the high amount of α -amylase, present in the mature kernels as a result of enzyme accumulation during desiccation, is composed of both high- and low-pI forms that are synthesized by the aleurone layer (Cornford and Black, 1985). Thus, it appears that α -amylase synthesized late in development in some cultivars is under a separate control from the low-pI enzymes of early development.

2.2.3. Plant Growth Substances Found During Kernel Development

The ABA and GA contents of seeds, which play an important role in seed development (King, 1989), have been determined by several workers employing various methods. For barley kernels, kernel ABA content increases following the pattern for dry weight (Goldbach and Michael, 1976; Tietz et al., 1981), and the greatest ABA content is attained as fresh weight reaches a maximum during stage IV and the content decreases rapidly with the onset of desiccation (Goldbach and Michael, 1976). Similar results are reported for wheat kernels (King, 1976 and 1979; King et al., 1979; McWha, 1975; Radley, 1976). During kernel maturation, 'free' ABA decreases rapidly accompanied by a rapid increase in 'bound' ABA, to about one tenth the level of 'free' ABA (Slominski et al., 1979). Both forms become very low in quantity at harvest.

Variation of the ABA contents can be seen among different cultivars, yet a pattern of ABA changes are maintained. For a given cultivar, higher temperature leads to a more rapid change in the level, yet the values for the maximal and mature ABA contents remain the same for barley (Goldbach and Michael, 1976) while slightly different for wheat (McWha, 1975).

An important consideration, when examining GA involvement, is that there are many forms of GAs present

that have varying biological activities. Peak GA levels are reached before the fresh weight maximum and decline rapidly prior to the ABA decrease (Radley, 1976; Slominski et al., 1979). The GA content in developing grains is less than one tenth of the ABA content in weight (Radley, 1976), yet this is much more than that found in germinating grain (Gaskin et al., 1984). Biological activities of these GAs are, however, very low, particularly lacking highly active GA₁. Germinating grain, on the other hand, contains smaller amounts of different, active forms of GAs (Gaskin et al., 1984).

2.3. Kernel Dormancy and Germination

Dormancy results from the inability of viable kernel to germinate under favourable environmental conditions (King, 1989). Evolutionary development to acquire dormancy is one of adaptational advantage in increasing physiological tolerance to limited water availability (Mapes et al., 1989).

Dormancy in kernels is an inherent character of the kernel as a result of its genetic background. The factors involved in dormancy mechanisms are complex and its regulation at the molecular level remains mostly unidentified (King, 1989). Numerous factors may modify the degree of dormancy during development, maturation and

germination. An achievement of the proper balance between the attainment of dormancy and an ability to germinate (germinability) is essential for cereal crop breeding. Considerable economical loss is experienced every year due to pre-harvest sprouting, a condition where cereal kernels germinate before processing.

2.3.1. Transition to Germination from Dormancy

In cereals, detached kernels exhibit variable germinability during development, maturation and after harvest (King, 1976; Mitchell et al., 1980; Slominski et al., 1979; Symons et al., 1983; Walker-Simmons, 1987). During development and maturation, kernels of some cereal cultivars are unable to germinate when imbibed under favourable conditions. This is referred to as primary dormancy. ABA acts in situ to regulate germination in cereals. Kernel tissue ABA, located in the palea and lemma, has been implicated in kernel dormancy during development, as kernel dormancy coincides with a period of high kernel ABA content (King, 1976) and removal of the outer pericarp increases germinability (Symons et al., 1983).

In addition to ABA, there have been other compounds contained within the kernel that have been implicated in kernel dormancy, such as catechin, catechin tannin and other water-soluble, yet unidentified compounds mainly in bran and seed coat (McCrate et al., 1982; Morris and Paulsen, 1988).

An application of these water-soluble extracts on isolated embryos prevents germination. An additional endogenous inhibitor identified is L-tryptophan in wheat grain and bran, but it is not as inhibitory as ABA, in bioassays on mature embryos, in preventing germination (Morris et al., 1988).

The onset of germinability coincides with the start of desiccation during development (Slominski et al., 1979; Walker-Simmons, 1987). Enforced drying of immature kernels induces premature germinability at earlier stages (Mitchell et al., 1980; Symons et al., 1983). These results imply that drying is one of the major factors that initiates the change from the development to the germination phase of kernels.

Coordinated with desiccation and the increase in germinability is the rapid decrease in kernel ABA levels (King, 1976; Slominski et al., 1979). Combined with the effects of ABA on embryo dormancy, ABA is considered to be important in regulating the germinability of kernels. In addition to the physical presence of ABA, there appear to be changes in the responsiveness to plant growth regulators, which are associated with germinability. The ability to produce α -amylase in response to exogenous GA₃ develops at about the same time as desiccation (King, 1976). Similar responsiveness can be artificially induced in, otherwise

non-responsive, half kernels that have been dried (Cornford et al., 1986; Evans et al., 1975; King, 1976).

2.3.2. Environmental Factors Modifying Kernel Germination

The transition from kernel development to germination is a normal part of a plant's growth cycle. However, the propensity to germinate can be modified by variable growing conditions.

Environmental conditions during seed development and maturation have a profound effect on the degree of dormancy. Cereal crops grown under cooler conditions are more dormant than those grown at higher temperature (Reddy et al., 1985). In particular, the last phase of kernel maturation determines the degree of dormancy at maturity, with low temperatures inducing a longer dormancy period (Belderock, 1971) or high temperatures reducing it (Reiner and Loch, 1976). This high temperature effect may be related to the rate of drying, since higher temperatures generally hasten the maturation process and drying. As mentioned above, germinability frequently accompanies grain drying. It is suggested that low temperatures may maintain high ABA levels for longer periods contributing to higher degrees of dormancy (Goldbach and Michael, 1976). In addition, low temperatures earlier in development confers lower dormancy, an opposite temperature effect to that found later in development (Reiner and Loch, 1976).

In addition, the degree of dormancy is affected by germination temperature. Stronger dormancy is imposed at higher germination temperatures for cereal kernels before after-ripening (Dunwell, 1981; George, 1967; Mares, 1984; Reddy et al., 1985). For after-ripened kernels, the optimum germination temperature is considerably higher (Mares, 1984) yet some non-dormant kernels fail to germinate at 30 °C (George, 1967).

2.3.3. Isolation and Characterization of Cereal α -Amylase

Alpha-amylase has been extensively studied due to its importance in the hydrolysis of kernel reserves during germination, its effect on baking quality of sprouting-damaged flour, and its significant role in the use of malting barley. The enzyme will be reviewed with regard to its relevance to the kernel germination process.

During the germination of cereal grains, many hydrolytic enzymes are synthesized and secreted by the aleurone layer to mobilize endosperm reserves for the embryo. Alpha-amylase, which is responsible for the degradation of endosperm starch, is the major protein synthesized (Varner and Chandra, 1964). The enzyme consists of a complex mixture of isozymes which can be separated by isoelectric focusing into two isozyme groups, characterized by different pIs (MacGregor and Ballance, 1980). The high-pI group is the dominant form synthesized by the aleurone

layers during germination, whereas the low-pI isozymes are found in smaller amounts in both immature and germinating barley (MacGregor, 1977; MacGregor and Ballance, 1980; MacGregor et al., 1974). The third group, α -amylase III, was identified to be a complex form of high-pI amylase group and an endogenous inhibitor of that enzyme (Mundy et al., 1983; Weselake et al., 1983a). Germinated barley kernels, green malt and kilned malt contain all three groups of the amylases of immunological identity, with a slight variation in quantitative distribution among them (MacGregor and Ballance, 1980; MacGregor and Daussant, 1981). The enzymes all have a molecular weight of about 44,000 to 45,000; their exact MW are determined by the amino acid sequence of each isozyme.

Studies of six euplasmic wheat-barley addition lines indicate that the structural genes of the low-pI group are on chromosome 1 while the high-pI group genes are on chromosome 6 (Muthukrishnan et al., 1984). Two unlinked loci were further characterized to examine the number of genes involved for each isozyme group. Southern blot analysis of barley DNA reveals the presence of multiple genes or pseudogenes coding for α -amylase transcripts (Chandler et al., 1984; Rogers and Milliman, 1984; Muthukrishnan et al., 1983b). An estimation of gene copy number indicates that there are about eight gene copies per haploid genome (Chandler et al., 1984). Use of low- and

high-pI specific cDNA probes enabled Rogers (1985) to identify five genes for low-pI and one gene for high-pI amylases based on differential hybridization intensities. The same number of high-pI genes are also predicted by primer extension studies (Rogers, 1985). Isolation of genomic clones, however, identified one low-pI and three high-pI genes from barley (Knox et al., 1987). Thus the exact number of genes encoding these isozymes remains uncertain.

Further molecular differences between low- and high-pI isozymes are found upon nucleotide sequence analysis of cDNA and genomic clones. Comparison of deduced amino acid sequences for one low- and two high-pI isozymes revealed 75% homology between the low- and high-pI isozymes and greater than 95% between the two high-pI cDNA clones (Chandler et al., 1984; Rogers, 1985). Both types of cDNA clones hybridize to a 1500 base mRNA. Sequence information also shows that α -amylase isozymes are translated with a signal peptide, 23 amino acids in length. The presence of the precursor form was predicted by in vitro translation and processing of RNA (Boston et al., 1982). Greater differences, however, are found within the signal peptides where only half of the amino acid sequence shows homology.

Possible differences in the regulatory mechanisms have been suggested on the basis of the analysis of genomic clones. Comparison of 5' regulatory sequences shows

marginally related promoter regions (Knox et al., 1987). Even within the low-pI isozyme genes, some differences are seen in the 5' region, indicating the presence of two separate genes (Whittier et al., 1987).

2.3.4. Regulation of α -Amylase Synthesis within the Aleurone

Regulation of α -amylase synthesis is important during germination. In relation to cereal germination, the mode of action of plant growth substances on gene regulation has been studied extensively using isolated barley aleurone layers as an experimental system based on the following initial reports on enzyme synthesis. During germination of barley kernels, hydrolytic enzymes are induced by gibberellic acid (Paleg, 1960a, 1960b and 1961; Varner, 1964). The site of enzyme synthesis is in the aleurone layers within the kernel (Varner and Chandra, 1964; Chrispeels and Varner, 1967a). The same isozyme groups and isozymes within each group are found in isolated aleurone layers treated with GA₃ and in germinating kernels (MacGregor, 1976).

The synthesis of α -amylase is regulated by plant growth substances in mature aleurone layers. GA₃ promotes synthesis whereas ABA inhibits synthesis (Chrispeels and Varner, 1966 and 1967b). The increase in amylase activity is due to de novo synthesis of the enzyme induced by GA₃,

(Jacobsen and Knox, 1974; Varner, 1964; Varner and Chandra, 1964), which in turn is the result of an increase in the level of translatable mRNA (Bernal-Lugo et al., 1981; Higgins et al., 1976 and 1982; Muthukrishnan et al., 1979). Investigation using the barley aleurone protoplast confirmed these findings and demonstrated that the protoplast is a useful system in the study of plant growth substances (Jacobsen et al., 1985). Combined with evidence from studies using cDNA clones for the enzymes (Chandler et al., 1984; Muthukrishnan et al., 1983b, Rogers and Milliman, 1983), it appears that events at the level of transcription are most affected by plant growth substances (Jacobsen and Beach, 1985).

Induction of enzyme synthesis by GA₃ occurs after an initial lag phase of about 6 hours, followed by a linear increase (Jacobsen, 1973; Moll and Jones, 1982; Mozer, 1980). A slightly shorter lag phase is observed for the appearance of the message (Chandler et al., 1984; Higgins et al., 1976 and 1982; Mozer, 1980; Muthukrishnan et al., 1979 and 1983a; Rogers and Milliman, 1983 and 1984). The extent of enzyme induction is GA₃ concentration dependent (Jacobsen 1973; Muthukrishnan et al., 1983a) and is also affected by the interaction between GA₃ and ABA. ABA can nullify the effects of GA₃ when ABA is applied with GA₃ and ABA effects decrease with longer time lapsed after the GA₃ addition (Higgins et al., 1982; Mozer, 1980; Muthukrishnan, et al.,

1983a; Nolan and Ho, 1988b; Nolan et al., 1987). A critical ABA concentration is required to be effective in inhibiting GA₃ effects (Nolan and Ho, 1988a). There is differential control on the expression of two isozyme group genes that is both GA₃ and time dependent. The GA₃ concentration requirement for the high-pI group is greater than that for the low-pI group (Jacobsen and Higgins, 1982; Nolan and Ho, 1988a). The lag time for the appearance of low-pI isozyms is longer than that for high-pI (Callis and Ho, 1983; Jacobsen and Higgins, 1982; Nolan and Ho, 1988a; Rogers, 1985).

Secretion of the enzyme is dependent upon the presence of Ca²⁺ in the medium (Moll and Jones, 1982). In addition, the cation has a regulatory role on α-amylase isozyme synthesis. Translation of the high-pI isozyme group requires the presence of Ca²⁺ in the medium (Bush et al., 1986; Carbonel and Jones, 1984; Deikman and Jones, 1985; Jones and Jacobsen, 1983), while the accumulation of high-pI α-amylase mRNA is not affected by Ca²⁺ (Deikman and Jones, 1985 and 1986). Ca²⁺ concentration in the cytoplasm, critical for high-pI α-amylase synthesis and secretion, is shown to be affected by both medium Ca²⁺ concentration and pH (Bush and Jones, 1988).

The effect of ABA on the GA₃ induction of α-amylase synthesis has been studied. In some studies, ABA reduced α-amylase synthesis in vivo more than the translatable mRNA

level, leading to the conclusion that ABA also acts at the level of translation of α -amylase mRNA (Higgins et al., 1982; Mozer, 1980). Other studies showed that both proteins and mRNA for α -amylase were reduced to a similar level (Bernal-Lugo et al., 1981; Chandler et al., 1984; Muthukrishnan et al., 1983a; Nolan et al., 1987). The discrepancy may have been due to a different response to ABA by the two isozyme groups which may have been overlooked due to the analytical methods used by earlier workers. ABA addition after GA₃ is less effective in inhibiting α -amylase synthesis. The longer the duration after GA₃ addition, the less effective ABA becomes (Ho and Varner, 1976; Nolan et al., 1987). This inhibitory effect of ABA on the promotional effect of GA₃ requires a continuous supply of RNA and protein synthesis since the addition of a transcriptional inhibitor abolishes the inhibitory effect of ABA (Ho and Varner, 1976; Nolan and Ho, 1988b).

Isolated aleurones are capable of taking up ABA which is metabolized into phaseic acid (PA), dihydrophaseic acid (DPA) and other metabolites (Dashek et al., 1979). The ABA metabolism is autocatalytic unique to barley aleurone layers (Uknes and Ho, 1984). The inhibitory effect of ABA added to the incubation medium can be duplicated by PA or DPA, indicating that PA or DPA may be the active substance to inhibit GA₃ effects (Dashek et al., 1979; Lin and Ho, 1986; Nolan and Ho, 1988b).

In addition to reducing α -amylase mRNA levels, ABA induces many ABA specific genes in barley aleurone layers (Higgins et al., 1982; Hong et al., 1988; Lin and Ho, 1986; Mozer, 1980). ABA induced proteins do not appear to be secreted (Lin and Ho, 1986), as profiles of secreted proteins are very similar to those secreted by aleurones incubated without plant growth substances (Higgins et al., 1982; Mozer, 1980). Although the function of the proteins remains unknown, one of the ABA induced proteins in the aleurones is a barley lectin (Lin and Ho, 1986), and another the α -amylase inhibitor (Mundy, 1984; Mundy et al., 1986).

Regulation of α -amylase synthesis in the aleurones has been discussed regarding plant growth substances. There are a few other factors controlling its synthesis. Heat-shock destabilizes α -amylase mRNA in barley aleurones (Belanger, et al., 1986). Reduction of α -amylase and induction of heat shock proteins are prominent at temperatures above 35 °C and are under transcriptional control. RNA complementary to α -amylase mRNA may also regulate α -amylase gene expression by altering the stability of the mRNA and/or by interfering with translation (Rogers, 1988). The complementary RNA is present in immature endosperm and aleurone, in mature aleurone incubated in the absence of plant growth substances, or in the presence of ABA. A proteinaceous factor induced by GA₃ in rice aleurone layers binds a 5' untranslated region of a rice genomic clone of α -amylase

(Ou-Lee et al., 1988). The proteinaceous factor is unique to GA₃ treated aleurones. A specific segment within the 5' region binds this factor and it contains putative regulatory sequences which share structural homologies with a barley gene (Knox et al., 1987). These results suggest that this factor acts as a mediator of the activation of the α -amylase gene by GA₃.

2.3.5. Responsiveness of the Kernel to Plant Growth Substances During Grain Maturation

Alpha-amylase is synthesized in response to GA during germination (Varner and Chandra, 1964). The kernel may have a varying degree of responsiveness to GA. The responsiveness can depend on factors such as artificial drying, grain maturation, environmental conditions during growth and maturation, and drying temperature. There are many contradictory findings but, so far there is no consensus of opinion on the relationship of these factors to GA responsiveness. Variability in genetic background of materials, assay methods, and/or treatments may contribute to this ambiguity. Immature grains do not respond to GA₃ with respect to α -amylase synthesis but will respond if the grain is dried prior to GA₃ treatment according to Evans et al. (1975). Nicholls (1986), on the other hand, reports that drying is not essential for the development of GA₃ sensitivity in immature kernels. Immature triticale

kernels, when dried prior to imbibition, will synthesize α -amylase and germinate in the absence of added GA₃ (King et al., 1979). In a high α -amylase-containing wheat cultivar, enzyme induction can occur during late maturation in the absence of artificial drying (Cornford and Black, 1985). For barley, environmental conditions during grain filling, and desiccation temperature during late maturation, affect GA₃ responsiveness (Nicholls, 1982). Aleurone responsiveness can be induced also by pre-incubation. Pre-incubated immature wheat aleurones in a medium can be made responsive to GA₃, producing α -amylase when transferred to a fresh medium containing GA₃ (Cornford et al., 1986).

Taken together, the interaction of many factors appears to contribute to the responsiveness to GA in cereal grains. If α -amylase synthesis is considered as a modulating factor for kernel germinability, interpretation of results becomes even more perplexing.

2.4. Embryo Development and Germination

Embryo tissues undergo some distinct changes during development that require some description apart from the overall process of grain development. In this section, embryogenesis and embryo germination of mostly monocots are reviewed. Unlike dicots, where the embryo includes the major storage organ, cotyledons, the embryo in cereal grains

represents the vital tissue that continues plant development separate from the major storage organ of monocots, the endosperm.

2.4.1. Cereal Embryogenesis and Germination

Embryogenesis is a period of embryo growth between ovule fertilization and embryo germination interrupted by developmental arrest or embryo quiescence (Goldberg et al., 1989; Quatrano, 1986). Metabolic or morphological changes of embryogenesis have been studied. Emphasis will be placed on metabolic changes in this section.

The embryo fresh and dry weights and protein content increases later in maturation (Duffus and Rosie, 1975; Grilli et al., 1986; Miller and Bowles, 1985; Walker-Simmons, 1987). Mature embryos contain large amounts of DNA and RNA (Duffus and Rosie, 1975; Grilli et al., 1986; Miller and Bowles, 1985), making the embryonic tissue metabolically active. During embryogenesis, the majority of embryonic proteins are constitutive and most of the mRNAs are continuously expressed throughout development (Goldberg et al., 1989). A profile of soluble proteins from immature embryos shows little qualitative change throughout embryo development (Grilli et al., 1986; Miller and Bowles, 1985).

A small number of proteins are, however, characteristic of embryogenesis. In cereals, three major embryogenesis specific proteins have been studied in detail;

lectin (Miller and Bowles, 1985; Morris et al., 1985; Raikhel et al., 1984 and 1988; Triplet and Quatrano, 1982), Em protein and 7S globulin (Berge et al., 1989; Williamson et al., 1985). Despite considerable characterization of these proteins and their genes, their plant function is largely unknown. They share a few common characteristics of gene expression. Their expression is temporally regulated during embryogenesis and the genes are expressed during late embryogenesis (Miller and Bowles, 1985; Morris et al., 1985; Raikhel et al., 1988; Triplet and Quatrano, 1982; Williamson et al., 1985). The expression is either exclusive to the embryo or it is several magnitudes lower in vegetative tissue (Berge et al., 1989; Raikhel et al., 1984), and the expression is spatially regulated both at the level of organs and cells. The cellular distribution is the same for the mRNA and the protein (Raikhel et al., 1988).

ABA plays a major role in maintaining embryogenesis in cereals and mediating the expression of embryo-specific genes (Quatrano, 1986). ABA content increases during embryogenesis in barley and wheat, as fresh weight increase, then rapidly decreases with desiccation (Goldbach and Michael 1976; Walker-Simmons, 1987). Embryogenesis proteins appear to be regulated by ABA since they are expressed during the period of high embryonic ABA and are induced by exogenous ABA (Goday et al., 1988; Miller and Bowels, 1985; Morris et al., 1985; Quatrano et al., 1983; Sanchez-Martinez

et al., 1986; Williamson, et al., 1985). Gene expression of these proteins usually occurs in coordination with the maintenance of an embryogenesis pathway, preventing germination (Triplet and Quatrano, 1982; Williamson and Quatrano, 1988). Further analysis of the Em gene shows that a regulatory element for synthesis of the protein in wheat is located in the 5' region since ABA is able to induce the expression of the chimeric gene containing this region and a GUS reporter (Marcotte et al., 1988).

During germination, metabolic processes within the embryo, including protein and RNA synthesis, increase markedly. A significant amount of translatable poly(A)-RNA can be found in dry, mature embryos (Brooker et al., 1978; Caers et al., 1979; Cuming and Lane, 1979; Grilli et al., 1986; Saitoh et al., 1988) and protein synthesis takes place upon the formation of polysomes using this stored mRNA during germination (Spiegel and Marcus, 1975). Although most of the stored mRNA is not required for germination, it may be important in selective adaptation to unfavourable conditions since a large number of different species of mRNA are readily available upon germination to be used without requiring transcription (Peumans et al., 1979). One of the messages stored is low-pI α -amylase (Saitoh et al., 1988), being immediately available during early germination events. Germinated embryos contained both high- and low-pI isozyme

forms (MacGregor and Marchylo, 1986), indicating that high-pI isozymes are transcribed after germination.

Following the initial phase of germination, active transcription to synthesize new RNA is essential to replace rapidly degraded poly(A)-RNA and continue protein synthesis in the embryo (Bhat and Padayatty, 1975; Brooker et al., 1978; Caers et al., 1979; Cuming and Lane, 1979; Sen et al., 1975). Inability to maintain a sufficient level of poly(A)-RNA has been associated with decreased embryonic viability (Roberts et al., 1973; Smith et al., 1986) and is attributed to selective inactivation of RNA polymerase II and poly(A) polymerase (Lakhani et al., 1987).

2.4.2. Precocious Germination and Embryo Dormancy

Cereal embryos in situ undergo a period of development and dormancy before germination. Immature wheat embryos at a certain stage of development are capable of germination when separated from the kernel (Dunwell, 1981; Symons et al., 1983; Walker-Simmons, 1987).

The precocious germination of isolated embryos can be blocked by an exogenous application of ABA, maintaining a status similar to in situ embryogenesis (Morris et al., 1985; Robichaud and Sussex, 1986; Triplett and Quatrano, 1982; Walker-Simmons, 1987). The role of ABA in restricting precocious germination has also been demonstrated in maize. Viviparous maize mutants germinate because of low endogenous

ABA levels (Robichaud and Sussex, 1986) while normal maize kernels can be induced to germinate during development by an application of fluridone to the intact kernel, inhibiting ABA synthesis (Fong et al., 1983). Fluridone inhibits carotenoid synthesis, and also decreases the endogenous level of ABA synthesized through the carotenoid biosynthesis pathway (Bartels and Watson, 1978; Gamble and Mullet, 1986; Raikhel et al., 1986).

An embryo that will not germinate, even when it is excised from the seed, is said to possess embryo dormancy (King, 1989). Embryo dormancy may arise from inhibitors within embryonic tissue, from a lack of promoters and/or from an incomplete embryo development. Frequently interaction among them, rather than any one factor, results in the onset of, and release from, dormancy (McCrate et al., 1982).

ABA is considered to be the most significant germination inhibitor (King, 1989). Application of ABA to isolated, mature embryos prevents germination (Williamson and Quatrano, 1988; Walker-Simmons, 1988). Immature embryos are able to germinate upon isolation from the kernel up until later in kernel maturation, when the embryos become dormant (Dunwell, 1981; Symons et al., 1983; Walker-Simmons, 1987). These embryos contain high embryonic ABA (Goldbach and Michael 1976; Walker-Simmons, 1987).

The degree of responsiveness of immature cereal embryos to ABA, as measured by germination, is affected by embryo age and genetic background. More mature embryos, in stage V, are less responsive to ABA than younger embryos, and a higher ABA concentration is required to arrest precocious germination (Morris et al., 1985; Robichaud and Sussex, 1986; Walker-Simmons, 1987). Embryos from a sprouting-resistant cultivar of wheat are more sensitive to ABA than those from a susceptible cultivar indicating that responsiveness may be more important than ABA content in maintaining dormancy (Walker-Simmons, 1987). Responsiveness to ABA by mature embryo also affects embryo germinability. Mature embryos are more responsive to ABA at a higher germination temperature (Walker-Simmons, 1988).

The importance of ABA in maintaining embryogenesis and preventing precocious germination, is a characteristic not only of monocots, but is shared by the embryos of dicots (Goldberg et al., 1989; Quatrano, 1986). The maintenance of embryogenesis and genes characteristic of this developmental stage are regulated by ABA (Ackerson, 1984; Bray and Beachy, 1985; Eisenberg and Mascarenhas, 1985; Finkelstein et al., 1985; Galau et al., 1986; Nielsen et al., 1989). Embryonic weight and protein content are greatly increased by exogenous ABA in dicots because seed storage protein synthesis in the cotyledons and embryo growth, measured by

fresh weight increase, are induced by ABA in dicot embryos (Ackerson, 1984; Eisenberg and Mascarenhas, 1985).

Exogenously applied GA₃ exerts an opposite effect to that of ABA, by promoting germination. It can partly overcome the inhibitory effects of ABA and water-soluble endogenous germination inhibitors, in promoting germination in mature cereal embryos (Dunwell, 1981; McCrate *et al.*, 1982). During cereal germination, embryonic GA is thought to be responsible for alleviating kernel dormancy since GA or a GA-like substance active in bioassays is produced by imbibing the cereal embryo (Cohen and Paleg, 1967; Radley, 1967). Isolated, mature embryos synthesize GA₃-dependent α -amylase isozymes both in the absence and presence of exogenously applied GA₃, indicating that there is sufficient endogenous GA present in these embryos (MacGregor and Marchylo, 1986).

2.5. Plant Response to Environmental Stress

Plants are continuously exposed to a multitude of biotic and abiotic stress conditions. Plant responses to these unfavourable conditions enable the survival and continuation of the species. Biotic stress involves the intrusion of other organisms. Abiotic stress involves the effects of unfavourable environmental conditions. This section will review plant biochemical responses to

environmental stress, with an emphasis on water stress. Changes in the physical water status of plants and their physiological response are relatively well characterized, but the biochemical changes are mostly unknown (Hsiao, 1973). Only recently have stress-induced proteins and genes been identified. Although their functions are currently largely unknown, their characterization will assist in understanding the mechanisms of plant stress tolerance. In the long term, controlled gene transformation and expression into desirable plants will benefit crop production (Sachs and Ho, 1986).

2.5.1. Plant Stress due to Limitation of Water Availability

Plants are placed under stress when insufficient water is available, due to drought and high soil salinity, resulting in a reduction in osmotic potential (Reid and Wample, 1985). Stress conditions often increase ABA in plant tissues and an application of ABA can precondition plants to an oncoming stress environment (Zeevaart and Creelman, 1988).

High soil salinity reduces soil osmotic potential, reducing water availability, in addition to causing specific salt effects in the plant. Plants respond to salinity stress by expressing stress-induced proteins specific to cell, tissue and genotype (Hurkman et al., 1988; Ramagopal, 1987a and 1987b). Induction of salinity proteins appears to

be mediated by ABA as both ABA and NaCl solutions induce the synthesis of a 26 kD protein, osmotin, in cultured tobacco cells (Singh *et al.*, 1987a). This protein shares a high amino acid sequence homology with thaumatin, a tobacco mosaic virus-induced protein, and maize trypsin/ α -amylase inhibitor (Singh *et al.*, 1987b).

The salt ion effect can be excluded by use of polyethylene glycol (PEG) or mannitol. The effects of osmotic potential and the salt ion can be differentiated, as salt ion-specific and water potential-specific genes are expressed. This has been demonstrated in soybean seedlings using NaCl and PEG (Czarnecka *et al.*, 1984). A large increase in the proline pool, a characteristic of water stressed cells (Hsiao, 1973), results from the low plant water potential caused by PEG in tomato suspension culture cells (Rhodes *et al.*, 1986). Overall protein synthesis is reduced by a controlled water stress condition imposed on barley leaves by PEG (Dasgupta and Bewley, 1984) or on oat coleoptile by mannitol (Dhindsa and Cleland, 1975). Protein synthesis reduction is greater, in both plants, under a higher solute concentration. Synthesis of a few proteins is induced by the stress and, in particular, a protein having a Mr of 60,000 is induced in barley leaves (Dasgupta and Bewley, 1984).

The response to other stress factors appears to share similarities with the water-stress response. Heat-shock

induces heat-shock proteins, hsp 70 in maize and hsp 27 in soybean, which are also induced by PEG-applied water stress (Czarnecka et al., 1984; Heikkila et al., 1984). Another stress, wounding, induces hsp 70 as well (Heikkila et al., 1984). ABA appears to be involved in the induction of these water, and other stress-induced genes, since an application of ABA induces hsp 70 and hsp 27 (Czarnecka et al., 1984; Heikkila et al., 1984).

Drought stress applied to whole plants increases the proteins unique to stress-induced morphological change. In the roots of Brassica napus, where water stress is encountered directly, a tissue-specific induction of proteins occurs in coordination with a formation of a type of roots induced only under stress (Damerval et al., 1988). Drought conditions, applied to soybean seedlings, affect growth. The reduced elongation in the hypocotyl region is accompanied by an increase in ABA and changes in the translatable poly(A)-RNA population (Bensen et al., 1988).

Cellular changes in gene expression relative to the thesis topic, also occur under water stress. Alpha-amylase activity increases in barley leaves during drought stress in coordination with an increase in ABA and a decrease in plant water potential (Jacobsen et al., 1986). The low-pI isozyme form is synthesized and preceded by an α -amylase mRNA increase. The regulation of enzyme synthesis, by ABA and stress, in leaves, is opposite to the well characterized

regulatory pattern in the aleurone system. This indicates the presence of a different control mechanism in leaves.

Controlled dehydration of plant parts is preferable to the use of whole plants since precise control can be applied to the experimental system. Using this system, Wright and Hiron (1969) showed an induction of ABA in wilted wheat leaves. ABA level increases in the shoots and roots of barley plants under dehydration stress, in coordination with an expression of one family of mRNA (Chandler et al., 1988). Dehydration or wounding of maize leaves also induces a mRNA for a glycine-rich protein in coordination with an increase in ABA (Gomez et al., 1988). A rice gene, encoding a protein rich in glycine, is induced in dehydrated shoots and ABA-treated shoots, roots and embryos (Mundy and Chua, 1988). The ability of cereal plants to survive water stress is linked to the presence of dehydration-induced proteins (Chandler et al., 1988).

Dehydration and ABA treatments induce the dehydration-inducible mRNA to the same degree in a GA-mutant and wild type of barley (Chandler, 1988). This implies that the ability to respond to ABA is not affected by a mutation in GA response, indicating the presence of a separate response pathway to ABA and GA in barley. However in a corn mutant which fails to accumulate ABA, a gene homologous to the barley water stress-induced gene is not induced under dehydration stress. The lack of induction can be overcome

with an application of ABA to the mutant, further supporting an involvement of ABA (Chandler et al., 1988).

The role of ABA in dehydration stress has also been examined in tomato ABA mutants (Bray, 1988). A set of proteins are induced in wild type tomato leaves by dehydration stress. The same proteins are synthesized to a lesser extent in an ABA-deficient mutant under stress. The proteins can be induced only after an ABA application to the mutant. These results indicate elevated ABA levels induced by stress are essential for the induction of these proteins.

The embryo and seed tissue generally undergo drying to enter a quiescent period of plant development. This process is a part of normal kernel maturation and it might be expected that the embryo expresses dehydration-induced genes late in grain development. Some of the dehydration-induced genes in vegetative tissue are present in mature cereal embryos and aleurone layers isolated late in kernel development (Chandler et al., 1988; Gomez et al., 1988; Mundy and Chua, 1988). Wheat germ contains another late maturation mRNA that appears to be induced by grain desiccation during late maturation (McElwain and Spiker, 1989). Along with the barley, rice and maize dehydration-induced genes that are abundant in mature embryos, these late embryogenesis genes may play an important role in kernel transition through the drying process.

The dehydration of embryos after germination can be detrimental to embryo survival. When dehydration occurs within a critical time limit, however, the process is beneficial as a pretreatment to enhance protein and RNA synthesis in rye (Sen and Osborne, 1974). If dehydration occurs later in the imbibition period, structural damage and a reduction in embryo growth occur (Sargent et al., 1981). Sensitivity to dehydration coincides with an increase in the protein synthesis rate and the onset of DNA replication during imbibition (Sargent et al., 1981). Embryos sensitive to dehydration are incapable of RNA and DNA synthesis upon rehydration following dehydration (Crevecœur et al., 1988). Upon rehydration, cellular disorganization increases with a longer imbibition period prior to dehydration (Marinos and Fife, 1972; Sargent et al., 1981).

MATERIALS AND METHODS

3.1. Chemicals

Canine microsomal membranes, ^{14}C -labelled marker proteins, a nick translation kit, Hybond-N nylon membranes and ^{35}S -methionine (>1000 Ci/mmol) were obtained from Amersham Canada Ltd., Oakville, Ontario. Bio-Gel P-30 (50-100 mesh), DNA grade agarose, Protein Assay Dye Reagent, goat-anti-rabbit horseradish peroxidase (GAR-HRP) and its substrate, 4-chloro-1-naphthol, were obtained from Bio-Rad Laboratories [Canada] Ltd., Mississauga, Ontario. DH5- α competent cells, guanidinium thiocyanate, RNA markers and a rabbit reticulocyte in vitro translation system were obtained from Bethesda Research Laboratories Life Technologies, Inc., Burlington, Ontario. Oligo(dT)-cellulose (type 3) was obtained from Collaborative Research, Lexington, Mass.. α - ^{32}P CTP (>3000 Ci/mmol) was obtained from ICN Biomedicals Canada, Ltd., Montreal, Quebec. Protein A-Sepharose CL-4B and ampholines (pH3.5-10, 4-6 and 6-8) were obtained from LKB-Pharmacia [Canada], Dorval, Quebec. ENHANCE, ^{35}S -sulphate (>300 mCi/mmol) and Du Pont Lightning Plus intensifying screens were obtained from

NEN Du Pont Canada Inc., Lachine, Quebec. Nitrocellulose was obtained from Schleicher & Schuell, Keene, New Hampshire. (+)-cis, trans-Abscisic acid (ABA) was obtained from Sigma Chemical Co., St. Louis, MO.. Other chemicals were reagent grade unless indicated otherwise.

3.2. Plant Material

Barley, Hordeum vulgare L. cv. Bonanza, was grown in the growth cabinet under 16 hour day length with 18/14°C day/night temperature. Kernels were planted in an artificial planting medium (Metro-mix 220, W. R. Grace & Co. of Canada Ltd. Ajax, Ontario, Canada) in 1L cartons. The seedlings were thinned to 2 plants per pot. The plants were fertilized with a fertilizer having a N-P-K composition of 20%-20%-20% once a week until maturity. Each head was tagged at mid-anthesis and was harvested at the required time intervals. Embryonic stages were determined according to Rogers and Quatrano (1983). At harvest, the samples were used immediately for experiments or stored whole at -75°C until required.

Young seedlings were obtained by germinating certified barley seeds. Seeds were surface sterilized with 1% hypochlorite with 0.1% Tween 20, rinsed with sterilized distilled water, then placed on a Whatman 3MM

paper and allowed to germinate with sterilized water, in the dark, for 2 days.

3.2.1. Effects of Plant Growth Substances on Kernel Proteins and on Embryos

The effects of plant growth substances on protein synthesis in barley kernels and embryos were studied through spike, endosperm and embryo culture in a nutrient medium with treatment substances.

Immature barley heads were harvested at 7 DPA. For spike culture, stems were sterilized with 1% hypochlorite solution for 5 minutes and rinsed with sterile distilled water. Spikes were cultured in basal medium of Murashige and Skoog (MS) (1962) except for KH_2PO_4 , which has been increased from 170 to 1200 mg/l to satisfy the requirement for K in the absence of KNO_3 (Donovan and Lee, 1977). The quantities of sucrose and vitamins were derived from the conditions for optimum growth of cultured grains (Gifford and Bremner, 1981). Casein hydrolysate contains about 14 amino acids and the addition of glutamine appears to satisfy the need of isolated kernel growth, thus they were used instead of NH_4NO_3 and KNO_3 or a mixture of amino acids. Culture medium contained ABA at concentrations of 0, 1, 10 and 100 μM and/or fluridone at 0, 1, 10 and 100 mg/l.

Culture tubes were covered with aluminium foil to exclude light and were immersed in a cooling bath at 4°C. Spikes were placed in a growth cabinet under the same conditions as for growing plants. At 13 DPA, or after 6 days under culture, each spike was fed with 80 μCi ^{35}S - Na_2SO_4 (NEN) then returned to the treatment medium. On 15 DPA, proteins were extracted from isolated endosperms and embryos.

Alternatively, 11 DPA kernels were isolated from plants and sterilized as above. They were cultured in a 24 well culture plate (Corning) at 1 kernel/well with 200 μl culture medium containing ABA and/or fluridone. The plates were wrapped in an aluminium foil and on 13 DPA, or after 2 days under culture, 15 μCi ^{35}S -Met (Amersham) was added to each kernel. Kernels were harvested at 14 DPA and proteins extracted.

Immature embryos were isolated aseptically from surface sterilized whole kernels. They were placed scutellum side down individually in a 96 well culture plate (Corning) with 50 μl of the medium used for the kernel culture. ABA concentrations used were 1, 10 and 100 μM . The embryos were cultured in the dark for 5 days. Then, the embryos were analyzed for germinability and inhibitor content.

3.2.2. Effects of Dehydration Stress on Embryos and Young Seedlings

Immature embryos and 2 day-old seedlings were placed in a desiccator in the dark which had been equilibrated with 25% (v/v) glycerol solution as described by Chandler (1988). The calculated relative humidity in this environment is 91% at 20°C. After the experiments, the embryos and seedlings were analyzed for inhibitor content. Duplicate samples were sent to Dr. M. Walker-Simmons (Washington State University, Pullman) for ABA analysis.

3.4. Analytical Methods

3.4.1. Protein Determination

In the kernel development study, barley grain was isolated on ice from frozen samples of 7 to 35 DPA. At maturity, whole kernels were used without the removal of husks. Crude protein extracts were prepared by homogenizing the kernels in 10x (v/w) 50mM Tris-Cl (pH 7.0) with 1mM CaCl₂ or 20 mM acetate (pH 5.5) with 1mM CaCl₂ using a glass homogenizer. These two buffers had similar protein extractability. Hordein fraction was obtained by extracting the pellet from the above extraction with 50% aqueous isopropanol. The cultured

and water stressed embryos were homogenized with 10 mM phosphate-buffered-saline (PBS : 10 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ at pH 7.0, 145 mM NaCl), 0.1% (w/v) Triton X-100, and 100 mM N-acetylglucosamine. Water stressed seedlings were divided into three parts after desiccation; segment containing the shoot, coleoptile and scutellum (thereafter referred to as SCS), the root, and the remaining kernel. Soluble proteins were extracted with 50 mM Tris (pH 7.5) and 1 mM NaCl. PBS and Tris buffers were equally effective in extracting the α -amylase inhibitor. The homogenates were kept on ice with an occasional shaking for 30 min, then microfuged at $15,000 \times g_{\text{max}}$ for 10 min.

Buffer extractable protein concentrations were determined by the method of Lowry et al. (1951), or Bradford (1976) (Bio-Rad) using BSA or γ -globulin as a standard. Total kernel protein content (% protein, dry weight basis) was calculated from Kjeldahl nitrogen determination multiplied by 6.25.

3.3.2. Protein Gel Electrophoresis

The crude protein samples were separated by discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 15% (T) gels by the method of Laemmli (1970), as modified by Maniatis et al. (1982) for one-dimensional electrophoresis.

Two-dimensional electrophoresis using IEF in the first direction and SDS-PAGE in the second was carried out according to O'Farrell (1975) with a slight change in ampholine composition (0.4% pH 3.5-10, 0.4% pH 4-6, 1.2% pH 6-8).

The proteins were visualized by a silver stain by Merril et al. (1981).

3.3.3. Western Blot of Protein Electrophoresis Gels

Protein gels were electroblotted onto nitrocellulose (Schleicher & Schuell) in a LKB transphor electroblotting unit (LKB-Pharmacia) according to the manufacturer's instructions. The antigen bands were visualized using specific rabbit antisera raised against the purified inhibitor or α -amylase (Weselake et al., 1985), GAR-HRP conjugate and 4-chloro-1-naphthol as a substrate according to the manufacturer's instructions (Bio-Rad). The inhibitor and α -amylase contents in the samples at various dilutions were quantified visually against the purified antigen standard on the same gel. Samples giving band intensities outside the range of the standards were appropriately diluted and the analysis repeated.

3.3.4 ABA Analysis

Dissected embryos of seedling tissues were frozen, lyophilized and powdered. Samples were extracted in methanol as described by Walker-Simmons (1987). ABA content of the extracts was measured by indirect ELISA utilizing a monoclonal antibody for (+)ABA as previously described (Walker-Simmons, 1987).

3.3.5. Total and Messenger RNA Isolation

Procedures were carried out in sterile disposable plasticware or glassware treated to eliminate RNase activity (Maniatis et al., 1982). Glass distilled water was treated with an ion exchange resin, diethylpyrocarbonate (DEPC), then autoclaved twice to eliminate residual DEPC.

Total RNA was isolated from dehusked whole kernels in a modified guanidinium thiocyanate procedure. Due to the high starch content in kernels, the guanidinium thiocyanate/CsCl method by Chirgwin et al. (1979) generally isolates intact RNA at very low yields. The isolation of RNA using a Tris buffer and phenol/chloroform by Brandt and Ingversen (1978) yields larger amounts of RNA, but the RNA showed some degradation. The modified method removes materials insoluble in the Tris buffer as a first step, followed by

the addition of guanidine thiocyanate to inactivate RNase completely.

The frozen sample was homogenized on ice in 0.1 M Tris, 0.1 M NaCl, 0.01 M EDTA (pH 9.0), 1.0% (w/v) SDS and 1.5% (v/v) β -mercaptoethanol using Polytron homogenizer (Brinkman Instruments. Rexdale, Ontario). The homogenate was centrifuged at 15,000 xg for 30 min at 4°C. The supernatant was pooled to measure its volume. Guanidinium thiocyanate salt was added to the supernatant to give a final concentration of 4.0 M. The homogenate was then layered on 5.7 M CsCl/EDTA and total RNA was isolated in the usual manner (Chirgwin et al., 1979).

Shoot samples were taken from the above ground portion of 2 week old green seedlings. Root tissues were collected from the root tip regions of mature plants. RNA was isolated from frozen samples of shoot and root by a guanidinium thiocyanate/CsCl method (Chirgwin et al., 1979).

Poly(A)-RNA was isolated by affinity chromatography on oligo(dT)-cellulose (Type 3) (Aviv and Leder, 1972).

3.3.6. In Vitro Translation and Immunoprecipitation

A rabbit reticulocyte in vitro translation system was programmed with 0.5 to 1 μ g poly(A)-RNA using 5 μ Ci 35 S-methionine (>1000 Ci/mmol) as a radio label.

Processing of the initial translation product was examined by including canine pancreas microsomal membranes in the in vitro translation system (Jackson and Blobel, 1977) and immunoprecipitating the labelled product with a specific antiserum raised in a rabbit against purified inhibitor (Weselake et al., 1985). The incorporation of the radiolabel into TCA-precipitable proteins was measured by a filter paper disc method (Mans and Novelli, 1961) and the activity was counted with 3 ml Aquassure (NEN Du Pont) in a Mark III liquid scintillation counting system (Searle Instrumentation, Oakville, Ontario).

Total translation products containing 300,000 to 450,000 cpm of TCA-precipitable proteins were used for immunoprecipitation. Cross-reacting proteins were removed by PRS/protein A sepharose. Immunoprecipitation buffer (50 mM Tris-Cl, pH=7.8, 0.1 M NaCl, 0.1% (w/v) SDS, 1% (v/v) Triton-X) was added to give a final volume of 0.5 ml and this was incubated with 5 μ l pre-immune rabbit serum (PRS) for 1 h at room temperature with shaking. Washed protein A-Sepharose CL-4B, 25 μ l, was added and the samples were incubated for a further 30 min. After separation in a microfuge at 15,000 xg, the supernatant was incubated with α -amylase inhibitor antiserum, overnight at 4°C. The incubation buffer

contained 0.5 M NaCl to reduce cross reactivity. The antigen/antibody complex was precipitated on protein A-Sepharose and, after several washes of the complex, the precipitate was denatured by the addition of Laemmli sample buffer (Laemmli, 1970) followed by boiling for 2 min. The total in vitro translated protein sample and the immunoprecipitated samples were separated on discontinuous SDS-PAGE as described above. The proteins were visualized by fluorography. They were fixed in 50% (v/v) methanol and 10% (v/v) acetic acid. The gel was treated with Enhance (NEN), dried and exposed to Kodak X-Omat AR film with a Du Pont intensifying screen at -75°C for 2 weeks.

3.3.7. Northern Analysis

An inhibitor cDNA clone was isolated by T. Hatton (Appendix 1). cDNA clones for an ABA induced protein (pHVA34) and B hordein [pHOR2-4(2.8)] were kindly provided by P. Chandler (Commonwealth Scientific and Industrial Research Organization) and A. Brandt (Carlsberg Laboratory), respectively. DH5- α competent cells were transformed with 5 or 10 ng plasmid (Maniatis et al., 1982) and screened on agar plates with appropriate antibiotics. The transformed single colony

was re-screened on another agar plate containing antibiotic.

Bacterial culture of E. coli was maintained in a glycerol stock kept at -75°C (Maniatis et al., 1982). The bacterial cultures were used to inoculate LB broth with an appropriate antibiotic, then they were grown over night at 37°C to increase plasmid. The plasmid was isolated according to Birnboim and Dolly (1979). The purified plasmids were digested with restriction enzymes and the inserts were electroeluted after separation on an agarose gel electrophoresis using dialysis membrane (Maniatis et al., 1982).

For gel blots, RNA was separated on a denaturing 1.5% (w/v) agarose formaldehyde gel electrophoresis and blotted onto nylon membranes (Hybond-N) (Maniatis et al., 1982). For dot blots, serial dilution of samples were made with TE. Denaturation of RNA was carried out in a buffer containing sample:formaldehyde:20xSSPE at 1:2:1, heated at 65°C . RNA was applied to Hybond-N in a 96 well dot blot apparatus (Bio-Rad). Filters were exposed to UV light, to covalently fix the RNA and prehybridized in BLOTTO (Siegel and Bresnick, 1987), 5XSSPE (1XSSPE=0.15 M NaCl, 0.01 M NaH_2PO_4 , 1mM EDTA pH 7.4), 0.1% (w/v) SDS and 50 $\mu\text{g}/\text{ml}$ yeast tRNA for at least three hours at 65°C .

The cDNA insert of the inhibitor clone was labelled with ^{32}P -CTP (>3000 Ci/mmol) by nick translation to a specific activity of $>1 \times 10^8$ cpm/ μg . Hybridizations were carried out under the same conditions as the prehybridization, with the addition of the nick translated insert. After hybridization, the blots were washed three times in $2 \times \text{SSPE}$, 0.1 (w/v) % SDS for 10 min at room temperature, followed by three washes in $0.2 \times \text{SSPE}$, 0.1% (w/v) SDS for 10 min at 65°C . The blots were exposed to Kodak X-OMAT AR film at -75°C with an intensifying screen (Du Pont Lightning Plus). The α -amylase inhibitor mRNA size was estimated by DNA fragment analysis operated on an IBM compatible computer adapted by J. Nash, based on an analysis of the least square fit of DNA length to gel migration (Schaffer and Sederoff, 1981).

Quantification of the hybridizing message was carried out by cutting out circles from the dot blot and ^{32}P activities were counted by liquid scintillation.

4. RESULTS AND DISCUSSION

4.1. Tissue Specificity of the α -Amylase Inhibitor

Analytical methods to detect and quantify the α -amylase inhibitor were developed to facilitate investigations using many distinct samples. An immunoblotting procedure, frequently referred to as a Western blot (Burnette, 1981), takes advantage of a specific antigen-antibody reaction to identify the protein of interest. Northern analysis utilizes a cDNA clone for the inhibitor as a probe to detect the inhibitor message among RNA populations (Meinkoth and Wahl, 1984).

4.1.1. Western Blot Analysis for the α -Amylase Inhibitor Protein

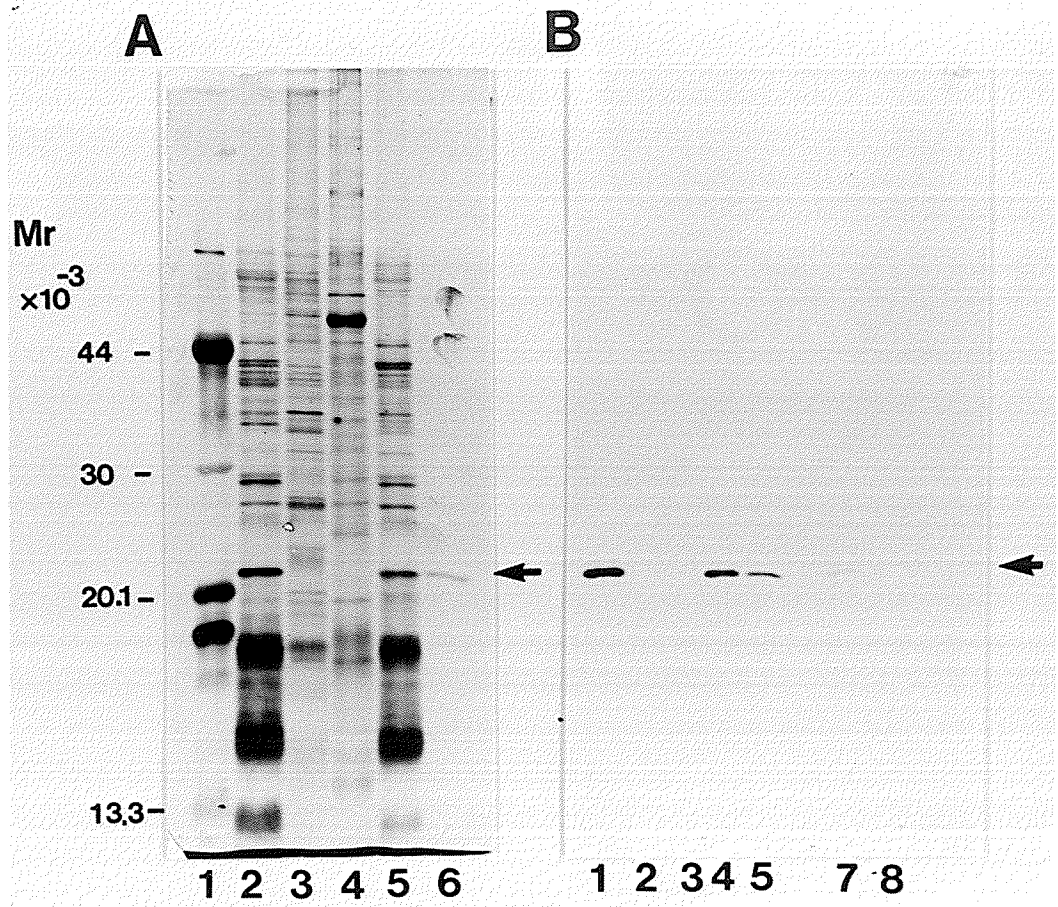
The antiserum used for development of the assay was previously employed to detect proteins that shared immunological identity with the inhibitor in various cereal grains (Weselake et al., 1985). By virtue of the nature of antibodies, recognition between antigens and antibodies is dependent on the concentration of both antigen and antiserum. Sensitivity and selectivity of an antibody is determined by antibody titre and these

parameters are mutually exclusive (Burnette, 1981). In order to establish a suitable detection range, having a minimum cross-reactivity with proteins other than the inhibitor, titration of the antiserum was carried out.

Within a dilution range between 1000:1 and 200:1, 100 ng purified inhibitor was visualized in greater intensity as more antiserum was used. In order to assess selectivity of the antiserum, a mixture of proteins was separated along with the purified inhibitor. At the lower antiserum dilution of 200:1, several cross-reacting proteins became visible, whereas these proteins were absent at dilutions of 1000:1. Weighing the balance between selectivity and sensitivity, a working antiserum dilution of 1000:1 was found suitable for use in Western blot experiments. Assessment of the antiserum at 1000:1 dilution revealed the detection range of the inhibitor to be between 10 and 250 ng (Appendix 2A). This technique has a wide working range from the application of appropriately diluted crude protein extracts of 5 to 100 μ g/lane to SDS-PAGE gels. Within this range, inhibitor contents as low as 0.01 percent and as high as 5 percent of total protein could be detected.

The rabbit antiserum directed towards the inhibitor had high specificity under the conditions used (Fig. 1). Crude extract from various tissues contained many

Figure 1. Western blot analysis of inhibitor protein in crude extracts of barley. The proteins were stained in panel A. Lane 1, Mr. standards, ribonuclease-A, 13.3, trypsin inhibitor, 20.1, carbonic anhydrase, 30, and ovalbumin, 44, $\times 10^{-3}$; lane 2, Bonanza endosperm extract; lane 3, Bonanza embryo extract; lane 4, Bonanza shoot extract; lane 5, Argyle kernel extract (10 μg each); lane 6, 100 ng purified inhibitor. Panel B is the Western blot for the inhibitor. Lane 1, Bonanza endosperm extract; lanes 2 and 7, Bonanza embryo extract; lanes 3 and 8, Bonanza shoot extract; lane 4, Argyle kernel extract; lane 5, 50 ng purified inhibitor; 5 μg total protein was applied to lanes 1 to 4, 100 μg to lanes 7 and 8.



proteins of a wide Mr range (Fig. 1A). Bonanza endosperm and Argyle kernel buffer-extracted proteins were similar in protein profile, whereas embryo and shoot proteins shared few similarities with the endosperm proteins. Although the endosperm proteins do not contain embryo proteins, endosperm and kernel proteins from a cultivar are qualitatively similar since embryo proteins constitute less than 10 percent of kernel proteins (sections 4.3. and 4.4.). Bands co-migrating with a purified inhibitor band were apparent in the endosperm and the kernel extracts. The existence of the inhibitor was confirmed by Western blot using the inhibitor antiserum (Fig. 1B). Endosperm and mature kernel contained high amounts of the inhibitor. More than 1 percent of the total buffer-extractable protein could be accounted for as the inhibitor. The inhibitor was not present as a major protein in the embryo or shoot extracts (lanes 2 and 3). When a large amount of buffer-extractable protein was loaded (lanes 7 and 8), however, a faint band was detected in the embryo sample, corresponding to less than 0.05 percent buffer-extractable protein.

The rabbit polyclonal antiserum directed towards the α -amylase inhibitor was specific for that antigen in endosperm and kernel extracts and did not cross react

with the numerous other proteins present in the crude extracts (Fig. 1). The inhibitor protein was not detected in vegetative tissues under the conditions used in these experiments, suggesting that the protein is specific to kernel tissue. The inhibitor constituted a higher proportion of buffer-extractable proteins in the endosperm than in the embryo.

When present, only one form of the inhibitor was observed in the kernel extract as shown by a Western blot of 2-D gel electrophoresis (Appendix. 3). Two independent separation parameters used in this system (O'Farrell, 1975) segregate individual protein species based on both pI and Mr. Observation of only one spot after immunoblotting was strong evidence that there is only one protein in the kernel that is recognized by the antibody.

4.1.2. Isolation of RNA from Kernel Tissue

In order to detect inhibitor mRNA, RNA was isolated from 14 DPA seeds, the most active period of inhibitor accumulation (section 4.3.2.). The guanidinium/CsCl method of Chirgwin et al. (1979) yielded very low amounts of RNA, varying from 0 to 300 µg/g fresh weight (Table 1). A Tris-buffer homogenization followed by a phenol/chloroform extraction (Brandt and Ingversen, 1978)

TABLE 1. RNA yield from 14 DPA barley kernels by three different methods.

Procedure	Yield
	($\mu\text{g/g}$ fresh weight)
Chirgwin <u>et al.</u> (1979)	95.4 (0-325) ¹
Brandt and Ingversen (1878)	480 (180-775)
Modified	1016 (730-1210)

¹ Numbers in brackets indicate range in value.

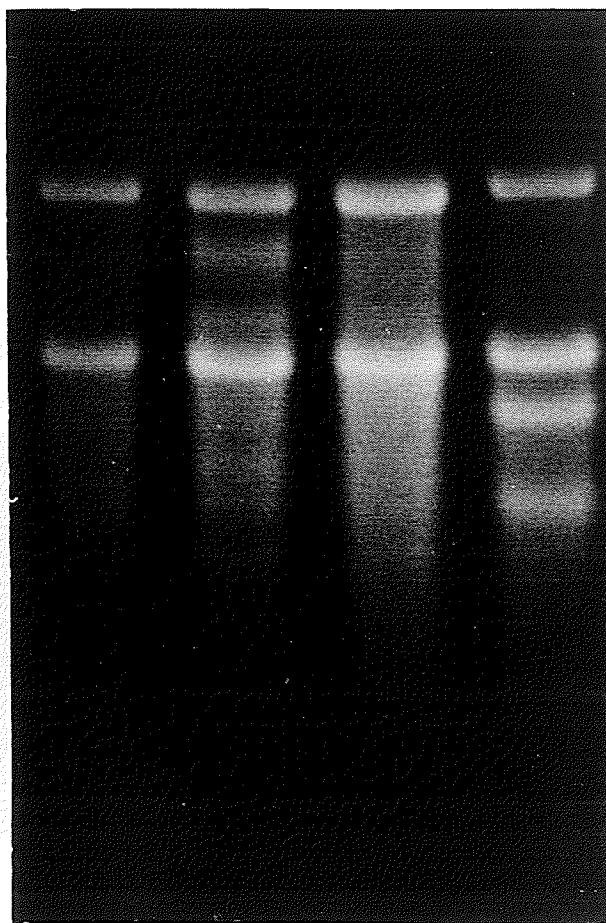
yielded larger amounts. Some degradation was apparent, however, as indicated by additional bands between the 26S and 18S rRNA bands and below the 18S rRNA band on formaldehyde gel electrophoresis (Fig. 2). A modified method, as outlined in Figure 3, yielded greater than 1 mg of total RNA per gram fresh weight which appeared to be undegraded when viewed after electrophoresis on the formaldehyde gel (Fig. 2). This yield is about 10 times that of the method by Chirgwin et al. (1979) and twice that by Brandt and Ingversen (1978) (Table 1).

The extraction of RNA from kernels is more difficult than from other plant parts because of the presence of large quantities of starch in the endosperm. The highly chaotropic character of guanidinium salts used in the procedure of Chirgwin et al. (1979) dissolves starch, resulting in a gelatinous homogenization buffer. This entraps RNA during ultracentrifugation through the CsCl cushion. In the modified procedure, the initial extraction of RNA in a Tris buffer allows the starch to remain insoluble. It can then be removed, along with other cell debris, by low speed centrifugation (Brandt and Ingversen, 1978). Phenol extraction on its own resulted in more degraded RNA (Fig. 2), probably because RNase was inactivated too slowly. Another problem with the Tris buffer procedure is that any soluble

Figure 2. Formaldehyde agarose gel (1.5 %) electrophoresis of total RNA isolated from 14 DPA barley kernels by three different procedures; Chirgwin *et al.* (1979) (Lane 1), Brandt and Ingversen (1978) (lane 2), modified (lane 3) and leaf total RNA (lane 4).

26s

18s



1

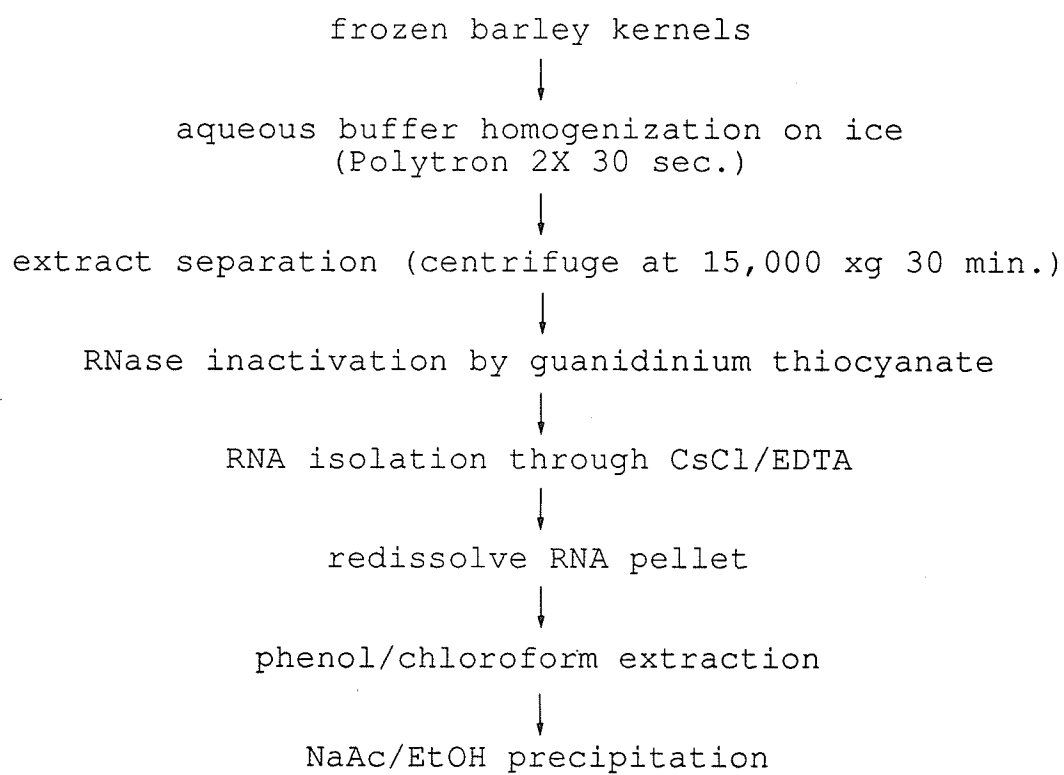
2

3

4

Figure 3. Outline of the modified RNA isolation scheme from kernels.

RNA Isolation Scheme



oligosaccharides present in the aqueous phase after the phenol extraction, can be precipitated by ethanol along with the RNA, requiring further purification by ultracentrifugation through a CsCl cushion.

The modified method takes advantage of the best features of these two procedures. The frozen sample was homogenized in a Tris buffer and the starch removed. The presence of EDTA, SDS and β -mercaptoethanol help to minimize RNase activity. The guanidinium thiocyanate salt subsequently added to the supernatant inactivated RNase activity completely. RNA was isolated through the CsCl cushion, and redissolved RNA was phenol treated to further remove any contaminating RNase. RNA, thus isolated, was shown to be intact (Fig. 2). This reliable, high-yielding procedure will be useful in RNA isolation from tissues containing a high amount of starch.

4.1.3. Northern Analysis of Barley Tissue for the α -Amylase Inhibitor

Tissue specificity for inhibitor gene expression was analyzed by Northern analysis (Maniatis et al., 1982). When poly(A)-RNA from kernel, shoot and root tissues was analyzed for the existence of inhibitor message, the transcript was present only in the kernel and not in the

shoot or the root (Fig. 4). Two separate preparations of kernel poly(A)-RNA, lanes 1 and 4, contained the same abundance of inhibitor message as shown by their equal hybridization intensities.

The size of the mature RNA transcript encoding the inhibitor was about 900 nucleotides in the developing kernel (Fig. 5). The mature inhibitor molecule contains 181 amino acid residues (Svendsen et al., 1986), indicating that about a third of the inhibitor message could represent untranslated regions. This is in agreement with a nearly full size cDNA isolated by Leah and Mundy (personal communication), which contained 609 bases of open reading frame within the 807 base cDNA. Relatively long untranslated sequences may be required for translational regulation of this message.

Figure 4. Tissue specificity of inhibitor mRNA. Poly(A)-RNA (2 μ g) from 14 DPA kernel (lane 1), shoot (lane 2), root (lane 3) and 13 DPA kernel (lane 4) was examined for the existence of inhibitor message by Northern analysis. Positions for the 26S and 18S rRNAs are indicated on the left and those for RNA molecular weight standards in kb are on the right.

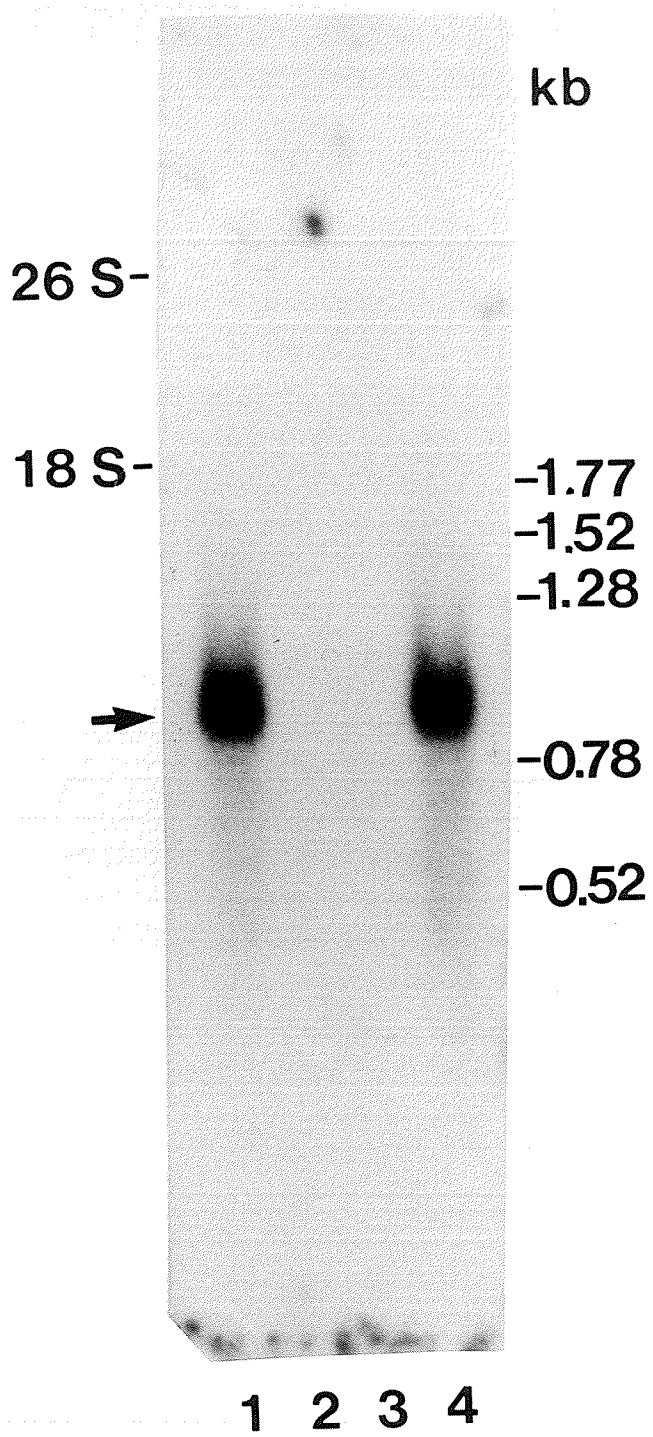
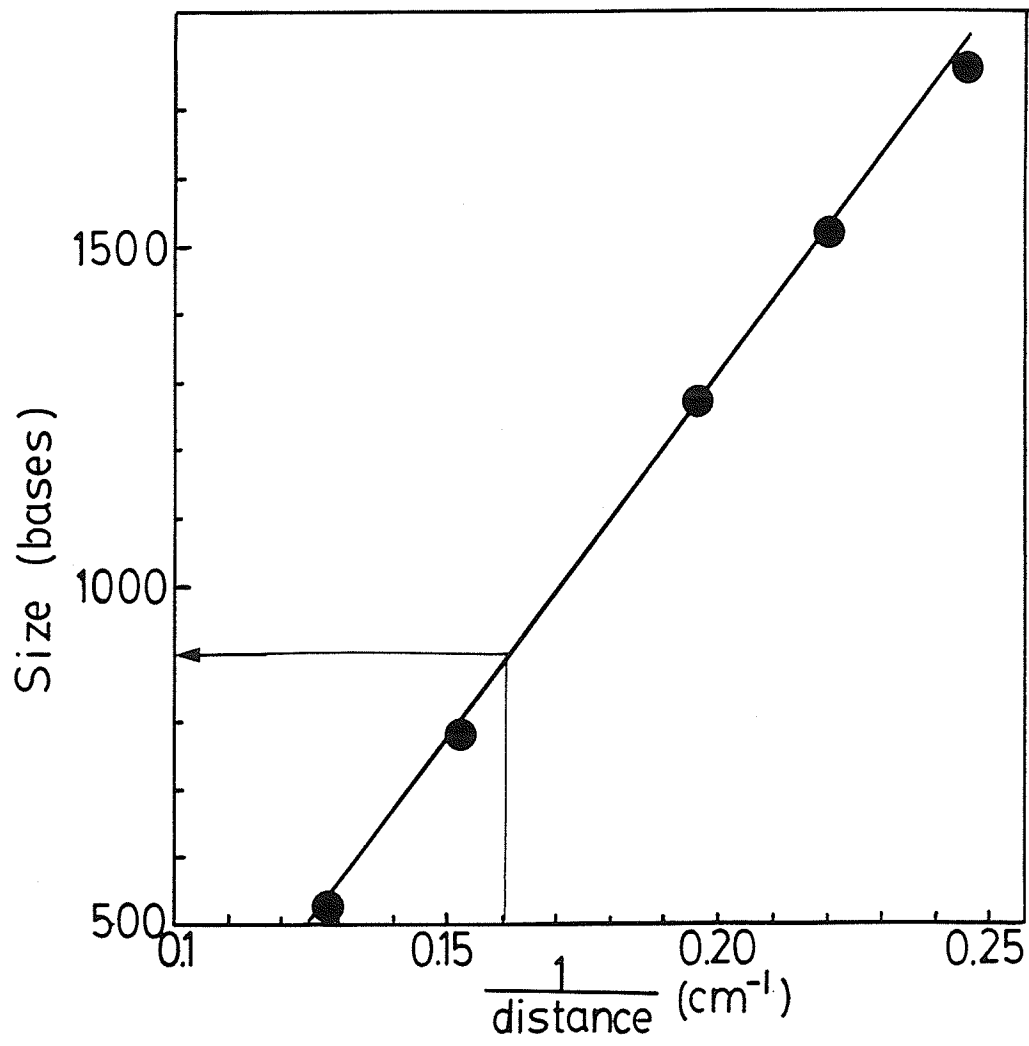


Figure 5. Estimation of α -amylase inhibitor mRNA size by Northern blot analysis. Migration distance and size of RNA standards are obtained from Fig. 4.



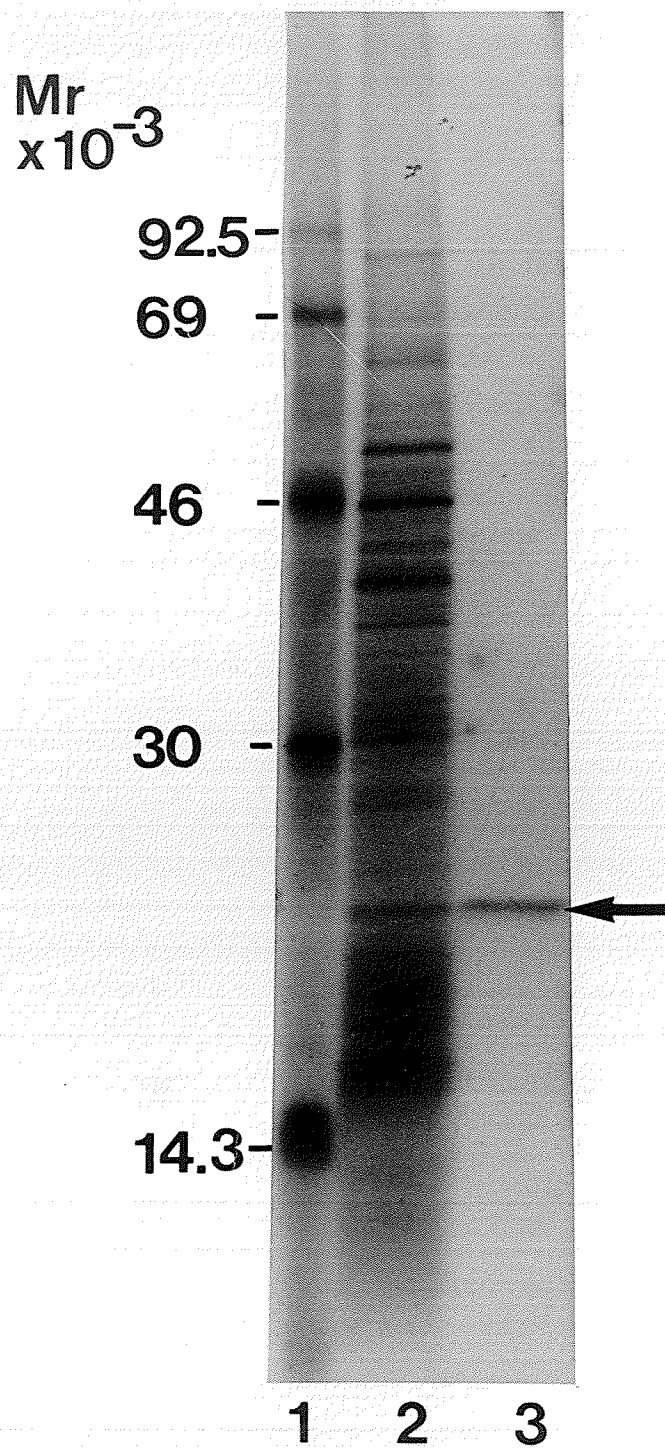
4.2. In Vitro Translation of Immature Barley Kernel RNA

RNA isolated from 14 DPA kernels was used to examine the presence of translatable inhibitor message by a rabbit reticulocyte in vitro translation system. Although the quality of the total RNA preparations appeared to be sound when examined in a formaldehyde gel (Fig. 2), this total RNA did not translate well in vitro. The translation system, however, was active in synthesizing proteins when poly(A)-RNA was used as a template. Translation was optimal with about 0.5 to 1 μ g poly(A)-RNA to synthesize TCA precipitable proteins at 10 to 20,000 cpm/ μ l. The system synthesized proteins of Mr from 10,000 to 80,000 (Fig. 6).

The inhibitor protein band was not visible among the total translation products. Immunoprecipitation of the total products with inhibitor antiserum produced a single band of about 21,500 (Fig. 6, lane 3). The estimated Mr was slightly larger than the reported size for the inhibitor protein (19,865) (Svendsen et al., 1986), which suggested the presence of a leader sequence.

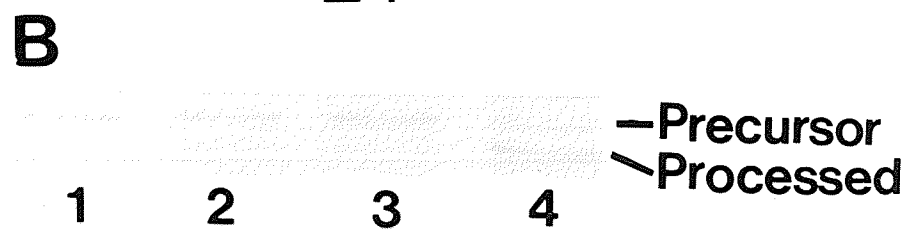
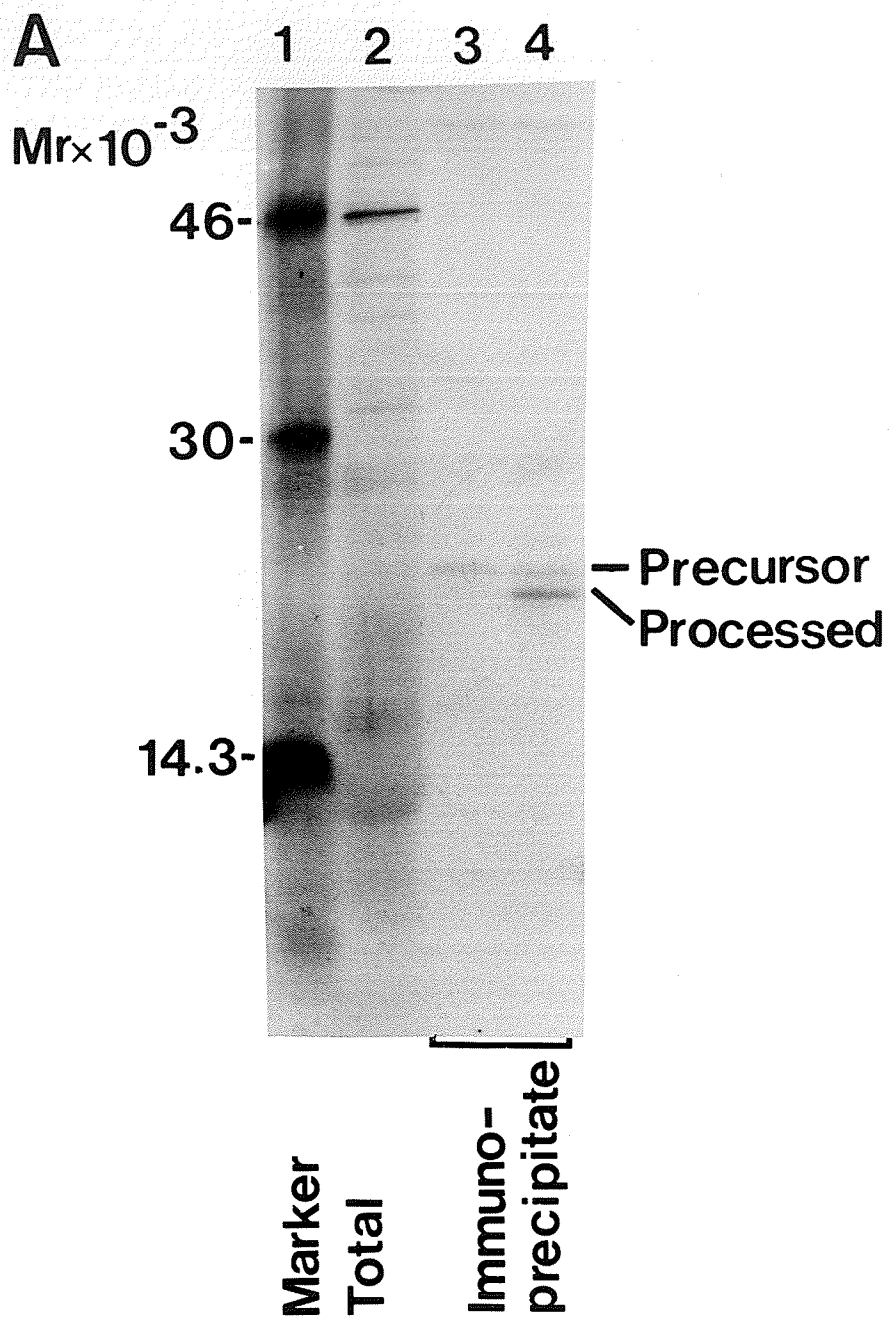
The possibility of a signal peptide presence was tested using canine pancreatic microsomal membranes (Jackson and Blobel, 1977). When the membranes were incubated with the translation system, immunoprecipi-

Figure 6. Fluorogram of in vitro translation products of 14 DPA kernel. A rabbit reticulocyte in vitro translation system was programmed with poly(A)-RNA and labelled. Total translation products were separated by SDS-PAGE (lane 2). The inhibitor protein was selected by immunoprecipitation (Lane 3). Lane 1 contains ^{14}C -labelled marker proteins.



tation of the products revealed a second band of smaller Mr, about 20,000 (Fig 7A). Signal peptidase dependent processing of the precursor protein was shown when an increasing proportion of the immunoprecipitated inhibitor was found in the processed, smaller Mr protein as larger amounts of the membranes were added (Fig. 7B). There, thus, appears to be a leader sequence of about 1500 which can be cleaved by the signal-peptidase activity of the membranes.

Figure 7. Signal peptidase processing of in vitro translation products of 14 DPA kernel poly(A)-RNA. Panel A : Total in vitro translation products (lane 2). The translation products without (lane 3) or with (lane 4) canine pancreatic microsome membranes were immunoprecipitated by inhibitor antiserum. Lane 1 contains labelled marker proteins. Panel B : Signal peptidase-dependent precursor protein processing. Different amounts of canine microsomal membranes were added to the translation system and the inhibitor immunoprecipitated. Lane 1, no membranes; lanes 2, 3 and 4, one, two and five μ l membranes were added, respectively.

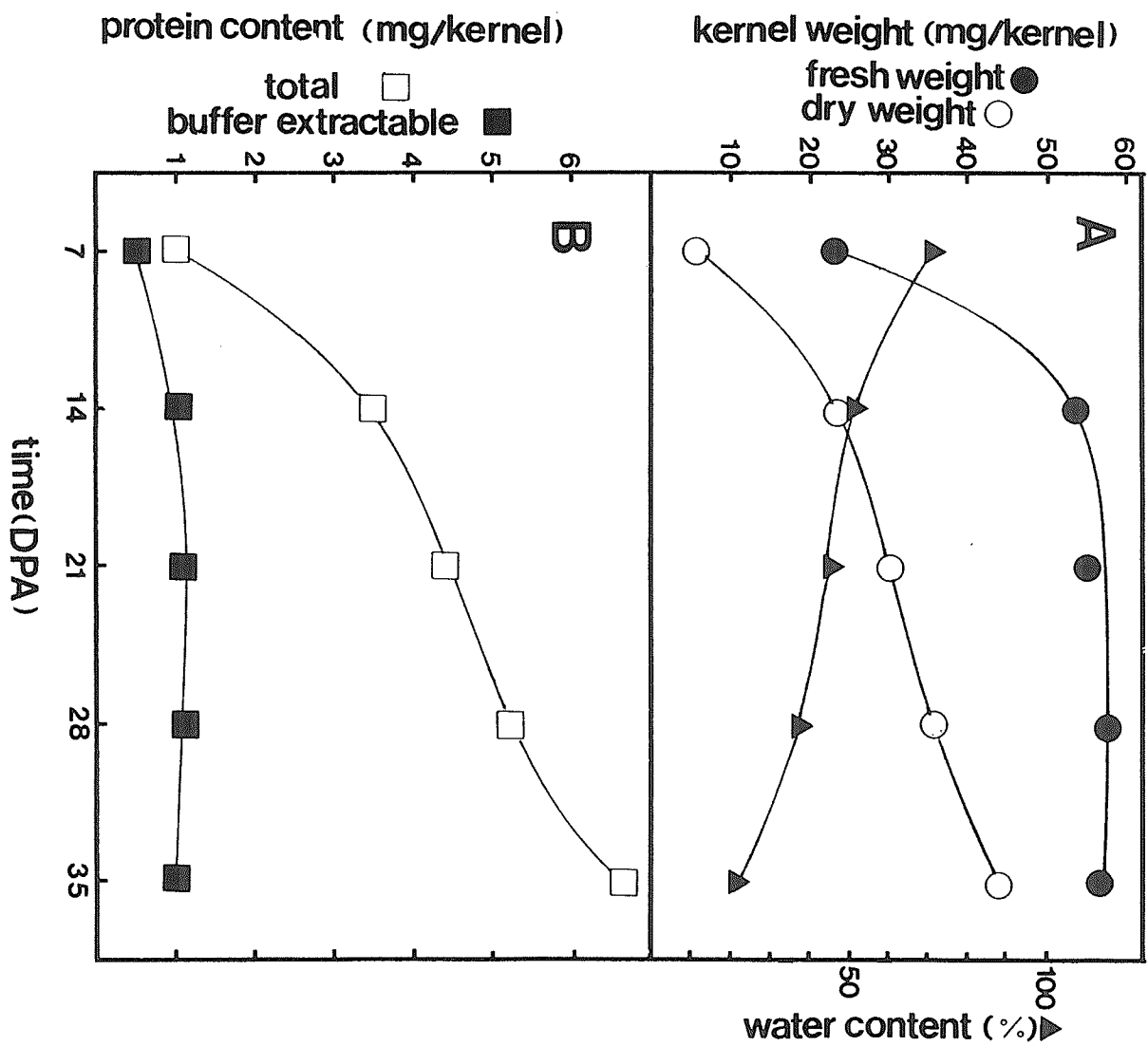


4.3. Barley Kernel Development

Developing barley kernel offers a suitable experimental system to study temporal regulation of kernel protein gene expression. Temporal control of α -amylase inhibitor synthesis was examined through its pattern of protein accumulation and the level of its message in developing kernels with the objective of establishing a physiological role for the inhibitor.

The general characteristics of barley kernel development observed in this study (Fig. 8) were similar to results from other studies (Brandt, 1976; Kirsi, 1973; MacGregor et al., 1974; Rahman et al., 1982). The level of Tris buffer-extractable proteins increased rapidly to one mg/kernel during the first 14 DPA and remained relatively constant thereafter (Fig. 8B). Fresh weight followed a similar pattern to this protein content change (Fig. 8A), resulting in a relatively constant 18 mg buffer-extractable protein/g fresh weight throughout grain development. Dry weight (Fig. 8A) and total kernel protein (Fig. 8B) increased over the period examined attaining maximum levels at 35 DPA of 44 mg and 6.66 mg, respectively. The water content of kernels at this time was 20 percent (Fig 8A) and kernels appeared to be at

Figure 8. Changes in protein, moisture contents, and weight of barley kernels during development. Panel A; fresh weight, dry weight and moisture content. Panel B; Tris buffer-extractable and total protein contents.



near maturity as assessed from these growth parameters.

4.3.1. Temporal Changes in the Protein Population During Kernel Development

Buffer-extractable protein species from immature barley were analyzed by SDS-PAGE (Fig. 9). At 7 DPA, many protein species of varying size were present. As the kernels matured, there was a general shift towards several more dominant proteins and a disappearance of other bands. One of the more dominant bands co-migrated with a purified inhibitor marker. This band became detectable at 14 DPA, then maintained its relative abundance from 21 to 35 DPA. No protein band co-migrated with the α -amylase marker throughout development. Overall, there were changes in protein species among 7, 14, and 21 DPA samples but the buffer-extractable protein distribution was relatively stable after 21 DPA. Several low Mr proteins (Mr 14,000 to 19,000) accumulated after 14 DPA.

More detailed observations were made by 2-D gel electrophoresis using IEF in the first dimension and SDS-PAGE in the second (Fig. 10). At 7 DPA, many low abundance proteins were present, which were mostly acidic (Fig. 10A). By 14 DPA, several proteins had increased in

Figure 9. Electropherograms of Tris buffer-extractable proteins from barley kernels during development. Lane 1, Mr standards as in Fig. 1; lanes 2, 3, 4, 5 and 6, 10 μg of 7, 14, 21, 28 and 35 DPA kernel extracts, respectively; lane 7, purified inhibitor and α -amylase.

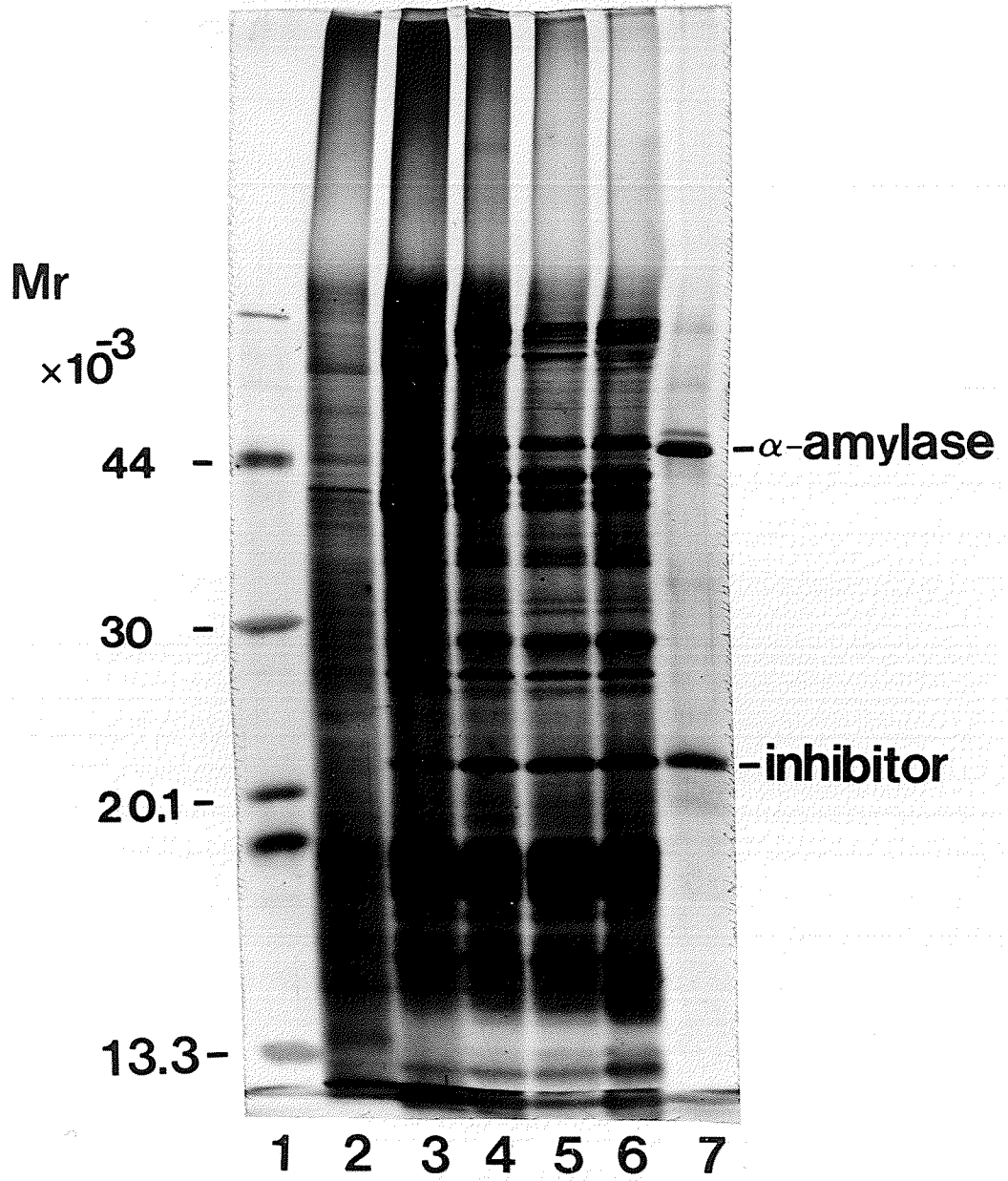
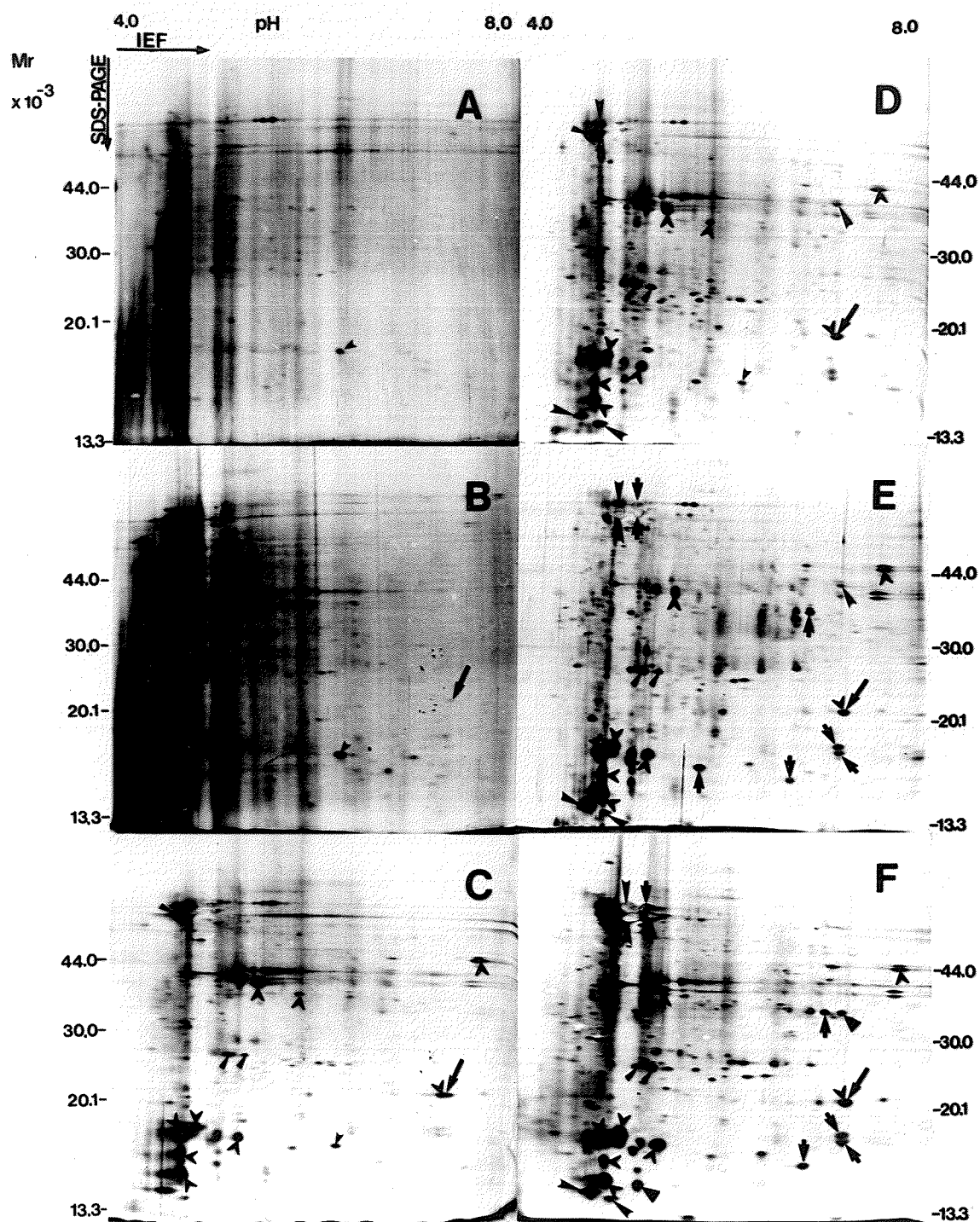


Figure 10. Two-dimensional gel electropherograms of Tris buffer-extractable proteins from developing barley kernels. Panel A, 7 DPA; panel B, 14 DPA; panel C, 21 DPA; panel D, 28 DPA; panel E, 35 DPA; panel F, mature kernels. Different arrows (▲), (▴), (⬆), (⬇), (↑) and (⬇) indicate proteins that have increased in relative abundance from the previous sampling date to 7, 14, 21, 28, 35 DPA, and maturity, respectively. When the relative abundance remained in the medium to high range, the various types of arrow are shown on one electropherogram. Only medium to high abundance proteins were examined. Inhibitor is indicated by (↑).



abundance (▲, Fig. 10B). The inhibitor could be detected (↑). There was one particularly dominant protein (Mr about 55,000, the protein is not clearly visible in this print), and it rapidly decreased in relative abundance thereafter. Between 14 and 21 DPA, many proteins had become of medium to high abundance (▲, Fig. 10C). This is the period of active synthesis of many proteins, and a changeover of protein species was observed. Proteins that were relatively abundant at 14 DPA all decreased in relative abundance. Several proteins that became abundant by 21 DPA were of low Mr.

The protein population remained similar at 21 and 28 DPA, yet a number of proteins increased in relative abundance (▲, Fig. 10D). By 28 DPA, the inhibitor had become one of the more prominent buffer-extractable proteins. Protein profile changes continued even after desiccation had begun at 28 DPA (Fig. 10E). Many of the proteins that increased in amount were neutral in nature. The late development proteins remained relatively abundant and the protein population changed little. The stability of these proteins was confirmed as the protein profile of a mature kernel extract very closely resembled that of the 35 DPA sample (Fig. 10F).

Use of a lysis buffer to extract total kernel proteins (Payne et al., 1986) revealed species other than

those in Fig. 10. Lysis buffer-extracted proteins in 7 DPA kernels were very similar to those in Tris-extracted proteins (Figs. 11A and 10A). By 28 DPA, however, the protein population changed considerably and additional, very abundant proteins were observed in the Mr range between 35,000 to 70,000 (Fig. 11B). These proteins are apparently hordeins since they were present in 2-D gel electropherogram (Fig. 12) of isopropanol extract of mature kernels, a procedure used to obtain the hordein fraction (Rahman et al., 1982). The stained hordein gel revealed A, B and C hordeins (Rahman et al., 1983) in high abundance in the 28 DPA kernel total protein extract. B hordeins were stained darkly whereas C hordeins were negatively stained. This is due to a lack of cysteine in C hordeins, a group of storage proteins nutritionally poor in S (Forde et al., 1985). Cysteine is required for silver staining (Chuba and Palchaudhuri, 1986).

Figure 11. Two-dimensional gel electropherogram of total protein extracts of developing kernels. Kernel proteins were extracted with lysis buffer and separated as in Fig. 9. Panel A, 7 DPA and panel B, 28 DPA.

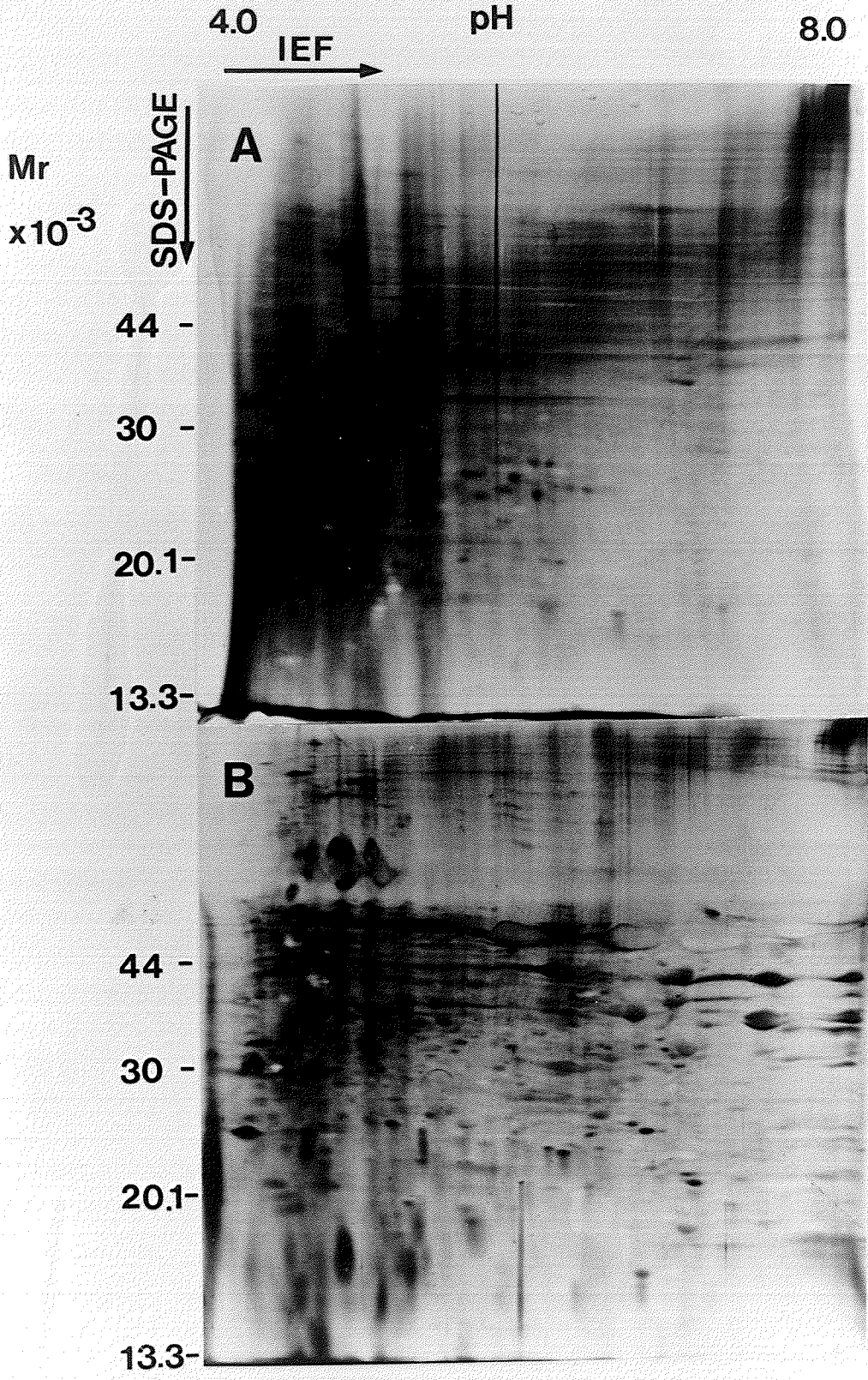
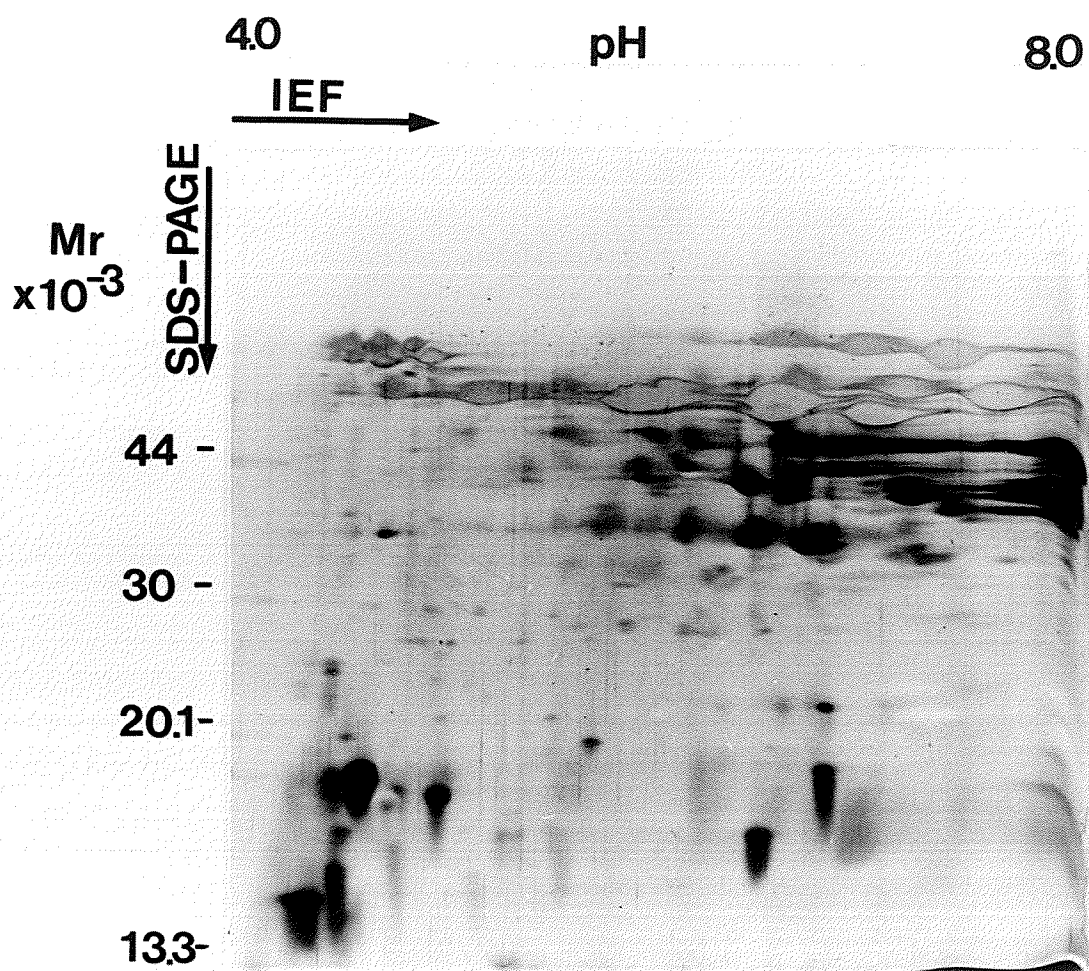


Figure 12. Two-dimensional gel electropherogram of hordeins. Isopropanol extract of mature kernels was separated as in Fig. 10.



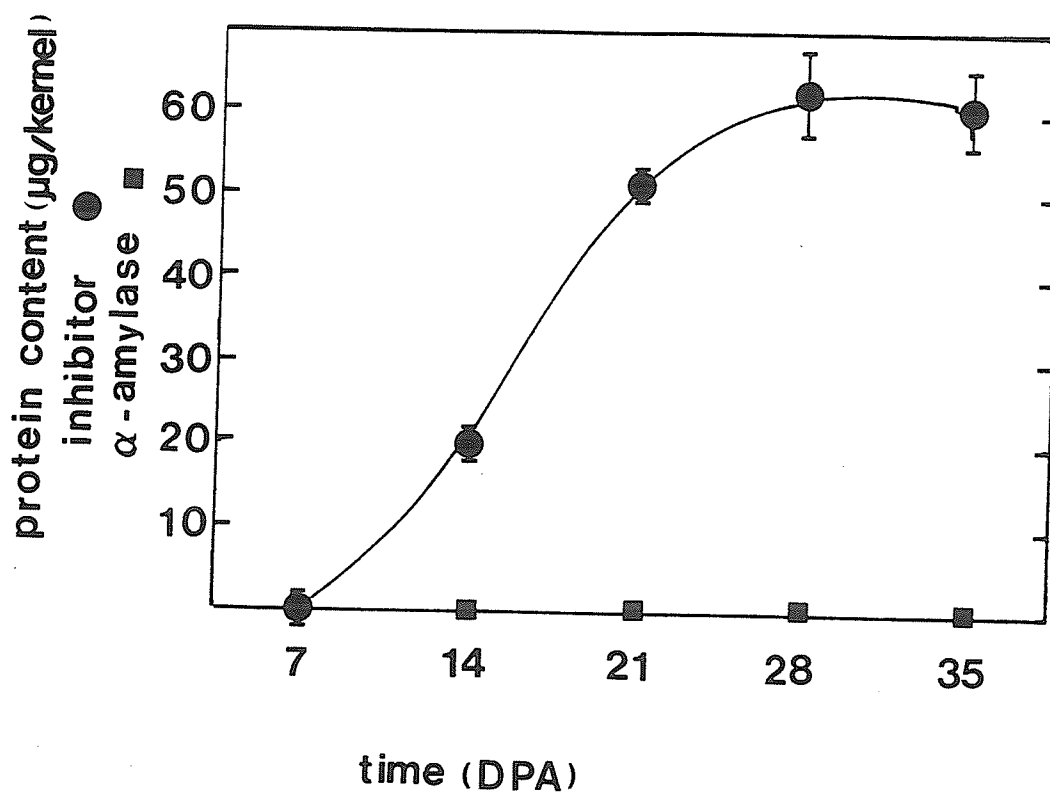
4.3.2. α -Amylase Inhibitor and α -Amylase Accumulation During Kernel Development

Protein accumulation of the α -amylase inhibitor and α -amylase was studied during barley kernel development using a Western blot of buffer-extractable proteins.

Inhibitor content increased during development (Fig. 13). Small amounts of the inhibitor were already present at 7 DPA as shown when an excess of crude protein extract was separated on SDS-PAGE (Appendix 2B). Rapid accumulation occurred from 7 to 21 DPA, with the most active accumulation taking place from 14 to 21 DPA (Fig 13). The inhibitor level remained relatively constant at 50 to 60 $\mu\text{g}/\text{kernel}$ from 21 to 35 DPA. This observation is in agreement with the 2-D gel electrophoresis results (Fig. 10). The initial increase of inhibitor protein to 14 DPA occurred concurrently with an increase in Tris buffer-extractable proteins (Fig. 8B). Since the buffer-extractable proteins remained relatively constant from 14 DPA on, inhibitor content increased, thereafter, in relative abundance among Tris buffer-extracted proteins (Appendix 2B).

Western blot analysis was also used to examine the presence of α -amylase in Tris buffer-extracted samples through use of an antiserum raised against a mixture of high- and low-pI α -amylase (Fig. 13 and Appendix 2C).

Figure 13. Changes in α -amylase and α -amylase inhibitor contents during barley kernel development. Extracts were examined for the existence of the inhibitor and α -amylase as in Fig. 1.

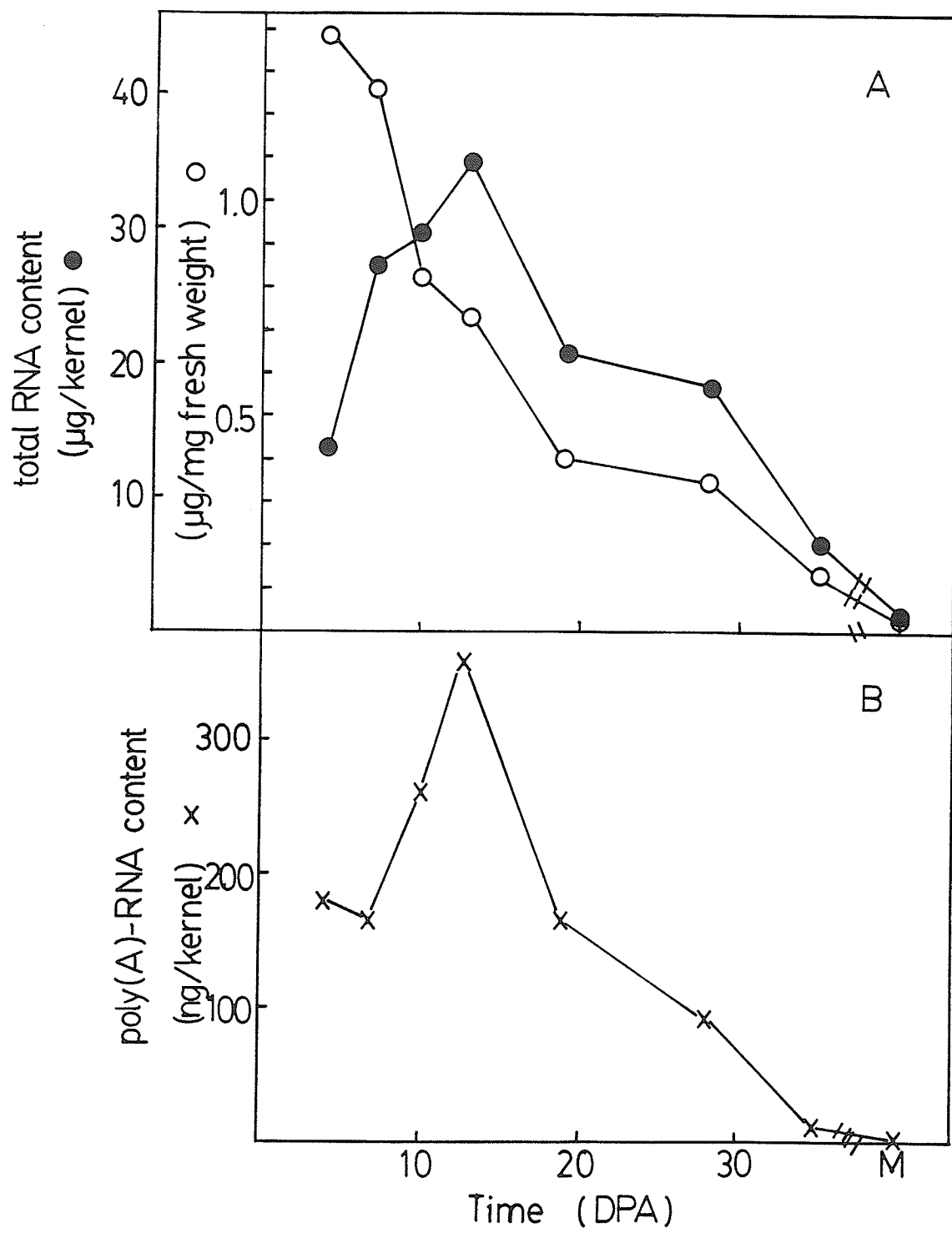


The enzyme was present in the 7 DPA sample in very low amounts, 0.01 percent of buffer-extractable proteins (Appendix 2C). It was also present in older kernels since it was detected when an excess amount of the crude extract was loaded on each lane. This antiserum was not as specific as the inhibitor antiserum, as higher and lower Mr proteins also cross-reacted with the antiserum even at a dilution at 4000:1. These cross-reacting proteins were temporally controlled as well.

4.3.3. Temporal Changes in RNA Contents During Kernel Development

Total RNA was extracted from developing and mature kernels using the previously described, modified procedure (Fig. 3). Total RNA content increased rapidly from 14 $\mu\text{g}/\text{kernel}$ at 4 DPA to 35 $\mu\text{g}/\text{kernel}$ at 13 DPA, then gradually declined as kernels matured (Fig. 14A). On a fresh weight basis, concentration of total RNA was highest, 1.4 $\mu\text{g}/\text{mg}$ fresh weight, at 4 DPA, on the earliest date sampled, then decreased due to the combined factors of fresh weight increase and kernel RNA decrease (Fig. 14A). The change in poly(A)-RNA content was similar to changes in total RNA content (Fig. 14B). A higher proportion of total RNA (greater than 1 percent) was in the poly(A) fraction at earlier stages than at

Figure 14. Total and poly(A)-RNA contents in developing barley kernels. Total RNA was isolated from immature and mature kernels by the modified method. Poly(A)-RNA was affinity isolated from total RNA. RNA contents were estimated by using a value of one absorbance unit/cm path/ml = 40 μ g.



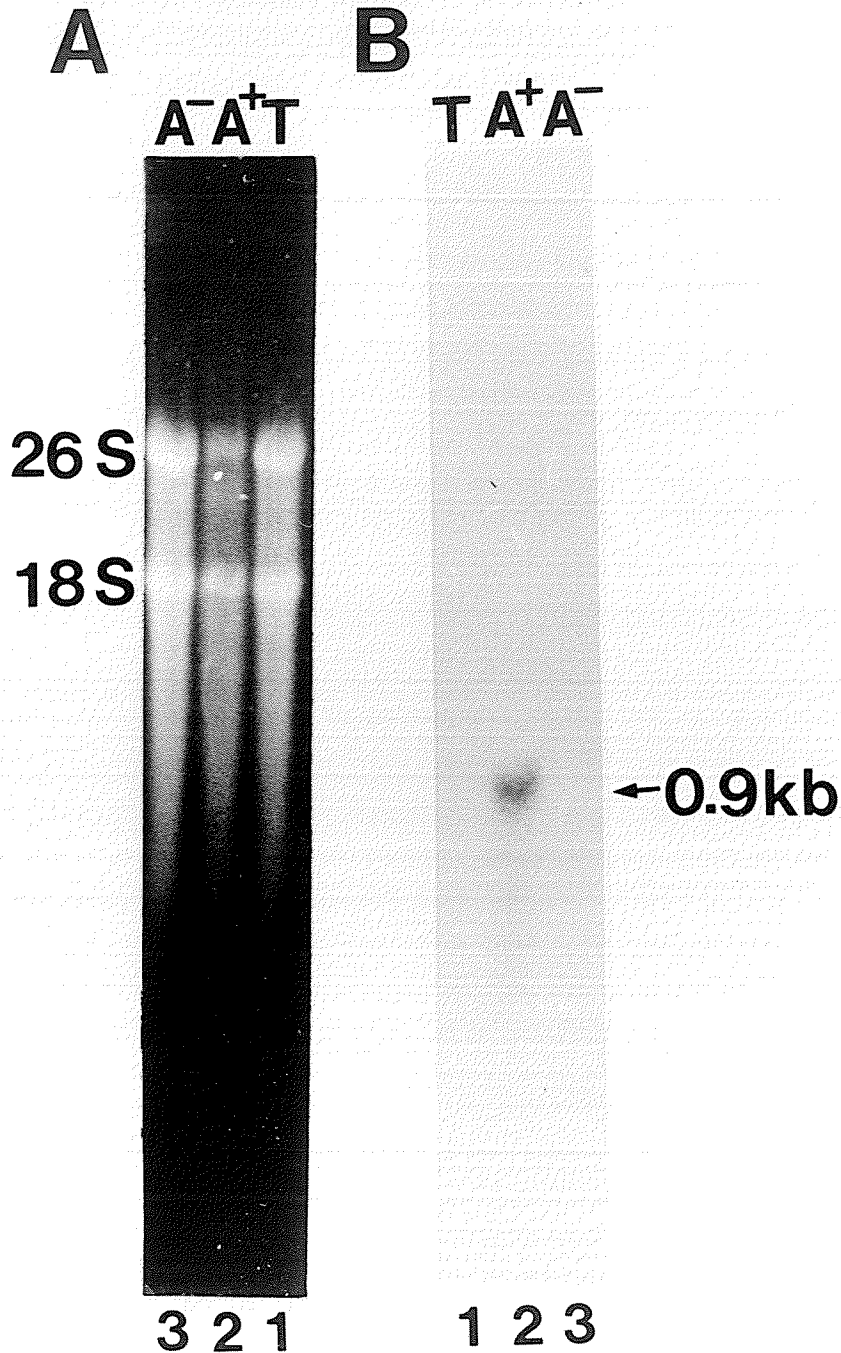
later stages, which may indicate that a higher portion of the RNA was available for translation during early kernel development.

4.3.4. Northern Analysis of Developmentally Regulated mRNAs

Inhibitor protein accumulation was temporally regulated during kernel development (Fig. 13). Relative abundance and the level of inhibitor message were also studied, using Northern analysis of gel and dot blots. The pattern of developmental regulation was then compared to other barley kernel proteins, B hordein and pHVA34. B hordein is the major group of barley storage proteins and its synthesis is developmentally regulated (Dailey *et al.*, 1988; Giese and Hopp, 1984; Giese *et al.*, 1983; Rahman *et al.*, 1982 and 1984). A protein characterized by a cDNA clone, pHVA34, is one of the ABA-induced proteins in the aleurone (P. Chandler, personal communication).

A cDNA clone for the inhibitor was used to carry out Northern analysis of 14 DPA barley kernel RNA for the existence of inhibitor message in total, poly(A⁺)- and poly(A⁻)-RNAs (Fig. 15). Poly(A)-RNA was intact with most of the rRNA removed (Fig. 15A). As can be seen, the inhibitor cDNA clone hybridized to a single transcript of

Figure 15. Northern analysis of total and poly(A)-RNA isolated from 14 DPA kernels. RNA was separated on a formaldehyde agarose gel (panel A) and transferred to Hybond-N, then hybridized to the ^{32}P -labelled insert of an inhibitor cDNA clone (panel B). Lane 1 (T), total RNA; lane 2 (A^+), poly(A)-RNA; lane 3 (A^-), poly(A^-)-RNA.



about 900 nucleotides in the 14 DPA kernel poly(A)-RNA. The inhibitor transcript was detectable in poly(A)-RNA, but not in total RNA (Fig. 15B, lane 2 vs. lane 1), suggesting a relatively low abundance of the inhibitor message in the RNA population. Since the observation that globin mRNA contained poly(A) sequences (Lim and Canellakis, 1970), most functional mRNAs are identified with similar poly(A) sequences and the inhibitor mRNA is not an exception.

Affinity isolation of poly(A)-RNA was necessary to detect a low abundance of inhibitor mRNA in Northern analysis (Fig. 15). The gel blot of 1 μ g poly(A)-RNA hybridized with the inhibitor cDNA insert showed that the message was already present in 4 DPA kernels and could be detected at higher abundance between 10 and 28 DPA (Fig. 16A). The presence of a single band on the blot also confirms that the washing stringency used eliminated any cross-reacting RNAs. Relative abundance was quantified using three separate dot blots of duplicate samples (Fig 17A). Each membrane also contained a set of internal standards consisting of a serial dilution of the isolated cDNA insert. From the blot, it can be clearly seen that the inhibitor message was present at very low relative abundance within the poly(A)-RNA population. In order to quantify inhibitor mRNA, each circle was cut out and the

Figure 16. Northern analysis of poly(A)-RNA gel blot analyzed for inhibitor (panel A) and pHVA34 (panel B) mRNAs during kernel development. One μg poly(A)-RNA/lane was used. Numbers at the bottom indicate sample DPA and M refers to the mature kernel sample. Molecular weight standards are indicated to the left and rRNA sizes to the right.

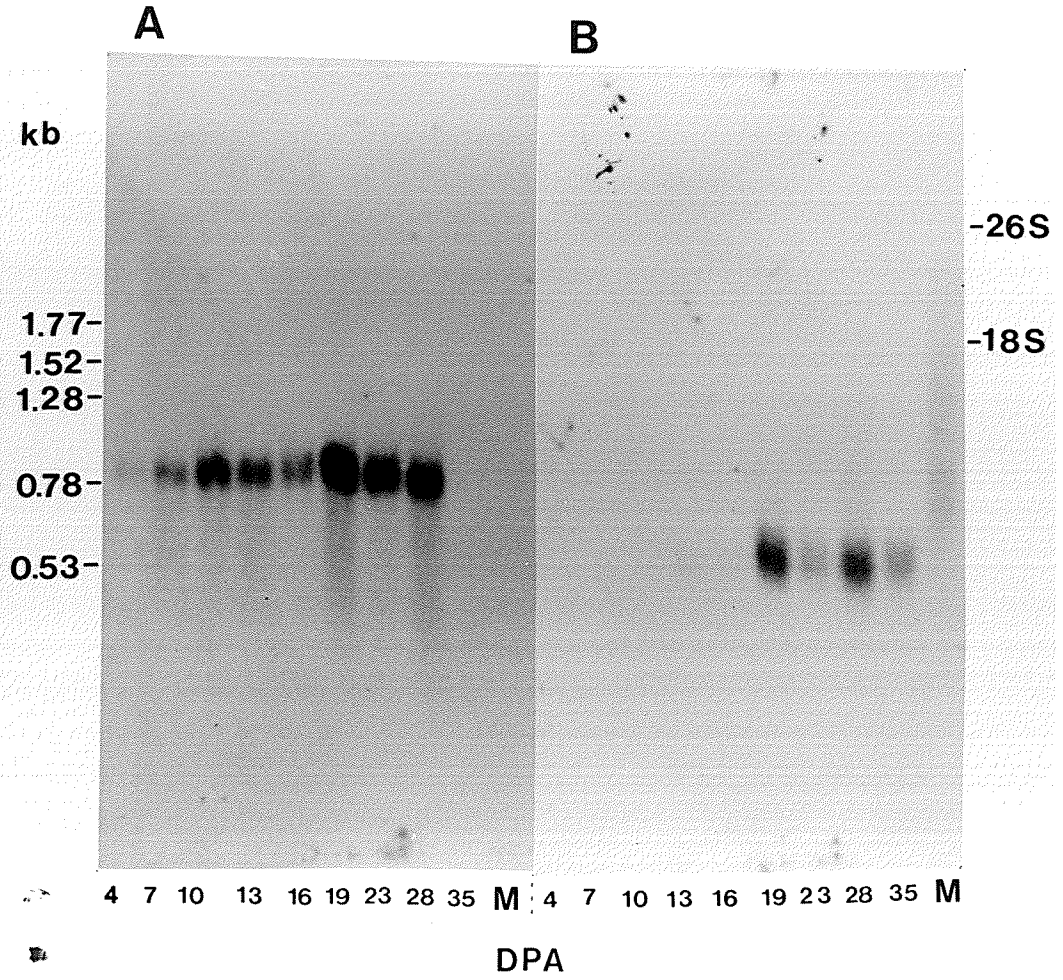
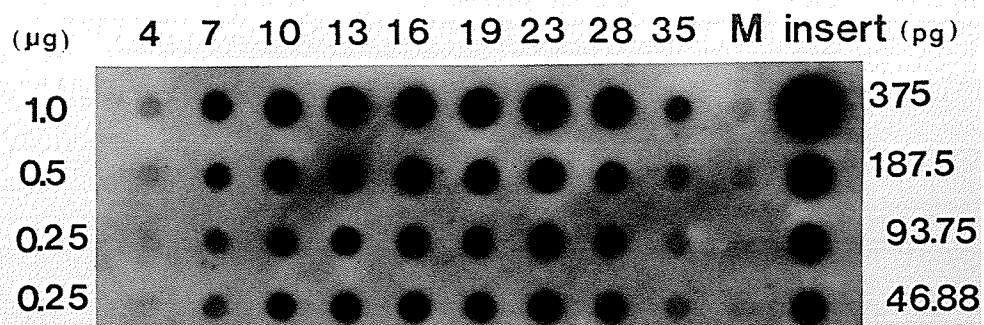


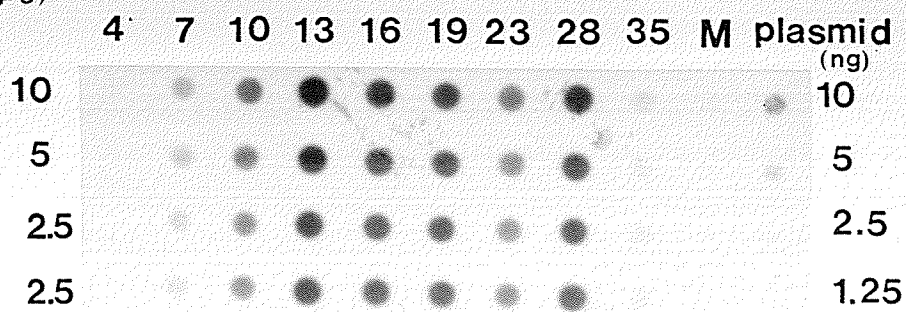
Figure 17. Dot blot analysis of poly(A)-RNA (panel A) and total RNA (RNAt) (panel B) for inhibitor and B hordein mRNAs, respectively, during kernel development. Serial dilutions of poly(A)-RNA at 1.0, 0.5 and 0.25 μg and RNAt at 10, 5 and 2.5 μg were used in Northern analysis. Numbers at the top indicate sample DPA, M refers to the mature kernel sample. The insert column contained the inhibitor cDNA and the plasmid column contained the B hordein cDNA at quantities indicated to the right.

Apoly A⁺

DPA

**B**RNA_t
(μg)

DPA



radioactivity determined. The quantity of the inhibitor message present in these tissues can be estimated based on a linear relationship between the known quantities of inhibitor cDNA insert applied to the blot and its activity after hybridization with the nick-translated probe (Fig. 18A). Inhibitor message abundance increased rapidly during the first 10 DPA, and, unlike the protein it codes, was nearly absent in the mature kernels (Figs. 16A, 17A and 19). Almost 20 percent of the maximum abundance was achieved by 4 DPA, indicating a very early commencement of gene expression (Fig. 19). The kernels reached a higher relative abundance between 10 and 28 DPA, after which, with the onset of kernel desiccation, there was a rapid decline in inhibitor mRNA. It was shown that even at the maximum abundance of inhibitor message on 23 DPA, the message was less than 125 pg per 1 μ g poly(A)-RNA or 0.0125 percent by weight (Fig. 19).

Using the relative abundance of the message (Fig. 19) and poly(A)-RNA content during development (Fig. 14), an estimation of the inhibitor message content in immature kernels during development can be calculated (Fig. 20). The inhibitor mRNA quantity increased seven fold between 4 and 13 DPA as a result of the combined effects of increased abundance and poly(A)-RNA contents between 4 and 13 DPA. Although the relative abundance of

Figure 18. Standard curve for an estimation of inhibitor and B hordein mRNAs based on hybridization activities of known amounts of the inhibitor cDNA insert (Panel A) and hordein cDNA plasmid (Panel B).

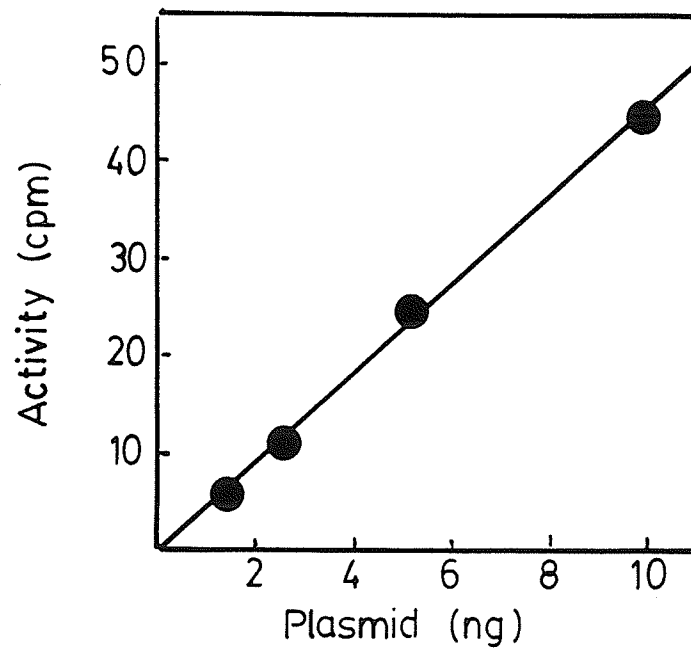
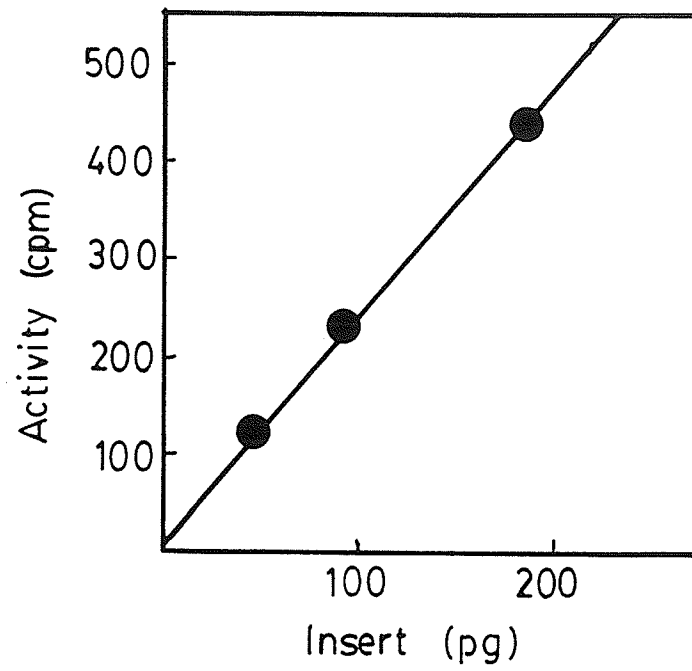


Figure 19. Relative abundance of inhibitor and B hordein mRNAs during kernel development. Relative abundance is expressed as an average of three duplicate dot blots in pg/ μ g poly(A)-RNA for the inhibitor and ng/ μ g RNAt for B hordein.

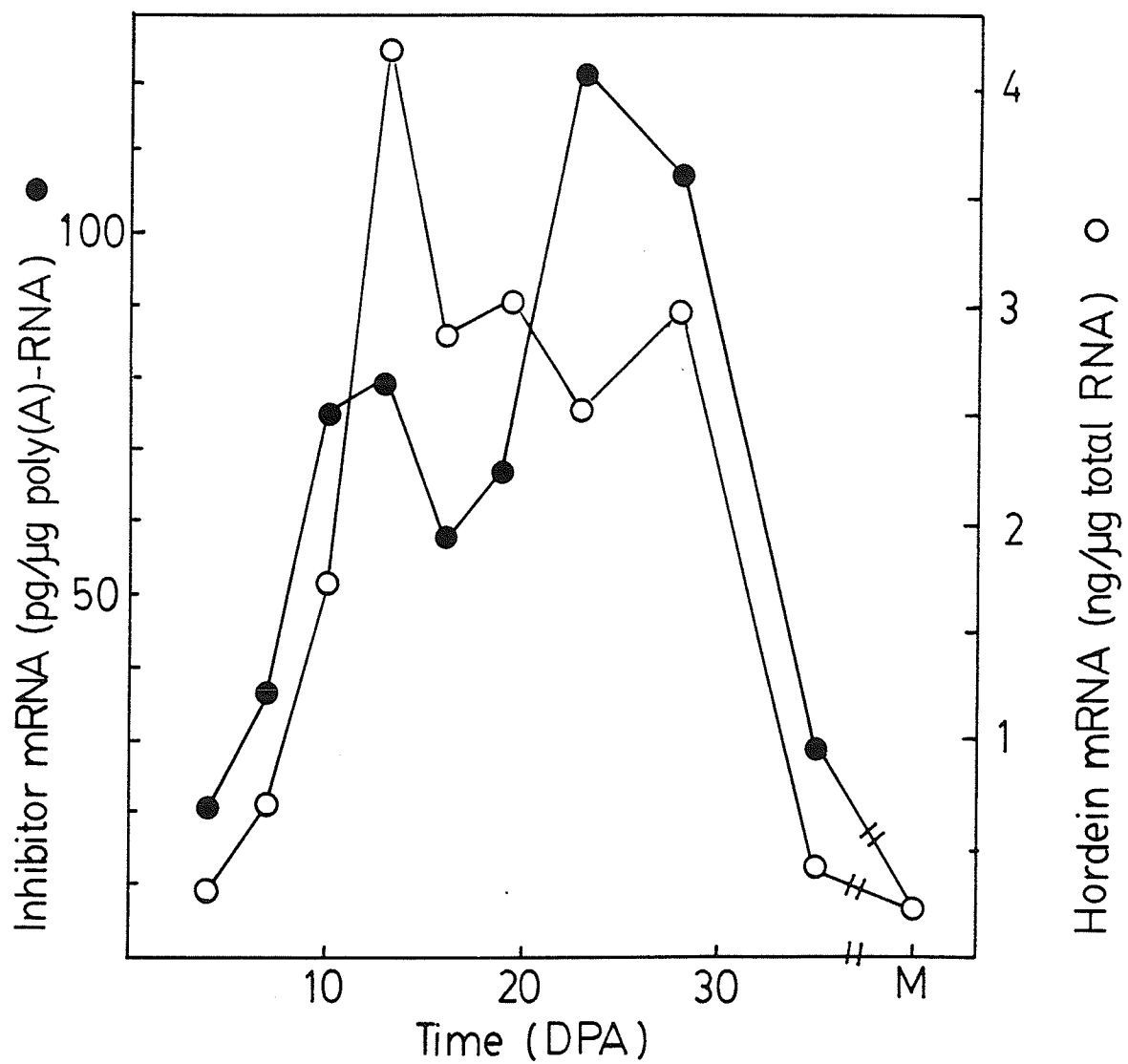
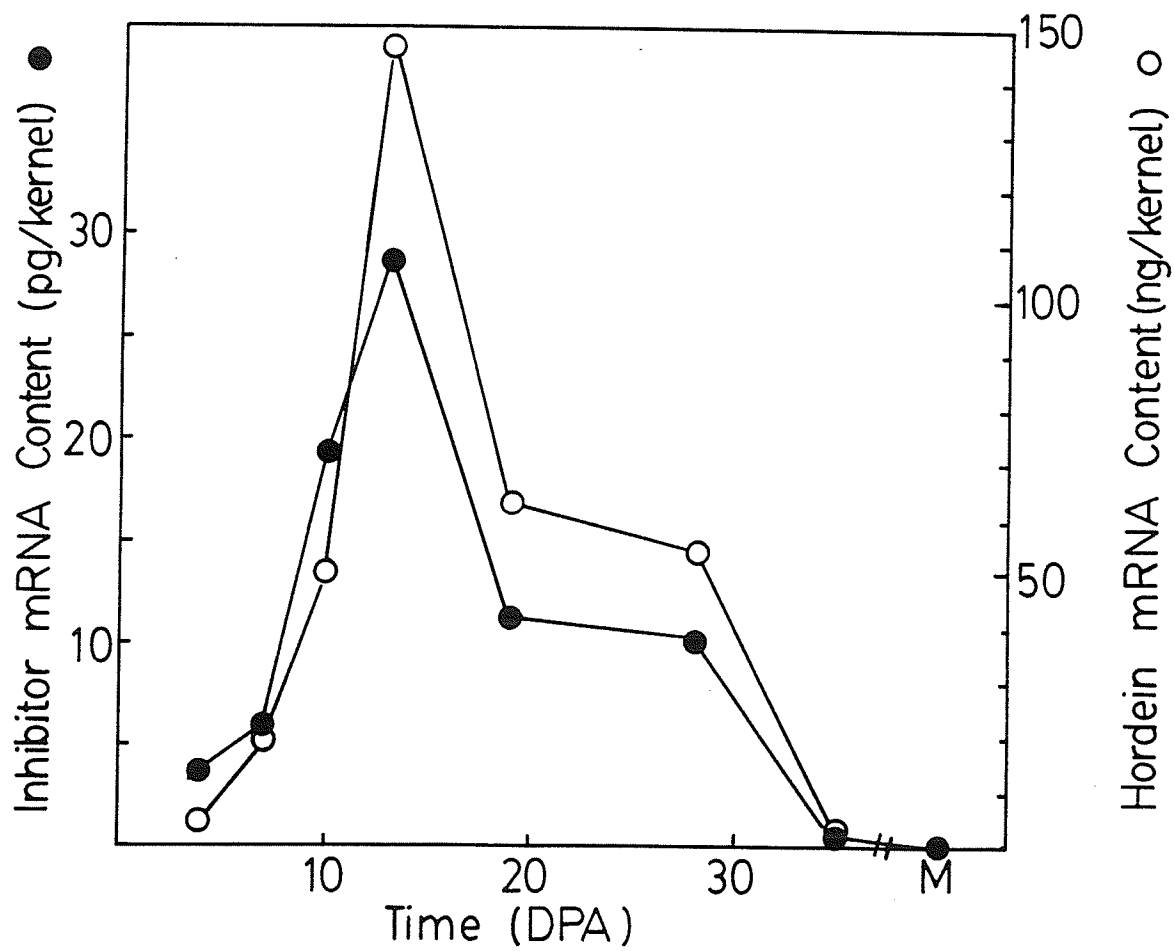


Figure 20. Inhibitor and B hordein mRNA contents in immature kernels during development.



inhibitor mRNA remained high between 10 and 28 DPA (Fig. 19), the inhibitor mRNA content was lower at 19 and 28 DPAs (Fig. 20).

The relative abundance of mRNA hybridizing to cDNA clone pHVA34 was also studied during kernel development. A poly(A)-RNA gel blot used for inhibitor Northern analysis was stripped of the inhibitor cDNA insert probe and re-hybridized with nick-translated pHVA34 plasmid (Fig. 16B). The message was barely detectable at 13 DPA, reached high abundance at 19 and 28 DPA, and was still present in 35 DPA kernels. Hybridization patterns over time for the inhibitor cDNA clone and pHVA34 were different (Fig. 16) suggesting that pHVA34 hybridizes to a mRNA representing a different type of developmentally regulated gene from that observed for the inhibitor.

B hordein mRNA was analyzed for its relative abundance and quantity in developing kernels by the same procedure as for the inhibitor mRNA (Figs. 17B, 18B, 19 and 20). The use of total RNA was adequate in the detection of hordein mRNAs due to very high abundance of the hordein mRNA among the RNA population (Fig. 17B). At maximum abundance on 13 DPA, 4.2 ng/ μ g total RNA can be accounted for by hordein mRNA (Fig. 19), or about one half of the poly(A)-RNA (Fig. 14). Hordein mRNA was barely detected in 4 DPA kernels (Fig. 17B) but its

quantity rapidly increased, remaining high between 10 and 28 DPA and changing parallel to the inhibitor mRNA (Fig. 20). The differences between the inhibitor and hordein mRNA expression are minor but suggest a slight delay in the initiation of the hordein gene (Fig. 20).

Increases in hordein mRNA in this experiment (Figs. 17 and 20) occurred earlier than reported by Rahman et al. (1984) and comparable to a result by Dailey et al. (1988). Bonanza barley kernel used in this study appears to have developed more rapidly based on the early detection of the message and an abrupt decline of its message at 35 DPA (Figs. 19 and 20).

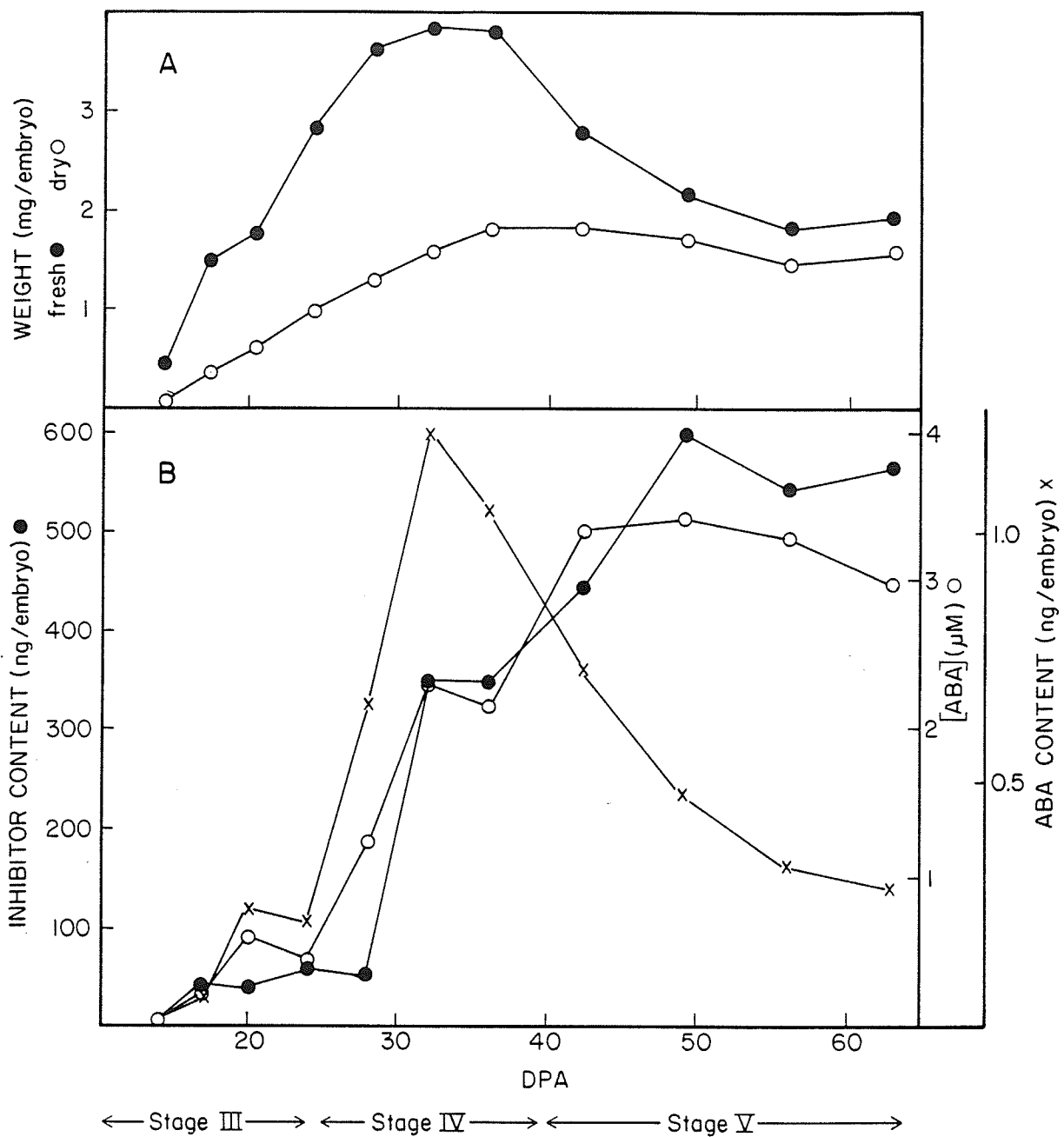
4.4. Regulation of α -Amylase Inhibitor Synthesis During Barley Embryo Development

The α -amylase inhibitor was expressed in kernel tissue (Figs. 1 and 4) with some protein found in embryos (Fig. 1). The protein synthesis could not be moderated in the endosperm (section 4.5.) but preliminary study showed an induction of the inhibitor by ABA in the embryo. Inhibitor synthesis in the embryo was studied to determine whether its regulation would be mediated by ABA in this tissue during development.

Fresh weight, dry weight, inhibitor, and ABA contents were determined during barley embryo development (Fig. 21). Fresh weight increased from less than 0.5 mg/embryo at 4 DPA to almost 4 mg at 32 DPA, after which fresh weight declined as a result of desiccation (Fig. 21A). Dry weight increased from 0.1 to 1.8 mg/embryo over the period from 4 to 42 DPA. Maximum dry weight was reached at 36 to 42 DPA, and remained near that level.

Large increases in the ABA content per embryo were found at 28 and 32 DPA (Fig. 21B). After that the ABA content per embryo declined. ABA concentration (μM) was estimated under the assumption that all of the embryonic water was available to ABA and that ABA was uniformly

Figure 21. Changes in weight, inhibitor and ABA contents of barley embryos during development. Panel A : fresh and dry weight. Panel B : ABA and inhibitor content.



distributed throughout the tissue. As with ABA content, ABA concentration increased up to 32 DPA. From then on, it continued to increase due to a decline in water content. Similar ABA results were reported for barley (Goldbach and Michael, 1976) and for wheat (Walker-Simmons, 1987). Increases in ABA concentration preceded the period of α -amylase inhibitor accumulation. The inhibitor content remained very low until 28 DPA when, over a period of four days, it increased six fold, from 50 ng to over 300 ng (Fig. 21B). Inhibitor accumulation continued until 49 DPA. Increases in both inhibitor abundance and buffer extractable protein content (Appendix 4) account for inhibitor accumulation. The final inhibitor content was 600 ng/embryo at 3 μ M ABA. Inhibitor content in the mature embryos was about 1 percent of that found in the whole kernel while embryo inhibitor concentration per unit of dry weight was about one quarter that of the whole seed; 0.3 μ g/mg dry weight in the embryos (Fig. 21) and 1.1 μ g/mg dry weight in the whole kernel (Figs. 8 and 13). Among the buffer extractable proteins, the protein profile remained relatively unchanged over the course of the development period (Appendix 5). The profile was characterized by the presence of many proteins with Mr ranging from 10,000

to over 100,000 with no one species very abundant. They probably function as metabolic proteins (Goldberg et al., 1989).

4.5. Effects of Plant Growth Substances on Kernel Proteins, Isolated Immature Embryos and Young Seedlings

Inhibitor synthesis appears to be mediated by ABA in developing embryos (Fig. 21) and exogenously applied ABA is effective in up-regulating the protein in aleurone layers (Mundy, 1984; Mundy et al., 1986). Further study was carried out to examine effects of exogenously applied plant growth substances on developing kernels, isolated immature embryos and young seedlings.

ABA and fluridone were applied to cultured spikes and whole, immature kernels. Protein synthesis was studied through comparison of protein profiles of de novo synthesized proteins labelled with ^{32}S on 2-D gel fluorography. Several attempts to study the effects of ABA and fluridone resulted in ambiguous observations. In brief, there was no conclusive evidence to suggest that either ABA or fluridone had any consistent effect on endosperm protein synthesis (Appendix 6). There have been no published reports that have shown an effect of

applied ABA on the regulation of cereal starchy endosperm proteins.

4.5.1. Effects of ABA on Isolated Immature Embryos

Isolated, immature embryos at various stages of development were cultured with and without ABA to examine embryo responsiveness to the plant growth substance as measured by precocious germination and inhibitor synthesis.

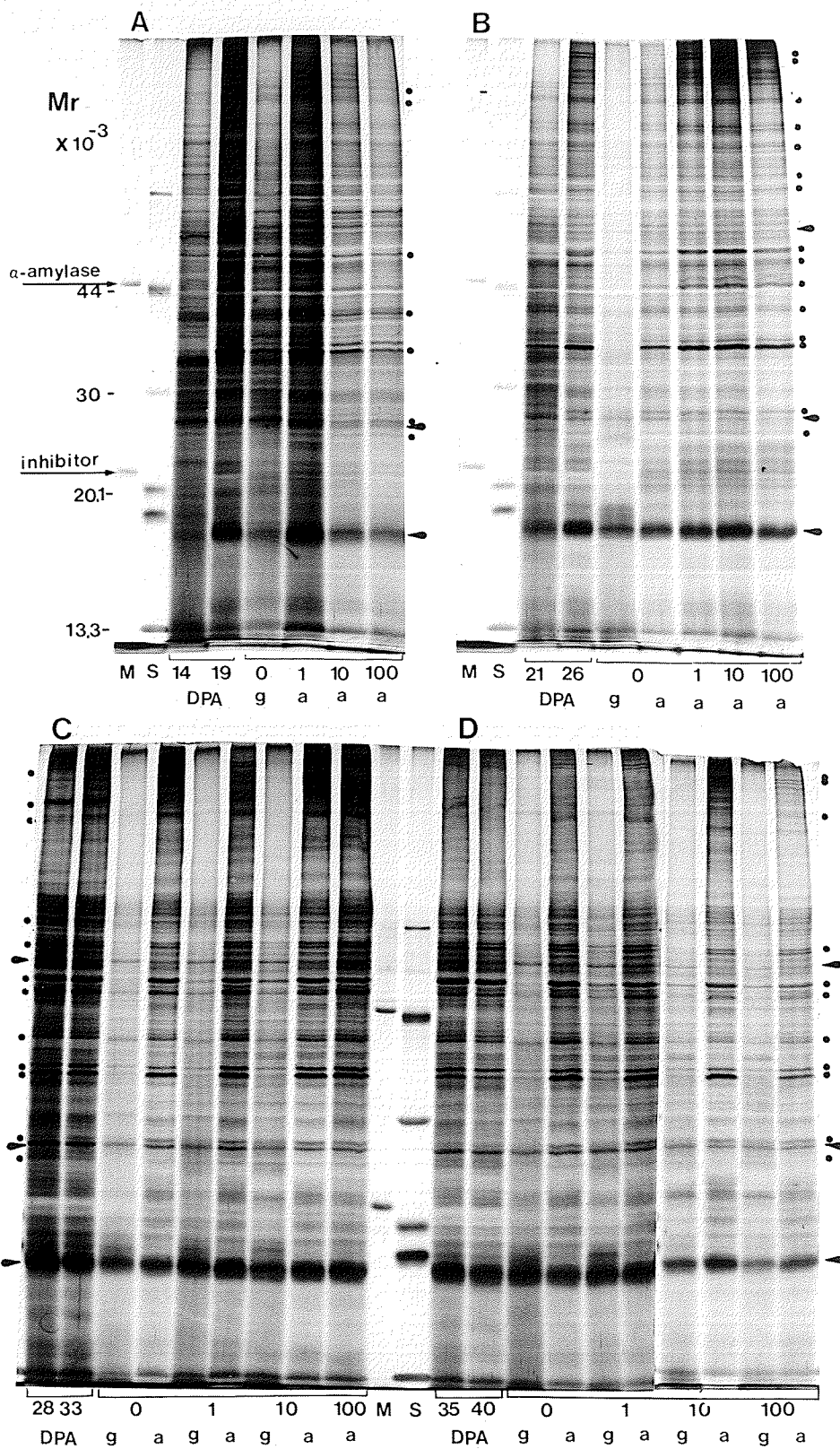
When stage III embryos (14 and 21 DPA) were cultured for 5 days without ABA, they germinated and grew into seedlings (Table 2). Inclusion of ABA in the medium at concentrations of 1, 10, 100 μM ABA prevented this precocious germination. As embryos approached stage IV, fewer embryos germinated in the absence of ABA, suggesting the presence of embryonic dormancy. The ability of ABA to inhibit precocious germination declined as embryos aged. Whereas 1 μM ABA inhibited germination by 95 percent in 21 DPA embryos, it only inhibited 2.2 percent of the germination in 35 DPA embryos (Table 2).

Protein profiles exhibited distinct characteristics of germinated and arrested embryos (Fig. 22). Proteins in the germination arrested embryos were present in in vivo embryos, indicating that these were embryogenic proteins. These include proteins having Mr 34,000,

TABLE 2. Effect of ABA on precocious germination of isolated embryos

DPA	ABA Concentration (μM)			
	0	1	10	100
	<hr/>			
	% Germination			
14	100	0	0	0
21	89.4	4.7	0	0
28	46.3	31.8	19.5	0
35	45.0	44.0	20.8	4.8

Figure 22. Effect of ABA on protein profiles of isolated embryos at 14 DPA (panel A), 21 DPA (panel B), 28 DPA (panel C) and 35 DPA (panel D). 0, 1, 10 and 100 refer to ABA concentration used (μM) for germinated (g) and arrested (a) embryos. Other numbers are DPA for embryos isolated from intact kernels and analyzed immediately. Lanes M contain protein standards as in Fig. 1. Embryogenesis-associated proteins are marked with • and proteins present in both germinated and arrested embryos are marked with †.



35,000, 39,000 and 49,000. Many polypeptides with Mr greater than 62,000 were present in the germination arrested and in vivo embryos. A few proteins were present in both germinated and arrested embryos, for example, a very abundant protein having Mr 15,000. Protein bands co-migrating with the inhibitor and α -amylase markers were not recognized among the population.

In the developing kernel, stage III embryos grew rapidly over the 5 day period, as fresh weight increased dramatically (Fig. 21). Inhibitor content rose by 30 ng during the 5 day development period (Table 3). ABA-cultured embryos, during the 5 day period, increased in fresh weight and protein content to a level similar to those left intact on the plant, although the protein content of 14 DPA embryos decreased as ABA concentration increased (Appendix 7). The inhibitor in these embryos increased to levels greater than those found in embryos left with the developing kernel over the same period (Table 3). This was due to an increase in relative abundance among soluble proteins since the soluble protein content remained about the same for 21 DPA embryos or decreased for 14 DPA embryos, indicating that increased accumulation of the inhibitor occurred at the expense of other proteins (Appendix 7). The level of inhibitor in isolated embryos increased with increasing

TABLE 3. Effect of ABA on the α -amylase inhibitor content of isolated embryos from developing kernels.

Embryo sample	ABA (μ M)	Inhibitor	
		Germinated ³	Arrested ⁴
(ng/embryo)			
<u>14 days post anthesis</u>			
kernel ¹ (14 DPA)	-	-	5.25
kernel (19 DPA)	-	-	38.7
cultured ²	0	18.3	-
cultured	1	-	43.4
cultured	10	-	73.9
cultured	100	-	50.3
<u>21 days post anthesis</u>			
kernel (21 DPA)	-	-	36.2
kernel (26 DPA)	-	-	69.2
cultured	0	87	78
cultured	1	113	299
cultured	10	-	713
cultured	100	-	835
<u>28 days post anthesis</u>			
kernel (28 DPA)	-	-	96
kernel (33 DPA)	-	-	176
cultured	0	24	107
cultured	1	28	359
cultured	10	108	444
cultured	100	-	427
<u>35 days post anthesis</u>			
kernel (35 DPA)	-	-	225
kernel (40 DPA)	-	-	376
cultured	0	113	394
cultured	1	264	521
cultured	10	776	1001
cultured	100	-	1328

¹ Embryos Excised from the kernel on the DPA indicated in brackets and analyzed directly

² Embryos excised from the kernel on the DPA indicated in heading and analyzed 5 days after designated treatment

³ Inhibitor contents in germinated embryos

⁴ Inhibitor contents in germination arrested embryos

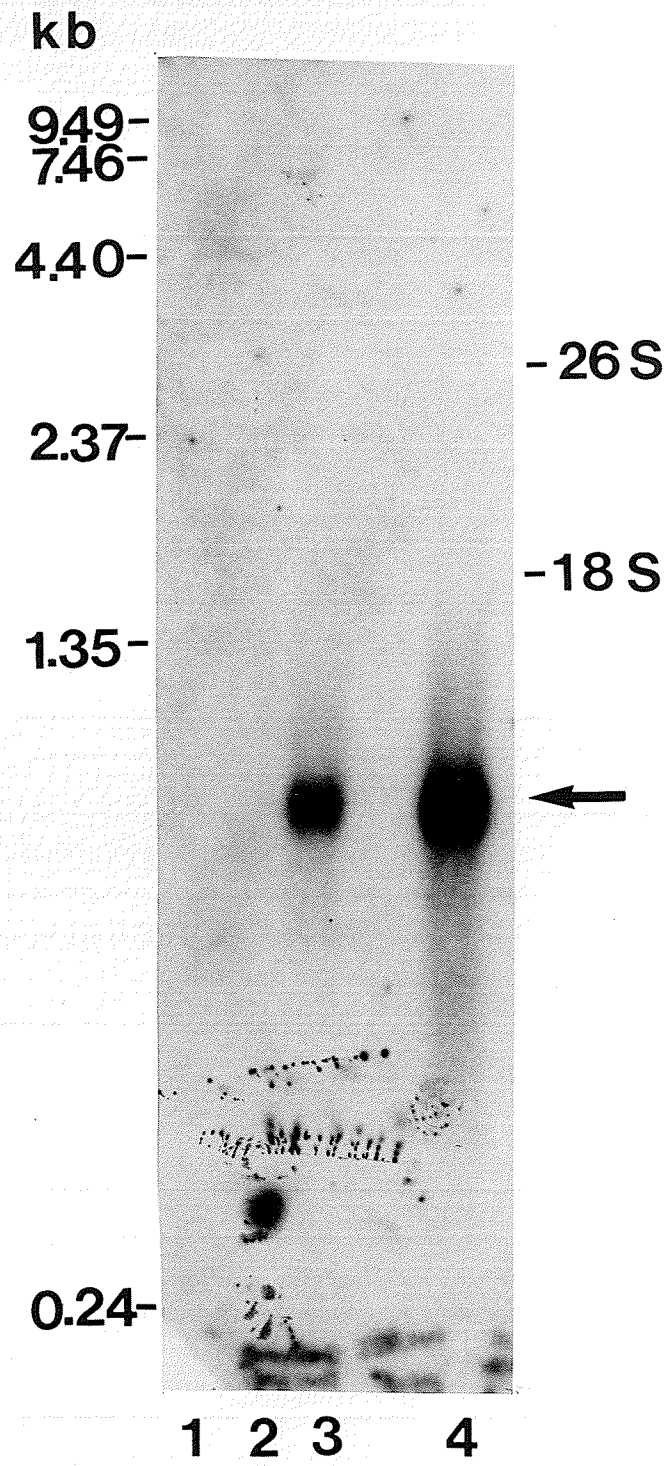
ABA concentrations from 1 to 100 μM . The isolated embryos incubated in the absence of ABA underwent germination, experiencing a large increase in fresh weight with little increase in α -amylase inhibitor in the seedlings (Table 3; Appendix 7).

For stage IV embryos, inhibitor synthesis was induced in the germination arrested embryos by ABA to levels similar to, or greater than, the intact embryos (Table 3). The relative abundance of the inhibitor among the buffer-extractable proteins increased during the culture in ABA, while little difference in the buffer-extractable protein content was observed between the cultured embryos and the intact embryos (Appendix 7). Some embryos, however, germinated even in the presence of ABA (Table 2). Under conditions where the embryos did germinate, inhibitor levels varied depending on the developmental age of the embryo. At 28 DPA, inhibitor levels for the germinated embryos were always lower than those left on the plant (Table 3). For the 35 DPA samples, inhibitor levels at 10 μM ABA concentrations and higher were greater in the germinated embryos than in the intact kernel embryo.

4.5.2. Effects of ABA on Young Seedlings

Two-day old barley seedlings were treated with 100 μM ABA to study whether inhibitor synthesis would be induced by the plant growth substance. Inhibitor mRNA was induced in ABA treated seedlings (Fig. 23). Northern analysis showed that a mRNA in ABA-treated seedlings hybridizing to the inhibitor cDNA was the same size as the transcript in the kernel (Fig. 23 lanes 3 and 4).

Fig. 23. Effect of ABA on inhibitor mRNA in young seedlings. Poly(A)-RNA from 2 day old untreated seedlings (lane 1), dehydrated seedlings (lane 2), ABA-treated seedlings (lane 3) and 14 DPA kernels (lane 4) were analyzed for inhibitor mRNA.



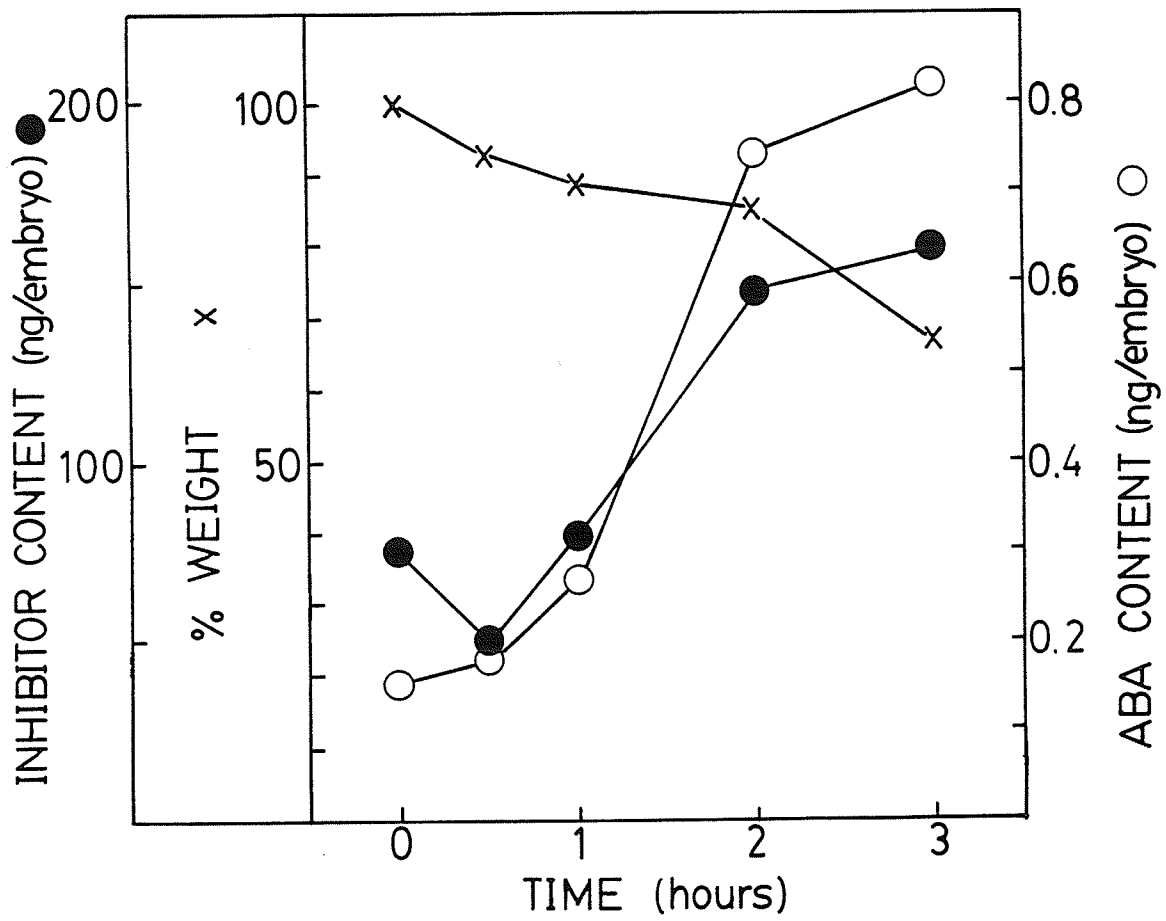
4.6. Effects of Dehydration Stress on Isolated Immature Embryos and Young Seedlings

Inhibitor synthesis was induced by ABA in immature isolated embryos (Table 3) and young seedlings (Fig. 23) and inhibitor synthesis in developing embryos appear to be mediated by endogenous ABA (Fig. 21). To determine whether the conditions that induce increased endogenous ABA content would also increase inhibitor levels, isolated immature embryos and young seedlings were subjected to dehydration stress, a condition that is known to elevate ABA content in plants (Hsiao, 1973).

4.6.1. Effects of Dehydration Stress on Isolated Immature Embryos

Isolated embryos were placed in a chamber of known humidity and were allowed to dehydrate. Twenty-one DPA embryos, which initially contained over 60 percent moisture, lost greater than 30 percent of the initial fresh weight within 3 hours from the dehydration stress (Fig. 24). The embryos remained viable during the stress as they were able to germinate upon rehydration. The immature embryos responded to the stress very rapidly.

Figure 24. Effect of dehydration stress on isolated immature embryos. 21 days post anthesis embryos were isolated and placed in a desiccation chamber and allowed to dehydrate for up to three hours. Contents of inhibitor and ABA were determined.



Endogenous ABA content increased almost five fold during the first two hours of stress, with the maximum change occurring between the first and second hour. Inhibitor content increased three fold within the first two hours of dehydration and remained at about the same level.

In immature embryos, inhibitor synthesis was induced by dehydration stress in conjunction with an ABA increase (Fig. 24), indicating that dehydration-induced inhibitor synthesis was mediated by ABA. This is the first evidence to show that a naturally occurring process, a dehydration stress, was able to regulate inhibitor synthesis.

4.6.2 Effects of Dehydration Stress on Young Seedlings

Since the induction of inhibitor synthesis may be one of many stress responses in barley mediated by ABA, an examination of the effect of a dehydration-induced ABA increase on α -amylase inhibitor synthesis in young seedlings was carried out.

The dehydration stress applied to young seedlings was relatively mild resulting in a final fresh weight loss of 28 percent (Fig. 25). Seedling growth ceased at the onset of the dehydration treatment and inhibitor

synthesis was induced in the SCS over the period of the stress. By 12 hours of dehydration stress, the ABA content increased 20 fold and an increase in the inhibitor content was detected. The ABA content continued to increase with longer stress application, eventually reaching a level of 2.1 ng/SCS, 80 times the levels in non-stressed, 2 day-old seedlings. The maximum level of inhibitor was reached on the 5th day of stress application, at greater than 20 times the non-stressed, 2 day-old seedling. The increase in inhibitor content in the SCS followed the observed increases in ABA content. In additional experiments, it was found that more than 90% of the inhibitor protein in the SCS sample was located in the scutellum. The inhibitor increase in the other parts examined, root tissue and the remaining seed, was minor compared to that in the SCS (Table 5).

When the stressed seedlings were allowed to rehydrate, ABA and inhibitor contents decreased rapidly (Fig. 26). During 2 days of rehydration following the 5 day stress, the ABA content in these tissues decreased from 2.1 ng/SCS in the dehydrated tissue to 0.31 ng/SCS in the rehydrated tissue. During the same period, the inhibitor content decreased from 2.3 μ g to 1.1 μ g, one half the stressed level. The SCS tissue isolated from

Figure 25. Effect of dehydration stress on young barley seedlings. 2 day old seedlings were placed in a desiccation chamber to dehydrate for up to 5 days. Seedlings were separated into three parts: shoots, coleoptile, and scutellum (SCS), roots, and remaining seeds. Inhibitor and ABA contents were determined for SCS samples.

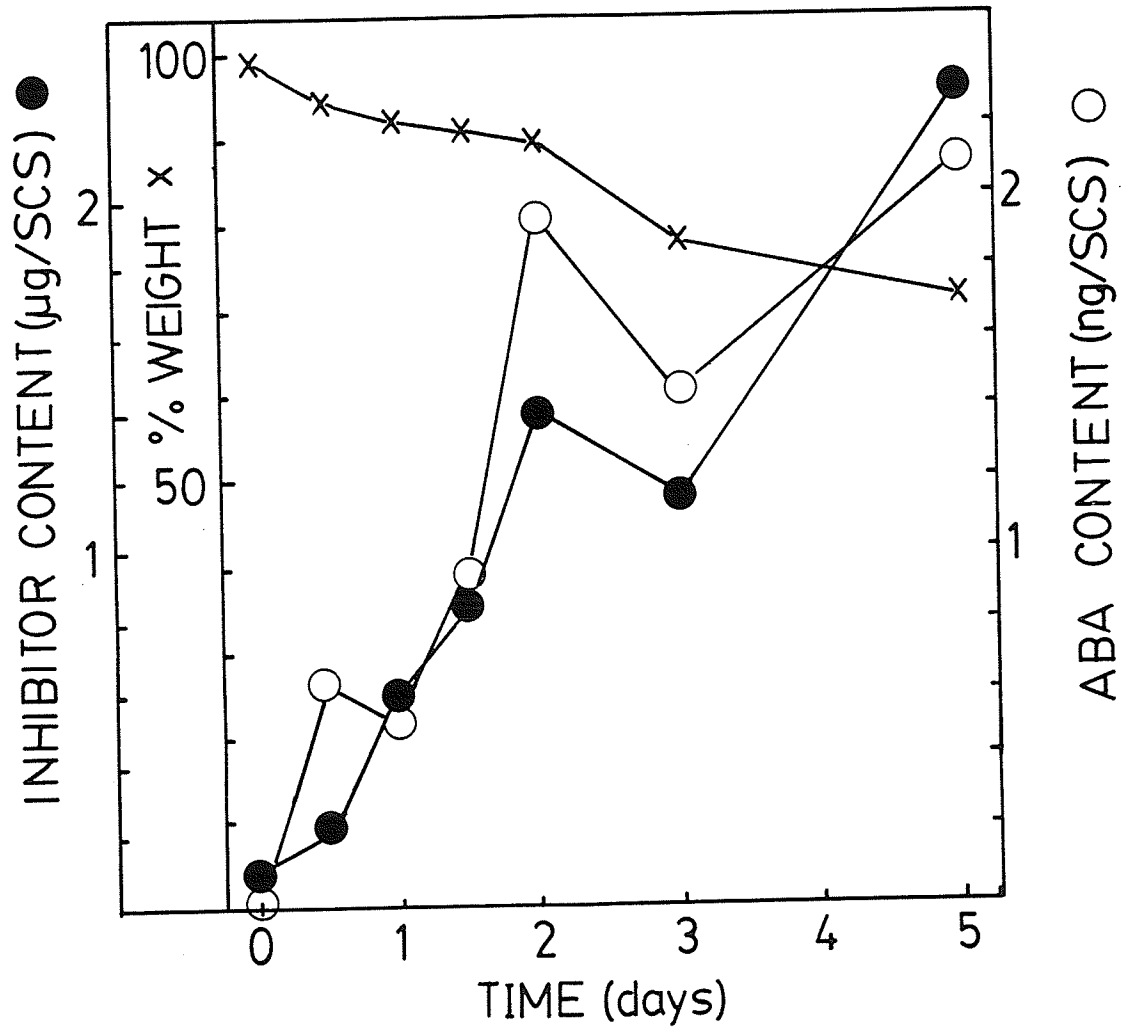


TABLE 5. Tris buffer-extractable protein and inhibitor contents of dehydrated young barley seedlings.

Time	Samples					
	SCS ¹		Root		RE ²	
	P ³	I ⁴	P	I	P	I
days	µg/SCS	ng/SCS	µg/root	ng/root	µg/RE	µg/RE
0	195	98	86	43	510	26
0.5	300	240	81	57	774	39
1.0	298	596	72	58	712	36
1.5	427	854	91	46	630	32
2.0	465	1395	96	29	735	37
3.0	414	1158	116	23	768	38
5.0	579	2316	105	105	664	33

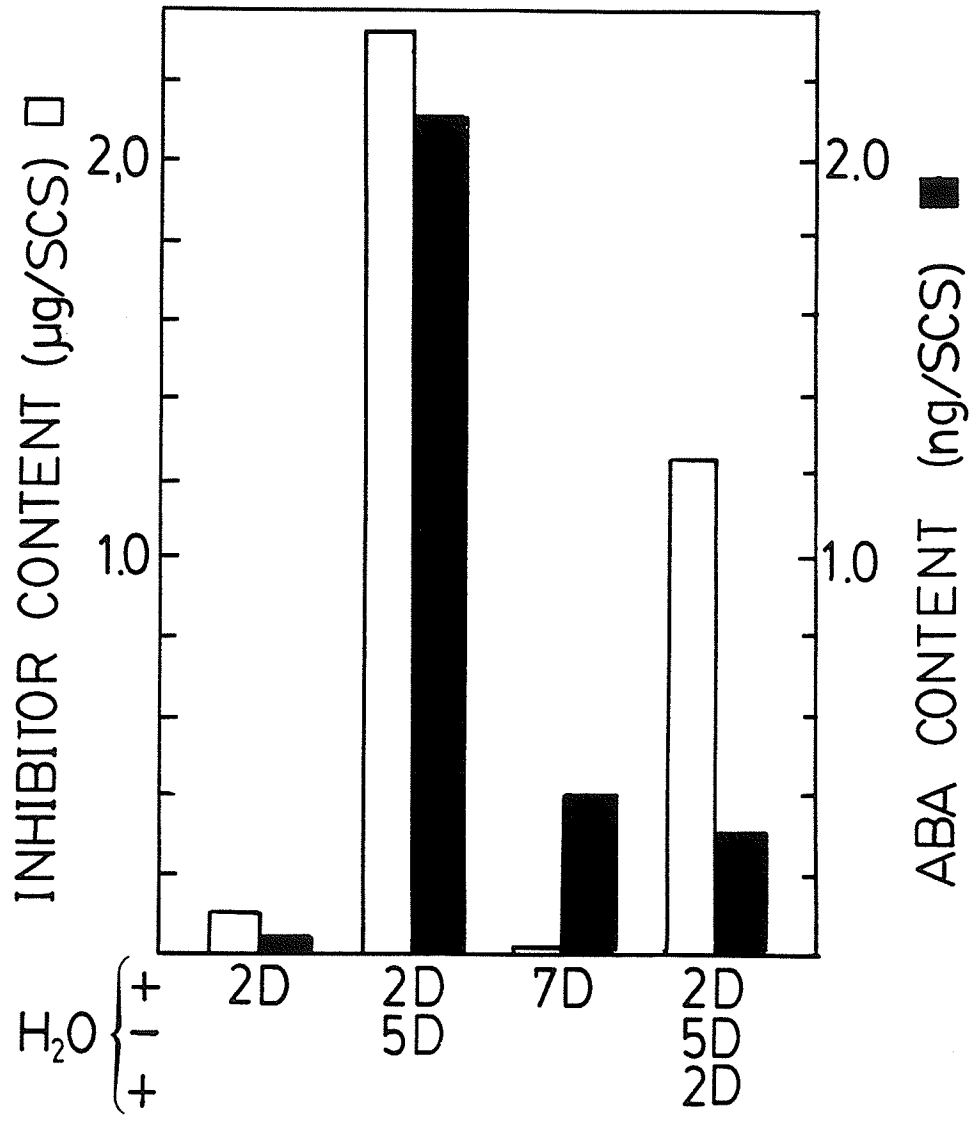
¹ Sample consisting shoot, coleoptile and scutellum isolated from dehydrated seedlings

² Sample consisting of remaining endosperm isolated from dehydrated seedlings

³ Tris buffer-extractable protein content

⁴ Inhibitor content

Figure. 26. Effect of rehydration on dehydrated seedlings. 2 day old seedlings were dehydrated for 5 days (2D+, 5D-), then rehydrated for 2 days (2D+, 5D-, 2D+). Unstressed seedling samples are shown at 2 days (2D) and 7 days (7D+).



etiolated seedlings grown without dehydration stress for 7 days had a low endogenous ABA content and contained very low levels of the inhibitor.

5. GENERAL DISCUSSION

5.1. Temporal Regulation of α -Amylase Inhibitor Synthesis During Barley Kernel Development

Barley kernel proteins and RNAs were examined during development to study patterns of temporal regulation and the presence of factors that might control inhibitor synthesis in the kernel. A comparison of the accumulation of the inhibitor protein and its mRNA with other endosperm proteins indicated that inhibitor synthesis was under temporal regulation.

Developmental regulation of the α -amylase inhibitor was studied by 2-D gel electrophoresis (Fig. 10), Western blot analysis (Fig. 13) and Northern analysis (Figs. 16A, 17A, 19 and 20). In this study using an immature barley grain system, it was found that inhibitor synthesis was initiated soon after fertilization and that the most rapid protein accumulation occurred early in kernel development in coordination with an increased level in the inhibitor mRNA (Figs. 10, 13 and 20). An indication of the very early onset of gene expression was shown by the detection of the inhibitor message at 4 DPA (Figs. 16A and 17A) and the protein in the 7 DPA sample (Fig.

13; Appendix 2B). An earlier detection of the inhibitor message before that of the protein, a slight deviation of a few days, is undoubtedly due to the sequential events involved in protein synthesis. Most of the inhibitor protein accumulated relatively early in development (Fig. 13) with accumulation being complete by the 21st day, before the commencement of kernel desiccation (Fig. 8). Similar accumulation patterns for the inhibitor protein have been reported for different barley cultivars (Rasmussen et al., 1988). Other reports indicated a continued accumulation persisting later into development (Lauriere et al., 1985; Munck, et al., 1985). The difference could be genetic or environmental. Genetic variations in the final inhibitor contents have been shown among cultivars (Fig. 1; Munck et al., 1985) that are due to dissimilar rates of accumulation (Munck et al., 1985; Rasmussen et al., 1988). In addition to cultivar differences, growing conditions affect the mature kernel inhibitor contents within cultivars (Audette and Hill, personal communication).

The pattern of inhibitor mRNA content (Fig. 20) during development was similar to that for inhibitor protein accumulation up to 14 DPA (Fig. 13), indicating that the temporal regulation of the inhibitor synthesis is at the level of the inhibitor message. Relative

abundance of the message after 19 DPA was greater, however, than what was expected from the small amount of protein accumulated after 21 DPA (Figs. 19 and 13). This discrepancy can be explained in part by estimating the inhibitor mRNA content per kernel, which decreased after 19 DPA (Fig. 20). Translatable inhibitor mRNA per kernel was also higher for 20 DPA kernels than 30 DPA (Mundy et al., 1986). The small amount of protein accumulation in the presence of inhibitor mRNA, however, may be due to translational control, as discussed later, or a more rapid turnover of the protein, if translation was to proceed at the same rate per mole mRNA. Lecommandeur et al. (1987) have shown that the protein is relatively stable, suggesting that protein turnover is not the explanation for the discrepancy between inhibitor protein and its mRNA levels. Protein turnover is prevented by acetylation of alanine at the N-terminus (Arfin and Bradshaw, 1988). The inhibitor may be stabilized by N^α-acetylation of alanine at the N-terminus (Svendsen et al., 1986).

The inhibitor protein accumulated earlier than storage or storage-type proteins, which accumulate most actively during the last half of kernel development. These endosperm proteins include the major barley storage protein, hordein (Giese and Hopp, 1984; Giese et al.,

1983; Rahman et al., 1982), salt-soluble proteins; such as protein Z (Giese and Hejgaard, 1984), β -amylase (Giese and Hejgaard, 1984; LaBerge and Marchylo, 1986), CI-1 and CI-2 (Kirsi, 1971; Rasmussen et al., 1988). Messenger RNAs for these proteins are present relative to the rate of protein synthesis, but were detected slightly earlier than the proteins (Dailey et al., 1988; Giese and Hopp, 1984; Mundy et al., 1986; Rahman et al., 1984; Rasmussen et al., 1988; Williamson et al., 1988). Since, in these studies, Northern analysis was not conducted during late maturation, when these proteins are reported to continue accumulation, a comparison of hordein mRNA levels to this study (Figs. 17B and 19) is incomplete. Hordein mRNA increased earlier (Figs. 17B and 19) than reported by Rahman et al. (1984) and comparable to the work by Dailey et al. (1988) by following similar stages of grain development (Rogers and Quatrano, 1983). The difference may be due to a variability in nutrient supply. Nitrogen and sulfur availabilities greatly influence hordein accumulation (Bottacin et al., 1985; Giese et al., 1983; Giese and Hopp, 1984; Rahman et al., 1983). Hordein mRNA content changed in a similar pattern to the inhibitor mRNA content (Fig. 20). Direct comparison between patterns of protein and mRNA contents for these proteins is difficult to make since a quantitative analysis for

hordein protein was not made. The majority of the very high abundance proteins in total kernel proteins extracted from 28 DPA kernels were hordein (Figs. 11 and 12), indicating that hordein protein accumulation could have been near completion by 28 DPA. An abrupt decline in its message at 35 DPA (Figs. 19 and 20) supports this suggestion. Temporal patterns of gene expression for the inhibitor and hordein appear to be distinctive in protein accumulation (Fig. 13; Rahman et al, 1982), yet similar in patterns of mRNA content change (Fig. 20), indicating that temporal regulation of these proteins may not only be on transcription of these genes, but also on their translation.

Temporal control of kernel protein synthesis is not limited to the inhibitor and hordein. The Tris buffer-extractable fraction changed in quantity and protein profile during development (Figs. 8 and 9). The changes in this fraction is very similar to changes observed in the albumins fraction reported by Brandt (1976).

In addition to temporal regulation on protein synthesis, a balance of translation system changed during development. Within RNA population, proportion of poly(A)-RNA related sequences decreased as kernels matured due to their reduction in transcription or stability relative to total RNA (Fig. 14). Functional

mRNAs usually contain poly(A) sequences (Aviv and Leder, 1972). The changes in balance among different RNA species, particularly a reduction in the poly(A)-RNA and an increase in poly(A⁻)-RNA sequences such as rRNA (Fig. 14), would suggest that translational machinery changed during development, affecting efficiency of translation (Tas and Martini, 1986).

5.2. Metabolic Function of the α -Amylase Inhibitor

Based on the known biochemical functions of the inhibitor and its target enzymes, the metabolic function of the inhibitor during barley kernel development and germination will be discussed. In the immature barley kernel, α -amylase protein was present at very low levels, representing a small fraction of the inhibitor levels throughout development (Fig. 13). Although the isozyme group detected in this study was not confirmed, the form of the enzyme present in developing barley kernels has been reported to be the low-pI group (MacGregor *et al.*, 1974). The inhibitor reacts only with the high-pI isozyme group (Weselake *et al.*, 1983a), which is the germination specific form of α -amylase isozymes (MacGregor, 1977). The inhibitor is, therefore, unlikely

to function to attenuate α -amylase during normal grain development.

The inhibitor protein may act as a serine protease inhibitor (Mundy et al., 1983; Yoshikawa et al., 1976) to protect barley kernel proteins against microbial attack during development. A relatively large quantity of the protein was rapidly accumulated by 21 DPA, being present shortly after fertilization, and eventually reaching about 1 percent of total proteins in mature kernel (Figs. 8, 10 and 13). It may be part of a defence strategy to synthesize the protease inhibitor early in development. Another protease inhibitor from barley kernels also shows prolonged existence during development (Kirsi, 1973).

In mature kernels, the α -amylase inhibitor may modulate high-pI isozyme activity on starch granules in cereal kernels during germination (Hill et al., 1987; Weselake et al., 1985b). In this study, the inhibitor was found mostly in the endosperm (Fig. 13), although a small amount was present in the embryo (Fig. 1 and 21). During barley germination, a large amount of high-pI α -amylase is synthesized (Jacobsen et al., 1970; Jacobsen and Higgins, 1982; MacGregor and Ballance, 1980; MacGregor and Daussant, 1981), the majority secreted into the endosperm with a relatively small amount present in the embryo (MacGregor et al., 1984). If the inhibitor

reduces enzyme activity, the majority of the inhibitor should be found in the endosperm.

In addition to modulating α -amylase activity during germination, the inhibitor may function to prevent starch granule degradation late in kernel development in some cultivars. In mature kernels of high α -amylase-containing cultivars, the high-pI isozyme group is found in wheat (Cornford and Black, 1985; Marchylo et al., 1980) and triticale (King et al., 1979). Enzyme increase in these cultivars occurs during the period of rapid water loss. This maturation associated pattern of synthesis is different from that generally observed early in grain development in quantity and quality (Macgregor et al., 1971). Synthesis can be induced by a high GA content during kernel desiccation as GA sensitivity is induced, or alternatively, the kernels are less responsive to ABA which prevents GA action (King et al., 1979). The inhibitor in the kernel may, then, interact with this high-pI isozyme group to prevent degradation of starch granules late in grain maturation.

The inhibitor expression is limited to the kernel tissue of barley (Figs. 1 and 4), which would fit with the function of the protein as an inhibitor of the high-pI isozyme group in cereal α -amylase (Weselake et al., 1983a). The inhibitor protein and mRNA were not detected

in vegetative tissues (Figs. 1 and 4) and no high-pI α -amylase isozyme types have been detected in barley leaf (Jacobsen et al., 1986). This spatial gene expression pattern is a characteristic common to many seed storage-type proteins (Goldberg et al., 1989) indicating the proteins of this type have functions confined to seeds.

5.3. ABA Regulation of α -Amylase Inhibitor Synthesis

The α -amylase inhibitor was induced by dehydration stress in barley (Figs. 24 and 25). This adds to a list of several water stress-induced proteins, such as hsp70 in maize (Heikkila et al., 1984), hsp27 in soybean (Czarnecka et al., 1984), osmotin in tobacco (Singh et al., 1987a and 1987b), and proteins identified by three cDNA clones which encode for relatively small proteins in barley (pHVA39) (Chandler, 1988; Chandler et al., 1988), maize (Chandler et al., 1988; Gomez et al., 1988) and rice (Mundy and Chua, 1988).

The induction of the inhibitor by dehydration stress is likely to be mediated by ABA. ABA is generally elevated in water-stressed plant tissues (Hsiao, 1973; Wright and Hiron, 1969). In this study, the inhibitor accumulated following increases in ABA content in isolated immature embryos (Fig. 24) and young seedlings

(Fig. 25) under dehydration. Furthermore, exogenous application of ABA to isolated immature embryos (Table 3) and young seedlings (Fig. 23) induced inhibitor synthesis. ABA induces inhibitor synthesis in the mature aleurones as well (Mundy, 1984; Mundy et al., 1986; Mundy and Rogers, 1986). Two other dehydration-induced genes are also induced in coordination with endogenous ABA increase (Chandler et al., 1988; Gomez et al., 1988). During normal embryo development, the inhibitor accumulated as embryonic ABA increased up to grain desiccation (Fig. 21). The maize gene identified by Gomez et al. (1988), a rice gene isolated by Mundy and Chua (1988) and barley pHVA39 (Chandler et al., 1988) are also expressed in embryos and aleurones during grain desiccation under natural conditions. The dehydration-induced proteins listed above are all induced by exogenous application of ABA (Chandler, 1988; Chandler et al., 1988; Czarnecka et al., 1984; Gomez et al., 1988; Heikkila et al., 1984; Mundy and Chua, 1988; Singh et al., 1987a and 1987b).

The inhibitor was induced not only in isolated immature embryos (Fig. 24) but also in young seedlings (Fig. 25) under dehydration. The degree of inhibitor induction was more pronounced for these seedlings than for the isolated embryos. Sen and Osborne (1974) have

speculated that seedlings have a greater ability to tolerate dehydration since the embryonic tissue has already gone through a desiccation process during maturation. The expression of the inhibitor, mediated by an elevated level of stress-induced ABA, may act as part of the tolerance mechanism in embryonic tissue during young cereal seedling establishment.

Speculation on the physiological function of the dehydration-induced inhibitor is based on the similarity of its mode of induction to other proteins and their function, which will be discussed in the following paragraphs.

Several water stress-induced proteins are induced by other stress parameters. One such parameter is heat-shock. Heat-shock proteins of maize (hsp70) and soybean (hsp27) are induced by water stress (Czarnecka et al., 1984; Heikkila et al., 1984). In addition to these heat shock-proteins, a late maturation protein, thought to have accumulated during grain desiccation, is present in wheat germ (McElwain and Spiker, 1989). A very high sequence homology, 79 percent, is observed between this protein and a 17 kD soybean heat-shock protein. Wounding is also capable of inducing proteins that appear under dehydration stress conditions. Soybean hsp27 and a maize dehydration-induced gene are both induced by wounding

(Czarnecka et al., 1984; Gomez et al., 1988). Although none of these proteins have a clearly established function, the broad spectrum of factors that are shown to induce these proteins would indicate that they may represent a general class of stress-induced proteins.

In contrast to these other stress-induced proteins whose function is largely unknown, two biochemical functions, inhibition of α -amylase and subtilisin activity, have been identified for the α -amylase inhibitor (Mundy et al., 1983; Weselake et al., 1983a). Although inhibitory activity against endogenous α -amylase is plausible during kernel germination, activity against proteases may be more important under stress. This inhibitor may function in a protective role against proteolysis in a similar manner to a protein described from tobacco. Osmotin is induced by NaCl and ABA (Singh et al., 1987a). The N-terminal of this protein is homologous to a sweet-tasting protein, thaumatin (Singh et al., 1987b). Thaumatin has shown strong sequence homologies to a tobacco mosaic virus-induced protein and a maize α -amylase/trypsin inhibitor (Richardson et al., 1987). Based on its relationship to a pathogenesis-related protein and an α -amylase/protease inhibitor, osmotin is thought to act as a defence mechanism against

external biotic stress caused by microorganisms, insects and mammals. Protease inhibitory activity of the barley inhibitor protein acts against bacterial proteases, subtilisin Carlsberg, BPN' and amylosaccharitics (Mundy et al., 1983; Yoshikawa et al., 1976). Although little sequence homology exists between other dehydration-induced protease inhibitors and the α -amylase inhibitor, the action of the barley α -amylase inhibitor represents, presumably, a protective mechanism against bacterial attack. The defensive requirement may increase when plants are placed under stress and become more vulnerable. The inhibitory activity against endogenous α -amylase remains relatively unique among cereal proteins. It may be indicative of this protein having evolved in order to regulate endogenous metabolic activities. Although the inhibitor failed to show any activity against other proteases tested (Yoshikawa et al., 1976), these proteases are microbial or animal in origin and no endogenous plant protease has been tested. The existence of inhibitory activity against endogenous proteases for this protein may establish an important regulatory role for the inhibitor as a mediator of cellular metabolic reorganization, necessitated by plants undergoing adaptation to water stress.

Embryonic ABA controls embryonic inhibitor accumulation during normal development (Fig. 21). It appears that ABA does not regulate embryonic germination through inhibitor induction, since the embryos respond to ABA differently for inhibitor induction and germination (Tables 2 and 3).

For isolated immature embryos in barley, exogenous ABA arrested germination, while in its absence, these immature embryos germinated and grew into normal seedlings (Table 2). The concentrations of exogenously applied ABA used to examine its effect on embryos have been found to be effective in modifying physiological functions in embryos (Zeevaart and Creelman, 1988). The phenomenon of ABA acting as a regulator of germination has been widely observed for both monocots and dicots (Zeevaart and Creelman, 1988). As the embryos aged, responsiveness to ABA decreased as more embryos germinated at a given ABA concentration. Loss of embryonic responsiveness to ABA, as measured by the capability of ABA to block precocious germination, has been reported for wheat embryos (Morris *et al.*, 1988; Walker-Simmons, 1987).

The α -amylase inhibitor was induced, in an age-dependent manner, by exogenously applied ABA in isolated immature embryos (Table 3). The regulatory effects of

plant growth substances on inhibitor synthesis have been reported in mature aleurone layers. Inhibitor synthesis is increased by ABA and decreased by GA (Mundy 1984; Mundy et al., 1986), which is opposite to what is observed for its target α -amylase isozyme group (Chrispeels and Varner, 1966 and 1967b). The effect of ABA on protein synthesis was not limited to the inhibitor. Other embryonic proteins were maintained in the germination arrested embryos as germinated embryos showed a significantly different set of proteins than arrested embryos (Fig. 22). This clearly shows that they are in distinctly different phases of plant development. The inhibitor (Table 3) and other embryogenic proteins (Fig. 22) belong to a group of germination arrest-associated proteins in cereals, such as, wheat- and barley-germ agglutinin (Morris et al., 1985; Triplett and Quatrano, 1982), and Em protein and 7S globulin (Williamson et al., 1985; Williamson and Quatrano, 1988), that are characteristic of late embryogenesis.

Both germinated and non-germinated embryos responded to ABA treatment by increasing the α -amylase inhibitor. In particular, the responsiveness of germinated stage IV embryos was greater, in terms of inhibitor accumulation, but the responsiveness to ABA was less, in terms of germination arrest, than stage III embryos (Tables 2 and

3). Embryos, thus, appear to possess independent mechanisms, regulated by ABA, for protein synthesis and germination arrest. This speculation is supported by the studies of Dashek et al. (1979) showing independent sensitivities to ABA for protein synthesis and germination arrest in barley kernels. Concentrations of ABA, effective in inhibiting the promotion of α -amylase activity by GA, were relatively ineffective in germination prevention.

5.4. Translational Control of α -Amylase Inhibitor Synthesis

Comparisons of the level of inhibitor transcript and the level of inhibitor protein during development suggest the possibility of translational control. Although the evidence for translational control of inhibitor synthesis is very limited at this time, the evidence available for other proteins is useful to speculate how translational control might act on the inhibitor.

Northern analysis of kernel RNA detected the inhibitor message only in poly(A)-RNA and not in total RNA (Fig. 14). In addition, a low frequency of message in the RNA population was suggested by the lack of a

detectable inhibitor band in total in vitro translation products (Fig. 6), even though the inhibitor protein was increasing rapidly at 14 DPA (Fig. 13). The protein may, therefore, accumulate from a small pool of mRNA over the course of grain development.

The similarity of the patterns of the inhibitor mRNA content and hordein mRNA content during development was noted, but there is a 5000 fold difference in the quantity of the two mRNA populations (Fig. 20). The inhibitor protein, on the other hand, comprises less than 1 percent of total kernel protein mostly accumulated prior to 21 DPA (Figs. 8 and 13) while hordeins make up about 30 percent to 50 percent (Brandt, 1976; Kreis et al., 1987; Rahman et al., 1982), which represents a 30 to 50 fold difference in protein between the two. Since their developmental patterns at the mRNA levels are very similar (Fig. 20) while their relative abundance differs by 5000 fold, the inhibitor message appears to be translated preferentially over that for hordein, suggesting the presence of a translational enhancer for the inhibitor mRNA up to 21 DPA in developing barley kernels.

The presence of a translational control was also suggested by the difference in the changes between protein accumulation and mRNA content during development.

Protein accumulates rapidly between 7 and 21 DPA, followed by little accumulation from 21 to 35 DPA (Fig. 13). The inhibitor mRNA content (Fig. 20) paralleled the pattern of protein accumulation (Fig. 13) up to 13 DPA, indicating that the temporal regulation of inhibitor synthesis is mostly at the level of the inhibitor message. Although the inhibitor mRNA level was much lower after 18 DPA (Fig. 20), its quantity was higher than that expected since there was little increase in protein accumulation (Fig. 13), indicating the involvement of post-transcriptional control or a translational repressor. Alternatively, the protein might be catabolized more quickly after 21 DPA. This, however, is unlikely since the inhibitor is shown to be very stable even during germination (Lecommandeur et al., 1987), a period of active proteolysis for storage proteins.

There are several translational control mechanisms identified. Some mechanisms offer steady state translational control. The 5'-leader sequence of a tobacco mosaic virus RNA enhances translation of many mRNAs (Gallie et al., 1987). Structural features other than the 5' end also control translation. An optimum sequence around the initiator codon has been identified (Kozak, 1986) and the leader sequence and mRNA secondary

structure affect translation (Kozak, 1988). Control factors that offer mechanisms amenable to modulation, being up- or down-regulated, are of greater interest. The biosynthesis of ferritin is translationally regulated through a binding between the 5' untranslated leader region of ferritin mRNA and a protein factor mediated by iron (Leibold and Munro, 1988). A yeast gene translation is repressed by the product of the 5' leader sequence, a case of feedback inhibition (Werner et al., 1987). Translation in transformed plant cells is increased by a minimum poly(A) tail of 25 adenylate residues (Gallie et al., 1989).

An investigation into the possible existence of any of the above mechanisms would involve detailed studies. A more plausible mechanism would entail antisense RNA. Many naturally occurring antisense RNAs function to control translation (Inoue, 1988; Simons, 1988). A small molecular weight antisense RNA isolated from the barley embryo inhibits translation by interfering with formation of the initiation complex (Gunnery and Datta, 1987). The existence of antisense RNAs for α -amylase isozymes has been detected and its expression is found to be regulated developmentally in the endosperm and aleurone, and by ABA in the mature aleurone (Rogers, 1988). The nick-translated cDNA insert would detect both sense and

antisense RNAs in Northern analysis used in this study (Figs. 16 and 17). In order to verify the presence of antisense RNA for the inhibitor and to analyze sense and antisense RNAs separately, Northern analysis using a single strand DNA or RNA must be carried out. Its repressing activity on inhibitor mRNA translation can be shown by the reduction in translatability of the exogenous antisense RNA in an in vitro translation system programmed with total cellular poly(A)-RNA.

The possibility of the existence of translational control may also be studied by comparing efficiencies of in vivo and in vitro translations. Less protein synthesized in an in vivo system than an in vitro translation of RNA from 21 DPA kernels would indicate the presence of a repressor factor in the in vivo system. A similar approach using homologous (chloroplastic) and heterologous translation systems showed the presence of some translation controlling factors in the chloroplast in senescing barley leaves, regulated by cytokinin (Martin and Sabater, 1989). In vitro translation systems derived from heat-shocked and normal cells retain selective translational control (Storti et al., 1980). Alternatively, in vitro translation of polysomal RNA and cellular RNA may reveal a translational control factor

contained in the polysomes (Fabijanski and Altosaar, 1985).

5.5. Cellular Transport of the α -Amylase Inhibitor

The in vitro translated inhibitor protein was processed to a smaller size mature protein with canine microsomal membranes, indicating the presence of a signal peptide (Fig. 7). A deduced signal peptide of 22 amino acids of a cDNA clone supports this finding (Leah and Mundy, personal communication). Microsomal membranes contain signal peptidase activity (Jackson and Blobel, 1977), and the processing of a precursor protein into a smaller, mature protein is strong evidence of the involvement of protein translocation across the endoplasmic reticulum (ER) membrane (Walter et al., 1984). Proteins translocated into the ER lumen must be sorted for their destinies which may take one of several forms; secretion out of the cell, translocation to be integrated in the membrane, deposition into cellular organelles such as the lysosome or vacuole, or residence in the lumen. Intracellular translocation into the mitochondria or the chloroplast involves post-translational processes and does not occur through the ER (Rothman and Kornberg, 1986). The presence of a signal

peptide in the inhibitor protein is positive evidence of its cellular transport through the ER. The pathway it may take, however, is unknown. Its synthesis during barley kernel development takes place in the endosperm (Figs. 1, 4 and 13; Mundy et al., 1986) and the protein is distributed throughout the mature endosperm (Weselake et al., 1985a). There is some evidence to suggest its presence between starch granules in the mature kernel (Lecommandeur et al., 1987). These results suggest the inhibitor is destined intracellularly and not secreted. After translocation into ER, the inhibitor may be intracellularly translocated into a cellular organelle, or may remain in the lumen to be dispersed among starch granules and protein bodies when other cellular structures disrupt as endosperm cells lose their cellular integrity. In experiments examining induction of inhibitor synthesis in aleurones and embryos, some of the protein was found in the medium (unpublished observations), suggesting that a secretory pathway may have been taken by the inhibitor in mature aleurones and immature embryos incubated with ABA, but it was not shown whether active secretion or protein leakage took place in these tissues.

Prior to discussing the possible translocation pathway for the inhibitor, it is appropriate to present

information available for other proteins. The signal peptide sequences of proteins destined for different locations are highly diverse. There is only one common denominator, a clustering of hydrophobic amino acids in the central region of the signal peptide (von Heijne, 1985). Since the signal peptides are cleaved off once the proteins are in the lumen (Jackson and Blobel, 1977), cellular transport sorting mechanisms must reside in the processed proteins. In a mammalian system, luminal ER proteins contain a specific C-terminal tetrapeptide signal (KDEL) and these proteins are retained in the ER by retrieval from a post-ER compartment (Munro and Pelham, 1987; Pelham, 1988). For the secretory and lysosomal proteins, bulk movement was suggested after the removal of the signal peptide (Warren, 1987). Intracellular transport signals for lysosomal and vacuolar proteins of yeast have, however, been identified. Lysosomal enzymes are recognized through a phosphomannosyl recognition marker introduced by co-translational glycosylation at asparagine and post-translational modification (Kornfeld, 1987). A sorting signal of yeast vacuolar protease is located at the N-terminal of the mature protein independent of modifications of glycosylation (Johnson et al., 1987; Valls et al., 1987).

In a plant system, protein translocation mechanisms are not as well understood as those of yeast and the mammalian systems, but, analogies between mammalian and plant systems do exist. Translocated plant proteins are secreted or deposited in the vacuole, an organelle that is the equivalent of the lysosomal compartment of the mammalian system. Cereal proteins, such as α -amylase and B1 and gamma-hordeins that are secreted or stored in the protein body, a differentiated form of the vacuole, contain hydrophobic signal peptides (Cameron-Mills and Brandt, 1988; Chandler et al., 1984; Forde et al., 1985). The translation of storage proteins is carried out at the membrane-bound polysomes (Brandt and Ingversen, 1978). The Golgi apparatus is used for α -amylase secretion since the enzyme is found in the Golgi fraction among subcellular fractions of aleurone cells (Heupke and Robinson, 1985). The signal recognition particle (SRP) recognizes a signal peptide and forms a complex, which then attaches to the ER membrane (Walter et al., 1984). Plant SRP has been isolated from wheat germ (Prehn et al., 1987) and its 7S RNA has 35 to 43 percent sequence homology with mammalian 7S RNAs (Marshallsay et al., 1989). Identification of similar components for plant protein translocation suggests similar sorting mechanisms may also operate in the plant system. One indication of

the requirement for sorting information has been identified in a plant system. A finding for a pea storage protein shows the transport is not by a bulk-flow mechanism and requires yet unknown information beyond that provided by the signal peptide (Dorel et al., 1989).

These factors, along with the known characteristics of the inhibitor, show that a further study of the sorting mechanisms, particularly those of plant proteins, is required to postulate the translocation pathway for the inhibitor. If the sorting mechanisms for the animal system are applied to the plant system, the lack of KDEL sequence at the C-terminus (Svendsen et al., 1986) suggests that the inhibitor is not a luminal ER protein. There may be different markers used for each organelle, since there have been at least two independent markers identified for translocation into two different eukaryotic organelles (Johnson et al., 1987; Kornfeld, 1987; Valls et al., 1987). Therefore no speculation can be made for the inhibitor, a plant protein not destined to either of the two organelles studied, based on the sequence information available. Post-translational modification required for the protein sorting mechanism (Johnson et al., 1987; Kornfeld, 1987; Valls et al., 1987), however, appears to be present in the inhibitor based on the estimated size for the mature protein.

Inhibitor Mr was estimated against Mr standards on a SDS-PAGE gel and the isolated inhibitor (Figs. 1 and 9) was larger, 22,000, than the in vitro translated protein, 20,000 (Fig. 7). The possibility of glycosylation may be examined on the purified inhibitor by an enzymatic digestion followed by an analysis on a SDS-PAGE (Genzyme) or by a use of a specific dye for glycoproteins (Munoz et al., 1988).

5.6. Conclusion

This study showed that the α -amylase inhibitor was synthesized under temporal control in barley kernels and embryos during development. Although the protein is regulated by ABA in the embryo, it is not likely to directly affect embryonic dormancy since embryo responsiveness to ABA is not the same for α -amylase inhibitor induction and germination. The inhibitor in the mature kernel is likely to play a secondary role in attenuating germination. Inhibitor induction under dehydration stress was mediated by ABA. Under stress, the protein may act as an inhibitor of a protease rather than of α -amylase, playing a protective role. With two known biochemical functions, the inhibitor is unique among dehydration-induced proteins regulated by ABA. The

inhibitor will be useful in further investigation of the role of dehydration-induced proteins in plant response to stress.

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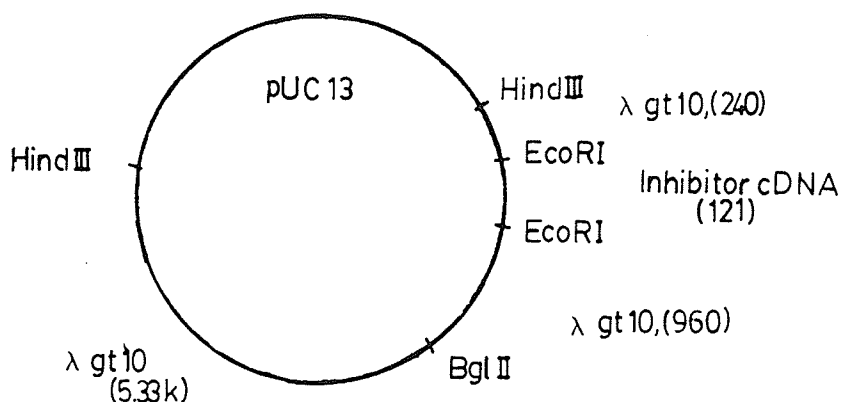
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Appendix 1.Inhibitor cDNA Clone

An inhibitor cDNA clone isolated from a lambda gt10 library was subcloned into a pUC13. The plasmid was double digested with HindIII and BglII to obtain an insert containing the inhibitor cDNA. This insert was nick translated and used in Northern analysis.



Nucleotide and deduced amino acid sequences of inhibitor cDNA

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439
TGG TGC CAG GAC CTC GGC GTG TTC AGG GAC CTC AAG GGT GGG
Trp Cys Gln Asp Leu Gly Val Phe Arg Asp Leu Lys Gly Gly

481
GCG TGG TTC TTG GGC GCC ACC GAG CCA TAC CAT GTC GTC GTG
Ala Trp Phe Leu Gly Ala Thr Glu Pro Tyr His Val Val Val

523
TTC AAG AAG GCG CCG CCC GCT TAA GGT CCA ATG ATC C
Phe Lys Lys Ala Pro Pro Ala END

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Appendix 2.Western Blots for the Inhibitor and α -Amylase

Western blot analysis of buffer-extractable proteins for the α -amylase inhibitor (A, B, D, E, F, G, H, I, J and K) and α -amylase (C).

A: Purified α -amylase inhibitor. Numbers indicate inhibitor quantity in ng.

B and C: Tris buffer-extractable proteins from barley kernels during development. 5 μ g was loaded per lane for the α -amylase inhibitor (B) and 100 μ g for α -amylase (C), except 50 μ g was applied to lane 7'. The numbers indicate DPA. The α -amylase blot also contains purified enzyme.

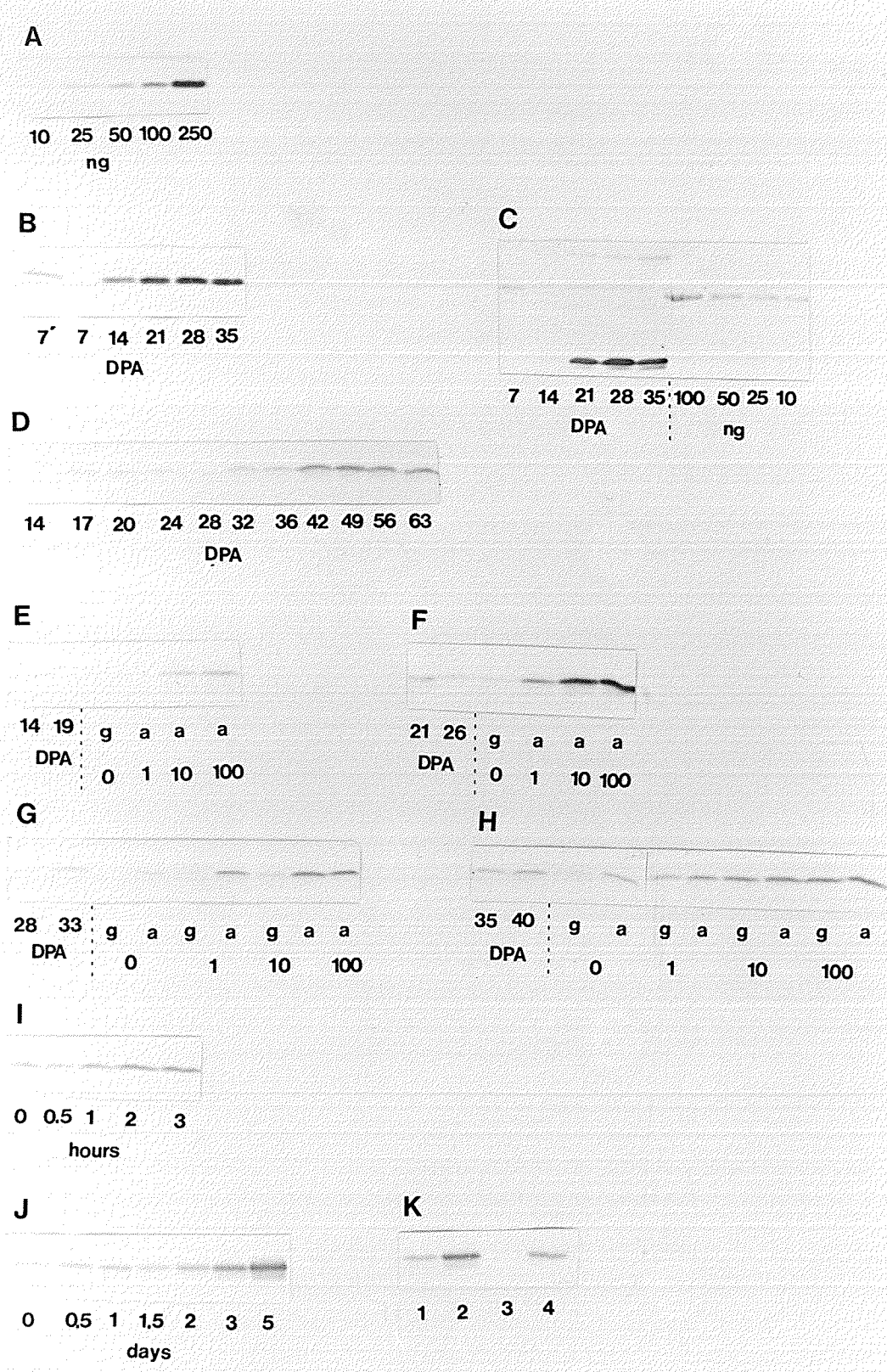
D: PBS buffer-extractable proteins from barley embryos during development. 100 μ g protein was loaded per lane. Numbers indicate DPA.

E, F, G and H: PBS buffer-extractable proteins from ABA treated embryos excised at 14 DPA (E), 21 DPA (F), 28 DPA (G) and 35 DPA (H). Numbers above DPA indicate age of in vivo embryos assayed directly without further treatment. Numbers, 0, 1, 10 and 100, indicate ABA treatment concentration in μ M. 100 μ g protein was loaded per lane. "g" refers proteins from germinated embryos and "a" from arrested embryos.

I: PBS buffer-extractable proteins from isolated immature embryos under dehydration stress. Numbers indicate duration of the stress applied in hours. 100 μ g protein was loaded per lane.

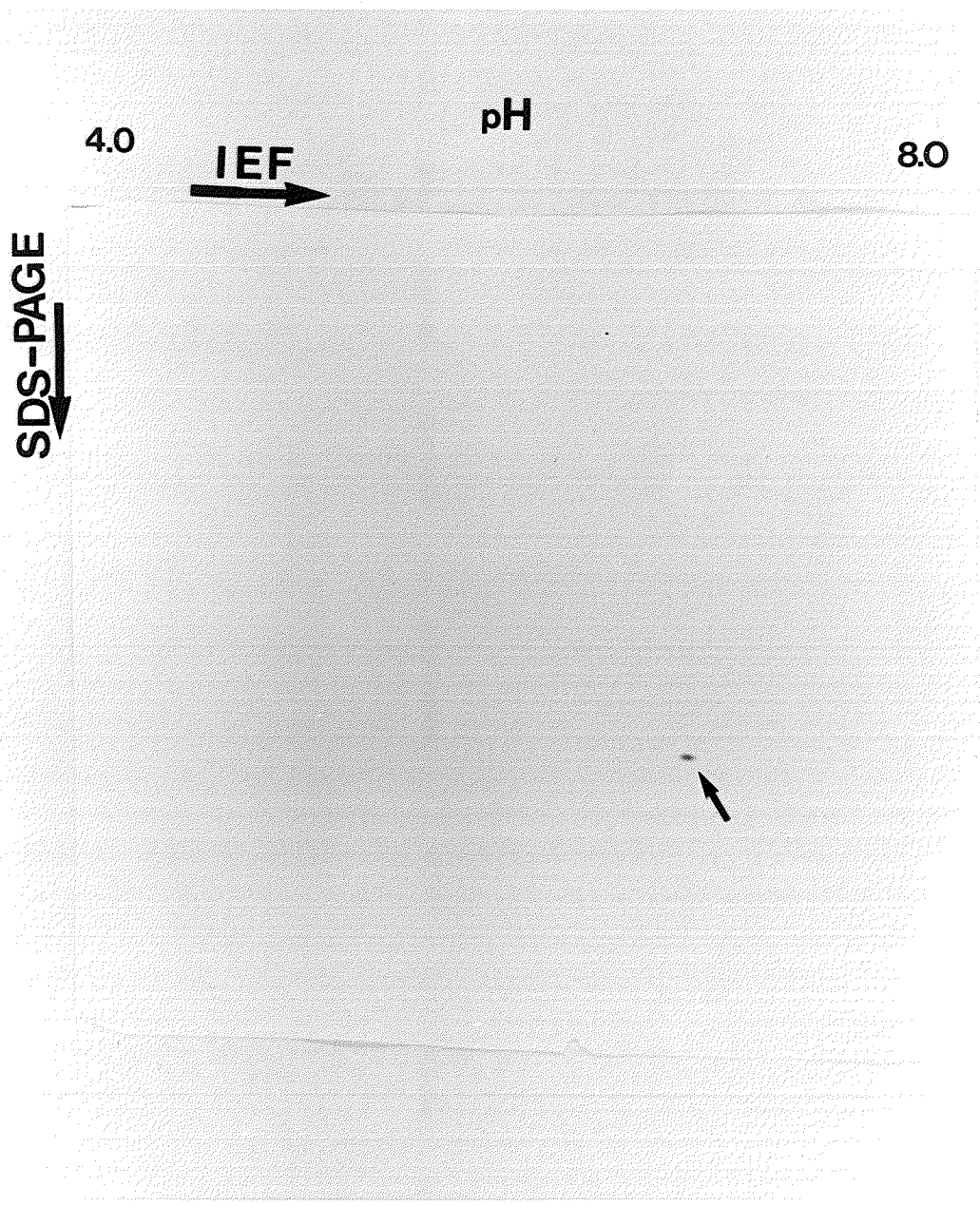
J: Tris buffer-extractable proteins from SCS under dehydration stress. Numbers indicate duration of the stress applied in days. 50 μ g protein was loaded per lane.

K: Tris buffer-extractable proteins from SCS after rehydration. Seedlings were dehydrated for 5 days (Lane 2), then rehydrated for 2 days (lane 4). Unstressed seedlings at 2 days (lane 1) and 7 days (Lane 3). 50 μ g protein was loaded per lane.



Appendix 3.Western Blot of 2-Dimensional Gel Electrophoresis

Tris buffer-extractable proteins from 14 DPA barley kernel was separated on 2-D gel and analyzed for the α -amylase inhibitor.



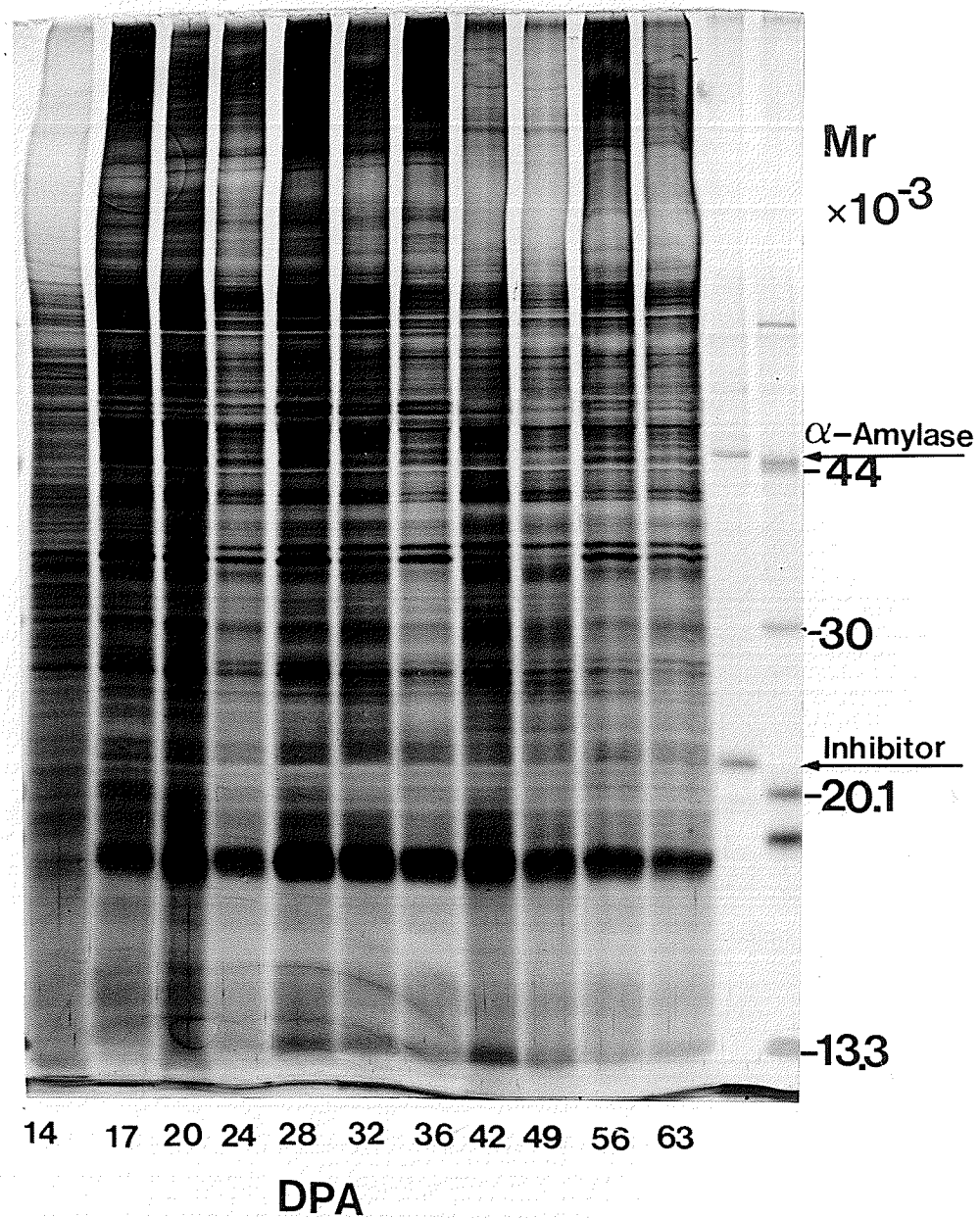
Appendix 4.

Inhibitor Abundance and Buffer-Extractable Proteins of
Embryos During Development

Embryo sample	Moisture	Buffer-extractable Proteins	Inhibitor abundance
(DPA)	(%)	($\mu\text{g}/\text{embryo}$)	($\text{pg}/\mu\text{g}$ protein)
14	80.9	42	69
17	72.4	184	200
20	68.9	170	215
24	64.9	392	149
28	62.7	342	149
32	57.3	542	642
36	53.2	552	625
42	34.7	542	817
49	20.9	467	1278
56	20.4	493	1101
63	20.4	580	974

Appendix 5.PBS Buffer-extractable Proteins ofEmbryos During development

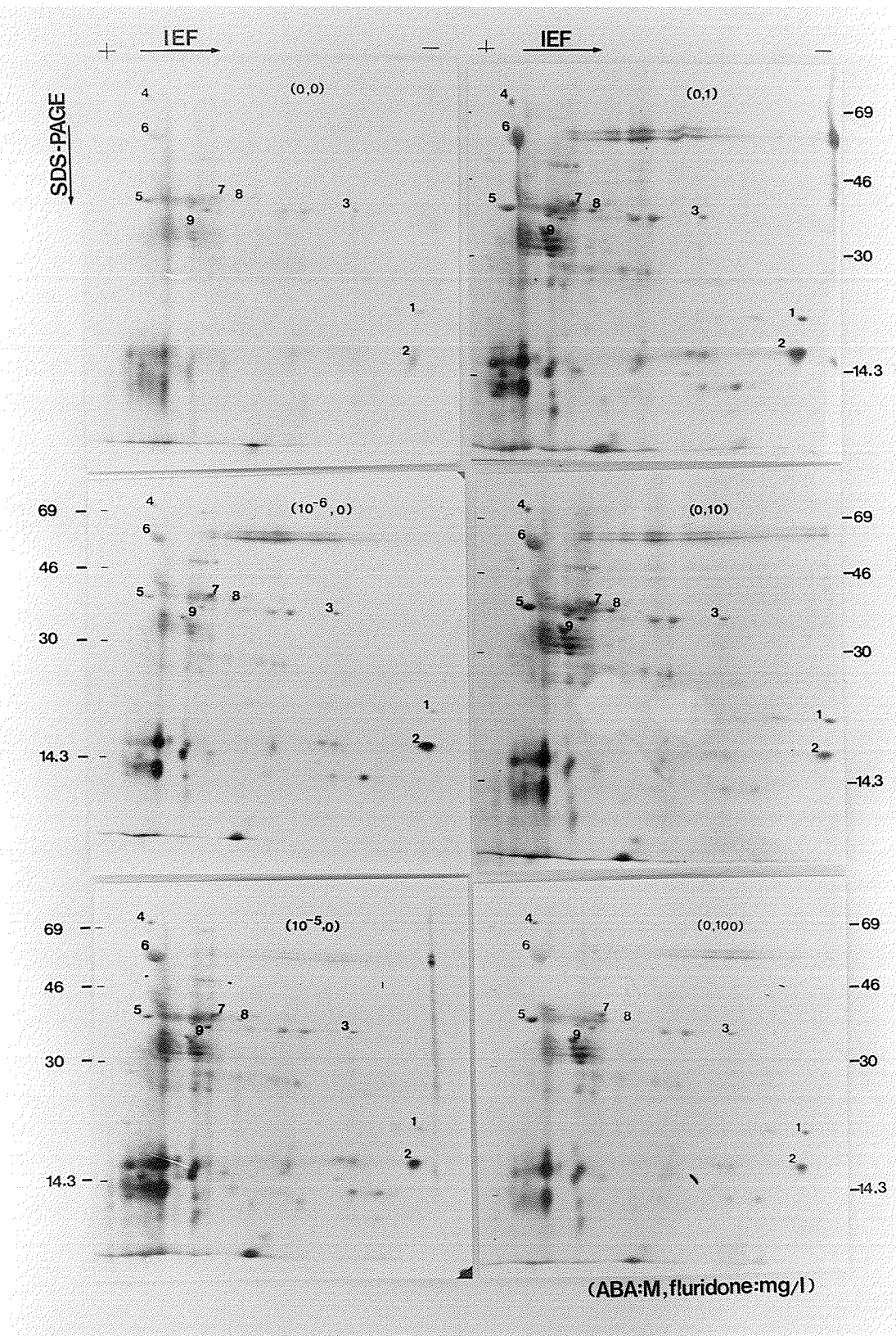
Electrophoreogram of PBS buffer-extractable proteins from barley embryos during development. 10 μ g of embryo extracts was applied per lane. Numbers refer to embryo age in DPA.



Appendix 6.Effect of ABA and Fluridone on Seed Proteins

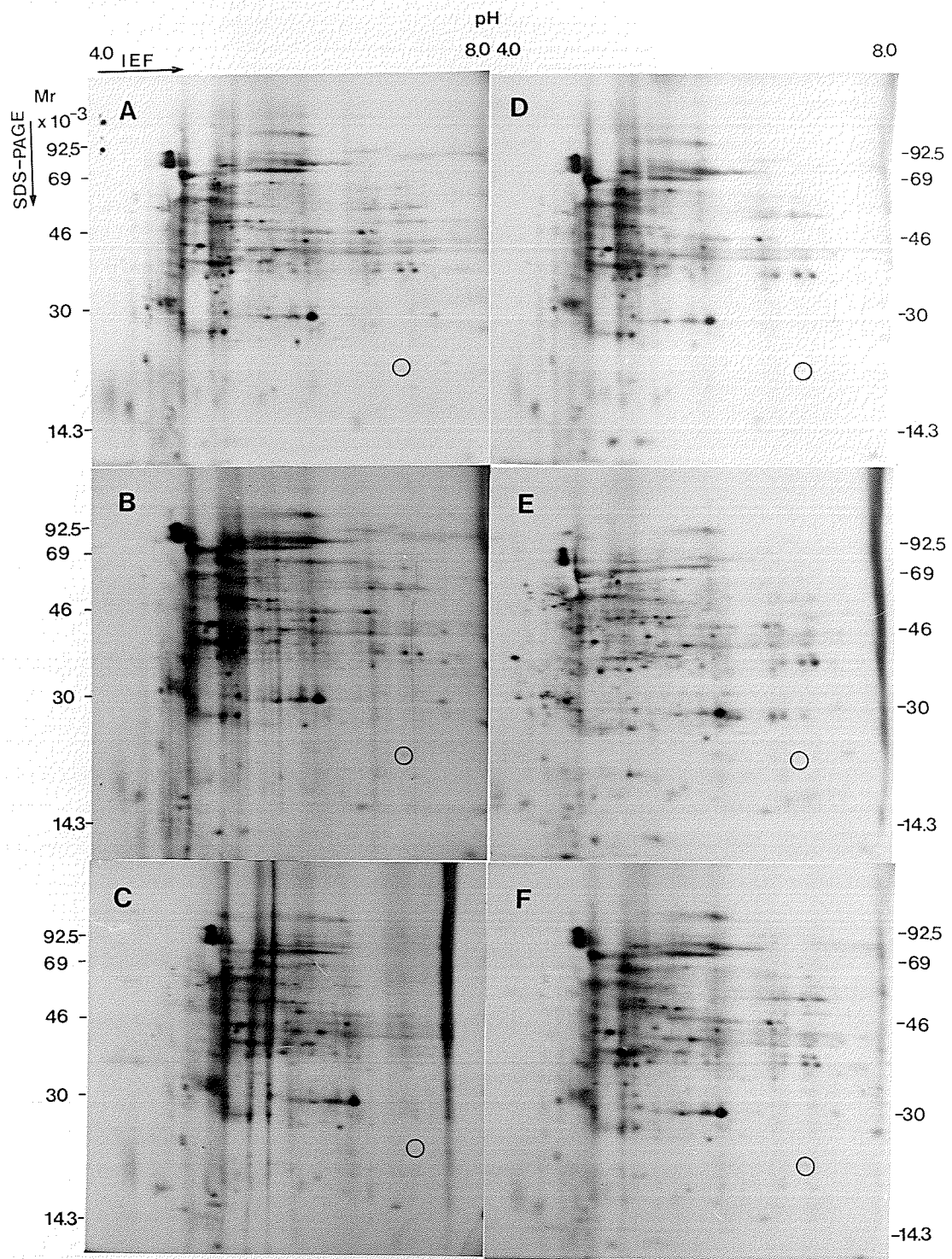
Appendix. 6.1. Fluorogram of Tris buffer-extractable endosperm proteins from cultured spikes treated with ABA and fluridone. Protein sample containing 100,000 dpm TCA-precipitable activity was separated per gel. Numbers in brackets refer to treatments applied for ABA in M and fluridone in mg/l. Some of the proteins were marked with numbers for comparison.

- 1: the inhibitor
- 2: a location reference protein.
- 3 and 4: medium abundance reference proteins.
- 5: a reference protein similar to the inhibitor in abundance.
- 6: very abundant protein among Tris buffer-extractable proteins from 14 DPA kernel.



(ABA:M, fluridone:mg/l)

Appendix. 6.2. Fluorogram of Tris buffer-extractable endosperm proteins from cultured kernels treated with ABA and fluridone. Protein sample containing 300,000 dpm TCA-precipitable activity was separated per gel. Panel A, control; panel B, 10 mg/l fluridone; panel C, 100 mg/l fluridone; panel D, 1 μ M ABA; panel E, 10 μ M ABA; panel F, 100 μ M ABA. Location of the inhibitor is indicated by a circle.



Appendix 6.Effect of ABA on Isolated Embryos from Developing Kernels

Embryo sample	ABA (μ M)	Fresh weight		Protein		Inhibitor	
		Germ. ³	Arr. ⁴	Germ.	Arr.	Germ.	Arr.
		(mg/embryo)	(μ g/embryo)	(μ g/embryo)	(ng/ μ g protein)		
<u>14 days post anthesis</u>							
kernel ¹ (14 DPA)	-	0.33	-	35	-	0.15	
kernel ² (19 DPA)	-	1.6	-	190	-	0.25	
cultured	0	3.4	-	121	-	0.15	-
cultured	1	-	1.4	-	75	-	0.55
cultured	10	-	1.2	-	93	-	0.80
cultured	100	-	0.7	-	55	-	0.85
<u>21 days post anthesis</u>							
kernel (21 DPA)	-	1.	-	181	-	0.20	
kernel (26 DPA)	-	2.5	-	346	-	0.20	
cultured	0	10.8	4.5	347	391	0.25	0.20
cultured	1	5.6	4.3	378	354	0.30	0.85
cultured	10	-	4.2	-	323	-	2.25
cultured	100	-	3.6	-	319	-	2.60
<u>28 days post anthesis</u>							
kernel (28 DPA)	-	3.1	-	521	-	0.20	
kernel (33 DPA)	-	3.8	-	507	-	0.35	
cultured	0	21.9	5.7	480	535	0.05	0.20
cultured	1	18.3	5.2	564	513	0.05	0.70
cultured	10	12.7	6.0	538	535	0.20	0.80
cultured	100	-	5.7	-	534	-	0.80
<u>35 days post anthesis</u>							
kernel (35 DPA)	-	4.0	-	750	-	0.30	
kernel (40 DPA)	-	3.4	-	751	-	0.50	
cultured	0	22.8	6.4	563	788	0.20	0.50
cultured	1	15.5	6.8	885	744	0.30	0.70
cultured	10	14.1	6.2	776	667	1.00	1.50
cultured	100	-	6.4	-	664	-	2.00

¹ Embryos Excised from the kernel on the DPA indicated in brackets and analyzed directly

² Embryos excised from the kernel on the DPA indicated in heading and analyzed 5 days after designated treatment

³ Germinated embryos

⁴ Germination arrested embryos