This is to certify that the

dissertation entitled

PROTEINASES IN SOFT WINTER WHEAT DURING
MATURATION, PREHARVEST SPROUTING AND GERMINATION

presented by

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has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Food Science
and human Nutrition

Major professor

Date Sept. 9, 1994

PROTEINASES IN SOFT WINTER WHEAT DURING MATURATION, PREHARVEST SPROUTING AND GERMINATION

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Food Science and Human Nutrition

1994

ABSTRACT

PROTEINASES IN SOFT WINTER WHEAT DURING MATURATION, PREHARVEST SPROUTING AND GERMINATION

By

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Along with alpha-amylase activity, proteinase activity has been linked with the detrimental effects of preharvest sprouting (PHS) on wheat product quality. However, many questions remain regarding these enzymes. Those addressed in this study are: is proteinases activity affected by environment or PHS; is proteinase activity correlated with alpha-amylase activity or rate of germination; what classes of proteinase are present in maturing and germinating soft winter wheat (SWW); and how do proteinases affect storage proteins during germination. Five red and four white SWW were grown in a greenhouse (GHG) and in Dansville, Michigan, in 1992. Samples were harvested at 21, 28, 35 and 42 days post anthesis. Additionally, kernels of GHG Augusta and Hillsdale were germinated for five days. Throughout kernel maturation, proteinase activity was significantly higher in the field-grown grain (FGG) compared to the GHG. At harvest maturity, proteinase and alpha-amylase activities were positively

correlated ($r = 0.669$, $p < 0.05$) in the FGG. Also, germination index was correlated with proteinase ($r = 0.553$, $p < 0.05$) and alpha-amylase activities ($r = 0.622$, $p < 0.05$). Crude extracts from GHG Augusta contained cysteine and aspartic proteinase activity during kernel maturation. As a percentage of total proteinase activity, cysteine proteolytic activity decreased as kernels matured, while the aspartic activity increased. During germination, cysteine proteinase activity increased, while aspartic proteinase activity decreased until the second day of germination. These trends then reversed. After one day of germination, serine and metallo-proteinase activity began to increase and peaked after four days. Ten bands of proteinase activity were visualized using native polyacrylamide gel electrophoresis with copolymerized gelatin and crude extracts from quiescent and germinated Augusta kernels. Two bands were present in quiescent grain at near neutral pH, suggesting that they contain serine or metallo-proteinase. The other eight bands had acidic pH optima and appeared after germination had commenced, indicating that they contain aspartic or cysteine proteinase. Glutenin subunits in germinated kernels appeared to be hydrolyzed by proteinases earlier than the gliadin proteins.

DEDICATION

I can do all things through Christ who strengthens me **(Phil. 4:13)**

I dedicate the completing of this dissertation to my Mom, Dad, my sister Lori, brother Jeff, Grandmas, Beulah and Olga, and Grandpa Oliver, whom I love dearly.

ACKNOWLEDGMENTS

"Life is like a box of chocolates. You never know what you're gonna get."

(Forest Gump's Mother)

The following people played various roles in helping me complete this dissertation: Daisy Gualberto, Lorraine Bergman, Karen Kane, Janice Harte, Bill Haines, Nirmal Sinha, Lee Bird, Pedro Maligo, Debi Klein, Jenny Bond, Patrick Finney, Arti Arora, Rick Bernsten, Eileen Van Tassel, Kitty O'Neil, Kati Loeffler, Luis Rayas, Scott Worthington, Sandy Daubermier, Dave Glenn, Kay Kresl, Cynthia Merriman and Jessalin Faulkner. A few of those mentioned above provided me technical help, but all offered thought provoking discussions and words of encouragement. I extend heartfelt thanks to all of them.

The financial assistance provided, in the form of fellowships and scholarships, by the Institute of Food Technologists, the American Association of Cereal Chemists, Michigan State University's Graduate School and the U.S. Government's Title IV Foreign Language Program is acknowledged.

Thanks also go to the following professors for serving on my committee and reviewing this dissertation: Jerry Cash, Perry Ng, Rick Ward and Mary Ellen Zabik. My gratitude is especially offered to Rick Ward for providing the wheat samples for this project, plus the field and greenhouse space.

TABLE OF CONTENTS

LIST OF TABLES xii

LIST OF FIGURES xiii

LIST OF ABBREVIATIONS xvi

CHAPTER ONE: INTRODUCTION 1

CHAPTER TWO: LITERATURE REVIEW 4

INTRODUCTION 4

PREHARVEST SPROUTING OF WHEAT 4

- *Geographic Occurrence and Impact* 4
- *Effect of Preharvest Sprouting on Cereal Product Quality* 5
  - Hard Wheat Products 5
  - Soft Wheat Products 9
- *Dormancy* 12
  - Physical Factors 12
  - Chemical Inhibitors and Promoters 14
  - Environmental Factors 16
- *Preharvest Sprouting Methodology* 16
  - Quantifying and Studying Preharvest Sprouting 16

Characterizing Resistance to Preharvest Sprouting....25

PHYSIOLOGY OF GERMINATION....29

Grain Maturation....29

Water Imbibition....30

BIOCHEMISTRY OF GERMINATION....31

Protein-Degrading Enzymes....31

Proteinase Biochemistry....32

Aspartic Proteinases....32

Cysteine Proteinases....34

Metallo-Proteinases....35

Serine Proteinases....36

Proteinases in Wheat....37

Proteinases Effect on Wheat Proteins....39

Proteinases Effect on Wheat Product Quality....41

Other Enzymes....42

REFERENCES....43

CHAPTER THREE: PROTEINASE ACTIVITY IN SOFT WINTER WHEAT DURING MATURATION AND PREHARVEST SPROUTING....55

ABSTRACT....55

INTRODUCTION....56

MATERIALS AND METHODS....58

Materials....58

Wheat Samples 58

Chemicals 58

Methods *58*

Wheat Growing Conditions and Harvest 58

Field-Grown Wheat 58

Greenhouse-Grown Wheat 59

Sample Preparation 59

Physical Methods 61

Moisture 61

Germination Index 61

Chemical Methods 62

Protein Content 62

Alpha-Amylase Activity 62

Proteinase Activity 62

Proteinase Inhibitors 63

Statistical Analysis 64

RESULTS 66

Inhibitor Studies *66*

Enzyme Activity During Maturation *66*

Field Environmental Conditions *77*

Field-Weathered Grain *77*

Greenhouse-Grown Grain *80*

DISCUSSION..80

REFERENCES 88

CHAPTER FOUR: PROTEINASES IN SOFT WINTER WHEAT DURING GERMINATION AND THEIR EFFECT ON STORAGE PROTEINS...................91

ABSTRACT..91

INTRODUCTION 92

MATERIAL AND METHODS..93

Materials..*93*

Wheat Samples 93

Chemicals..94

Methods *94*

Wheat Growing Conditions and Harvest 94

Sample Preparation..94

Controlled Germination 94

Milling..95

Germination Index 95

Proteinase Activity...95

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Glutenins 96

Acid Polyacrylamide Gel Electrophoresis (PAGE) of Gliadins 96

Proteinase Characterization...96

Enzyme Extraction 96

Native PAGE with Copolymerized Substrate 96

Glutenin Preparation 97

Proteinase Inhibitor Studies 97

Statistical Analysis 97

RESULTS 98

Germination Index *98*

Proteinase Activity *98*

Visualization of Proteinase Activity *98*

Inhibitor Studies *103*

Proteinase Effect on Storage Proteins *111*

DISCUSSION 116

REFERENCES 127

CHAPTER FIVE: CONCLUSIONS 130

CHAPTER SIX: FUTURE WORK 133

APPENDIX A 135

APPENDIX B 136

APPENDIX C 147

LIST OF TABLES

TABLE I. TEMPERATURE AND PRECIPITATION IN THE VICINITY OF DANSVILLE, MICHIGAN, DURING THE SUMMER OF 1992 60

TABLE II. CONDITIONS FOR PROTEINASE INHIBITOR STUDIES 65

TABLE III. CORRELATION ANALYSIS OF MATURING SOFT WINTER WHEAT ENZYME ACTIVITIES WITH DPA AND PROTEIN CONTENT 78

TABLE IV. CORRELATION ANALYSIS OF ENZYME ACTIVITIES WITH GERMINATION INDEX AND PROTEIN CONTENT OF MATURE SOFT WINTER WHEAT GROWN IN DANSVILLE, MICHIGAN, IN 1992 AND HARVESTED AT 42 DPA 79

TABLE V. ALPHA-AMYLASE AND PROTEINASE ACTIVITY IN SOFT WINTER WHEAT CULTIVARS GROWN IN DANSVILLE, MICHIGAN, IN 1992 AND HARVESTED AT 42 DPA 81

LIST OF FIGURES

FIGURE. 1. GRAPH OF RELATIVE PROTEINASE ACTIVITY OF THE CULTIVAR AUGUSTA GROWN IN A GREENHOUSE AND HARVESTED AT 21, 28, 35, AND 42 DAYS POST ANTHESIS, WITH AND WITHOUT PEPSTATIN A. 67

FIGURE. 2. GRAPH OF RELATIVE PROTEINASE ACTIVITY OF THE CULTIVAR AUGUSTA GROWN IN A GREENHOUSE AND HARVESTED AT 21, 28, 35, AND 42 DAYS POST ANTHESIS, WITH AND WITHOUT IODOACETIC ACID.. 68

FIGURE. 3. GRAPH OF RELATIVE PROTEINASE ACTIVITY OF THE CULTIVAR AUGUSTA GROWN IN GREENHOUSE AND HARVESTED AT 21, 28, 35, AND 42 DAYS POST ANTHESIS, WITH AND WITHOUT N-ETHYLMALEIMIDE.. 69

FIGURE. 4. GRAPH OF RELATIVE PROTEINASE ACTIVITY OF THE CULTIVAR AUGUSTA GROWN IN A GREENHOUSE AND HARVESTED AT 21, 28, 35, AND 42 DAYS POST ANTHESIS, WITH AND WITHOUT P-CHLOROMERCURIPHENYL SULFONIC ACID 70

FIGURE. 5. GRAPH OF RELATIVE PROTEINASE ACTIVITY OF THE CULTIVAR AUGUSTA GROWN IN A GREENHOUSE AND HARVESTED AT 21, 28, 35, AND 42 DAYS POST ANTHESIS, WITH AND WITHOUT 3-CARBOXYL-2, 3-L-*TRANS*-EPOXYPROPYL-LEUCYLAMIDO (4-GUANIDINO) BUTANE (E-64).. 71

FIGURE. 6. GRAPH OF RELATIVE PROTEINASE ACTIVITY OF THE CULTIVAR AUGUSTA GROWN IN A GREENHOUSE AND HARVESTED AT 21, 28, 35, AND 42 DAYS POST ANTHESIS, WITH AND WITHOUT 1, 10 PHENANTHROLINE.................................72

FIGURE. 7. GRAPH OF RELATIVE PROTEINASE ACTIVITY OF THE CULTIVAR AUGUSTA GROWN IN A GREENHOUSE AND HARVESTED AT 21, 28, 35, AND 42 DAYS POST ANTHESIS, WITH AND WITHOUT 3,4 DICHLOROISOCOUMARIN............................73

FIGURE. 8. GRAPH OF RELATIVE PROTEINASE ACTIVITY IN SOFT WINTER WHEAT GROWN IN A GREENHOUSE AND IN DANSVILLE, MICHIGAN, IN 1992.. 75

FIGURE. 9. GRAPH OF ALPHA-AMYLASE ACTIVITY IN SOFT WINTER WHEAT GROWN IN A GREENHOUSE AND IN DANSVILLE, MICHIGAN, IN 1992.......................................76

FIGURE. 10. GRAPH OF THE RELATIVE PROTEINASE ACTIVITY OF THE CULTIVARS AUGUSTA AND HILLSDALE GROWN IN A GREENHOUSE AND EXPOSED TO CONDITIONS CONDUSIVE TO GERMINATION IN A LABORATORY.. 99

FIGURE. 11. PAGE WITH COPOLYMERIZED GELATIN OF SOFT WHEAT CRUDE EXTRACTS PREPARED FROM GRAIN AT DIFFERENT GERMINATION STAGES.. 100

FIGURE 12. GRAPH OF RELATIVE PROTEINASE ACTIVITY OF THE CULTIVAR AUGUSTA, GROWN IN A GREENHOUSE, WITH AND WITHOUT PEPSTATIN A.........................104

FIGURE. 13. LINE GRAPH OF RELATIVE PROTEINASE ACTIVITY OF THE CULTIVAR AUGUSTA, GROWN IN A GREENHOUSE, WITH AND WITHOUT IODOACETIC ACID...105

FIGURE. 14. GRAPH OF RELATIVE PROTEINASE ACTIVITY OF THE CULTIVAR AUGUSTA, GROWN IN A GREENHOUSE, WITH AND WITHOUT 3-CARBOXYL-2, 3-L-*TRANS*-EPOXY PROPYL-LEUCYLAMIDO (4-GUANIDINO) BUTANE (E-64)..................................106

FIGURE. 15. GRAPH OF RELATIVE PROTEINASE ACTIVITY OF THE CULTIVAR AUGUSTA, GROWN IN A GREENHOUSE, WITH AND WITHOUT N-ETHYLMALEIMIDE.. 107

FIGURE. 16. GRAPH OF RELATIVE PROTEINASE ACTIVITY OF THE CULTIVAR AUGUSTA, GROWN IN A GREENHOUSE, WITH AND WITHOUT P-CHLOROMERCURIPHENYL SULFONIC ACID 108

FIGURE. 17. GRAPH OF RELATIVE PROTEINASE ACTIVITY OF THE CULTIVAR AUGUSTA, GROWN IN A GREENHOUSE, WITH AND WITHOUT 1, 10 PHENANTHROLINE.. 109

FIGURE. 18. GRAPH OF RELATIVE PROTEINASE ACTIVITY OF THE CULTIVAR AUGUSTA, GROWN IN A GREENHOUSE, WITH AND WITHOUT 3,4 DICHLOROISOCOUMARIN .. 110

FIGURE. 19. SDS-PAGE OF REDUCED HMW-GLUTENINS FROM THE CULTIVARS AUGUSTA AND HILLSDALE AT VARIOUS STAGES OF GERMINATION.. 112

FIGURE. 20. SDS-PAGE OF UNREDUCED HMW-GLUTENINS FROM THE CULTIVARS AUGUSTA AND HILLSDALE AT VARIOUS STAGES OF GERMINATION.. 114

FIGURE. 21. ACID-PAGE OF GLIADIN PROTEINS FROM THE CULTIVARS AUGUSTA AND HILLSDALE AT VARIOUS STAGES OF GERMINATION.. 117

LIST OF ABBREVIATIONS

ABA	abscisic acid
A-CL	Augusta control
A-1 D, A-2 D......... A-5 D	Augusta kernels germinated for one day, two days and subsequent days up to five
CMPS	p-chloromercuriphenyl sulfonic acid
DAN	diazoacetyl amino acid esters in the presence of cupric ion
3, 4 DIC	3, 4 dichloroisocoumarin
DMSO	dimethyl sulfoxide
DFP	diisopropyl fluorophosphate
DPA	days post anthesis
DTT	dithiothreitol
E-64	3-carboxyl-2, 3-L-*trans*-epoxypropyl-leucylamido (4-guanidino) butane
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay

FDB	fluorescein dibutyrate
FGG	field-grown grain
GA	gibberellic acid
GHG	greenhouse-grown grain
H-Cl	Hillsdale control
H-1 D, H-2D........ H-5 D	Hillsdale kernels germinated for one day, two days and subsequent days up to five
HM	harvest maturity
HMW	high molecular weight
kDa	kilodalton
LMHAA	late maturity high alpha-amylase
2-ME	2-mercaptoethanol
MUH	4-methylumbelliferyl
MW	molecular weight
PAGE	polyacrylamide gel electrophoresis
1, 10 PA	1, 10 phenanthroline
PHS	preharvest sprouting
PM	physiological maturity
PMSF	phenylmethane sulfonylfluoride
RH	relative humidity

SDS-PAGE	sodium dodecyl sulfate-PAGE
TCA	trichloroacetic acid
TEMED	N,N,N',N'-Tetramethylethylenediamine

CHAPTER ONE

INTRODUCTION

Preharvest sprouting (PHS) damage, which occurs when cereal grains with limited dormancy are exposed to precipitation just prior to harvest, is of concern world wide due to the economic losses which result from decreased yield, loss of seed viability, and poor flour quality (Tipples et al 1966).

Preharvest sprouting consists of root and shoot growth, as well as starch and protein modification, from a cascade of events involving hormones and enzymes. These changes are thought to be regulated at the molecular level, as increased mRNA synthesis occurs within 24 h of wheat kernel imbibition (Weidner and Kulka 1980). Concurrently, gibberellic acid travels from the embryo to the aleurone layer triggering reserve mobilization through several enzyme responses: increased *de novo* synthesis, secretion, and activation of preexisting forms. Increased activity has been reported for carbohydrate, protein, and lipid hydrolytic enzymes during sprouting, with the most concentrated research efforts being on delineating the role of alpha-amylase (Ashford and Gubler 1984).

Proteases in wheat have been studied since the early part of this century (Balls and Hale 1936). However, much about these enzymes still eludes us. Such as: what classes of proteinases are present during the various stages of a kernel's life cycle; where are they located in the kernel; what are their substrate specificities; what are their temperature and pH optima; and where are the genes located which code for them?

Proteinase activity has been reported to increase approximately 20-fold after three to five days of germination. These enzymes are known to have the ability to hydrolyze gliadins and glutenins, and thus, in addition to alpha-amylase conceivably may play a role in the effects of PHS on product quality (Preston 1978, Okamota et al 1981, Kruger 1990).

The study of proteinases, specifically in soft wheat, is of interest, in part, because of the potential role they may play in the end-product quality of sprouted grain. Although increased alpha-amylase activity in sprouted grain has been thought to be of greater consequence than other enzymes present, a few studies have linked proteinase activity with a change in product quality (Lorenz et al 1983, Edwards et al 1989). Also, greater protease activity compared to alpha-amylase activity, in rain-damaged grain, has been reported to be carried over from grain to flour after milling (Edwards et al 1989).

The focus of this study was to investigate several questions related to the hypothesis that proteinase activity, in sprouted soft wheat, results in biochemical changes which modify end-product quality. These questions included: (1) what classes of proteinase are present in maturing soft winter wheat; (2) is proteinase activity during kernel maturation affected by environment; (3) is proteinase activity at harvest maturity correlated with alpha-amylase activity, or degree of sprouting; and (4) does proteinase activity increase when grain sprouts prior to harvest? In addition, this project sought to gain a "big-picture" perspective of the proteinases in quiescent and germinating soft wheat, and to characterize their effects on soft wheat storage proteins.

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CHAPTER TWO

LITERATURE REVIEW

INTRODUCTION

Preharvest sprouting (PHS) is a phenomenum where physiologically ripe cereal grains begin the germination process prior to being harvested. This situation tends to occur when genotypes with limited dormancy are exposed to steady rains followed by cool temperature, as opposed to short rains followed by hot dry periods. Preharvest sprouting consists of increased hydrolytic enzyme activity and, as a result, grain which is less suitable for processing into food products. Although PHS is defined as the germination of grain still in the plant ear, for ease of experimentation and control of the environment, laboratory-germinated material is often used to study the effects of PHS.

Preharvest Sprouting of Wheat

Geographic Occurrence and Impact

Sprouting damage is of concern world wide due to the economic losses which result from decreased yield, loss of seed viability, and poor flour quality (Tipples et al 1966). Areas of the world particularly susceptible to preharvest sprouting are Argentina, Brazil, Chile, Eastern New Zealand, England, Northern Europe, Kenya, South Africa, Zimbabwe, and the Canadian regions of Saskatchewan and Manitoba. For example, in 1987 England realized a $114 Million loss due to winter wheat sprouting (Derera 1990). In the United States, white wheat growing areas, including the Pacific Northwest (Finney et al 1980), New York (Briggle 1980), and Kansas (McCrate et al 1981), are affected most often. Michigan, primarily a soft white wheat producer, has also experienced PHS, with an estimated 50-60% of its acreage containing sprouted grain in 1980 (Copeland et al 1980).

Effect of Preharvest Sprouting on Cereal Product Quality

Hard Wheat Products

Leavened bread, hard wheat's primary product, is consumed world wide. For example, bread consumption in Hungry, France, United States, Brazil, and Japan is 120, 67, 37, 19 and 10 kg/person/year, respectively (Orth and Shellenberger 1988). With the protein and kilocalorie needs of so many dependent on this one product plus the potential for economic losses, the effect of PHS on hard wheat has received considerable attention.

Millers have concerns about sprouted grain not only because of its effect on product quality, but also due to its potential for changes in milling quality. Soaking and to a lesser extent sprouting have been reported to result in hard wheat acquiring some of the milling traits of soft wheat (Lukow and Bushuk 1984a). That is, break-stage yields increased to the same extent that reduction flours decreased with no change in overall yield. Depending on the intended use of the flour, millers may find this a benefit or a drawback.

For the baker, the first manifestation of sprouting is generally identified by dough that is sticky and hard to handle (D'Appolonia 1983). However, with a method that uses a short fermentation time, such as the Chorleywood process, signs of sprouting may not be seen until the product reaches the slicing machine

(Chamberlain et al 1983). Loaves made from sprout-damaged wheat can become deformed as they travel through a slicer. The slices become uneven, stick together, and sticky dough builds up on the blades. In time, this build-up of gummy material results in a loaf being ripped apart and slicing is brought to a halt. With several hours of production backed up at this point, the economic losses can be substantial (Meredith and Pomeranz 1985).

Examination of the effect of sprouting on bread quality began with work by Kozmin (1933), when the author reported compromised dough handling, crumb color, grain, and texture. Later studies tend to agree that sprouting results in a sticky dough, very dark crust, open grain, either a gray or yellowish crumb color, and in extreme cases, collapsed loaves (Ibrahim and D'Appolonia 1979, Lorenz et al 1983, Ariyama and Khan 1990).

The effect of sprouting on loaf volume is complex. Ranhotra et al (1977) reported that at levels less than five percent, sprouting results in larger loaf volumes; at higher levels, loaf volumes were said to decrease. Work by Lorenz et al (1983) determined that bread volume increased at all levels of sprouting. Ariyama and Khan (1990) found greater loaf volumes for laboratory sprouted hard wheat. However, grain sprouted for 12 h produced loaves with larger volumes than that sprouted for 40 h It has been suggested that sprouting displays a concomitant rise in loaf volume, because the sugars hydrolyzed by alpha-amylase provide superior levels of fermentable sugars and thus more CO_2 is produced (Lorenz et al 1983).

Suggestions have been made as to the amount of sprouting that can be tolerated by a bakery (Ranhotra et al 1977). However, a study of commercial bakeries in England concluded that tolerable levels of alpha-amylase activity, using the Chorleywood process, varied according to the type of product being produced and the equipment used. For example, one bakery's slicing operation was successful until 20 Farrand units was reached while another began to have problems at 10 Farrand units (Buchanan and Nicholas 1980).

Determining tolerable levels of sprouting is thus a rather arduous task considering the many variables possibly involved in the effect of sprouting on product quality: variety of wheat, type of product, formulation, processing time, and equipment used. This issue is further complicated when it is realized that the degree of sprouting is generally reported based on alpha-amylase activity. If a high percentage of a lot of grain were exposed to water prior to harvesting, other germinative enzymes which may also affect product quality may or may not be present in significant amounts. For example, proteolytic enzymes have been reported to be involved in the effect of germination on leavened bread quality (Kruger 1990). Thus, traditional methods of reporting PHS may not be able to adequately predict product quality.

As it is currently accepted that PHS is a detriment to leavened bread quality, more recent work has focused on establishing the physicochemical changes which result in the modified functional properties of wheat components. The hope is that this type of work will lead not only to a better understanding of sprouting, but also may determine specific applications where germinated grain can be used without loss of quality.

Dough stickiness has, by tradition, been attributed to starch modification during sprouting. Evidence for this was not available until Lorenz et al (1983) individually recombined starch and gluten from flour milled from sprout-damaged grain with a control flour's starch or gluten. Stickiness occurred when starch from sprouted grain was recombined with sound gluten. In contrast, dough produced from sprout-damaged gluten with a control starch demonstrated no stickiness.

Lorenz and Kulp (1981) demonstrated that laboratory-sprouted hard wheat starch exhibits reduced swelling power, is more soluble, gelatinizes at a lower temperature over a narrower temperature range, and has a lower falling number and amylograph viscosities.

Four varieties of hard wheat, which contained from 2 to 80% field-sprouted kernels, did not display swelling power to be a function of degree of sprouting (Kulp et al 1983). Nor was PHS found to affect starch solubility of samples that had undergone an enzyme inactivation step after extraction. However, when enzyme inactivation was not performed, considerable increases in solubility accompanied samples with greater levels of sprouting. The authors suggested that, during the solubility measurement, alpha-amylase on the starch granule surfaces was exposed to temperatures ideal for this enzyme's activity. Thus, the difference in solubilities was due to hydrolysis that occurred after initiation of the solubility measurement. Thus, it appears that alpha-amylase damage does not occur *in situ,* but rather after the dough has been hydrated. As with laboratory sprouting, field sprouting was associated with slight increases in water binding capacity, with no difference noted between enzyme inactivated and noninactivated starch. In opposition to laboratory-sprouted grain, field-sprouted wheat displayed no change in gelatinization temperature ranges.

Starch granules from wheat varieties containing 0, 40, and 78% germinated grain displayed no sign of damage from higher amylase activity, using scanning electron microscopy and x-ray diffraction patterns (Kulp et al 1983). This was contrary to findings by Dronzek et al (1972) and Bean et al (1974), who determined that laboratory sprouting resulted in extensive starch granule damage. Kulp et al (1983) suggested that the differences between starch granules and their functionality, in field- versus laboratory-sprouted wheat, is because field sprouting does not achieve the same level of starch degradation that laboratory-sprouted grain does. That is, conditions for germination (temperature and moisture levels) are not held constant, and thus, are less than ideal.

Along with increases in carbohydrate-degrading enzymes, germination also results in greater protease activity. As a result, there has been some question as to the role protein modification may play in the detrimental effects of sprouting on product quality. This topic will be covered in detail under the proteinase section.

Soft Wheat Products

Studies have established that sprouting of hard wheat results in sticky dough and bread with inferior crumb and coarse texture (Kulp and Lorenz 1983). However, the PHS potential of soft wheats and its effects on soft wheat product quality has, in comparison, received limited attention. Perhaps in part due to the large variety of products soft wheat is used for or because soft wheats, in general, have received less research attention than hard wheats.

Triticum aestivum varieties which have soft-textured kernels are known as soft wheats. Generally lower in protein than hard wheats, soft wheat cultivars are known as the wheat of choice for a large variety of products. Soft wheat product categories include: biscuits, some breakfast cereals, breadings, cakes, cookies, crackers, doughnuts, flat breads, oriental noodles, pancakes, pastries (including pie crusts), pretzels, some snack foods, thickeners, waffles, and wafers.

Although the effect of sprouting on product quality has primarily focused on hard wheat leavened bread, a few studies have examined its effect on the quality of soft wheat products. A soft white wheat cultivar, which was laboratory sprouted for four days, was reported to have flour extraction rates within the commercial range for soft wheat applications (Lorenz and Valvano 1981). Sorrells et al (1989) subjected sprouting resistant and susceptible soft wheat varieties to controlled field sprouting. Sprouting susceptible cultivars yielded the same amount of flour as the control while those resistant to sprouting had lower flour yields.

Lorenz and Valvano (1981) found improved spread ratios and subjective grain score measurements from cookies made using a laboratory sprouted soft wheat. The cookies were darker in color apparently due to increased sugar levels, which provided favorable conditions for the maillard reaction. Sprouting had no effect on the cookies flavor score, but a sensory panel did give the sprouted products lower scores for texture and appearance. Sorrells et al (1989) found no difference between a control and field sprouted soft wheat cookie spread and appearance.

From these studies it is difficult to draw conclusions about the effect of sprouting on cookie quality because different sprouting methods were used, and the methods used to report degree of sprouting cannot be compared. Lorenz and Valvano (1981) reported falling number while Sorrells et al (1989) determined percent germination and alpha-amylase activity. Finney et al (1981) reported that using falling number or visual reports of sprouting greater than 30% does not accurately reflect alpha-amylase activity.

At low levels of soft wheat sprouting, Finney et al (1981) reported an increase in Japanese-type sponge cake volume. Greater than 0.35 dextrinizing units per gram, however, resulted in extreme volume losses. The authors proposed that no adverse effects on sponge cake quality could be assured if the amount of sprouted wheat were limited to 2.5%, or if the process were modified to include 28 rather than 40% water.

Lorenz and Valvano (1981) produced high-ratio yellow cake from one, two, and four day sprouted soft wheat. One day of sprouting caused increased cake volume, whereas with two and four days, not only did the volume decrease, but all external and internal cake characteristics were also compromised. These findings were explained by the authors in light of work by Lorenz and Kulp (1981), who demonstrated the ability of alpha-amylase to decrease starch swelling power, and to allow gelatinization at a lower temperature and over a narrower range.

Thickening power of laboratory-sprouted soft wheat was found to decrease with greater time of sprouting prior to flour milling. Gels stored at room temperature displayed syneresis after three days of sprouting and after five days when stored at 10°C (Lorenz and Kulp 1981).

Finney et al (1980), using field-sprouted soft wheat and composites of sound and sprouted wheat, examined the product quality of nine International breads including: fermented, unfermented, whole grain, and white flour products. Chapatti made from either a moderately or highly sprouted whole grain flour composite was coarse, dark in color, and unacceptable to a sensory panel. A fermented Iranian bread, barbari, was noted to have increased sensitivity to dough water content. Water absorption was lower for sprouted flour dough and when the optimum was not used, the breads color was very pale and baking times were increased. Changing proof time from 7 to 11 minutes improved the crust color. Moroccan sour-dough bread, made from highly sprouted grain was whiter, its crumb grain was gummy and it contained many air pockets. Several Indian breads, Egyptian balady, and Moroccan straight-dough whole wheat bread were determined to be acceptable products when made with sprouted soft wheat.

Dormancy

If merely quiescent, a seed provided with the appropriate environmental conditions will germinate, while dormant seeds resist germination under such conditions. When and for how long a kernel is dormant appears to be controlled by a complex set of factors. Potential relationships examined have included: spike and seed characteristics, germination inhibitors, germination stimulators, and environmental conditions (Nielson et al 1984, Reddy et al 1985). Also, numerous reports have documented white colored genotypes as having greater tendency to sprout (Gfeller and Svejda 1969). A more complete understanding of the factors which cause dormancy is paramount to unveiling the mechanisms of and eventually controlling PHS.

Physical Factors

Huang et al (1983), using scanning electron microscopy and autoradiography, reported that white wheat cultivars possessed more separation between the seed coat and tube cells of the inner pericarp, and a looser integument structure than red wheat cultivars. Also, the white wheat cultivars imbibed water faster which penetrated the kernel more than in the red wheat cultivars.

King (1984 a,b), using 49 wheat cultivars, determined that a two-fold difference in water uptake over the first two h of imbibition was correlated with whether or not a seed germinated after 30 h. The following were reported to not be associated with varietal differences in water imbibition rates: seed coat color, protein content, pericarp thickness, thickness of testa or suberized layer. However, a study by Moss (1973) has implicated grain hardness and thickness of the lignified sub-testa suberin layer. In addition, smaller seeds have been reported to imbibe water faster than larger seeds, but this did not correlate with the germination rate (rate with which kernels germinated after exposure to water) (King 1984a).

When examining differences in germination rate for seeds within whole ears, King and Richards (1984) found that awned wheat ears imbibed water faster than awnless and germinated 10 to 15 h sooner. However, Mares (1983) who controlled ear wetting and corrected for differences in grain wetting, also found differences in seed hydration rates. Thus, suggesting that differences in ear structure might also affect kernel hydration rates.

The path water takes during seed imbibition has been suggested as the source of variation in rate of water uptake. The greatest resistance to transverse water movement in wheat has been reported to be the testa (Hinton 1955). In barley endosperm, water uptake lags behind hydration achieved by the husk, pericarp and embryo (Reynolds and MacWilliam 1966). Apparently, water first travels through the husk to the pericarp and is wicked laterally to the embryo. Briggs and MacDonald (1983) have provided evidence for this pathway in barley. They submerged the end distal to the embryo in water while either maintaining high or low humidity at the proximal end. In the high humidity treatment, germination occurred, while not in the low. Allowing evaporation to occur in the low humidity environment apparently prevented the wicking of water to the embryo.

Others have suggested that differences in the amount of grain surface wax might be a factor related to speed of imbibition. However, primarily visual reports of the degree of waxiness were initially used to base this correlation on (Pool and Patterson 1958). Isogenic lines that differed in surface waxiness were reported to show no difference in hydration rate (King and Richards 1984). Work has indicated that differences may lie in the fact that waxy lines show a 30-fold greater rate of evaporation than nonwaxy lines. Recently, wax crystals were reported to be present in the embryo cavity of red wheat varieties stored for 25 years, but were not seen in white cultivars stored an equal time (Evers and Kratochvil 1993). These authors hypothesized that the wax, from which these crystals formed, may play a role in modulating the rate of PHS of wheat; and further, that the waxy material, which contains over 200 hundred components, may exist in an amorphous condition, in a region of the kernel which would prevent water or oxygen from entering the embryo. Over time, this waxy substance may progressively move toward a crystalline state, and thus create areas of the kernel with less of a water or oxygen barrier, making germination more likely. To date, the complete mechanism behind varietal differences in speed of water imbibition by different grain has not been fully elucidated.

Chemical Inhibitors and Promoters

Exogenously applied gibberellic acid (GA) is able to initiate the germination of dormant grain kernels. Although GA-responsive genes which result in increased hydrolytic enzyme activity have been identified, none which are thought to actually initiate germination have been identified (Jacobsen and Chandler 1987, Morris et al 1991).

Suggested inhibitors of germination have included: phenolics, coumarins, tryptophan and abscisic acid (ABA) (Miyamoto et al 1961, Paulsen and Heyne 1983, Bewley and Black 1985, Walker-Simmons 1987, Morris and Paulsen 1988, Weidner et al 1993). Currently, however, a direct correlation between the quantity of any of these substances and dormancy has not been established.

Of the substances mentioned above, ABA has received the most attention. Beyond a direct correlation to dormancy, others have hypothesized that a specific balance of GA to ABA may be what controls dormancy (King 1989). This appears doubtful in light of work by Karssen and Lacka (1986). They identified GA-deficient mutants which develop typical kernel dormancy periods, that are broken by a prolonged storage period or cool temperatures.

Abscisic acid-deficient or ABA-nonresponsive mutants have demonstrated that without the effects of ABA, dormancy does not occur (Karssen et al 1983, Karssen and Lacka 1986). When exposed to applied ABA, germination of embryos from dormant and nondormant seeds has been reported to be prevented; the nondormant embryos, however, required more ABA (Walker-Simmons 1987). Interestingly, however, levels of ABA are similar in dormant and nondormant mature kernels (King 1989).

Heat-stable proteins are reported to be synthesized for a prolonged period of time in dormant but not in nondormant kernels exposed to an ABA solution (Ried and Walker-Simmons 1990). They are very hydrophilic and are hypothesized to protect proteins during times of hydration or repeated drying and rehydration. Based on this observation, Morris et al (1991) have suggested that dormancy may be positively controlled. These authors, using cDNA cloning and differential screening to examine this phenomenon, have reported identifying mRNAs from five gene families which are present in greater amounts in imbibing dormant versus nondormant seeds. All of these transcripts increased during exposure to ABA. Studies have been initiated to map these ABA-sensititive clones which appear to be related to dormancy (Rayfuse et al 1993).

Environmental Factors

A correlation between higher temperatures during grain filling and low dormancy has been reported in several instances (Belderok and Habekotte 1980, Briggle 1980, Hagemann and Ciha 1981). That is, ears harvested and kept at room temperature lost dormancy faster than those kept outdoors at cooler temperatures. However, Briggle (1980) and Mares (1984) have reported that the reverse appears to be true when mature grain becomes wet; higher temperatures during wetting resulted in greater dormancy. Similarly, Plett and Larter (1986) found developing seed, harvested at from the second to the seventh week post-anthesis, to have a greater loss of dormancy when exposed to germination conditions at 17°C, compared to 25°C.

In terms of differences in levels of dormancy between cultivars, George (1967) reported no difference between cultivars when the germination temperature was 10°C, while at 30°C all had a deep persistent dormancy, and at 20°C the dormancy period ranged from 20 to 60 days.

Preharvest Sprouting Methodology

Quantifying and Studying Preharvest Sprouting

Rain-damaged grain presents the potential for economic loss to farmers, millers and processors. Thus, it is paramount to the cereal industry's financial viability that accurate, fast, inexpensive methods of classifying sprouting damage be developed and adopted. For example, an intense evaluation of

Australia's 1983/84 harvest, using the Hagberg falling number resulted in the down-grading of 2 million tonnes of rain-damaged grain at a cost of over $30 million to growers (Keene 1987).

A large variety of measurements have been employed in the evaluation of preharvest sprouting. All possess positive and negative attributes depending on the objectives of the measurement, i.e., what is of interest: the potential use of a grain in a specific end product, the percentage of kernels that have sprouted, or the degree to which grain components have been modified by sprouting. In addition, there is generally a trade off between methodology speed, cost, technical skill required, ease of use in the field, and accuracy. Grain sprouting has traditionally been evaluated visually (i.e., the percentage of kernels with a ruptured seed coat). Although still used by breeders, visual identification of sprouting is generally used today merely as an early guide to wheat classification. That is, visual examination is used to determine if objective measurements are needed to characterize the grain. The advantage of visual inspection for signs of sprouting is of course its speed and low cost. However, as fast as this method is, it is equally as inaccurate in predicting grain's alpha-amylase activity or the effect of sprouting on product quality (Moot and Every 1990). This, in part, is due to changes in enzyme activity over time. That is, after hydration occurs levels of alpha-amylase increase slowly at first, then very rapidly. As a result, grain that appears to contain a low percentage of sprouted kernels may pose no problem or may contain such high enzyme activity as to prevent its use in products destined for human consumption. Additionally, sprouted grain easily lose their germ during handling, thus increasing the chance of inaccurately characterizing the degree of sprouting.

Grain test weight (weight per unit volume) generally decreases with sprouting due to previous loss from the rupture of seed coats and embryo growth. However, other grain stress such as disease and temperature extremes can also decrease grain test weight. The degree to which grain test weight is correlated with sprouting can also be confounded by variables such as environmental conditions, grain genotype, and handling conditions. The use of test weight for judging sprouting damage thus appears to be less than reliable (Orth and Shellenberger 1988).

The Brabender amylograph has long been used to measure the hot-paste characteristics of starches and flours and, because such functional properties are affected by the amount of alpha-amylase present, it is also used to measure sprouting damage. In the AACC method #22-10, a flour or starch slurry is slowly heated from 30 to 95°C, and a viscosity curve is recorded with the initial peak said to represent alpha-amylase activity (AACC 1983). Amylograph pasting peaks, however, are also related to other variables: inherent pasting ability of the starch granules, damaged starch content, susceptibility of undamaged granules to attack by amylase and the protein and gum content (Meredith 1970, Dengate 1984). Pasting characteristics of isolated starch and of flour treated with acid to denature alpha-amylase have been used to differentiate between a flour's inherent pasting ability and its enzyme activity. However, this is a multi-step, expensive method of determining the degree of sprouting. Furthermore, amylograph tracings consist of arbitrary units, which collaborative studies have demonstrated should not be compared from one amylograph to another (Wilson et al 1974, Mattewson and Pomeranz 1978). Meredith (1970) suggested that the amylograph can be considered a useful investigative tool, but it is inappropriate for screening grain for sprout damage or its potential effect on product quality.

The penetrometer test is a method designed to predict the baking potential of flour or meal (Hagberg 1961, Mitchell 1968). Flour and water slurries are cooked in a boiling water bath with subsequent cooling. The distance a cone-shaped penetrometer falls through the cold gel is converted into sprout units. This is considered to predict bread-baking quality better than the amylograph, because the strength of cold cooked gel is more related to bread quality than hot-paste viscosity (Meredith and Pomeranz 1985).

Certainly the most widely used predictor of alpha-amylase activity has been the Hagberg falling number (Hagberg 1960, Hagberg 1961, Perten 1964). After milling the grain in a falling number mill to a consistent particle size, a sample plus water are placed in a glass tube. A stirrer is placed in the tube and the tube is then placed into a boiling water bath. After the slurry is stirred, the stirrer is then released. The time required, in seconds, for the stirrer to fall through the gelatinized suspension plus the stirring time is reported as the falling number value. As alpha-amylase activity increases, falling number decreases. As with amylograph measurements, falling number values are dependent on grain genotype, environment, inherent viscosity of the starch, temperature of starch gelatinization, quantity of other grain components present, and the susceptibility of the starch to enzyme attack. Falling number values have resulted in sprout-susceptible lines having a falling number lower than its alpha-amylase content would have predicted (McCaig and DePauw 1983). Work by Moot and Every (1990) found that falling number identified most, but not all, sprout damaged wheat that would pose quality problems for commercial bakers. The grain, inaccurately categorized as sound using the falling number method, produced test breads with a slightly doughy crumb. Also, Ringlund (1983) reported differences in falling number even when alpha-amylase activity was constant. This was attributed to differences in starch properties.

Treatment of flour with acid, silver nitrate, or mercuric chloride have been used to limit the shortcomings of the falling number method. That is, the treatments were done to separate a grain's inherent viscosity from the effects of alpha-amylase (Meredith 1970, Noll 1985). Finney (1985) developed a method of eliminating the effect of genotype on falling number. A standard curve was established which related known spiked alpha-amylase activity with falling numbers of an unknown flour. Although such methods are an improvement over the standard falling number method, time and level of training certainly prohibit their use.

Even with its known inherent shortcomings, falling number evaluation of grain has remained the U.S. standard method of evaluating sprouting damage (AACC 1983). It has established its respect as a suitable method, because of its speed, simplicity, reliability in the field, and reproducibility (Tipples 1971).

The inherent difficulties with methods previously discussed have, in part, resulted in the development of direct alpha-amylase assays. Such measurements have also been desired for the study of the germination process.

Early alpha-amylase methodology measured enzyme activity using starch as the substrate. Starch hydrolysis was then determined by measuring a decrease in viscosity, increase in reducing power, or increase in the color of the slurry from release of a dye from a dye-bound starch.

Of the direct alpha-amylase measurements, the dye liberation techniques undoubtedly have been most used. These methods are based on the release of a dye covalently bound to starch. As the starch is enzymatically cleaved, the dye is released into the solution and measured colorimetrically. The major differences between the dye liberation methods lies in the dyed substrate used, and whether extraction and the enzyme reaction is performed in one or two steps.

The method described by Barnes and Blakeney (1974) uses an insoluble cross linked dyed (Cibachron Blue) starch substrate, in a two-step procedure. After being extracted, samples are filtered using a special filtration system, incubated in a heating block along with the substrate for 15 min, filtered again, and measured colorimetrically.

Another two-step alpha-amylase method was developed by McCleary and Sheehan (1987). This method employs a soluble, blocked p-nitrophenyl maltoheptaoside and requires approximately 15 min. The benefit of this method lies in that the effects of beta-amylase are removed.

AACC method #22-06 based on work by Mathewson and Pomeranz (1977, 1978), utilizes substrate tablets containing buffer salts and calcium. This technique requires only three min to perform, because filtration is not required, and the enzyme extraction and reaction step take place in one container. However, because the slurry is not filtered, more care must be taken to ensure that a finely ground sample is used, than is needed in the dye methods discussed above.

Henry (1989) examined the use of an insoluble cross-linked dye-labeled starch and a soluble blocked p-nitrophenyl maltoheptaoside in both, a one- and a two-step method. The one-step methods required approximately two min and displayed alpha-amylase activity, which correlated with that of the two-step assay and the falling number method.

Gel diffusion techniques combine a starch substrate into an agarose gel. Wells are cut into the agar, enzyme extract is placed in the well, and the radial distance the enzyme diffuses is measured after an incubation period of four to 12 h, depending on the temperature used. The diffusion distance is proportional to the logarithm of alpha-amylase activity.

Several gel assays allow alpha-amylase diffusion to be visualized by using iodine staining (Mottonen 1970, Gothard 1976). For example, Hejgaard and Gibbons (1979) included a dyed starch substrate in an agarose gel, and measured the diffusion zone after washing away the dye that had become soluble after incubation. A modification of the basic gel diffusion technique measures alpha-amylase by pressing halved kernels into the gel surface (Hayter and Allison 1972).

The gel diffusion techniques offer several advantages. Variations in alpha-amylase activity from one kernel to another can readily be identified for less money than the methods previously discussed. Perhaps more importantly, these methods offer the ability to screen many samples at one time.

Nephelometric determination of alpha-amylase activity relays on the rate of decrease in turbidity (light scattering) of a solution containing a dextran and an alpha-amylase extract of the sample being evaluated. Collaborative testing demonstrated that a nephelometric method exhibited sufficient accuracy and reliability to be approved as an official AACC method (AACC 1983 Method #22-07). The Perkin-Elmer Corporation has made an instrument (Grain Amylase Analyzer) designed for this procedure. Since its conception this assay has received several modifications including a substrate change to beta-limit dextran to avoid measuring beta-amylase activity, a recorder to follow turbidity, and fungal alpha-amylase as a reference (Kruger et al 1979, Campbell 1980, Kruger and Tipples 1981). Although the nephelometric method requires a good deal of laboratory skill and is relatively expensive to perform, its apparent advantage over other methods is its ability to reliably measure alpha-amylase at low levels (Kruger 1987).

Techniques have also been reported which use fluorescent substrates or products to either quantify hydrolase activity or to examine location-specific enzyme activity. Hydrolase activity is of interest, because both lipase and protease activities increase during germination and these changes in activity have been suggested to correlate with alpha-amylase activity during germination (Tavener and Laidman 1972, Pancholy and Lynd 1972, Jenson and Heltved 1982).

One such method relies on lipase hydrolase of a fluorochrome, fluorescein dibutyrate (FDB) to butyrate and fluorescent fluorescein (Jenson and Law 1983, Jenson et al 1984). After cut grain halves are dipped into a solution of FDB, the degree of fluorescence is examined under low power magnification using a conventional fluorescence microscope or a Carlsburg Seed Quality Analyzer. Fluorescence can be quantified with a TLC fluorescence scanner. This technique has been proposed as a technique for quantifying sprouting damage.

Henry and McClean (1986) measured endosperm modification due to lipase activity using the FDB seed method, and found it to be unable to predict sprouting levels of individual wheat and barley varieties. This may in part be due to cell wall modification being a function of enzyme activity and the level of resistance to hydrolytic breakdown the cell wall exhibits due to thickness and compactness. For example, a soft endosperm barley mutant (M-737 from Minerva barley) displays the same level of alpha-amylase and beta-glucanase activity as its parent variety, but exhibits greater cell wall modification during germination (Aastrup 1983).

Alternative applications suggested for this technique include a method to follow the malting process and to predict sprouted grain storage longevity (Jensen and Heltved 1982). Munck (1987) suggested that the FDB seed method might be exploited as a means of sorting out light sprouted seeds from sound seeds, using density gradient tables.

Lipase activity as measured in flour samples using spectrofluorimetric assays of 4-methyumbelliferyl heptanoate (MUH) and FDB hydrolysis are techniques suggested as rapid (two to five min), sensitive indicators of germination (Heltved 1984). Flour samples are suspended in a buffer, MUH or FDB substrates are added and the fluorescence intensity measured. MUH was reported to hold the following advantages: greater stability in solution and a better predictor of lipase and alpha-amylase activity. The authors suggested that FDB would be more applicable to work requiring visual identification of hydrolytic activity in sprouted grain, because its fluorescence is much easier to see by eye.

Various immunochemical techniques have also been employed in the study of sprouting. For example, monoclonal antibodies raised against alpha-amylase II and labeled with fluorescein were used to examine alpha-amylase transport and cell wall breakdown in germinating barley seeds (Gibbons 1979, Gibbons 1980, Gibbons 1981). Enzyme linked immunosorbent assay (ELISA) was developed to follow the synthesis of an inhibitor of barley alpha-amylase II during grain filling (Munck et al 1985). Such methods have the advantage of being highly specific, and thus, appropriate for investigative work. The use of an ELISA as a tool for screening grain for sprouting damage has yet to be examined.

There is some concern that traditional methods of reporting PHS, that is alpha-amylase activity measurements and falling number, may not adequately predict product quality (Kruger 1990). If grain is exposed to water prior to harvest, other germinative enzymes with the potential to modify product quality may also be present in significant amounts. Many of these enzymes have not been rigorously studied and their potential importance to cereal processing should not be underestimated.

Characterizing Resistance to Preharvest Sprouting

One approach to reducing the impact of preharvest sprouting has been to develop wheat genotypes with improved resistance to sprouting. As a result, a variety of methods have been developed to quantify the sprouting tolerance of breeding lines and cultivars. In order to make comparisons within and between studies, grain maturity, germination, and sprouting resistance must be defined.

Physiological maturity (PM) of grain is generally said to occur when seed attains maximum dry weight. Identifying a kernel's stage of maturity is difficult because there is no clear visual distinction between when grain development has ended and maturity is reached. However, Hanft and Wyck (1982) have reported that PM is correlated with the time when glumes loose their green pigment, and also with the appearance of the pigment strand in the kernel crease. These authors suggested that the bottom spikelets be used as an indicator of PM, because they are the last to mature.

Harvest maturity (HM), assessed by subjectively evaluating grain firmness, is another point sometimes ascribed to grain maturity in sprouting studies. Countries, such as Australia and Sweden, define HM by grain moisture content. However, moisture content at maximum grain dry weight is said to range from 32 to 44% depending on the environment (Hanft and Wyck 1982).

The number of days post-anthesis has also been used to report a grain's stage of maturity (Mitchell et al 1980). This method may be appropriate when evaluating the potential a material has for sprouting prior to development of dormancy, and when studying high prematurity alpha-amylase, apart from sprouting (Mares et al 1987, Gale and Lenton 1987). However, as Derera (1989) has pointed out, environmental factors can result in significant differences in the rate of kernel development after anthesis, thus making it very difficult to use this method to screen lines based on a given level of maturity.

A method which has received praise for its accurate depiction of maturity has been eosin ripeness (Derera 1989). Weilenmann (1976) describes eosin ripeness to be the point when water, labeled with eosin red dye, ceases to move into the stems of a harvested ear. The principle behind this technique is that the cessation of water flow from plant to kernel correlates with the cessation of dry matter translocation to the kernel from the plant.

Water uptake, increased respiration, and enzyme level can all be used to define germination. However, for ease of measurement, termination of germination is generally used. That is, either when the testa over the embryo is ruptured or when a shoot or a root can be seen.

Resistance to sprouting has been reported to be 49 to 64%, dependent on seed dormancy, with the following factors causing 36 to 51% of the variation: chaff inhibitors, moisture conditions, and experimental error (Strand 1980). Most studies of sprouting resistance have focused on the dormancy factor. In such cases, sprouting resistant genotypes are defined as those with kernels displaying a reduced rate of germination under conditions favorable for germination. Degree of sprouting resistance is generally described by comparing genotypes being studied to varieties with known sprouting tolerance.

Having defined maturity, germination, and sprouting resistance, the next step in a screening program is to establish the conditions that will be used to favor sprouting and how sprouting will be measured. When studying preharvest sprouting, several questions must be answered prior to initiating the study. Will grain be examined in the intact plant, in the ear apart from the plant or as individual kernels apart from the ear? If the grain is left with the plant will natural rain or artificial precipitation be used to wet the kernels? If kernels are removed from the plant how will they be wet?

Answers to these questions of course are dependent on the aspect of sprouting under examination. That is, if grain germinability is of interest, then kernels apart from the plant should be used. However, if the vegetative structures (e.g., germination inhibitors in the chaff) impact on grain germinability are in question, then intact plants should be used.

Although grain sprouted under natural precipitation has been used to examine preharvest sprouting, the usefulness of such data is certainly in question due to the many variables not controlled. For example, frequency and intensity of rain would all need to be recorded if grain sprouted under natural conditions were to be used to establish a variety's propensity to sprout.

Grain in the ear has been examined using a variety of rain simulators. For example, Sorrells et al (1989) used a sprinkler irrigation treatment in the field. Others have reported variations of spray being applied to spikes held upright in a chamber which possesses temperature and humidity control (King and Richards 1984, Mares 1987). Providing support for the validity of the misting methods mentioned above, Mares (1983) reported that the ranking of 11 wheat cultivars' propensities to germinate was not affected by the wetting methods used. However, the rate that roots appeared was dependent on the method.

Although it has been suggested that misting systems should simulate natural rainfall in droplet size and velocity, practically speaking, creating conditions similar to a specific location would be an arduous task (King 1987).

Both subjective and objective measures have been employed to screen the sprouting tolerance of lines. A sprouting score developed by McMaster and Derera (1976) ranks lines using values from one (no visible sprouting) to 11 (fully sprouted). Mean scores of each line are determined after a fixed period of wetting. A similar method described by Mares (1987) determines a line's relationship between percent sprouted ears and length of treatment. Rate of sprouting has also been measured by recording the time required for an arbitrarily chosen percentage of seeds to germinate.

Assessment of sprouting resistance can also be performed using methodology developed to study germination. That is, a population's germination capacity, uniformity, and rate are calculated after placing kernels at a constant temperature, in excess water, either on filter paper or sand. For example, germination capacity is the proportion of seeds in a population that are able to germinate while the degree of germination synchrony is known as germination uniformity. Bewley and Black (1985) have described germination synchrony numerically as the coefficient of uniformity. The mean rate of germination of a population, also described by Bewley and Black (1985), is determined by calculating the coefficient of the rate of germination. In addition, Goodchild and Walker (1971) suggested that a polynomial regression method of curve fitting, using total germination and rate of germination, is an appropriate method to use when studying seed germination plus biochemical and physiological components of seeds.

Alpha-amylase levels and falling number have also been used in the screening of genotypes for sprouting resistance. These techniques are especially important because visual sprouting is not always correlated with alpha-amylase activity levels.

A more extensive objective measure is the sprouting index developed by Weilenmann (1976, 1987). Grain is harvested after eosin ripeness is established and falling number is measured before and after a wetting treatment (falling number dry and falling number wet). These values are then used in a formula which allows sprouting resistance to be reported on a scale of one to nine (one = high degree of resistance; nine = no resistance). When a more detailed description of sprouting resistance is desired, a sprouting index value is determined at several different times after eosin ripeness.

Prior to beginning a germination study, it has been suggested that seed viability be established. Gordon (1980) describes a method which includes the use of several dormancy breaking conditions: cool temperature, dilute potassium nitrate, and alternate exposure to light and dark. Other dormancy breaking techniques that have been employed include seed rupture, seed abrasion and application of GA or ammonia (Taylorson and Hendricks 1977, Cairns and deVilliers 1987).

Physiology of Germination

Grain Maturation

Seed formation begins following fertilization. One haploid male nuclei fuses with the egg nucleus, and another with two polar nuclei forming the diploid zygote (embryo) and the triploid endosperm nucleus, respectively. The process of mitosis then leads to cell division with the process, for example, in wheat endosperm completed 14 to 20 days after fertilization (Briaty et al 1979). Cell differentiation, which results in the formation of tissues and organs, begins prior to completion of cell division.

Seed development is often divided into three stages, each involving different biochemical events (Dure 1975). Stage I consists of pollination, fertilization, and zygote formation. Tremendous metabolic activity during this period is facilitated by the precursors needed for development (saccharides, fatty acids, amino acids, organic acids, nucleosides, inorganic ions, and water), being provided by the parent plant. This stage is finished when cell division is completed and the embryo is fully differentiated.

During Phase II, storage materials (phytin, starch, protein, and fat) are layed down and the aleurone layer is formed between the endosperm and the developing seed coat. The aleurone is a layer of densely packed living cells consisting predominately of protein. Water content, during the deposit of storage material, is from 50 to 60%.

Stage III begins when the seed moisture level starts to decrease. The point of lowest moisture level in unweathered grain correlates with seed maturity and is from 10 to 15%. During this stage, vascular connections between the seed and parent are severed and water loss may be accelerated through membrane modification. During this desiccation process polysomes become single ribosomes and metabolic activity decreases. Low moisture levels result in the seed entering either a quiescent or dormant state. In both cases, further development of the embryonic axis ceases.

Water Imbibition

Nondormant seeds experience renewed embryonic growth when exposed to appropriate water, air, temperature, and light conditions. The seeds also must not be exposed to excess levels of inorganic salts or toxins.

Germination is said to begin with the imbibition of water by quiescent seeds and ends with the emergence phase, that is, when a portion of the growing embryo penetrates the seed coat. Initial water uptake is temperature-dependent. With each 10°C rise in temperature, water uptake is reported to increase by 5% (Murphy and Nolan 1982). Under constant conditions, Mayer and Poljakoff-Mayber (1989) determined the optimum temperature for wheat germination to be from 15 to 31°C, while the minimum and the maximum ranges were from 3 to 5°C and 30 to 43°C, respectively. The values for barley and rye were similar to wheat while temperature ranges for maize and rice germination were slightly higher. As seeds aged, germination temperature ranges broadened.

On a fresh weight basis, wheat has been shown to require approximately 45% water in order for 50% germination to occur (Lush et al 1981). However, maximal germination has been shown to occur only when relative humidities are greater than 97% at 20°C (Owen 1952).

Biochemistry of Germination

Protein-Degrading Enzymes

Proteolytic enzymes catalyze the hydrolytic cleavage of peptide bonds. These enzymes can be placed into two large groups based on their ability to cleave external (peptidases or exopeptidases) or internal (proteinases or endopeptidases) bonds.

Peptidases are distinguished based on the size of the substrate (di- or tripeptidases) they cleave, or on the identity of the terminus (amino- or carboxypeptidases; omega peptidase for those with a blocked terminus) of the oligo- or polypeptide they attack. These hydrolases are also categorized according to the size of the residue they release and from the terminus released from (aminopeptidases, dipeptidyl peptidases, tripeptidyl peptidases, carboxypeptidases, peptidyl dipeptidases, dipeptidases, and tripeptidases).

Proteinase Biochemistry

Of the two groups of proteolytic enzymes, proteinases are generally the rate-limiting hydrolases (Barrett 1986a). That is, the degradation of a protein begins through the action of a proteinase and then proceeds, unhindered, rapidly to completion. The description of the four types of proteinases was initially based on the catalytic mechanism employed (acid, metal, serine, and thiol proteinases). However, these names have evolved to reflect the increased details known about the catalytic sites of these enzymes (aspartic, metallo-, serine, and cysteine proteinases).

Aspartic Proteinases

Aspartic proteinases (EC 3.4.23) contain an aspartic acid residue in their active site and in general possess an acidic pH optima (pH 3.5 to 5.5). Found only in eukaryotes, aspartic proteinases, have been reported to have molecular weight range (M_r) values ranging from 30,000 to 45,000. In general they have a pI below pH 5.1, however, forms have been reported with pI values up to 6.5 (Barrett 1986a).

Aspartic proteinases seem to act through a general acid-base mechanism. That is, water attacks the carbonyl of the peptide bond to be cleaved, and the carboxylates in the active site assist with the proton transfer (Rich et al 1984). These enzymes, in general, prefer to act on peptides bonds existing between bulky hydrophobic amino acid residues. However, beyond this general trend in selectivity, this group of proteinases contains those which will cleave only one specific bond in a given substrate, while others show little selectivity (Barrett 1986a).

Several aspartic proteinases have been reported to be synthesized as zymogens which are converted to their active form by hydrolytic cleavage of an N-terminal extension. For example, pepsin, an enzyme secreted as the zymogen pepsinogen into the stomach of mammals is responsible for protein digestion. The increased activity of this enzyme on certain substrates, at as low a pH as two, is thought to occur not only because the pK of its two catalytic carboxyl groups is in this region but also as a result of pH stimulated substrate unfolding. In comparison the pH optima for renin's ability to cleave angiotensinogen in blood is near neutral depending on the species (Barrett 1986a).

Known inhibitors of aspartic proteinases include diazoacetyl amino acid esters in the presence of cupric ion (DAN) and 1,2-epoxy-3-(p-nitrophenoxy)-propane (Stauffer 1987). These inhibitors inactivate aspartic proteinases by forming an ester with a carboxylate group. An irreversible tight binding inhibitor of aspartic proteinases is pepstatin, which is synthesized by various strains of *Streptomyces*. Enzymes with a low pH optima which are inhibited by DAN and pepstatin can be fairly comfortably labeled an aspartic proteinase.

Doi et al (1980) isolated an aspartic proteinase from resting rice (*Oryza sativa*) with a molecular weight (MW) of 60,000-65,000 and a pH optima of 2.5-3.5. Utilizing isoelectric focusing, the enzyme was found to consist of several fractions, all with similar properties. Aspartic proteinases have also been reported in ungerminated (Wrobel and Jones 1992, Sarkkinen et al 1992, Siuro et al 1988) and germinating barley (Wrobel and Jones 1992).

Cysteine Proteinases

Animals, plants, eukaryotic microorganisms and bacteria have been reported to contain cysteine proteinases (EC 3.4.22) (Barrett 1986a). A free sulfhydryl group and a histidine imidazole group, present in the active site of a proteinase are the distinguishing characteristics of cysteine proteinase. Cysteine proteinase have molecular masses from 20 to 35 kDa, however there are reports of masses up to 110 kDa. Optimum pH tends to be in the acidic range, but reports of cysteine proteinases with optima at 7.5 have also been reported (Barrett 1986b). In addition to the denaturation of protein substrates, the acidic pH optima of cysteine proteinase is thought to be due to the low pKa of the thiolate-imidazolium ion pair in the active site.

Four superfamilies of proteinases have been identified: the papain superfamily, the streptoccal superfamily, the calpain superfamily, and the clostripain superfamily (Barrett 1986b). The papain superfamily is named after the most studied proteinase of the family, namely, the enzyme papain. This proteinase is found in the fruit, leaves, and trunk of *Carica papaya L.*

The activity of cysteine proteinases can be inhibited by thiol-blocking agents (organomercurial compounds and iodoacetate) and activated by low molecular weight thiol compounds (dithiothreitol and 2-mercaptoethanol). Leupeptin and antipain (aldehydes), isolated from *Streptomyces* strains, and E-64 (3-carboxyl-2,3-L-*trans*-epoxypropyl-leucylamido(4-guanidino) butane, an epoxide made by *Aspergillus japonicus,* have also been reported as cysteine proteinase inhibitors. The former also have an inhibitory effect on serine proteinase, while the latter binds, irreversibly, to cysteine proteinase only. In addition a group of polypeptides, known as cystatins, have been identified which inhibit cysteine proteinase (Rich 1986). Of these naturally occurring inhibitors, egg-white cystatine is the best understood.

Unlike the animal kingdom, plants primarily use cysteine proteinase rather than serine proteinase for protein degradation. Of the cereal grains, barley cysteine proteinases have received the most attention; M_r of 29,000 to 37,000 have been reported for cysteine proteinase isolated from germinating barley (Koehler and Ho 1989, Koehler and Ho 1990, Phillips and Wallace 1989).

Metallo-Proteinases

Metallo-proteinases (EC 3.4.24) are found in fungi, bacteria, and higher organisms (Barrett 1986b). They contain zinc in their active site, are stabilized by calcium ions, and generally have a pH optimum near seven. As with aspartic proteases many mammalian metallo-proteinases initially are in the form of a zymogen, which can be activated either by proteolysis or exposure to organomercurial compounds.

Metallo-proteinases frequently hydrolyze a protein between amino acids with nonpolar side chains (Barrett 1986a). In addition a large hydrophobic residue (leucine and phenylalanine) is often preferred. Extreme substrate specificity (e.g. mammalian collagenase) as well as limited selectivity (e.g. thermolysin from *Bacillus thermoproteolyticus*) exists in the metallo-proteinase group.

Identification of metallo-proteinases can be performed using synthetic substrates such as N-terminus blocking agents like furylacryloyl and phenylazobenzoyl, as well as with various inhibitors. The metal chelator 1,10-phenanthroline (1,10 PA) has been reported to be an effective inhibitor of metallo-proteinases (Barrett 1986b). Although also a metallo-proteinase inhibitor, the use of ethylenediaminetetraacetic acid (EDTA) alone can produce false results, as serine proteinases are also inhibited by this compound (Powers and Harper 1986). Other inhibitory compounds considered useful in detecting metallo-proteinase activity are dithiothreitol (DTT), n-phosphoryl-Leu-Trp, phosphoramidon, hydroxamic acid derivatives and mercaptoacetyldipeptides (Barrett 1986a).

A barley proteinase thought to be a metallo-proteinase, with pH optima of 5.5 and 8.5, and inhibited by EDTA, has been reported (Mikola and Enari 1970). Using EDTA, 1,10-phenanthroline, and DTT, Wrobel and Jones (1992,1993) have recently demonstrated persuasive evidence for the presence of five metallo-proteinases in germinating barley.

Serine Proteinases

Serine proteinases (EC 3.4.21) are undoubtedly the most studied and widespread of the four classes of proteinases (Barrett 1986b). They have been identified in prokaryotic and eukaryotic microorganisms, invertebrate and vertebrate animals, and plants. Two types of serine proteinases have been identified, namely the trypsin-like (secreted by animal pancreas as a zymogen) and the subtilisin-like (made by *Bacillus* sp.), with the former found only in bacteria. Optimum pH's for these enzymes are generally at seven or higher. Calcium ions are sometimes required for serine proteinase stabilization, as well as the activation of their zymogen forms.

Serine proteinase substrate specificities are very dissimilar. However, there are several irreversible inhibitors which show little nonserine proteinase inhibition; namely, diisopropyl fluorophosphate (DFP), phenylmethane sulfonylfluoride (PMSF), and blocked aminoacyl and peptidyl chloromethanes (Powers and Harper 1986). Examples of protein serine proteinase inhibitors includes: soya bean trypsin inhibitor, chymotrypsin inhibitor, and elastase inhibitor (Ryan and Walker-Simmons 1981).

Barley is the only grain that has been reported to contain serine proteinases (Wrobel and Jones 1993). The activity of these enzymes was reported to increase with higher pH conditions and their M_r was from 47,000 to 91,000.

Proteinases in Wheat

Proteases in wheat have been studied since the early part of this century (Balls and Hale 1936). During kernel ripening proteolytic activity in three hard wheat cultivars were reported to decrease 3.4-fold (Bushuk et al 1971). Work by Kruger (1973) found proteolytic activity in two hard wheat varieties to reach a maximum 16 to 18 days after flowering. At maturity these grains had 50% of their maximum activity and it was located primarily in the pericarp, seed coat, and aleurone layer.

McDonald and Chen (1964) found much of the proteolytic activity in wheat to be extractable using acetate buffer (pH 3.8), water (pH 8.0) or 10% NaCl. Kaminski and Bushuk (1969), however, reported most proteolytic activity to be in the alcohol fraction compared to the other Osborne fractions. Using 0.2 M acetic acid Wang and Grant (1969) were able to extract 75% of total flour proteolytic activity. Kruger (1973) reported that even after four days of germination, unextractable proteolytic activity remains in wheat. These differences in reports on proteinase solubility may indicate the following: more than one proteinase is present and/or bound, as well as, free proteinase is present.

Optimum activity of extractable proteinase from wheat has been reported to be at pH 3.8 (McDonald and Chen 1964, Wang and Grant 1969). However, maximum activity for flour was found to be at pH 4.4 (McDonald and Chen 1964). Mountfield (1936a, 1936b) reported proteinase in germinating wheat to have a pH optimum of 4.1, at 40°C. All of the proteolytic activity of a dilute acetic acid extract was reported to be gone at 70°C, while only 50% of the activity was lost after exposure to 50°C (Wang and Grant 1969).

McDonald and Chen (1964) described the proteinase activity of mature wheat to be unaffected by trypsin or chymotrypsin inhibitors, while mercury was reported to have an inhibitory effect. Sulfhydryl blocking agents had a greater impact on the unextractable than the extractable proteinase. Proteinase activity in wheat has also been reported to be inhibited by DFP (Belitz and Lynen 1974). Kawamura and Yonezawa (1982) reported that the addition of pepstatin completely inhibited the decrease in viscosity of a gluten solution, found after a 24 h incubation. Proteolytic activity in germinating wheat was found to be increased by exposure to cyanide, while addition of the chloride ion had an inhibitory effect (Mountfield 1936a, 1936b, 1938).

Attempts to purify the proteinase in wheat appear to have begun with work by Wang and Grant (1969) and Kaminski and Bushuk (1969). Wang and Grant (1969) separated an acetic acid wheat extract using gel filtration chromatography. Two fractions with proteinase activity were identified, one with a MW of 50,000 and the other was reported to be complexed with glutenin. Four groups of proteinases, reported to be heterogeneous, were identified using starch-gel electrophoresis by Kaminski and Bushuk (1969). Skupin and

Warchalewski (1971) extracted wheat flour with 10% NaCl, partially purified it with ammonium sulfate, and separated it into two homogenous, active fractions, using gel filtration chromatography. The amino acid composition of one fraction was found to contain disulfide and predominantely unreactive sulfhydryl groups. Proteinase activity of this fraction was reduced using sulfhydryl blocking agents. Germinated and quiescent grain extracts were partially purified by Preston (1978), using affinity chromatography. One peak of proteinase activity was identified from the quiescent grain, while the germinated wheat extract was resolved into two heterogeneous peaks. More recently, Lin et al (1993) produced a crude extract containing proteolytic activity, from a soft wheat, using ammonium sulfate. One of the two proteolytically active fractions, separated using gel filtration chromatography, was found to be related to the change which occurs in elongational viscosity of cracker sponge during fermentation and was inhibited by pepstatin.

Intervarietal differences in proteolytic activity have been reported (Bushuk et al 1971). Proteinases have been reported to not be synthesized *de novo*; Mikola and Kolehmainen (1972) suggested that upon tissue hydration they are activated. On the contrary, however, others have reported that increased proteinase activity during germination is at least in part a result of hormonally induced *de novo* synthesis (Preston and Kruger 1979, Hammerton and Ho 1986).

Proteinases Effect on Wheat Proteins

After eight days of sprouting, protease activity in hard red spring wheat endosperm has been reported to increase 17-fold (Hwang and Bushuk 1973). During this time the amount of insoluble protein decreased and the number of amino groups increased, while no change was found in the amount of sulfhydryl and disulfide groups.

Kruger (1980) identified an endoproteolytic system as the hydrolytic factor involved in storage protein changes during sprouting. Three days of germination were needed prior to protein changes, as measured by protein solubility.

Several authors have reported similar trends in Osborne protein fractionation of sprouted wheat: increased albumin and glutenin fractions; decreased globulin and residue protein fractions; and no change in the gliadin fraction (Hwang and Bushuk 1973, Ariyama and Khan 1990). The only difference found in fractionation studies has been that of Lukow and Bushuk (1984b) who reported that sprouting resulted in increased gliadin proteins.

Polyacrylamide gel electrophoresis (PAGE) has also been used to try and further delineate the changes which occur to wheat proteins during germination. Two minor albumin bands were reported to appear during germination (Hwang and Bushuk 1973). Ariyama and Khan (1990) reported only a decrease in intensity of a few low-mobility albumins with their complete demise occurring after an extended period of germination. Using sodium dodecyl sulphate polyacrilamide gel electrophoresis (SDS-PAGE) these investigators also noted that germinated grain revealed lower intensities for some albumin bands in the high molecular weight (HMW) range.

Germination has also been reported to result in reduced intensity of a few HMW globulin bands (Hwang and Bushuk 1973, Ariyama and Khan 1990). Reduced globulins from sprouted grain showed no quantitative or qualitative changes compared to sound grain (Ariyama and Khan 1990).

Lukow and Bushuk (1984b) reported an increase in intensity of reduced gliadins of 118,000, 92,000, and 69,000 daltons while Ariyama and Khan (1990) found no changes in reduced or unreduced gliadin bands after sprouting.

No changes were found by Lukow and Bushuk (1984b) in glutenin bands after grain sprouting. However, Ariyama and Khan (1990) reported various changes in the intensity of several bands.

Attempting to resolve the apparent disagreement between several of the studies cited above, Ariyama and Khan (1990) suggested that differences may have resulted from differences in varieties and/or germination procedures used.

Proteinases Effect on Wheat Product Quality

Many agree that grain would not be fit for food product production, due to high amylase levels, long before increased levels of proteinase would become a factor in loss of product quality (Kruger 1990). However, evidence strong enough to support or reject this opinion is not available. An alternative hypothesis to the situation is as follows. According to Kulp and Lorenz (1983) alpha-amylase hydrolyzes grain starch during processing, while proteinases act both during storage and processing. Therefore it is possible that certain products may run a greater risk of damage due to proteinase activity. Products which require little dough handling and are easily penetrated by heat would limit alpha-amylases effect on product quality. Proteinases, however, having been active *in situ* and perhaps during processing, depending on their temperature tolerance, could become the primary cause of loss of product quality rather than the alpha-amylase. Examples of products with a potentially greater propensity to be affected by increased proteinase activity are: flat breads, noodles, and sugar wafer cookies.

Extending the conjecture described above, soft wheat products compared with hard, may also be more inclined to show changes in quality due to proteinase activity in sprouted grain because of differences in the quality and quantity of storage proteins.

Edwards et al (1989) reported that defects in the quality of Cantonese (yellow alkaline) and Korean (white salted) noodles, and Arabic flat breads, made using five wheat varieties subjected to laboratory wetting, were related to the flours protease activity. Pan bread, also made in this study, demonstrated improved quality characteristics. The authors concluded that the amount and quality of the protein in the sprouted grain may have influenced the effects seen. They also reported that protease activity may have played as large a role as alpha-amylase activity in the loss of product quality. In addition, several authors have noted that stronger mixing varieties are able to tolerate slight germination without negatively affecting bread quality (Lukow and Bushuk 1984a, Ariyama and Khan 1990).

Lorenz et al (1983) examined the functionality of gluten from sprouted hard wheat varieties, recombined with starch from a control. Gluten from grain that had undergone low levels of sprouting produced dry extensible dough. More highly sprouted grain contained gluten which produced a dry inelastic dough. With greater sprouting, the grain of bread produced from the recombined gluten and starch became more open and grayer in color. Amino acid analysis revealed no differences between sprouted and sound grain. The authors concluded that the changes in gluten functionality, resulting from sprouting, appears to be related to modification of protein conformation, which occurred primarily *in situ,* rather than during the bread making process.

Other Enzymes

As seeds germinate, whether in the field or laboratory, many of their enzymes develop greater levels of activity. Of these, alpha-amylase has been reported to be responsible for most of the deleterious effects of sprouting on cereal grain quality. Alpha-amylase *de novo* synthesis is thought to occur in the aleurone layer and scutellum, with subsequent release to the endosperm. Its action on starch results in glucose being released, then converted to sucrose in the scutellum and transported to the embryo axis to be used for growth (Bewley and Block 1985, Jacobson 1983, Edelman et al 1959). In addition to alpha-amylase and proteinases, which were discussed above, the following have also been implicated as participants in the quality loss resulting from PHS: arabinoxylanase (Marsh et al 1988), catalases (Kruger 1977), polyphenoloxidases (Kruger 1976) and lipases (Tavener and Laidman 1972).

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CHAPTER THREE

PROTEINASE ACTIVITY IN SOFT WINTER WHEAT DURING MATURATION AND PREHARVEST SPROUTING

ABSTRACT

Along with alpha-amylase activity, increased proteinase activity has been linked with the detrimental effects of preharvest sprouting (PHS) on wheat product quality. However, many questions remain regarding these enzymes. Those addressed in this study are: what classes of proteinases are present in maturing soft winter wheat (SWW); is proteinase activity affected by environment or PHS; and is proteinase activity correlated with alpha-amylase activity or rate of germination? Five red and four white SWW were grown in a greenhouse (GHG) and in Dansville, Michigan, in 1992. Samples were harvested at 21, 28, 35, and 42 days post anthesis. Throughout kernel maturation, proteinase activity was significantly higher in the field-grown grain (FGG) compared to the GHG. At harvest maturity, proteinase and alpha-amylase activities were positively correlated ($r = 0.669$, $p < 0.05$) in the FGG. Also, germination index was correlated with proteinase ($r = 0.553$, $p < 0.05$) and alpha-amylase activities ($r = 0.622$, $p < 0.05$). Utilizing class-specific inhibitors, crude extracts from the cultivar Augusta demonstrated cysteine and aspartic proteinase activity during kernel maturation. The cysteine proteolytic activity decreased as kernels matured, while the aspartic activity increased.

INTRODUCTION

Sprouting damage, which occurs when rain precedes harvest, is of concern world wide due to the economic losses which result from decreased yield, loss of seed viability, and poor flour quality (Tipples et al 1966). Areas of the world particularly susceptible to preharvest sprouting are Argentina, Brazil, Chile, Canadian regions of Saskatchewan and Manitoba, Eastern New Zealand, England, Kenya, Northern Europe, South Africa, and Zimbabwe. For example, in 1987 England realized a $114 Million loss due to winter wheat sprouting (Derera 1990). In the United States, white wheat growing areas including the Pacific Northwest (Finney et al 1980), New York (Briggle 1980), and Kansas (McCrate et al 1981), are affected most often. Michigan, primarily a soft white wheat producer, has also experienced PHS with an estimated 50-60% of its acreage containing sprouted grain in 1980 (Copeland et al 1980).

The study of proteinases, specifically in soft wheat, is of interest, in part, because of the potential role they may play in the end-product quality of sprouted grain. Although increased alpha-amylase activity in sprouted grain has been thought to be of greater consequence than other enzymes present, a few studies have linked proteinase activity with a change in product quality (Lorenz et al 1983, Edwards et al 1989). Also, greater protease activity compared to alpha-amylase activity in rain-damaged grain has been reported to be carried over from grain to flour after milling (Edwards et al 1989).

According to Kulp and Lorenz (1983) alpha-amylase performs its damaging activities during processing, while proteinases act both during storage and processing. Therefore, it is possible that certain products may run a greater risk of damage due to proteinase activity. Products which require little dough handling and are easily penetrated by heat would limit alpha-amylases effect on product quality. Proteinases, however, having been active *in situ* and perhaps during processing, depending on their temperature tolerance, could then become the primary cause of change in product quality rather than alpha-amylase. Examples of products with a potentially greater propensity to be affected by increased proteinase activity are: flat breads, noodles, and sugar wafer cookies (Kulp and Lorenz 1983).

In addition, wheat with lower protein strength has been reported to be less tolerant to the effects of preharvest sprouting (Ariyama and Khan 1990, Edwards et al 1989, Lukow and Bushuk 1984). Therefore, soft wheat storage proteins may be particularly affected by proteinase activity in sprouted grain. What this means in terms of product quality certainly is not known, as the role of storage proteins in the plethora of products made from soft wheat is not completely understood.

The focus of this study was to investigate several questions related to the hypothesis that proteinase activity, in sprouted soft wheat, results in biochemical changes which modify endproduct quality. These questions included: (1) what classes of proteinase are present in maturing soft winter wheat; (2) is proteinase activity, during kernel maturation, affected by environment; (3) is proteinase activity, at harvest maturity, correlated with alpha-amylase activity, or degree of sprouting; and (4) does proteinase activity increase when grain sprouts prior to harvest?

MATERIALS AND METHODS

Materials

Wheat Samples

Samples for this work consisted of five soft red (Cardinal, Mendon, Hillsdale, Pioneer 2548, Twain) and four soft white (Augusta, Lowell, Frankenmuth, Geneva) winter wheat varieties (*Triticum aestivum*). They were grown during 1991 to 1992, in Dansville, Michigan, and in 1993, in a greenhouse on the campus of the Michigan State University. The pedigrees of these varieties are listed in Appendix A.

Chemicals

All chemicals were reagent grade or better and were purchased from Sigma Chemical Co. (St. Louis, MO).

Methods

Wheat Growing Conditions and Harvest

The experimental design used was two replicates of a completely randomized block design. One replicate was the field and the other the greenhouse. Each replicate had two blocks.

Field-Grown Wheat

The field-grown wheat was planted in seven row plots, seven 12 feet long, with seven inches between rows. The seed was fertilized with 90 lbs N, as urea, per acre in the spring. Summer temperature and precipitation data collected in the vicinity of Dansville, Michigan by the Michigan Climate Bureau, are reported in Table I. In each plot, sixty heads were tagged at random throughout the plot, at anthesis, and 15 heads were harvested from each plot, at 21, 28, 35, and 42 (also called harvest maturity) days post-anthesis (DPA). Upon harvest, 10 heads from each plot, destined for enzyme work and storage protein analysis were placed on ice, in polypropylene bags, and within 1 h, stored at -20°C. The remaining five heads were transported at room temperature to be used later that day for moisture and germination index measurements.

Greenhouse-Grown Wheat

Seedlings were vernalized for seven weeks, transplanted, and transferred to a greenhouse. Two blocks containing each genotype were grown. They consisted of three rows of 10 pots (three plants per pot) per genotype, placed at random on three benches. Common cultural practices were followed with a 20 h photoperiod and the temperature ranged from 17 to 25°C. Head tagging and harvesting were performed as described in the field-grown wheat section.

Sample Preparation

Heads to be used for enzyme analysis were freeze-dried to 4% moisture or less (freezing time required to reach 4% moisture was first established using a trial sample from 21, 28, 35 and 42 DPA), threshed in a single-head thresher and ground using a UDY Cyclone mill with a 0.05 mm screen. Ground samples were then stored in polypropylene bags at -20°C.

Table I. Temperature and Precipitation in the Vicinity of Dansville, Michigan, during the Summer of 1992[a]

Month	Temperature (°F)[b]			Precipitation (in)	
	Avg. Max.	Avg. Min.	Departure From Normal[c]	Total	30 Yr Avg.[d]
May	69.9	42.4	-1.0	2.09	2.88
June	73.8	50.6	-3.4	2.07	3.81
July	76.6	55.5	-4.7	6.43	3.91

[a] Information provided by the Michigan Climate Bureau.

[b] Average maximum and average minimum temperatures.

[c] NOAA (1982).

[d] 30 year average.

Physical Methods

Moisture

Moisture was determined by drying 20 kernels, randomly chosen from the freshly harvested heads, overnight at 100°C and 30 psi.

Germination Index

The rate of germination was measured using 25 kernels from primary or secondary florets selected at random from the freshly harvested heads. Kernels were shaken in 100 ml of 1% sodium hypochlorite for 20 min. They were then rinsed with 200 ml of water three times and placed, crease side down, on two layers of Whatman #1 filter paper in a disposable petri dish (100 x 15 mm). Four ml of water were added to each petri dish prior to placing them into a darkened cabinet at 20°C and 96% relative humidity (RH). The number of kernels commencing germination were recorded daily for a period of five days. Germination was defined as the rupture of the pericarp with at least 1 mm of growth. A germination index value, for each replication, was calculated by employing a formula (see Appendix C) reported by Walker-Simmons (1987). This formula was shortened to five days and values were not divided by the number of kernels used, because all replications used a consistent number of kernels. Visible sprouting was evident at 42 DPA in some of the varieties grown in the field. In spite of this, kernels showing visible signs of sprouting had an equal chance of being chosen for the germination index and were counted as being sprouted only after one day in the germination cabinet.

Chemical Methods

Protein Content

The protein content of the grain was determined using the AACC micro-Kjeldahl method #14-13 (AACC 1990).

Alpha-Amylase Activity

Alpha-amylase activity was determined utilizing the AACC method 22-06 (AACC 1990). Reactions were run at 40°C for three min using a dyed Azurine-cross-linked amylose substrate. After running 10 samples, a sample of Augusta was analyzed, as a standard, having previously had a mean value plus standard deviation established. Results for the samples were reported only if the standard fell within the previously determined mean and standard deviation. Alpha-amylase activity was reported as dextrinizing units per gram, using a standard curve established with malt samples spiked with alpha-amylase.

Proteinase Activity

Proteinase activity was measured using azocasein as the substrate. The basic principles behind this method were first described by Charney and Tomarelli (1947). One g samples were homogenized with three ml of cold 50 mM acetate buffer (pH 5.4, 10 mM 2-mercaptoethanol) each, using a vortex for five sec. Enzyme extraction took place by shaking the homogenized mixture at 4°C for 1 h, in 10 ml tubes placed horizontally in a Barnstead Thermolyne rotatory shaker (1,200 speed). Samples were then centrifuged at 4°C for 20 min at 25,000 x g, and the supernatants filtered using Whatman GF/A filters. The substrate solution consisted of 1% azocasein in 75 mM acetate buffer (pH 5.4, 10 mM 2-mercaptoethanol), dialyzed against the same buffer overnight at 4°C and stored in 1 ml portions at -20°C. In 10 sec increments, 150 µl of thawed substrate was added to the enzyme extract (20 to 150 µl). The amount of enzyme extract used was established based on the linearity of this assay. After an incubation period of 3 h at 40°C, the reaction was terminated by adding 0.7 ml of 5% TCA in the same order as the assay initiation. Tubes were then stored at 4 °C for 20 min, followed by centrifuging at 10,000 x g for 25 min in a micro-centrifuge. Samples were then read in a spectrophotometer at 366 nm. Blanks were made by incubating the substrate and sample-extract buffer separately, and then combining them just prior to terminating the assay. Each time this assay was run, a standard sample of Augusta was included. Results for the samples were reported only if the value for the standard fell within the previously determined mean and standard deviation. Proteinase activity is reported relative to the value obtained for a sample of the cultivar Augusta germinated for five days. That is, absorbance values were divided by the value found for the germinated Augusta and the result multiplied by 100. Alpha-amylase and relative proteinase values are analyzed in the results section using the mean enzyme activity values for the GHG at each stage of maturity as 100% of activity.

Proteinase Inhibitors

Inhibitor studies were performed using the proteinase activity assay. Crude proteinase extracts of green-house grown Augusta were incubated with inhibitors at 0°C for 0.5 h in the concentrations listed in Table II. The degree of inhibition of proteinase activity was calculated by dividing the values from the assays, which included the inhibitors, by the values from the assays run without the inhibitor but with the inhibitor solvent (i.e., water, dimethyl sulfoxide - DMSO, ethanol).

Statistical Analysis

The data were analyzed using the statistical software package Systat (Systat 1992). Mean separation was performed using the Tukey Test, while correlation analysis was done by calculating Pearson's product-moment correlation coefficients. Tabulated data from this study can be found in Appendix B.

Table II. Conditions for Proteinase Inhibitor Studies

Inhibitor	Concentration	Solvent
Aspartic Proteinases		
Pepstatin A	20 μM	Ethanol
Cysteine Proteinases		
Iodoacetic acid	10 mM	Water
E-64[a]	10 μM	Water
N-ethylmaleimide	10 mM	Water
CMPS[b]	5 mM	Water
Metallo-Proteinases		
1, 10 Phenanthroline	5 mM	DMSO[e]
EDTA[c]	10 mM	Water
Serine Proteinase		
3, 4 Dichloroisocoumarin	0.1 mM	DMSO
PMSF[d]	5 mM	Ethanol

[a] trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane(L-trans-3-Carboxyoxiran-2-carbonyl-L-leucylagmatine)

[b] p-chloromercuriphenyl sulfonic acid

[c] Ethylene diaminetetraacetic acid

[d] Phenylmethyl sulfonyl fluoride

[e] Dimethyl sulphoxide

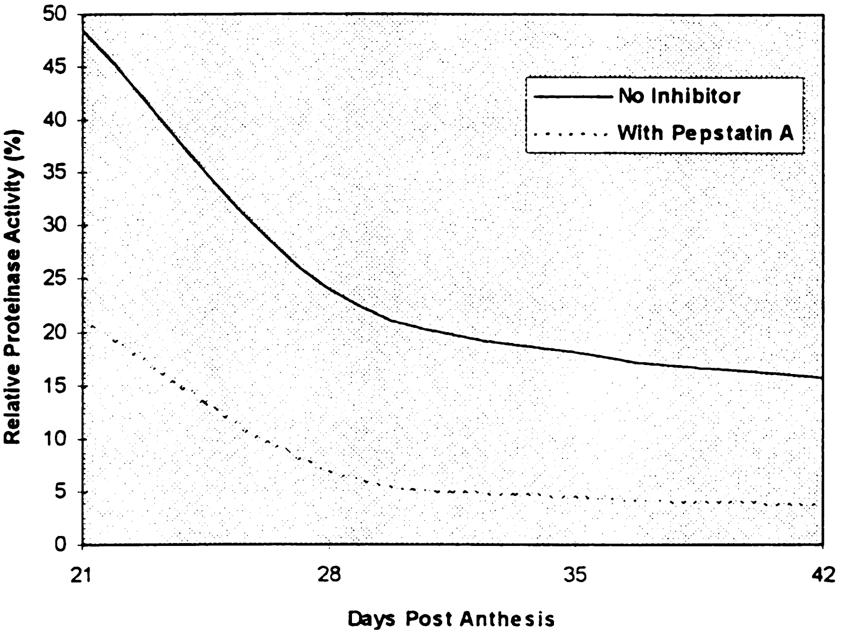
RESULTS

Inhibitor Studies

Proteinase activity, as affected by class-specific inhibitors, is presented in Figures 1 to 7. The solvents ethanol and DMSO had an inhibitory effect on total proteinase activity. Thus the control assays run to compare to the proteinase activity which existed with the proteinase inhibitors, which were soluble in ethanol or DMSO, had an equal amount of solvent added to them. Inhibition of proteinase activity by Pepstatin A was 56% at 21 DPA and increased to 76% by 42 DPA (Fig. 1). Iodoacetic acid inhibited proteinase activity by 36% at 21 DPA while its effect decreased to 15% by 42 DPA (Fig. 2). Similar to iodoacetic acid, N-ethylmaleimide and p-chloromercuriphenyl sulfonic acid (CMPS), both showed a decrease in their effect from 21 to 42 DPA; 49 to 35% and 25 to 20%, respectively (Figs. 3 and 4). E-64 (3-carboxyl-2, 3-L-*trans*-epoxypropyl-leucylamido(4-guanidino) butane) also decreased proteinase activity at 21 and 28 DPA, albeit to a lesser degree than the other cysteine proteinase inhibitors (Fig. 5). However, 1,10 phenanthroline (1,10 PA), and 2,3 dichloroisocoumarin (2,3 DIC) had no affect on proteinase activity (Figs. 6, 7).

Enzyme Activity During Maturation

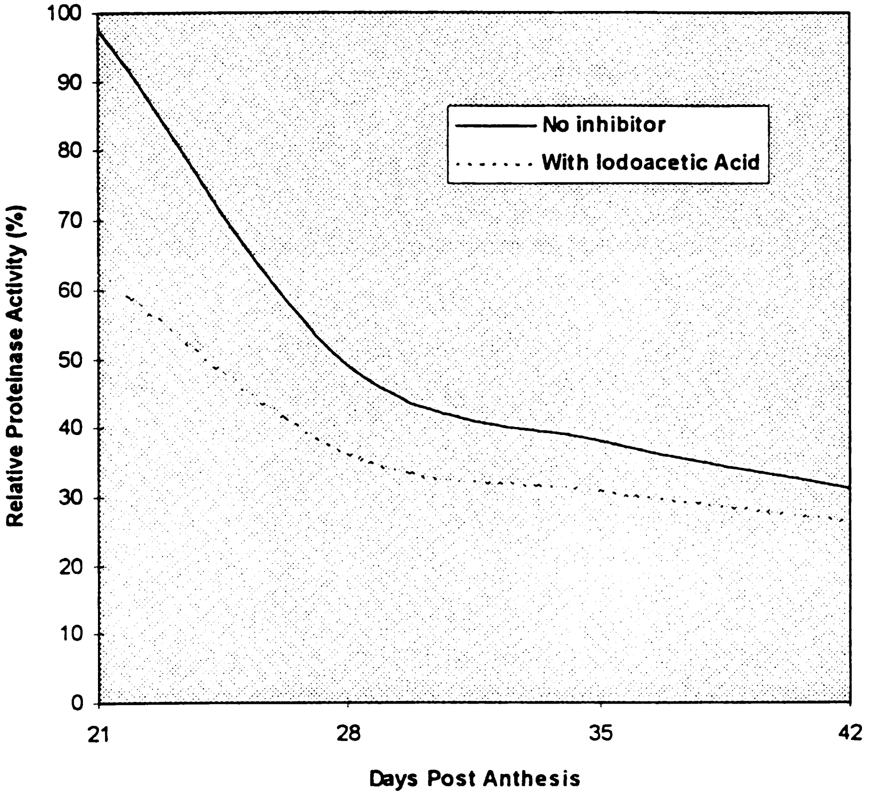
Mean relative proteinase activity of greenhouse-grown soft wheat cultivars (GHG) versus the field-grown grain (FGG), reported on a dry weight



Inhibitor Effect

DPA	% Decrease
21	56
28	71
35	75
42	76

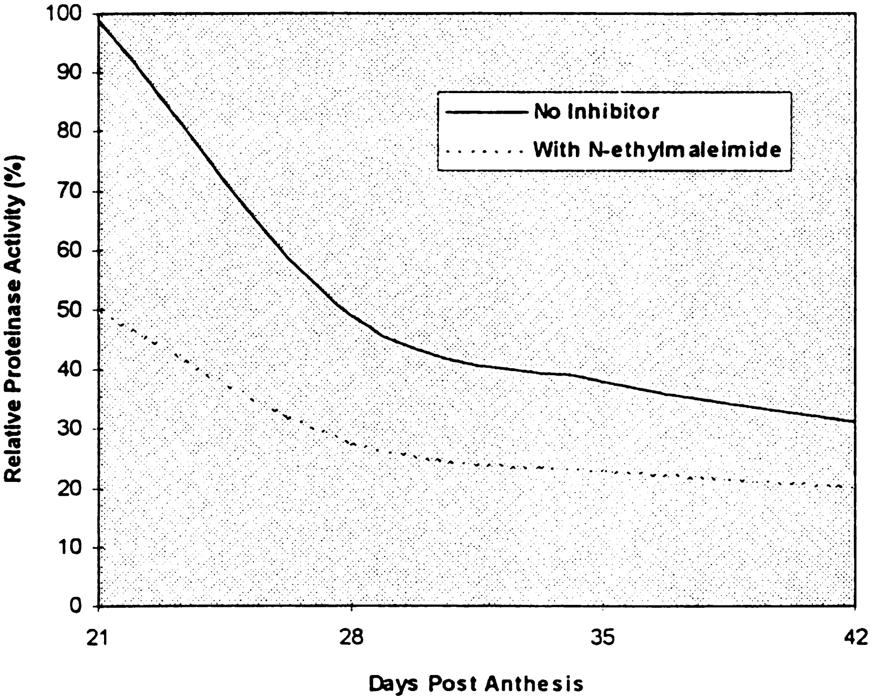
Fig. 1. Graph of relative proteinase activity of the cultivar Augusta grown in a greenhouse and harvested at 21, 28, 35, and 42 days post anthesis, with and without pepstatin A. All assays included ethanol, the inhibitor solvent. The table on the right of the figure lists the percentage decrease in proteinase activity which resulted from the incorporation of the inhibitor.



Inhibitor Effect

DPA	% Decrease
21	36
28	26
35	18
42	15

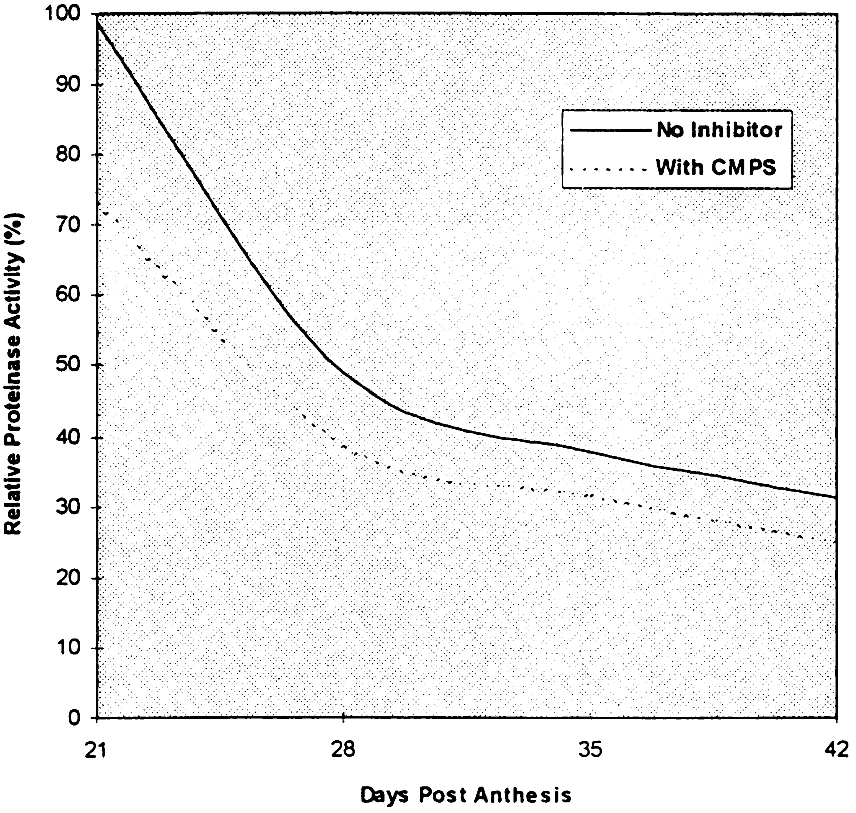
Fig. 2. Graph of relative proteinase activity of the cultivar Augusta grown in a greenhouse and harvested at 21, 28, 35, and 42 days post anthesis, with and without iodoacetic acid. The table on the right of the figure lists the percentage decrease in proteinase activity which resulted from the incorporation of the inhibitor.



Inhibitor Effect

DPA	% Decrease
21	49
28	43
35	40
42	35

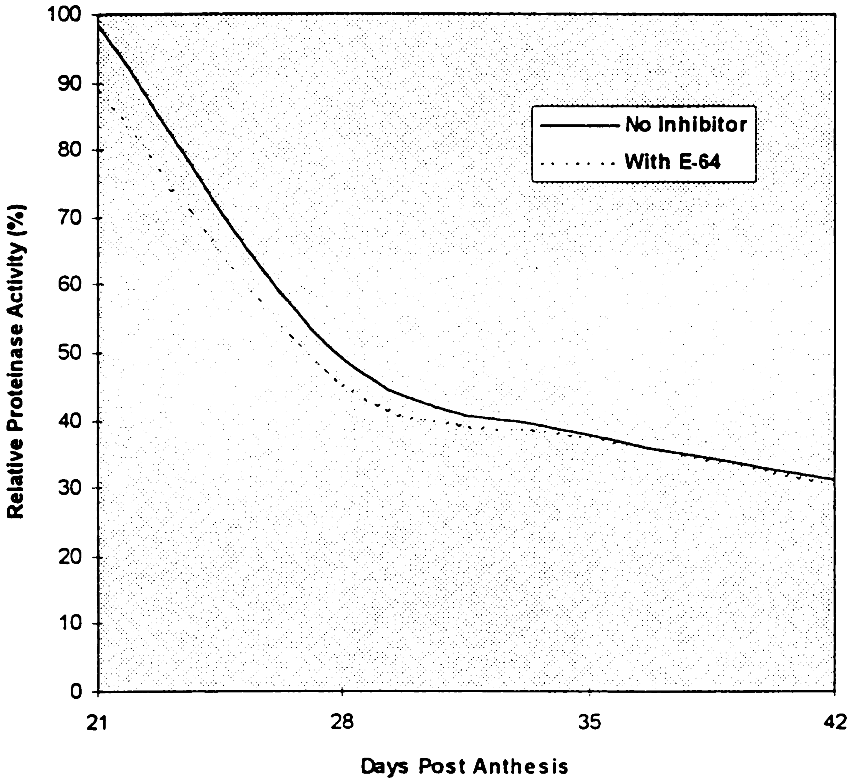
Fig. 3. Graph of relative proteinase activity of the cultivar Augusta grown in greenhouse and harvested at 21, 28, 35, and 42 days post anthesis, with and without N-ethylmaleimide. The table on the right of the figure lists the percentage decrease in proteinase activity which resulted from the incorporation of the inhibitor.



Inhibitor Effect

DPA	% Decrease
21	25
28	21
35	17
42	20

Fig. 4. Graph of relative proteinase activity of the cultivar Augusta grown in a greenhouse and harvested at 21, 28, 35, and 42 days post anthesis, with and without p-chloromercuriphenyl sulfonic acid (CMPS). The table on the right of the figure lists the percentage decrease in proteinase activity which resulted from the incorporation of the inhibitor.



Inhibitor Effect

DPA	% Decrease
21	10
28	8
35	1
42	1

Fig. 5. Graph of relative proteinase activity of the cultivar Augusta grown in a greenhouse and harvested at 21, 28, 35, and 42 days post anthesis, with and without 3-carboxyl-2, 3-L-*trans*-epoxypropyl-leucylamido (4-guanidino) butane (E-64). The table on the right of the figure lists the percentage decrease in proteinase activity which resulted from the incorporation of the inhibitor.

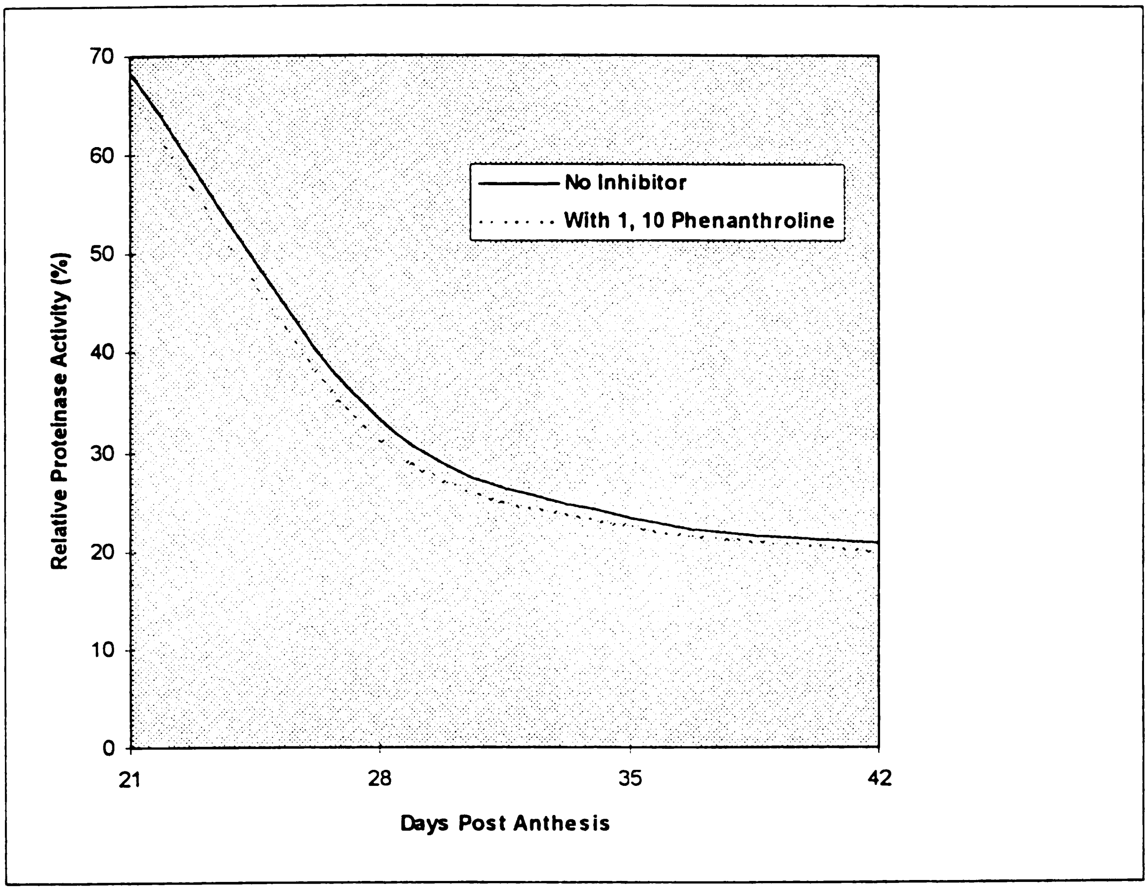


Fig. 6. Graph of relative proteinase activity of the cultivar Augusta grown in a greenhouse and harvested at 21, 28, 35, and 42 days post anthesis, with and without 1, 10 phenanthroline. All assays included dimethyl sulphoxide, the inhibitor solvent.

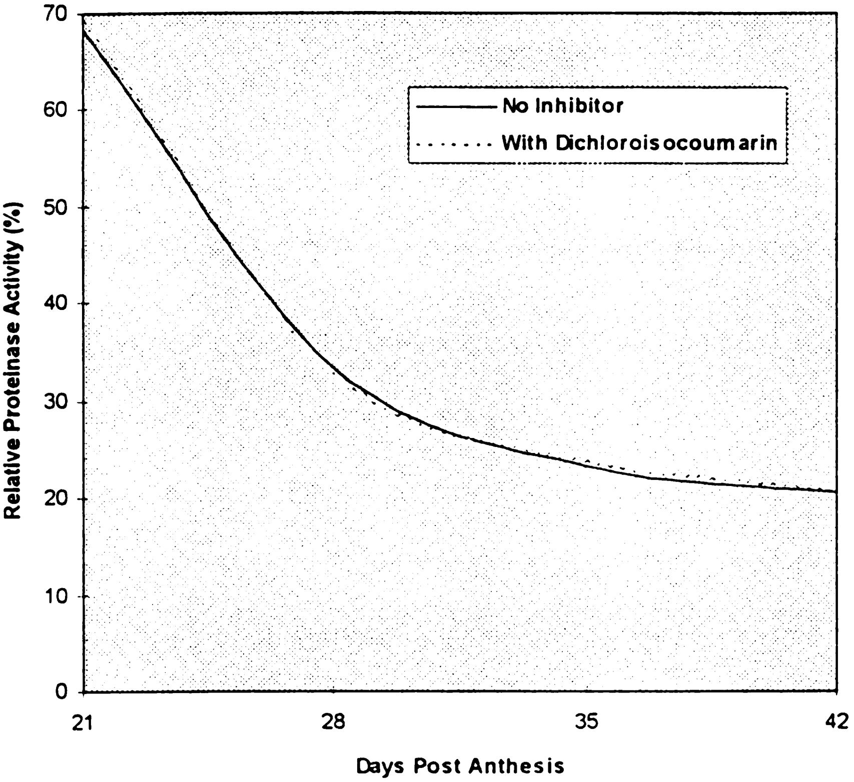


Fig. 7. Graph of relative proteinase activity of the cultivar Augusta grown in a greenhouse and harvested at 21, 28, 35, and 42 days post anthesis, with and without 3,4 dichloroisocoumarin. All assays included dimethyl sulphoxide, the inhibitor solvent..

basis, is displayed in Figure 8. Throughout kernel maturation, proteinase activity was significantly higher in the FGG compared to the GHG. At 21 DPA, the FGG had 31% more proteinase activity than the GHG, while at 28, 35, and 42 DPA (i.e., harvest maturity) this difference was 11, 20, and 30%, respectively. In both the GHG and the FGG, the proteinase activity was 3.3-fold greater at 21 DPA than at 42 DPA. However, the decrease in proteinase activity from 35 to 42 DPA was less in the FGG (14.6%) than the GHG (21.1%).

Figure 9 displays the mean alpha-amylase activity, reported on a dry wet basis, which was 4-fold higher in the FGG, and 8.5-fold higher in the GHG, at 21 DPA compared to 42 DPA. In the FGG, compared to the GHG, proteinase activity was significantly higher at 21 and 28 DPA, the same at 35 DPA and significantly greater at 42 DPA (Fig. 9). From 35 to 42 DPA, the alpha-amylase activity in the FGG increased 2.8-fold while the activity in the GHG remained constant.

Using the moisture data, alpha-amylase and proteinase activity were calculated on a wet weight basis. Proteinase activity was one-fold higher at 21 DPA compared to the grain at 42 DPA in the FGG, whereas the GHG showed 0.7-fold greater activity. Alpha-amylase activity was found to be two-fold higher in the FGG and 3.5-fold greater in the GHG at 21 compared to 42 DPA.

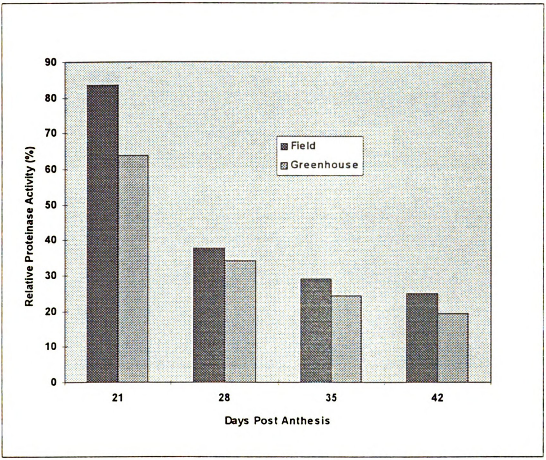


Fig. 8. Graph of relative proteinase activity in soft winter wheat grown in a greenhouse and in Dansville, Michigan, in 1992. Values for each location represent the mean activity of five white and four red cultivars. The relative proteinase activity of the field and greenhouse grown grain were significantly different ($p < 0.05$) at all stages of maturity.

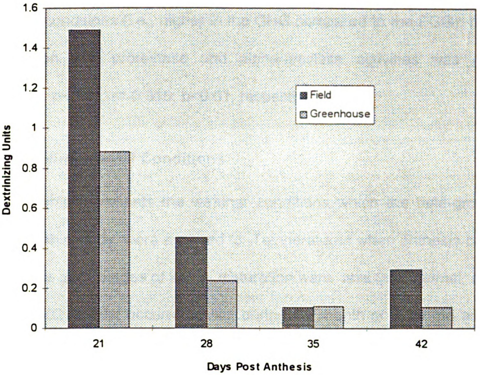


Fig. 9. Graph of alpha-amylase activity in soft winter wheat grown in a greenhouse, and in Dansville, Michigan, in 1992. Values for each location represent the mean activity of five white and four red cultivars. Alpha-amylase activity of the field and greenhouse grown grain were significantly different ($p < 0.05$) at all stages of maturity, except at 35 days post anthesis.

Considering all stages of growth and all genotypes, proteinase activity was positively correlated ($r = 0.875$, $p<0.01$) with alpha-amylase activity (Table III). The protein content of the grain was highly correlated ($r=0.930$, $p<0.01$) with the growing conditions (i.e., higher in the GHG compared to the FGG), however, the correlation with proteinase and alpha-amylase activities was much lower ($r=0.297$, $p<0.01$; $r=-0.310$, $p<0.01$, respectively).

Field Environmental Conditions

Table I presents the weather conditions which the field-grown kernels, used for this study, were exposed to. Temperatures when anthesis occurred and during the early stages of kernel maturation were less than normal, and on June 22nd, 1992, a frost occurred. Also, during the month of June the area received 46% less precipitation than average. As the kernels moved into the later stages of maturity, during the month of July, temperatures continued to be lower than the 30 year average, while precipitation was 65% greater than the 30 year average.

Field-Weathered Grain

At harvest maturity, the correlation between proteinase and alpha-amylase activity, in the FGG, was lower (Table IV, $r = 0.669$) than the relationship in the grain during maturation, as noted above. Also, germination

Table III. Correlation Analysis of Maturing Soft Winter Wheat[a] Enzyme Activities with DPA[b] and Protein Content[c]

	AMYLASE[d]	DPA	PROTEIN	PROTEINASE[e]
AMYLASE	1.000			
DPA	-0.683**	1.000		
PROTEIN	-0.310**	0.144	1.000	
PROTEINASE	0.875**	-0.852**	-0.297**	1.000

[a] Four white and five red varieties were grown in a greenhouse and in Dansville, Michigan, in 1992.

[b] Days post anthesis.

[c] (*) Significant at the $p < 0.05$; (**) Significant at the $p < 0.01$.

[d] Alpha-amylase activity.

[e] Relative proteinase activity.

Table IV. Correlation Analysis of Enzyme Activities with Germination Index and Protein Content of Mature Soft Winter Wheat Grown in Dansville, Michigan, in 1992 and Harvested at 42 DPA[a,b]

	AMYLASE[c]	GERMINATION INDEX	PROTEIN	PROTEINASE[d]
AMYLASE	1.000			
GERMINATION INDEX	0.622**	1.000		
PROTEIN	-0.412	-0.286	1.000	
PROTEINASE	0.669**	0.553*	-0.287	1.000

[a] (*) Significant at the $p < 0.05$; (**) Significant at the $p < 0.01$.

[b] DPA = Days post anthesis.

[c] Alpha-amylase activity.

[d] Relative proteinase activity.

index was correlated with proteinase ($r = 0.553$) and alpha-amylase activities ($r = 0.622$). Table V displays alpha-amylase and proteinase in the FGG, at 42 DPA. The alpha-amylase activity found for the cultivar Lowell was significantly different from all other cultivars. Hillsdale and P2548 had significantly less proteinase activity than the other seven cultivars, while Augusta and Lowell had significantly more. Besides varieties, the source of variation found for the alpha-amylase ($p < 0.01$) and proteinase ($p < 0.01$) activities in the FGG, was the blocks. Appendix B contains the analysis of variance tables for this analysis.

Greenhouse-Grown Grain

At harvest maturity, relative proteinase activity in the nine cultivars grown in a greenhouse was not significantly different from each other. However, the cultivar Twain did have significantly greater alpha-amylase activity than the other cultivars.

DISCUSSION

From the inhibitor studies it appears cysteine and aspartic proteinases are present during the soft winter wheat maturation process. Cysteine proteinase activity, as a percentage of total proteinase activity, decreased as the maturation process proceeded, while aspartic proteinase activity increased. Previous reports on the classes of proteinase which exist in wheat during maturation are not available. However, Preston and Kruger (1976) have reported that as wheat kernels develop, they contain at least two groups of

Table V. Alpha-Amylase and Proteinase Activity in Soft Winter Wheat Cultivars Grown in Dansville, Michigan, in 1992 and Harvested at 42 DPA[a,b]

Cultivar	Alpha-Amylase Activity (DU)[c]	Relative Proteinase Activity (%)[d]
White		
Augusta	0.38 [b]	30.69[a]
Frankenmuth	0.18 [b]	26.83 [b]
Geneva	0.29 [b]	26.72 [b]
Lowell	0.81[a]	29.57 [a]
Red		
Cardinal	0.11 [b]	27.40[b]
Hillsdale	0.07 [b]	25.62 [c]
Mendon	0.23 [b]	27.11[b]
P2548	0.06 [b]	25.31[c]
Twain	0.15 [b]	27.71 [b]

[a] Values followed by different letters in a column are significantly different ($p < 0.05$).

[b] DPA = Days post anthesis.

[c] Dextrinizing units.

[d] Proteinase activity relative to the activity found in the cultivar Augusta after five days of germination.

proteinases. One group, of acid proteinases, which was more active on azocasein than hemoglobin, was said to be most active during early development. Another group which contained neutral proteinases, increased in activity as maturity approached, and had little activity on azocasein. In terms of proteinase activity in mature wheat kernels, other authors have reported it to be partially inhibited by sulfhydryl blocking agents (Skupin and Warchalewski 1971, McDonald and Chen 1964), while others have found pepstatin A to be effective (Dunaevskii et al 1990, Kawamura and Yonezawa 1982). These studies, along with the present work, seem to provide ample support for the presence of both cysteine and aspartic proteinase in mature grain. Work by McDonald and Chen (1964) found proteinase to be active both in aqueous solvents and in the flour left after extraction. They reported the nonextractable proteinase to be more susceptible to sulfhydryl blocking agents. Kaminski and Bushuk (1969) have reported most of the proteolytic activity of mature wheat kernels to be in the alcohol-soluble fraction. The present work, in light of these previous observations, suggests that during the early stages of maturation, cysteine proteinases are in a free state (i.e., water soluble). However, as maturation approaches they become bound to storage proteins, rendering them less soluble. Thus, saying that proteolytic activity decreases as maturation of a kernel approaches may not be completely true. A more appropriate statement may be that the cysteine proteolytic activity of aqueous extracts of wheat decreases as a kernel matures, while the aspartic proteolytic activity increases.

As found by others and validated by this study, proteinase activities, reported on a dry weight basis, peak and begin to decline around 20 DPA in maturing wheat (Cornford and Black 1985, Kruger 1973, Bushuk et al 1971). The enzyme activity, on a wet weight basis, allows activity at each stage of maturity to be compared on an equal basis. Although alpha-amylase and proteinase activities in the present study decreased during maturation, when considered on a wet weight basis, the decrease was much less than previously reported.

The proteinase activity of the FGG at 21 DPA in this study, was close to that found for the cultivar Augusta, after five days of germination (reported in chapter four). Evidence that proteinase activity during kernel maturation is affected by the environment was provided by this study. Research has been reported which found a correlation between higher protein content and greater alpha-amylase activity and susceptibility to sprouting (Morris and Paulsen 1985, Huang and Marston 1980). However, work by Bhatt et al (1981) did not validate the work mentioned above, nor was a relationship between protein content and protease activity found. Although all of the GHG had significantly more protein than their FGG counterparts in the present study, other aspects of the environment were not controlled. Thus, the effect of a greater protein content cannot be examined. However, it is fair to say that it appears that if protein content is correlated with enzyme activity and susceptibility to sprouting as measured by germination index, the effect must be an interaction with other environmental factors and not nitrogen nutrition alone. That is, the present study found enzyme activity to be lower in the unweathered, higher protein grain. Apparently, the environmental factors which influence susceptibility to sprouting and enzyme activity remain to be fully understood.

Certainly, a question this study raises is: does environment, along with its effect on proteolytic activity during kernel maturation, affect the synthesis of wheat storage proteins? Because little is known about the proteinases present in grain during maturation, such as what metabolic function(s) they perform, the degree of compartmentalization, and the degree of control by inhibitors present, it is difficult to postulate about the question posed above. Nevertheless, it may be speculated that different levels of proteinase in maturing grain can influence storage protein biosynthesis, as a result of their potential role in peptide processing during translation. Cereal storage proteins are known to be translated on ribosomes attached to the endoplasmic reticulum, where cotranslationally, the signal sequence is cleaved and the polypeptide is released (Brinegar and Peterson 1982, Yanagata et al 1982). Furthermore, legume storage protein subunits have been shown to be first cleaved at the N-terminal signal sequence, and then endoproteolytically cleaved into subunits which are then linked together with disulfide bonds (Chrispeels et al 1982, Bollini et al 1982). Interestingly, in the current study, proteinase activity in soft wheat was highest during the time wheat storage protein biosynthesis is known to be completed (i.e., in terms of specific protein patterns on SDS-PAGE being present, but not in terms of band intensity which increased until 35 DPA); more specifically, around 17 to 21 DPA (Ng et al 1990). Oxidation of these proteins, that is, formation of inter- and intra-disulfide linkages between them has recently been reported to occur after storage protein formation is complete and up until kernel maturation is complete (Gobin et al 1994). Although the proteinase enzymes involved in the posttranslational cleavage mentioned above have yet to be identified, the aspartic and/or cysteine proteinases measured in this project during kernel maturation may be involved. In addition, allelochemical (i.e., nonmetabolic) roles for nongerminating proteinase have been suggested. Bell (1981) proposed a role for plant proteinases similar to that of tannins, that is, they may participate in defense by degrading parasitic enzymes or toxins.

The summer of 1992, in Dansville, Michigan, was considerably dryer during the early stages of kernel development while the later period was exposed to very wet conditions. Such conditions have previously been reported to be conducive to preharvest sprouting, and its concomitant increased levels of alpha-amylase activity. In the present study, alpha-amylase activity at harvest maturity was not as closely correlated to a germination index, as others have reported (Bhatt et al 1981). Proteinase activity was found to be more closely correlated with alpha-amylase activity, than a genotype's propensity to sprout. By selecting for low alpha-amylase levels, it appears that lower proteolytic activity should also be expected. Thus reinforcing, as previously suggested, the value of measuring alpha-amylase, after grain has been exposed to PHS conditions, during cultivar development, in addition to sprouting susceptibility (Derera 1989).

The need for proper sampling prior to measuring alpha-amylase or proteinase activity is reinforced by this study. As previously reported for other grain traits, this study found a micro-environment effect (i.e., differences in enzyme activity between blocks) to exist for alpha-amylase and proteinase activity (Finney and Campbell 1994). As a result, the standard deviations for alpha-amylase were quite large. This resulted in the cultivar Lowell having the only significantly different level of alpha-amylase activity, even though the cultivars Augusta, Frankenmuth, Geneva, Mendon, and Twain were found to have values which would be considered to indicate that sprouting had occurred (P. L. Finney - personal communication).

Intervarietal differences in proteolytic activity have been reported, as have increases in proteolytic activity during laboratory germination of grain (Preston et al 1978, Bushuk et al 1971). However, although many have alluded that increased proteolytic activity exists in grain due to PHS, limited evidence for this is available in the literature (Edwards et al 1989). In this study, mean proteinase activity in the FGG did not increase as maturation approached in response to PHS conditions, as did the alpha-amylase activity. However, when comparing the proteinase activity in the grain between 35 and 42 DPA, the activity in the FGG decreased less than in the GHG. In addition, proteinase activity remained at a higher level in the FGG at harvest maturity compared to in the GHG. This suggests that grain exposed to PHS conditions will have greater proteinase activity than unweathered grain. The findings reported by Edwards et al (1989) appear to be similar to those found in the present study. Three out of five genotypes exposed to PHS field conditions were reported to have had greater proteinase activity compared to their controls. However, Bhatt et al (1981) reported no correlation between field sprouting and proteolytic activity.

A comment regarding the level of alpha-amylase activity in the cultivar Twain (GHG) at 42 DPA should be made, as it contained significantly more activity ($p < 0.05$) than the other eight varieties. The activity in the cultivar Twain (GHG) increased from 35 DPA to 42 DPA, while it decreased during this time in the other genotypes. In comparison to the other cultivars, Twain grown in the field did not display an unusual level of alpha-amylase. This appears to be an example of a wheat cultivar with high alpha-amylase activity in mature grain, even when sprouting has not occurred. Such a late maturity high alpha-amylase (LMHAA) syndrome has previously been reported (Cornford and Black 1985, Gale et al 1983). Work by Mares and Gale (1990), using the cultivar Spica, demonstrated that LMHAA is controlled by a single, recessive gene located on the group six chromosomes and modulated by the environment and the genetic background. Mares and Mrva (1992) have suggested that greenhouse-ripening provides better discrimination between high and low LMHAA genotypes, because some cultivars with LMHAA, depending on the conditions, display low alpha-amylase in the field.

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CHAPTER FOUR

PROTEINASES IN SOFT WINTER WHEAT DURING GERMINATION AND THEIR EFFECT ON STORAGE PROTEINS

ABSTRACT

Characterization of proteinases is needed to understand their role in kernel physiology, thus opening up possibilities of exploiting them for the benefit of the food industry. The objectives of this project were to gain a "big-picture" perspective of proteinases in germinating soft winter wheat (SWW). Two SWW varieties were germinated for five days. The variety with a greater susceptibility to sprouting contained more proteinase activity at all stages of germination. Utilizing class-specific proteinase inhibitors, cysteine proteinase activity increased, while aspartic proteinase activity decreased until the second day of germination. These trends then reversed. After one day of germination serine and metallo-proteinase activity began to increase and peaked after four days. Ten bands of proteinase activity were visualized using native polyacrylamide gel electrophoresis with copolymerized gelatin and crude extracts from quiescent and germinated kernels. Two bands were present in quiescent grain at near neutral pH, suggesting that they contain serine or metallo-proteinase. The other eight bands had acidic pH optima and appeared after germination had commenced, indicating that they contain aspartic or cysteine proteinase. Glutenin subunits in germinated kernels appeared to be hydrolyzed by

proteinases earlier than the gliadin proteins. This study demonstrates that germinating soft wheat contains a complex set of proteinases.

INTRODUCTION

Proteases in wheat have been studied since the early part of this century (Balls and Hale 1936). However, much about these enzymes still eludes us. Such as: what classes of proteinases are present during the various stages of a kernel's life cycle; where are they located in the kernel; what are their substrate specificities; what are their temperature and pH optima; and where are the genes located which code for them?

Endogenous wheat enzymes have the potential to create desirable, as well as deleterious changes in a product, depending on the product's different functional property requirements, and thus, different needs for biochemical components. For example, durum wheat varieties have been selected for low lipoxygenase activity in order to limit carotenoid pigment oxidation (Hoseney 1994). A potential application in soft wheat, previously identified, lies within saltine cracker production. Endogenous proteolytic enzymes in flour have been reported to be related to the desired changes the rheological properties of cracker doughs undergo during fermentation (Pizzinatto and Hoseney 1980). Also, increased proteolytic activity which accompanies preharvest sprouting (PHS) has been linked to changes in product quality (Edwards et al 1989, Lorenz et al 1983). The examples cited above indicate that proteinases in wheat play a role in product quality, and thus, warrant further investigation.

Attempts to purify the proteinases in wheat appear to have begun with work by Wang and Grant (1969), and Kaminski and Bushuk (1969). Wang and Grant (1969) separated an acetic acid wheat extract using gel filtration chromatography. Two fractions with proteinase activity were identified, one with a MW of 50,000 and the other was reported to be complexed with glutenin. Four groups of proteinases, reported to be heterogeneous, were identified using starch-gel electrophoresis by Kaminski and Bushuk (1969). Skupin and Warchalewski (1971) extracted wheat flour with 10% NaCl, partially purified it with ammonium sulfate, and separated it into two homogenous active fractions using gel filtration chromatography. Proteinase activity of one of these was reduced using sulfhydryl blocking agents. Germinated and quiescent grain extracts were partially purified by Preston et al (1978) using affinity chromatography. One peak of proteinase activity was identified from the quiescent grain, while the germinated wheat extract was resolved into two heterogeneous peaks. More recently, Lin et al (1993) separated a crude extract from soft wheat using gel filtration chromatography. They reported two fractions with proteolytic activity, one of which was inhibited by pepstatin.

The objectives of this study were to gain a "big-picture" perspective of the proteinases in quiescent and germinating soft wheat, and to characterize their effects on soft wheat storage proteins.

MATERIAL AND METHODS

Materials

Wheat Samples

Augusta (a soft white winter wheat) and Hillsdale (a soft red winter wheat) provided by the Department of Crop and Soil Sciences, Michigan State University, were used for this study.

Chemicals

All chemicals were of reagent grade or higher and were purchased from Sigma Chemical Co. (St. Louis, MO).

Methods

Wheat Growing Conditions and Harvest

Seedlings were vernalized for seven weeks, transplanted, and transferred to a greenhouse. Two replications of each genotype were grown, consisting of three rows of 10 pots (three plants per pot) per replication, placed at random on three benches. Common cultural practices were followed, with a 20 h photoperiod and the temperature ranged from 17 to 25°C. Heads were tagged at anthesis, harvested 42 days later, placed in paper bags, and stored at room temperature.

Sample Preparation

Controlled Germination

Grain samples of greenhouse-grown (GHG) Augusta and Hillsdale (70 g each) were surface sterilized according to Hoy et al (1981) with some modifications. Kernels (10 g per container) were shaken in 200 ml of 0.1% (w/v) silver nitrate for 20 min, then washed three times with 200 ml of sterile 0.5 M NaCl, followed by the same routine using sterile water. The kernels were then soaked for 24 h under agitation, using 100 ml of sterile water per container. The sterile water contained 10,000 U penicillin, 10 mg streptomycin, and 25 µg amphotericin B per ml. After draining the antibiotic/antimycotic soak water, each 10 g lot of kernels was placed on two layers of blotting paper in a flat bottomed container and germinated in the dark at 20°C and 96% RH. At the end of each germination period, samples were freeze-dried, root and shoot growth was manually removed and the kernels were ground in a UDY Cyclone mill (O.05 mm screen).

Treatments for this study consisted of the two controls, Augusta and Hillsdale (A-CL, H-CL), kernels soaked 24 h (A-24h, H-24h), and kernels exposed to conditions condusive to germination for 1 (A-1D, H-1D), 2 (A-2D, H-2D), 3 (A-3D, H-3D), 4 (A-4D, H-4D), and 5 days (A-5D, H-5D). It should be understood that, in the text, when referring to grain exposed to conditions conducive to germination, not all kernels at a given time were germinating. However, for ease of discussion, the samples will be referred to as having been germinating for a given number of days.

Milling

Heads to be used for enzyme and storage protein work were freeze-dried to 4% moisture, threshed in a single-head thresher, and ground using a UDY Cyclone mill with a 0.05 mm screen. Ground samples were then stored in polypropylene bags at -20°C.

Germination Index

A germination index was measured for the cultivars Augusta and Hillsdale following the method decribed in chapter three of this dissertation. This index was measured one month after the grain was harvested at 42 DPA.

Proteinase Activity

Proteinase activity was measured using the method described in chapter three of this dissertation. All values are expressed relative to the maximum value obtained in this study, which was for A-5D.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Glutenins

A method by Ng and Bushuk (1987) was used to examine the effects of sprouting on glutenin subunits and is described in Appendix C.

Acid Polyacrylamide Gel Electrophoresis (PAGE) of Gliadins

Gliadins were examined using the first dimensional procedure of the two-dimensional PAGE method described by Lafiandra and Kasarda (1985). The extraction component of this method was not used; instead samples were extracted following the method by (Ng and Bushuk 1990). The modification of this method consisted of the use of 50% sucrose and 3% w/v methyl green dye in the extraction solution. A description of the acid-PAGE procedure can be found in Appendix C.

Proteinase Characterization

The wheat samples used to characterize the proteinase in soft winter wheat were Augusta and Hillsdale, germinated from 1 to 5 days.

Enzyme Extraction

Samples were homogenized with 50 mM acetate buffer (pH 5.4, 2 mM cysteine) in a 1:2 ratio (g/ml), using a polytron for 2 min. Slurries were then extracted by shaking for 1 h, centrifuging at 5,000 x g for 20 min, and dialyzing (MWCO 12-14,000) overnight, against 10 volumes of 5 mM acetate buffer (pH 5.4). The crude extract was then pipetted into 100 μl lots and stored at -20°C. All procedures mentioned above were carried out at 4°C.

Native PAGE with Copolymerized Substrate

Proteinases were separated and visualized using PAGE with copolymerized gelatin (0.1% in water) or glutenin (0.1% in 0.05 M acetic acid), according to Wrobel and Jones (1992). Modifications to this method included the use of 25 μl of sample extract per well, gel incubation at pH 3.8 (11 h), 5.4 (18 h), and 6.5 (18 h), and the use of glutenin as a copolymerized substrate. This method can be found in its entirety in Appendix C. Only crude extract from the Augusta samples were run on the gels with copolymerized glutenin.

Glutenin Preparation

The preparation of glutenin for use as a substrate in the proteinase gels involved several steps. First, crude gluten was prepared according to AACC method 38-10 (AACC 1990) from 10 g of Augusta meal which had been grown in a greenhouse and harvested at 42 DPA. Second, the crude gluten was stirred with 50 ml of 0.05 M acetic acid for 1 h at 4°C. The solubilized glutenin was then separated from the insoluble material by centrifuging the mixture at 5,000 X g for 10 min at 4°C. The supernatant was then dialyzed (MWCO 12,000 - 13,000) against distilled water, overnight at 4°C, and freeze-dried.

Proteinase Inhibitor Studies

Inhibitor studies were performed according to the method described in chapter three of this dissertation. The crude extract used was from the ungerminated and germinated Augusta grain described above.

Statistical Analysis

The data were analyzed using the statistical software package Systat (Systat 1992).

RESULTS

Germination Index

The germination index calculated for the cultivar Augusta was 130. However, the germination index found for the cultivar Hillsdale (40) was less than one third of that found for Augusta.

Proteinase Activity

Proteinase activity is reported in Figure 10. All values are expressed relative to that which was obtained for A-5D, that is, as a percentage of the highest absorbance found during this study. After the 24 h soak through five days of germination, the Augusta samples displayed significantly ($p < 0.05$) greater proteinase activity than the Hillsdale samples. From one to five days of germination, activity in Augusta increased at a faster rate than in Hillsdale. Proteinase activity was 6.7-fold greater in the A-5D compared to A-CL, while H-5D had 3.5-fold greater activity than the H-CL. After five days of germination, Augusta was found to have 1.7-fold greater activity than Hillsdale.

Visualization of Proteinase Activity

The proteinase activity bands (zones of hydrolysis) of the gels with copolymerized gelatin were numbered in the following manner: the band which had the fastest mobility was labelled B-10 (A-2D incubated at pH 3.8). All others, appearing in ascending order toward the top of the gels, were labeled B-9 through B-1 (Fig. 11).

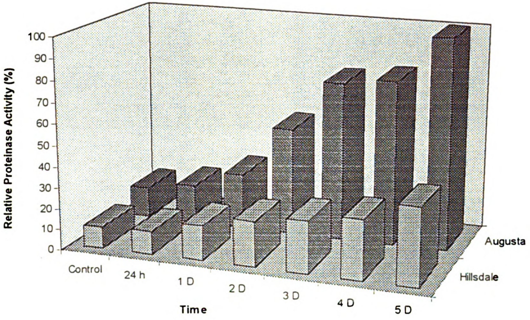
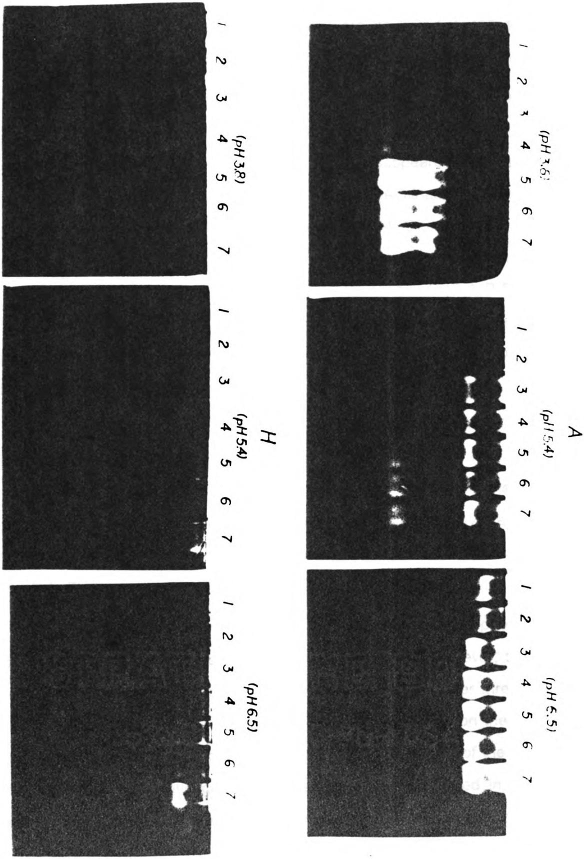


Fig. 10. Graph of the relative proteinase activity of the cultivars Augusta and Hillsdale grown in a greenhouse and exposed to conditions condusive to germination in a laboratory. Control is the ungerminated grain, 24 h is the grain after soaking for 24 h, and 1 D through 5 D is the grain germinated from one to five days. All Augusta values are significantly greater ($p < 0.05$) than the Hillsdale values, except for the controls.

Fig. 11. PAGE with copolymerized gelatin of soft wheat crude extracts prepared from grain at different germination stages. A, cultivar Augusta; H, cultivar Hillsdale; lane 1, ungerminated; lane 2, 24 h soak; lanes 3, 4, 5, 6, and 7, 1 through 5 days of germination, respectively; pH 3.8 (0.1 M acetate buffer, 11 h), pH 5.4 (0.1 M acetate buffer, 15 h), and pH 6.5 (0.1 M sodium phosphate buffer, 15 h), pH of gel incubation buffers; B1 to B11, enzyme band code. Buffers all contained 2 mM cysteine. Twenty-five microliters of extract loaded per gel.


A
(pH 3.6)
1 2 3 4 5 6 7
(pH 5.4)
1 2 3 4 5 6 7
(pH 6.5)
1 2 3 4 5 6 7
H
(pH 3.8)
1 2 3 4 5 6 7
(pH 5.4)
1 2 3 4 5 6 7
(pH 6.5)
1 2 3 4 5 6 7

Two bands (B-1 and B-2) of proteolytic activity in both quiescent grains (A-CL and H-CL) could be seen when the gels were incubated at pH 5.4 and 6.5. Increased activity of these bands became evident as germination progressed.

The Augusta gel, incubated at pH 3.8, first showed signs of activity after one day of germination (A-1D). After two days of germination (A-2D), two additional bands (B-3 and B-10) appeared which disappeared at three days of germination (A-3D). From day two to day three of germination, an increase in the proteinase activity of the five bands present (B-4, B-5, B-6, B-7 and B-8) was noted. This level of activity for all five bands appeared to remain constant throughout the duration of the germination period. Four bands (B-4, B-5, B-6 and B-8) were noted in the Augusta gel incubated at pH 5.4 after three days of germination. There seemed to be no change in these bands after four or five days of germination.

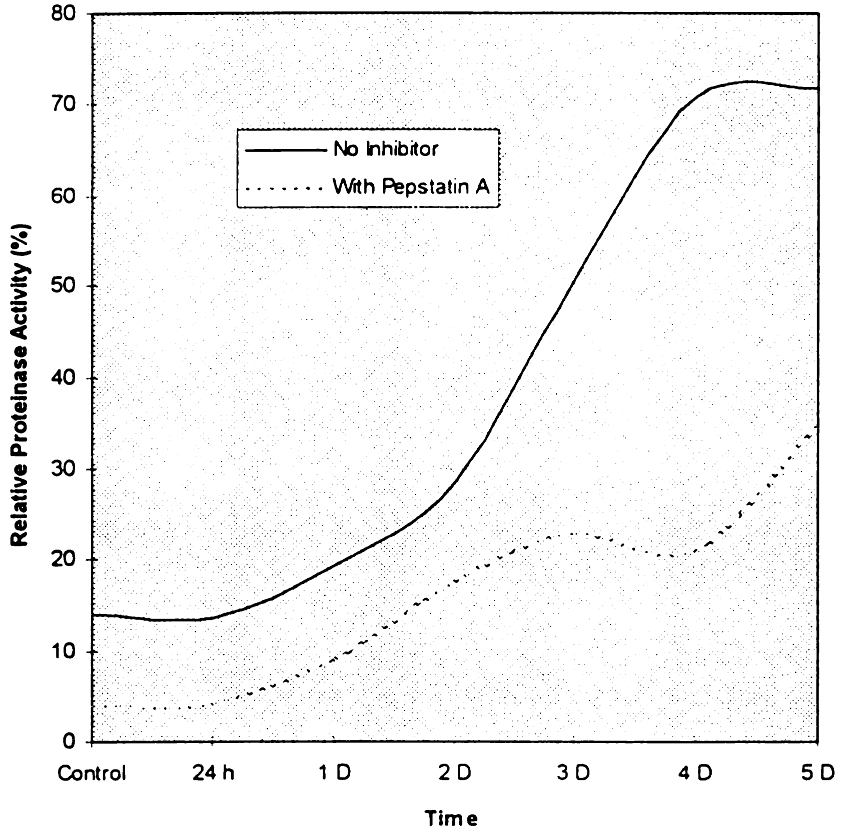
Proteinase activity first appeared in the Hillsdale gel incubated at pH 3.8 after three days of germination. These two bands of activity (B-5 and B-8) remained during the fourth and fifth days of germination. At pH 5.4, the Hillsdale gel showed only a slight amount of activity, in the faster mobility area, at five days of germination. A small amount of hydrolysis occurred here, but it is not visible in Figure 11.

The gels containing copolymerized glutenin photographed poorly due to low activity, and thus, are also included in this document. However, three faint activity bands were evident on the gel incubated at pH 3.8 after three days of germination. These bands were more diffuse than the bands on the gels with copolymerized gelatin. One band had faster mobility than any of the bands on the copolymerized gelatin gels. The other two bands appeared in the same regions as B5 and B6. Proteinase activity was evident on the gel incubated at pH 5.4, but only after four days of germination. No proteinase activity was seen on the gel incubated at pH 6.5.

In summary proteinases detected in the quiescent grain displayed increased activity with germination, had slow rates of mobility on a native PAGE gel, and appeared to have pH optima close to neutral. Proteinases with activity bands that appeared after germination had faster mobilities and acidic pH optima.

Inhibitor Studies

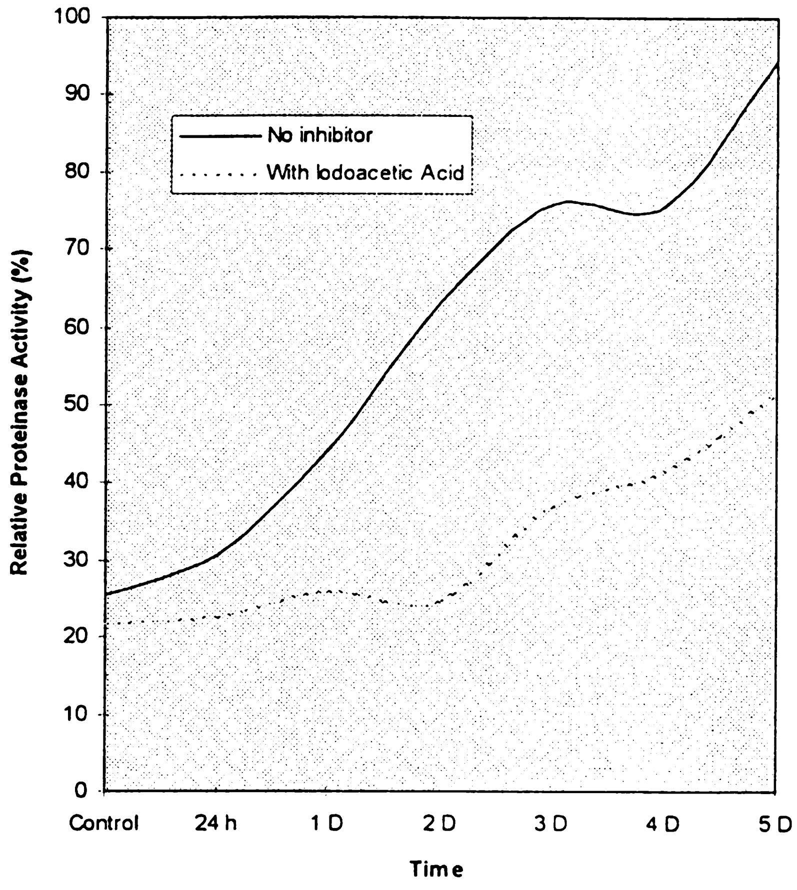
Figures 12 to 18 contain graphs displaying the effect of various inhibitors on the proteinase activity of Augusta crude extract from quiescent grain, grain soaked for 24 h, and grain germinated for one to five days. Crude extract proteinase activity when incubated with pepstatin A was inhibited 72% in the control. The level of inhibition decreased to a low of 38% by the second day of germination, then increased to 70% after four days of germination, and fell to 52% after five days of germination. Iodoacetic acid, 3-carboxyl-2, 3-L-*trans*-epoxypropyl-leucylamido(4-guanidino) butane (E-64) and N-ethylmaleimide all demonstrated increased inhibitory effectiveness from the ungerminated stage (14, 1, and 30%, respectively) until day two of germination (61, 45, and 50%, respectively). After the second day of germination, their percentage of inhibition continued to fall until the final day of germination. After five days of germination, however, their inhibitory ability was greater than the degree of inhibition seen at quiescent stage. Similarly, p-chloromercuriphenyl sulfonic acid (CMPS) increased in its ability to inhibit proteinase activity beginning during the 24 h



Inhibitor Effect

Time	% Decrease
Control	72
24 h	69
1 D	53
2 D	38
3 D	55
4 D	70
5 D	52

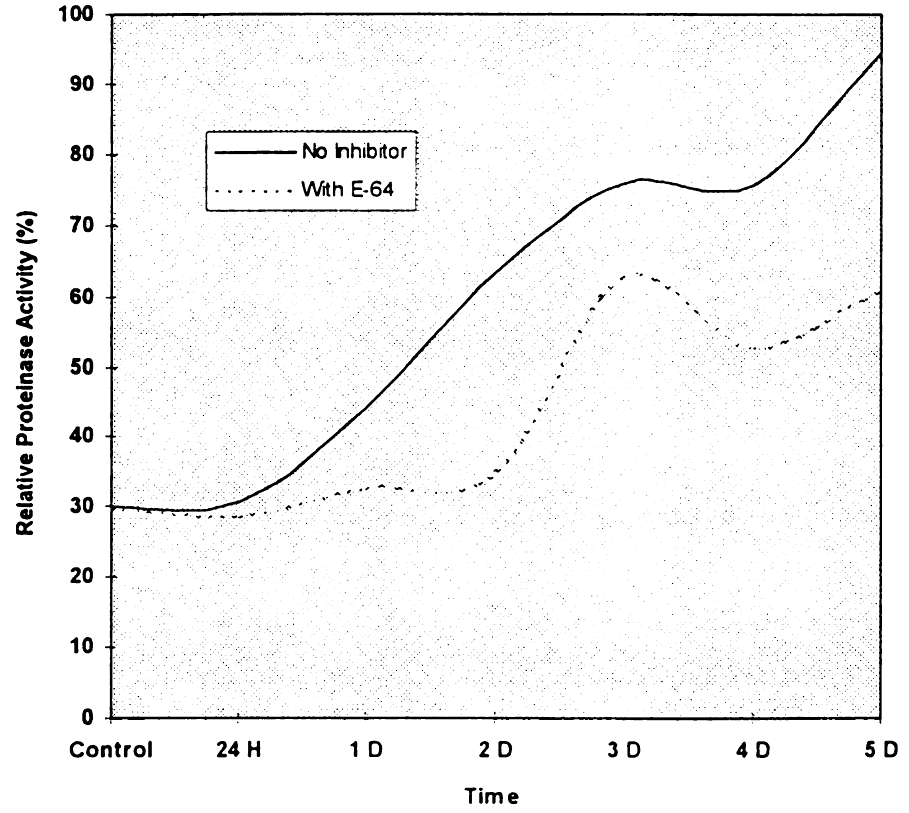
Fig. 12. Graph of relative proteinase activity of the cultivar Augusta, grown in a greenhouse, with and without pepstatin A. All assays included ethanol, the inhibitor solvent. Control, ungerminated grain; 24 h, grain soaked for 24 h; 1 D, 2 D, 3 D, 4 D, and 5 D, grain germinated for one to five days. The table on the right of the figure lists the percentage decrease in proteinase activity which resulted from the incorporation of the inhibitor.



Inhibitor Effect

Time	% Decrease
Control	14
24 h	23
1 D	40
2 D	61
3 D	51
4 D	45
5 D	46

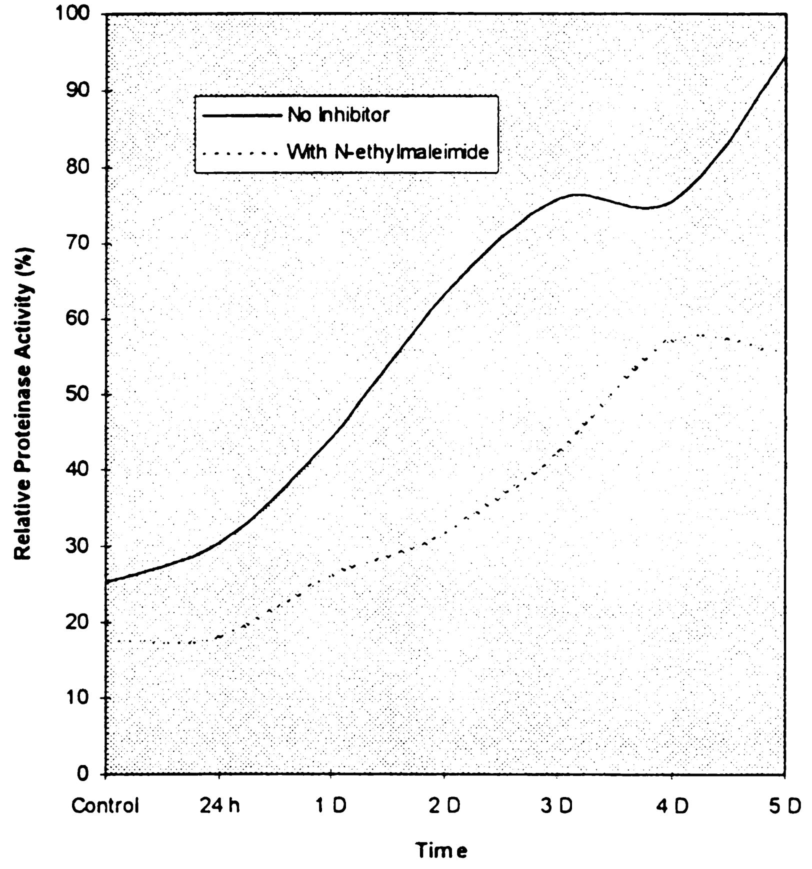
Fig. 13. Line graph of relative proteinase activity of the cultivar Augusta, grown in a greenhouse, with and without iodoacetic acid. Control, ungerminated grain; 24 h, grain soaked for 24 h; 1 D, 2 D, 3 D, 4 D, and 5 D, grain germinated for one to five days. The table on the right of the figure lists the percentage decrease in proteinase activity which resulted from the incorporation of the inhibitor.



Inhibitor Effect

Time	% Decrease
Control	1
24 h	6
1 D	24
2 D	45
3 D	17
4 D	30
5 D	36

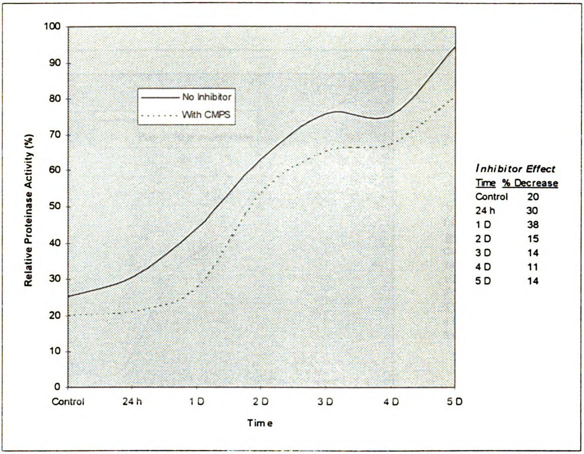
Fig. 14. Graph of relative proteinase activity of the cultivar Augusta, grown in a greenhouse, with and without 3-carboxyl-2, 3-L-*trans*-epoxy propyl-leucylamido (4-guanidino) butane (E-64). Control, ungerminated grain; 24 h, grain soaked for 24 h; 1 D, 2 D, 3 D, 4 D, and 5 D, grain germinated for one to five days. The table on the right of the figure lists the percentage decrease in proteinase activity which resulted from the incorporation of the inhibitor.



Inhibitor Effect

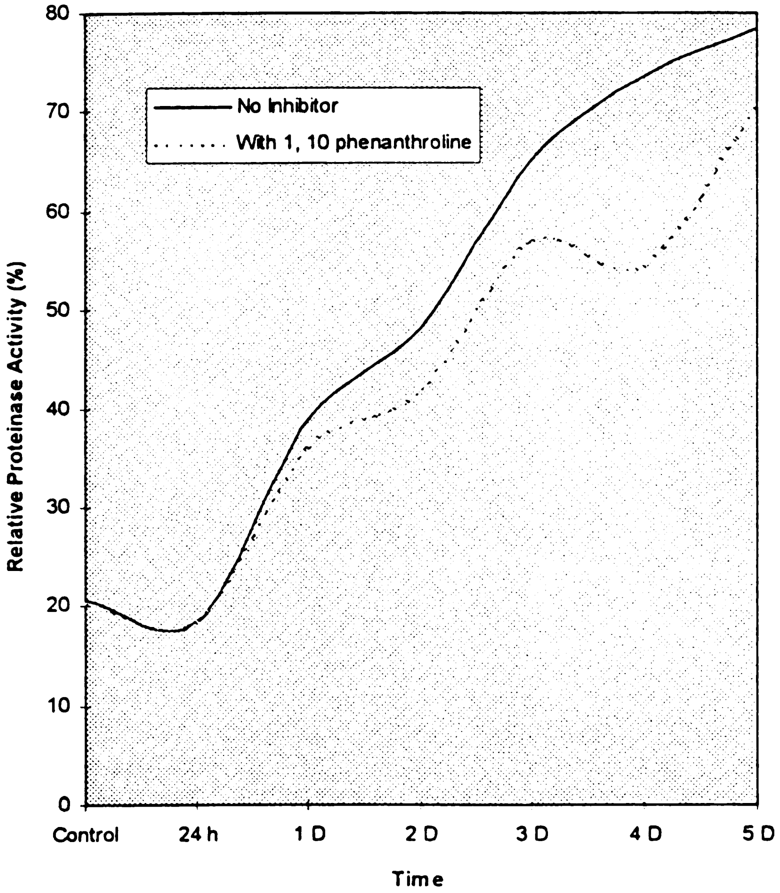
Time	% Decrease
Control	30
24 h	40
1 D	41
2 D	50
3 D	44
4 D	25
5 D	41

Fig. 15. Graph of relative proteinase activity of the cultivar Augusta, grown in a greenhouse, with and without N-ethylmaleimide. Control, ungerminated grain; 24 h, grain soaked for 24 h; 1 D, 2 D, 3 D, 4 D, and 5 D, grain germinated for one to five days. The table on the right of the figure lists the percentage decrease in proteinase activity which resulted from the incorporation of the inhibitor.



Time	% Decrease
Control	20
24 h	30
1 D	38
2 D	15
3 D	14
4 D	11
5 D	14

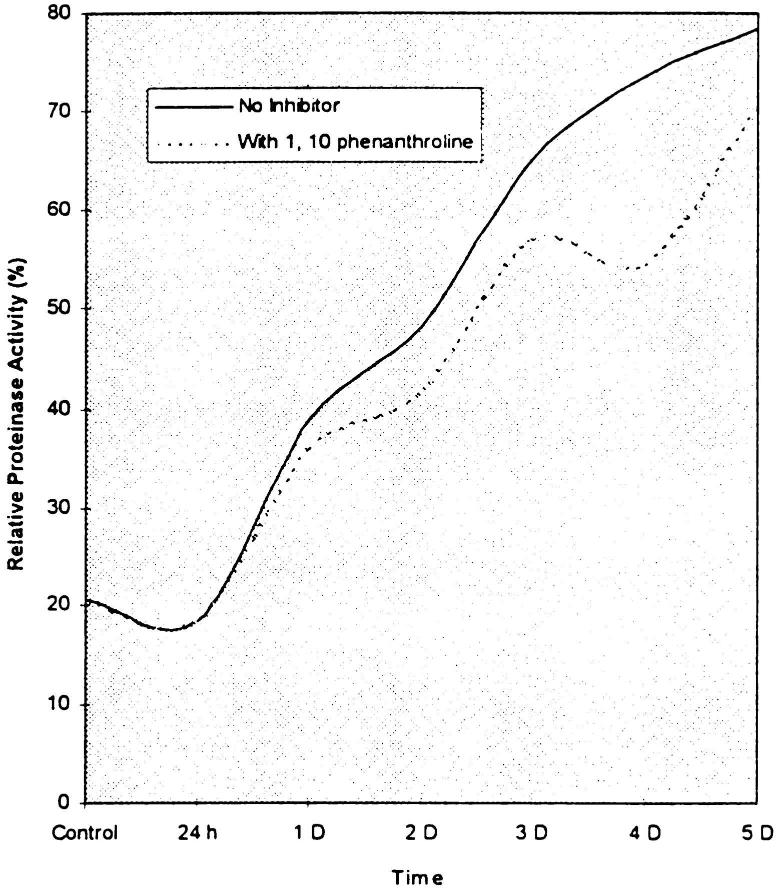
Fig. 16. Graph of relative proteinase activity of the cultivar Augusta, grown in a greenhouse, with and without p-chloromercuriphenyl sulfonic acid (CMPS). Control, ungerminated grain; 24 h, grain soaked for 24 h; 1 D, 2 D, 3 D, 4 D, and 5 D, grain germinated for one to five days. The table on the right of the figure lists the percentage decrease in proteinase activity which resulted from the incorporation of the inhibitor.



Inhibitor Effect

Time	% Decrease
Control	1
24 h	1
1 D	7
2 D	13
3 D	13
4 D	26
5 D	10

Fig. 17. Graph of relative proteinase activity of the cultivar Augusta, grown in a greenhouse, with and without 1, 10 phenanthroline. All assays included dimethyl sulphoxide, the inhibitor solvent. Control, ungerminated grain; 24 h, grain soaked for 24 h; 1 D, 2 D, 3 D, 4 D, and 5 D, grain germinated for one to five days. The table on the right of the figure lists the percentage decrease in proteinase activity which resulted from the incorporation of the inhibitor.



Inhibitor Effect

Time	% Decrease
Control	1
24 h	1
1 D	7
2 D	13
3 D	13
4 D	26
5 D	10

Fig. 18. Graph of relative proteinase activity of the cultivar Augusta, grown in a greenhouse, with and without 3,4 dichloroisocoumarin (3,4 DIC). All assays included dimethyl sulphoxide, the inhibitor solvent. Control, ungerminated grain; 24 h, grain soaked for 24 h; 1 D, 2 D, 3 D, 4 D, and 5 D, grain germinated for one to five days. The table on the right of the figure lists the percentage decrease in proteinase activity which resulted from the incorporation of the inhibitor.

soak, but its maximum inhibitory effect was found after one day of germination (38%). Both, 1, 10 phenanthroline (1,10 PA) and 3, 4 dichloroisocoumarin (3,4 DIC) began to have an inhibitory effect some time between the 24 h soak and the first day of germination. Maximum inhibition for 1,10 PA and 3,4 DIC, that is, 26% and 14%, respectively, was found to occur after four days of germination.

Proteinase Effect on Storage Proteins

Figure 19 displays the SDS-PAGE patterns of high molecular weight (HMW) glutenins of the varieties Augusta and Hillsdale. Degradation of these protein subunits began in the Augusta samples after two days of germination. At the same time, several bands below the HMW glutenins became darker. By day five of germination, almost complete loss of the HMW glutenins had occurred in the Augusta grain.

Similar to the Augusta HMW glutenin bands, the band intensity in the Hillsdale gel appeared to decrease after two days of germination. However, throughout the complete period of germination, evidence of HMW glutenin subunits remained. Similar to the Augusta gel, the Hillsdale gel also showed bands in the lower portion of the gel which became darker, as germination progressed.

Unreduced protein extracts, from the cultivars Augusta and Hillsdale, examined using SDS-PAGE, are shown in Figure 20. Protein too large to enter the gel can be seen at the top of both the Augusta and Hillsdale gels. However, after one day of germination of the Augusta grain, a loss in the intensity of the stain at the top of this gel can be seen. This loss continues throughout the period of germination, but evidence for protein remains up to and including day

Fig. 19. SDS-PAGE of reduced HMW-glutenins from the cultivars Augusta and Hillsdale at various stages of germination. A, cultivar Augusta; H, cultivar Hillsdale; lane 1, ungerminated; lane 2, 24 h soak; lanes 3, 4, 5, 6, and 7, 1 through 5 days of germination, respectively; NP, standard Neepawa; arrows, protein bands appearing during germination. Eight microliters of protein extract loaded for NP and twelve for each of the other samples.

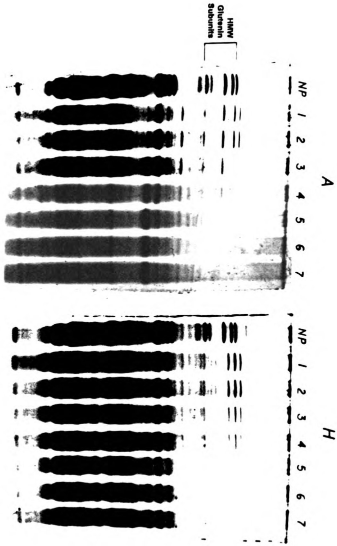
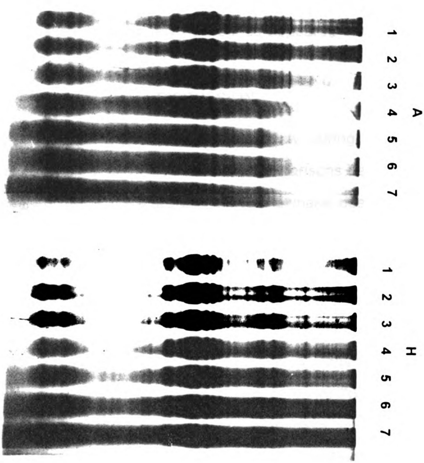

HMW Glutenin Subunits
A
NP 1 2 3 4 5 6 7
H
NP 1 2 3 4 5 6 7

Fig. 20. SDS-PAGE of unreduced HMW-glutenins from the cultivars Augusta and Hillsdale at various stages of germination. A, cultivar Augusta; H, cultivar Hillsdale; lane 1, ungerminated; lane 2, 24 h soak; lanes 3, 4, 5, 6, and 7, 1 through 5 days of germination, respectively. Twelve microliters of protein extract were loaded for each sample.


A
1
2
3
4
5
6
7
H
1
2
3
4
5
6
7

five of germination. The gel containing unreduced protein from Hillsdale samples displays no loss of the protein which did not enter the gel.

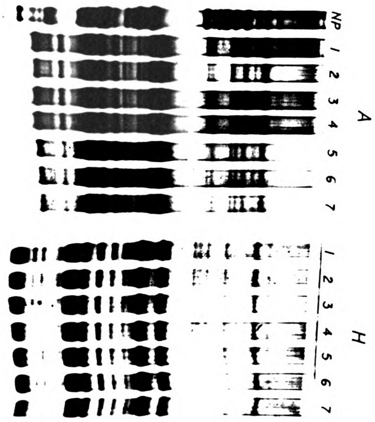
Gliadin proteins from the Augusta and Hillsdale treatments are shown in Figure 21. There appeared to be some degradation of these proteins in the Augusta kernels after three days of germination. From day three to day five, however, there was apparently little change.

In contrast to the Augusta gliadin gel, the Hillsdale gel seems to have maintained the same band intensity during the five days of germination. As found for the Augusta grain, a few new bands appeared in the Hillsdale acid-PAGE gel during germination.

DISCUSSION

Intervarietal differences in proteolytic activity during germination have been reported (Bushuk et al 1971). However, comparisons between the various cultivars examined in the literature are difficult to make due to differences in methodology. For example, Marsh (1988) reported a 1.5-fold increase in proteinase activity of a NaCl extract of two-day germinated wheat. In this case, proteinase activity was measured using azocasein at 25°C and pH 5.8. After eight days of germination, Hwang and Bushuk (1973) found a 17-fold increase in proteinase activity. A two-fold increase in proteinase activity was reported by Lukow and Bushuk (1984) for two cultivars germinated for 70 h using azocasein at pH 6.0 for 2 h at 40°C. In addition to the proteinase activity methods used being different in the studies cited above, rates of germination for the various cultivars used where not reported. Thus adding to the difficulty of making comparisons between the results reported for these studies.

Fig. 21. Acid-PAGE of gliadin proteins from the cultivars Augusta and Hillsdale at various stages of germination. A, cultivar Augusta; H, cultivar Hillsdale; lane 1, ungerminated; lane 2, 24 h soak; lanes 3, 4, 5, 6, and 7, 1 through 5 days of germination, respectively; NP, standard Neepawa; arrows, protein bands appearing during germination. Eight microliters of protein extract loaded for NP and twelve for each of the other samples.


A
NP
1
2
3
4
5
6
7
H
1
2
3
4
5
6
7

The two varieties used for the present study were chosen because of their differences in susceptibility to PHS as supported by the germination index results. Augusta which had limited dormancy had greater proteinase activity than Hillsdale which possess relatively more dormancy. In addition, Chapter Three of this dissertation reports differences found between the proteinase activity of several field-sprouted soft wheat cultivars. The proteinase activity results in the present study and those found in Chapter Three, indicate that genotypes with little resistance to PHS, when exposed to PHS environmental conditions, will have greater activity than those with less resistance.

The pH of the PAGE (with copolymerized substrate) incubation buffers was chosen to approximate the pH optima of wheat proteinases as well as to simulate several situations. These situations included the previously reported pH optima of proteinases in sound wheat (pH 3.8), the pH of wheat endosperm (pH 5.4), and the pH of various soft wheat product doughs and batters (pH 6.5) (McDonald and Chen 1964, Hoseney 1994)

The proteinases which were active, both in the quiescent and germinating grain (B-1 and B-2), are also those which would be most likely to have their activity enhanced during the processing of soft wheat products, such as cakes and cookies (Fig. 11). Possessing pH optima close to neutral suggests that these bands contain serine and/or metallo-proteinases. However, this is not supported by the inhibitor work: crude extracts of the ungerminated Augusta displayed no inhibition by either 3,4 DIC or 1,10 PA. Possible explanations are as follows: (1) either aspartic and/or cysteine proteinase with neutral pH optima are present, (2) the enzymes are serine and/or metallo-proteinases which are not inhibited by the inhibitors employed in this study, or (3) the serine and/or cysteine proteinase activity was so low that the azocasein with inhibitor assays were not sensitive to detect their presence. Aspartic proteinases in general have acidic pH optima, but some, such as renin, act at neutral pH (Barrett 1986). Similarly, cysteine proteinases tend to have acidic pH optima, however, pH optima near seven are also known to exist (e.g., papain). Although 3,4 DIC is said to be a general serine proteinase inhibitor due to its inhibition of many trypsin-like, chymotrypsin-like, and elastase-like proteinases, it has also proven to be ineffective at inhibiting serine proteinases such as papain (Harper and Powers 1985). A similar situation exists for 1,10 PA as well. Thus both number one and two stated above cannot be ruled out; however, examining previous inhibitor work seems to help clarify the situation.

McDonald and Chen (1964) reported that the proteinase activity of ungerminated wheat was not affected by trypsin or chymotrypsin inhibitors, thus suggesting that either serine proteinases were not present, or the reversible protein inhibitors used lacked specificity for the serine proteinase present. Evidence suggesting the presence of serine proteinase in wheat has been provided by Belitz and Lynen (1974). These authors reported that proteinase activity was inhibited by diisopropylfluorophosphate (DFP), a proteinase inhibitor which shows little nonserine proteinase inhibition. However, DFP has also been reported to inhibit plant carboxypeptidases which are known to exist in ungerminated grain (Kruger and Preston 1977). Salgo (1981) has suggested that metallo-proteinases are present in wheat. However, this support was weak at best because only the nonspecific proteinase inhibitor EDTA was used to base this conclusion on. Recently, the first persuasive evidence for the presence of metallo-proteinases and serine proteinases in barley seed has been reported (Wrobel and Jones 1992). Initially using a crude extract separated with PAGE gel copolymerized with gelatin, these proteinases appeared in the same region as B-1 and B-2 in the present study. The activity in ungerminated barley was reported to very low, but increased with germination. Results from serine or metallo-proteinase inhibitor studies were not reported for the ungerminated barley. Further work by these authors included separating the extract from barley germinated for four days, using carboxymethyl cellulose chromatography, and then, with the PAGE system (Wrobel and Jones 1993). Five serine and five metallo-proteinases which were identified using phenylmethylsulfonyl fluoride (serine proteinase inhibitor), DFP, EDTA, and dithiothreitol (a metallo-proteinase inhibitor) possessed a close to neutral pH optimum. If it is assumed that the neutral enzymes, in the four day germinated barley, are the same as the enzymes in the ungerminated barley, then it would appear that serine and metallo-proteinases are present in ungerminated barley. As a result, it seems possible that B-1 and B-2 from ungerminated soft wheat contain serine and/or metallo-proteinases. Perhaps the inhibitors used only showed significant inhibition after an increase in activity was noted for B-1 and B-2 during germination, because, as suggested above, the serine and/or metallo-proteinase activities were too low to measure in the ungerminated wheat.

Along with evidence for serine and/or metallo-proteinase in ungerminated soft wheat discussed above, the inhibitor studies added support to previous work reporting cysteine (Skupin and Warchalewski 1971, McDonald and Chen 1964) and aspartic proteinases (Lin et al 1993, Dunaevskii et al 1990, Kawamura and Yonezawa 1982) in sound wheat.

Regarding the work by Lin et al (1993), it should be mentioned that the aspartic proteinase from soft wheat, partially purified by these authors, had a pH optimum of 4.1. In the present study, no activity was visualized using gelatin as the substrate at pH 3.8 in the quiescent grain. However, using azocasein in the activity assay did show proteinase activity, which was inhibited by an aspartic proteinase inhibitor found to be present in the quiescent wheat samples. This certainly furnishes evidence that substrate specificities exist for wheat proteinases.

The initiation of HMW glutenin subunit mobilization coincided with the increase in proteinase activity determined using the azocasein assay, as well as with the first proteinase activity bands seen on the native PAGE Augusta gel incubated at pH 3.8. These proteinases span a rather large range in mobility on a native PAGE gel, and their acidic pH optima suggest that these bands contain aspartic and/or cysteine proteinase. An interesting question raised by the gel mentioned above is, what is the role of the germinative proteinases present in A-2D, but not in A-3D (B-9 and B-10)? Mayer and Shain (1974) have suggested that proteinase present early in the germination process may have the regulatory function of activating or releasing bound enzymes. Quiescent wheat is known to have cysteine proteinases which make up a large portion of its total proteinase activity and are associated with storage proteins (Kaminski and Bushuk 1969, McDonald and Chen 1964). Perhaps the enzymes in B-9 and B-10 serve to release these cysteine proteinases. This would agree with the finding in this study that cysteine proteinase activity peaks near the second day of germination. Another possibility would be that these proteinases may also have the task of cleaving bonds in proteins, making the proteins better substrates for other proteases present. Thus, these enzymes would only be needed during the early stages of storage protein degradation.

Concomitant, with the largest increase in proteinase activity found using the azocasein assay, between day one and two of germination, was the appearance of the germinative enzymes from the cultivar Augusta on the PAGE gel incubated at pH 3.8, and the beginnings of storage protein mobilization. The inhibitor studies demonstrated that during this time aspartic proteinases are at their lowest point of activity, cysteine proteinase are at their highest level, and serine and metallo-proteinase activities are increasing. Thus, as it has been previously suggested, cysteine proteinases appear to be the "work-horses" of storage protein degradation, beginning some time near the second day of germination (Shutov and Vaintraub 1987).

The role of the aspartic proteinases present in germinating grain appears to be much more controversial. Some have suggested that aspartic proteinases initiate storage protein mobilization prior to the *de novo* biosynthesis of cysteine proteinases (Doi et al 1980, Mikola 1983). Contrarily, Shutov and Vaintraub (1987) have indicated that aspartic proteinases may quicken protein degradation after it is initiated by cysteine proteinases. Recently, a barley grain aspartic proteinase has been found to be related to mammalian lysosomal cathepsin D, which is known to regulate the activity of other enzymes by cleavage of regulatory enzymes (Sarkkinen et al 1992). Thus, the authors have suggested a similar role for aspartic proteinase in cereal grains.

The inhibitor studies on extract from germinating soft wheat in the present investigation, lend support to the findings of Wrobel and Jones (1993, 1992); that is, that serine and metallo-proteinase are present in germinating cereal grains. These authors reported having evidence of one EDTA-inhibited proteinase after one day of germination, which is approximately the same time that the present study using 1,10 PA and 2,3 DIC demonstrated evidence for serine and metallo-proteinase in soft wheat.

The gel containing unreduced Augusta proteins (Fig. 20) suggests that after one day of germination, reduction of glutenins begins. This correlates with work by Kobrehel et al (1992) which found glutenin as well as gliadin reduction in a durum wheat cultivar began after one day of germination. This reduction peaked after two to three days of germination with glutenins experiencing a five-fold increase in reduction and gliadins a two-fold increase. Interestingly, evidence of unreduced glutenins remained even up to five days of germination of Augusta. Why the cultivar Hillsdale appeared to have no loss of unreduced glutenins as seen at the top of the SDS-PAGE gel in Figure 20 is unclear.

A question raised by this study is why the HMW glutenins are apparently cleaved earlier than the gliadins. Are there proteinases whose substrate specificities include only the gliadins, or the glutenins, or is the structure of the glutenins more suited to attack by the germination proteinase? Previous work by Masson et al (1986) suggests the latter may be the case. Their work demonstrated that glutenin was more rapidly hydrolyzed by porcine pepsin, a serine proteinase, than was gliadin. Also, the digests of each were composed of polypeptides differing in molecular weight, amino acid composition, and surface hydrophobicity, thus confirming previous work by Bietz and Rothfus (1970). Masson et al (1986) suggested that differences in peptic cleavage site location, that is, differences in site accessibility in gliadins versus glutenins, could explain these results.

Previous work has suggested that once initially cleaved during germination, storage proteins quickly are reduced to amino acids with little time spent as smaller proteins or peptides (Preston et al 1978, Coulson and Sim 1965). Although this may be true in terms of relative quantities of protein degraded, the present study does demonstrate that small proteins, i.e., those with faster mobility on SDS-PAGE and Acid-PAGE gels (see Fig. 19 and 21), are formed during the germination process presumably as a result of the cleavage of larger proteins. Lukow and Bushuk (1984) reported an increase in intensity of several reduced gliadin bands during germination of wheat, while Ariyama and Khan (1990) found no changes in reduced or unreduced gliadin bands after germination. Attempting to resolve the apparent disagreement between these studies, Ariyama and Khan (1990) suggested that divergences may have resulted from differences in varieties or germination procedure used. The former suggestion would agree with the present study which demonstrates varietal differences in the effect of germination on storage proteins.

Little can be said regarding the differences noted for the PAGE gels with copolymerized gelatin, loaded with extract from the cultivar Augusta versus Hillsdale as well as the differences found in germination influence on these cultivars storage proteins. It could be assumed that the lower proteinase activity found for the cultivar Hillsdale resulted in such investigations as B-9 and B-10 not being evident on the Hillsdale proteinase gel incubated at pH 3.8, no signs of Hillsdale gliadin protein mobilization, and apparently, no change in the amount of unreduced glutenins during five days of germination. However, it is also possible that the presence of different proteinase isoenzymes or differences in the structure of the storage proteins played a role in the differences noted above.

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CHAPTER FIVE

CONCLUSIONS

1. Throughout kernel maturation, proteinase activity was significantly higher in the field-grown grain (FGG) compared to the greenhouse-grown grain (GHG).

2. Alpha-amylase activity was significantly higher in the FGG compared to the GHG at 21 and 28 days post anthesis (DPA), the same at 35 DPA, and greater at 42 DPA.

3. Considering all stages of growth, proteinase activity was positively correlated ($r = 0.875$, $p<0.01$) with alpha-amylase activity. The protein content of the grain was highly correlated ($r=0.930$, $p<0.01$) with the growing conditions (i.e., higher in the GHG compared to the FGG). However, the correlation with proteinase and alpha-amylase activities was much lower ($r=0.287$, $p<0.01$; and $r=-0.310$, $p<0.01$).

4. At harvest maturity, the correlation between proteinase and alpha-amylase activity in the FGG, was weaker ($r = 0.669$, $p<0.05$) than the relationship in the grain during maturation. Also, germination index was correlated with proteinase ($r = 0.553$, $p<0.05$) and alpha-amylase activities ($r = 0.622$, $p<0.05$).

5. The cultivars Hillsdale and P2548 grown in the field and harvested at 42 DPA had significantly less proteinase activity than the other seven cultivars, while Augusta and Lowell had significantly more. The alpha-amylase activity found for the cultivar Lowell was significantly higher than for all other cultivars.

6. In the laboratory-germinated grain, Augusta, the variety which had a greater germination index, displayed greater proteinase activity than Hillsdale, the variety with more dormancy.

7. Using class-specific proteinase inhibitors, soft winter wheat was found to contain cysteine and aspartic proteinase activity during kernel maturation. The cysteine proteolytic activity of aquatic extracts decreased as kernels matured, while the aspartic proteolytic activity increased.

8. Through the use of native polyacrylamide gel electrophoresis (PAGE) with copolymerized gelatin, it also appears likely that mature soft winter wheat contains serine and/or metallo-proteinase.

9. Soft winter wheat aspartic proteinase activity declined as a percentage of total activity from the quiescent stage to the second day of germination; this activity then increased, ending after five days of germination at a lower level than in the quiescent kernel.

10. Soft winter wheat cysteine proteinase activity increased as a percentage of total activity from the quiescent stage to the second day of germination; this activity then decreased until five days of germination. The cysteine proteinases

thus appear to be those responsible for the early stages of storage protein mobilization.

11. Beginning after one day of germination, serine and metallo-proteinase activity began to increase as a percentage of total proteinase activity in germinating soft winter wheat; these activities peaked after four days of germination.

12. Ten bands of proteinase activity were visualized using native PAGE copolymerized with gelatin and crude extracts from quiescent and germinating Augusta kernels. Two bands were present in quiescent grain at close to neutral pH, thus suggesting that the bands contain metallo and/or serine proteinase. The other eight zones which were present during germination had acidic pH optima, indicating that they contain either aspartic or cysteine proteinase.

13. The glutenin storage proteins in germinating soft winter wheat appeared to be degraded sooner during germination than the gliadins.

CHAPTER SIX

FUTURE WORK

Much remains to be understood about the proteinases in wheat. Answering the questions still unanswered rests on initially purifying and characterizing each of the classes of proteinases found in wheat during the present study. Determining their location in the kernel during the various stages of the life cycle would then lead to understanding the role(s) these enzymes play in maturing and germinating wheat. With this information in hand, various applications for the food industry could arise which could be exploited by using the tools of biotechnology to produce wheat proteinases. A specific example that comes to mind is saltine cracker production. Endogenous proteolytic enzymes in flour are thought to be important to the desired changes the rheological properties of cracker doughs undergo during fermentation. Although commercial preparations of fungal and bacterial proteolytic enzymes have been used to try to reduce fermentation times during cracker processing, none have been completely successful. This situation could perhaps be improved with the availability of proteinases whose substrate specificities include the wheat proteins which are cleaved during cracker dough fermentation.

Having determined in this study that genotypic differences exist for proteinase activity in preharvest sprouted grain, a next step would be to determine the effect of these differences on the quality of various soft wheat products.

APPENDIX A

Varieties Used to Investigate Enzyme Activity in Soft Winter Wheat

Genotype	Pedigree	Grain Color	Origin
Cardinal	Virginia 635212 x Logan//Blue boy x (Logan x 2)	Red	Ohio
Hillsdale	Asosan/Genesee *4//VA 66-54-10	Red	Michigan
Twain	Knox 62/SRW 14-74	Red	Indiana
Mendon	(Genesee/Winoka,xo467)/5/(B2141, (Suwon 92/Brevor/2/5*Genesee, A6506)/4/(A4528, Norin 10/Brevor/2/Yorkwin/3/Genesee*4/2/Norin 10/Brevor))	Red	Michigan
P2548	Hadden*2/4/Georgia 1123 /3/Norin 10/Brevor/2/Tenmarq/5/(Vigo/Clarkan/2/Norin 66, M06582/3/Redcoat/6/Coker 68-15/5/(M07910, Etoile de Choisy/2/Thorne/Clarkan/4/Pawnee/3/(Pd3848A5-5-26, Citr12454, Trumbull/W38/2/Fultz/Hungarian))	Red	Indiana
Augusta	Genesee/Redcoat B2747//Yorkstar	White	Michigan
Frankenmuth	Norin 10/Brevor 14//Yorkwin/3/2 *Genesee A3141/4/Genesse *3/Redcoat A5115	White	Michigan
Geneva	Burt/5/Genesee/4/Frondoso/3/Trumbull//Hope/Hussa R/6/Ross/7/Genesee	White	New York
Lowell	(Genesee/Winoka,xo467)/5/(B2141, (Suwon 92/Brevor/2/5*Genesee, A6506)/4/(A4528, Norin 10/Brevor/2/Yorkwin/3/Genesee*4/2/Norin 10/Brevor))	White	Michigan

APPENDIX B

Tabulated Data

Moisture Content of Soft Winter Wheat Grown in Dansville, Michigan, in 1992

Wheat Cultivar	21 DPA[a]	28 DPA	35 DPA	42 DPA
	Moisture (%)			
White				
Augusta	59.27	44.98	39.66	32.39
Frankenmuth	58.99	47.08	42.50	32.66
Geneva	55.14	49.05	41.03	33.46
Lowell	57.99	50.09	39.38	23.38
Red				
Cardinal	56.89	46.29	40.11	30.60
Hillsdale	56.11	43.56	41.08	30.90
Mendon	57.27	51.75	40.40	30.93
P2548	55.81	47.06	38.34	25.56
Twain	58.84	48.92	42.96	27.21

[a] Days post anthesis

Moisture Content of Soft Winter Wheat Grown in a Greenhouse

Wheat Cultivar	Moisture (%) 21 DPA[a]	28 DPA	35 DPA	42 DPA
White				
Augusta	52.36	43.43	18.99	11.51
Frankenmuth	50.37	43.96	18.61	10.60
Geneva	53.98	44.56	21.93	11.85
Lowell	51.56	47.45	27.56	11.82
Red				
Cardinal	51.55	43.22	26.29	11.80
Hillsdale	48.20	44.08	21.68	11.28
Mendon	50.81	46.12	34.14	11.32
P2548	56.72	48.21	26.53	11.08
Twain	56.37	47.88	30.77	11.74

[a] Days post anthesis

Protein Content of Soft Winter Wheat Grown in Dansville, Michigan, in 1992

Wheat Cultivar	21 DPA[b]	28 DPA	35 DPA	42 DPA
	Protein (%)[a]			
White				
Augusta	9.58	9.74	10.28	10.32
Frankenmuth	10.18	10.23	10.51	10.76
Geneva	11.03	11.89	11.42	11.98
Lowell	10.02	9.98	9.50	9.94
Red				
Cardinal	9.43	9.79	10.28	10.73
Hillsdale	10.23	9.99	11.64	10.00
Mendon	10.21	9.78	9.55	9.77
P2548	9.14	9.67	9.71	10.12
Twain	10.46	12.05	11.66	11.69

[a] Values are means on a dry wt basis

[b] Days post anthesis

Protein Content of Soft Winter Wheat Grown in a Greenhouse

Wheat	Protein (%)[a]			
Cultivar	21 DPA[b]	28 DPA	35 DPA	42 DPA
White				
Augusta	14.81	15.49	17.39	16.87
Frankenmuth	15.22	15.64	17.53	17.65
Geneva	15.43	16.59	17.43	17.54
Lowell	16.44	17.37	18.17	18.12
Red				
Cardinal	17.54	16.56	18.19	18.31
Hillsdale	14.34	16.22	18.04	18.07
Mendon	16.94	18.66	19.36	18.88
P2548	14.67	14.80	16.01	17.04
Twain	19.29	20.25	20.62	21.14

[a] Values are means on a dry wt basis

[b] Days post anthesis

Alpha-Amylase Activity in Soft Winter Wheat Grown in Dansville, Michigan, in 1992

Wheat Cultivar	Alpha-Amylase Activity (DU)[a,b] 21 DPA[c]	28 DPA	35 DPA	42 DPA
White				
Augusta	1.84	0.52	0.09	0.38
Frankenmuth	2.05	0.63	0.09	0.08
Geneva	1.46	0.45	0.13	0.29
Lowell	1.03	0.34	0.08	1.31
Red				
Cardinal	1.32	0.46	0.10	0.11
Hillsdale	1.87	0.57	0.13	0.07
Mendon	1.47	0.45	0.07	0.23
P2548	1.08	0.34	0.07	0.06
Twain	1.33	0.33	0.19	0.15

[a] Dextrinizing Units

[b] Values are means on a dry wt basis

[c] Days post anthesis

Alpha-Amylase Activity in Soft Winter Wheat Grown in a Greenhouse

Wheat	Alpha-Amylase Activity (DU)[a,b]			
Cultivar	21 DPA[c]	28 DPA	35 DPA	42 DPA
White				
Augusta	0.74	0.15	0.10	0.11
Frankenmuth	0.78	0.17	0.10	0.10
Geneva	1.28	0.26	0.11	0.11
Lowell	0.81	0.21	0.10	0.09
Red				
Cardinal	0.71	0.23	0.10	0.10
Hillsdale	0.67	0.16	0.10	0.10
Mendon	0.77	0.27	0.16	0.09
P2548	1.03	0.30	0.08	0.09
Twain	1.16	0.40	0.13	0.16

[a] Dextrinizing Units

[b] Values are means on a dry wt basis

[c] Days post anthesis

Proteinase Activity in Soft Winter Wheat Grown in Dansville, Michigan, in 1992

Wheat Cultivar	Proteinase Activity [a,b]			
	21 DPA[c]	28 DPA	35 DPA	42 DPA
White				
Augusta	3.451	1.546	1.233	1.089
Frankenmuth	3.218	1.478	1.210	0.942
Geneva	3.647	1.629	1.064	0.948
Lowell	3.018	1.401	1.137	1.049
Red				
Cardinal	2.733	1.493	1.026	0.899
Hillsdale	3.895	1.574	1.067	0.909
Mendon	3.239	1.382	1.135	0.962
P2548	3.262	1.387	1.096	0.898
Twain	2.873	1.389	1.292	0.983

[a] Absorbance at 366 nm

[b] Values are means on a dry weight basis

[c] Days post anthesis

Proteinase Activity in Soft Winter Wheat Grown in a Greenhouse

Wheat Cultivar	Proteinase Activity [a,b] 21 DPA[c]	28 DPA	35 DPA	42 DPA
White				
Augusta	2.479	1.258	0.900	0.722
Frankenmuth	2.325	1.210	0.824	0.719
Geneva	2.928	1.324	0.999	0.771
Lowell	2.672	1.330	0.909	0.742
Red				
Cardinal	2.285	1.300	0.981	0.788
Hillsdale	2.152	1.275	0.908	0.695
Mendon	2.615	1.457	1.088	0.787
P2548	2.585	1.476	0.957	0.782
Twain	2.371	1.335	0.999	0.757

[a] Absorbance at 366 nm

[b] Values are means on a dry weight basis

[c] Days post anthesis

Germination Index for Soft Winter Wheat Grown in Dansville, Michigan, in 1992

Wheat		Germination Index[a]		
Cultivar	21 DPA[b]	28 DPA	35 DPA	42 DPA
White				
Augusta	0	10	70	290
Frankenmuth	0	0	7	257
Geneva	4	14	60	308
Lowell	3	1	55	313
Red				
Cardinal	0	1	34	200
Hillsdale	0	0	2	82
Mendon	0	1	50	235
P2548	0	0	8	91
Twain	0	0	2	95

[a] Germination Index is the number of kernels which germinated during 5 days, with greater weight given to those germinating earlier

[b] Days post anthesis

Germination Index for Greenhouse Grown Soft Winter Wheat

Wheat		Germination Index[a]		
Cultivar	21 DPA[b]	28 DPA	35 DPA	42 DPA
<u>White</u>				
Augusta	3	5	1	36
Frankenmuth	0	1	0	0
Geneva	0	1	2	1
Lowell	3	3	0	1
<u>Red</u>				
Cardinal	1	2	0	1
Hillsdale	0	1	0	0
Mendon	1	3	0	8
P2548	0	0	0	0
Twain	1	1	0	1

[a] Germination Index is the number of kernels which germinated during 5 days, with greater weight given to those germinating earlier

[b] Days post anthesis

Analysis of variance of alpha-amylase activity in nine soft winter wheat cultivars grown in Dansville, Michigan, 1992, and harvested at 42 days post anthesis

Source of Variance	DF	Sum of Squares	Mean Square	F-Ratio	P
Variety	8	4.982	0.623	4.969	0.001
Block	1	1.210	1.210	9.656	0.005
Error	26	3.258	0.125		

Analysis of variance of proteinase activity in nine soft winter wheat cultivars grown in Dansville, Michigan, 1992, and harvested at 42 days post anthesis

Source of Variance	DF	Sum of Squares	Mean Square	F-Ratio	P
Variety	8	0.121	0.015	10.385	0.000
Block	1	0.016	0.016	11.233	0.002
Error	26	0.038	0.001		

APPENDIX C

Germination Index Formula

(Walker-Simmons 1987 - Chapter Three)

Germination Index = $(5 \times n1 + 4 \times n2 + 3 \times n3 + 2 \times n4 + 1 \times n5)$

Where $n1$, $n2$, $n3$, $n4$ and $n5$ are the number of seeds germinated on the first, second and subsequent days until the fifth day, respectively. The numbers 5, 4, 3, 2 and 1 are the weights given to the number of seeds germinated on the first, second and subsequent days, respectively.

SDS-PAGE Protocol

(Ng and Bushuk 1987 - Chapter Four)

Glutenin Extraction

Let sit at room temperature 40 g of sample plus 1 ml extraction buffer. Vortex periodically. Heat in boiling water for 2.5 min. Aliquots of the supernatant are used as the protein extract.

Extraction buffer stock solution

glycerol	20.0 ml
stacking-gel buffer (below)	12.5 ml
MilliQ water	24.1 ml
SDS	4.0 g
Pyronin Y	20.0 mg

Extraction buffer (made just prior to use)

MilliQ water	24.0 ml
extraction buffer stock solution	10.2 ml
mercaptoethanol	1.8 ml

Vertical electrophoresis unit (140 mm x 160 mm x 1.5 mm)

	Per Two Gels (ml)
Separating Gel	
Acrylamide (35%)	49.30 0
Bisacrylamide (2%)	3.880
Separating-gel buffer (1 M Tris, pH 8.8)	37.600
(Degas)	
SDS solution (10%)	1.000
Ammonium persulfate (1%)	2.500
TEMED	0.050
Stacking Gel	
Acrylamide (35%)	1.710
Bisacrylamide (2%)	0.430
Stacking-gel buffer (1.0 M Tris, pH 6.8)	2.500
MilliQ water	14.400
(Degas)	
Ammonium persulfate (1%)	0.750
N,N,N',N'-Tetramethylethylenediamine (TEMED)	0.015

Running Buffer (pH 8.3)

Tris	24.228 g
Glycine	115.308 g
SDS solution (10%)	80 ml
MilliQ water	complete to 8 L

Electrophoresis Running Conditions

8 µl of sample extract

4°C

5 mA per gel for 2 h, then 10 mM per gel for 18 h, then 15 mM per gel for 2 h

Gel Rinsing

Agitate gel in rinsing solution for 1 h, drain, repeat 2 times

Rinsing Solution

TCA (100%)	100 ml
Methanol	330 ml
MilliQ water	570 ml

Acid-PAGE Protocol

(Ng et al 1990 - Chapter Four)

Gliadin Extraction

100 mg of sample plus 200 μl of 70% ethanol, vortex briefly. Let sit at room temperature for 15 min. Centrifuge for 2 min, at 10,000 x G, at room temperature. Remove supernatant and add 1.25 times its volume with dilution solution (0.25% w/v aluminum lactate, adjusted to pH 3.1 with lactic acid, 50% w/v sucrose and 3% w/v methyl green).

(Lafiandra and Kasarda 1985 - Chapter Four)

Apparatus

Vertical electrophoresis unit (140 mm x 160 mm x 1.5 mm)

Solutions

A: Acrylamide (28%) and bisacrylamide (1.2%)

B: Potassium hydroxide (3.5 g) and lactic acid (25 ml of 85%) per 100 ml

C: Aluminum lactate (6.25 g) and lactic acid (10 ml of 85%) per 100 ml

D: Silver nitrate (17 mg per ml)

E: Ammonium persulfate (90 mg per 100 ml)

F: Hydrogen peroxide (100 μl of 30% H_2O_2 diluted to 3 ml)

G: Solution A (17 ml), solution B (2 ml), ascorbic acid (20mg), and ferrous sulfate (2.5 mg) per 100 ml

Separating Gel	Per Two Gels (ml)
Solution A	20.00
Solution B	1.60
Solution D	0.80
Solution E	40.00
MilliQ water	complete to 80 ml
(Degas 2 min)	

Stacking Gel

Solution G (Thaw)	10.000
Solution F (Degas)	0.015

Running Buffer

50-fold dilution of solution B	5 L

Electrophoresis Conditions

Prerun stacking gels for 1 h at 4°C and 45 mM Amp per gel

Use 0.5 L running buffer in upper chamber and 4.5 L in the lower chamber. Discard the upper chamber buffer after prerun.

After pouring stacking gel, rinse with solution C (diluted 50-fold and adjusted to pH 3.1 with lactic acid)

Load 7-25 μl aliquot of protein extract

PAGE with Copolymerized Substrate Protocol

(Wrobel and Jones 1992 -Chapter Four)

Apparatus

mini-gel with 1.5 mm spacers

Separating Gel	Per Two Gels (ml)
Acrylamide (30%) and bisacrylamide (1%)	7.420
1.5 M Tris buffer, pH 8.8	5.060
Substrate (1%)	2.020
MilliQ water	5.730
Ammonium Persulfate (10%)	0.045
(Degas)	
TEMED	0.025

Stacking Gel	
Acrylamide (30%) and bisacrylamide (1%)	1.000
0.5 M Tris buffer, pH 6.8	1.250
MilliQ water	7.750
Ammonium persulfate (10%)	0.100
(Degas)	
TEMED	0.050

Running Buffer

0.025 M Tris, 0.192 M Glycine, pH 8.5

Electrophoresis Running Conditions

25 µl of sample buffer (1 M Tris, pH 6.8, 2% sucrose, trace bromophenol blue) and crude extract (1:1)

10 mA per gel, 4.5 h, at 4°C

Gel Incubation Conditions

0.1 M acetate buffer (pH 3.8), 2 mM cysteine,15 h, 40°C

0.1 M acetate buffer (pH 5.4), 2 mM cysteine,18 h, 40°C

0.1 M phosphate buffer (ph 6.5), 2 mM cysteine,18 h, 40°C

Gel Staining

1 h in 0.1% amido black in acetic acid:methanol:water (10:30:60)

Gel Destaining

6 h (3 changes) in acetic acid:methanol:water (10:30:60)