SOME ASPECTS OF NITROGEN METABOLISM OF WHEAT WITH SPECIAL REGARD TO NITRATE REDUCTASE AND PROTEASE SYSTEMS EFFECTS ON GRAIN PROTEIN

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CHAPTER I

INTRODUCTION

The research reported in this dissertation is divided into four chapters. Each chapter is a manuscript prepared for publication in a professional journal. The manuscripts appear as they will be submitted to the journals for publication, except for modifications to comply with publication standards.

The world at the present time is facing a need for additional protein. Many areas of the world are faced with a critical shortage of protein. Since wheat is a major crop throughout the world, an increase in the levels of protein in wheat could be a major step toward procuring the protein needed. With the discovery of high protein genotype in the variety, 'Atlas,' by Middleton (1954) the possibility of high grain protein development exists. However, the efficiency of breedings for high grain protein is difficult to measure due to the large influence environment has on the protein character. The second chapter in this dissertation presents information on the inheritance and the relationship of nitrate reductase and protease levels to grain protein. If it is found that NR and proteases are regulated by simpler genetics, then these enzymes could be used as breeding tools to improve grain protein. The third chapter involves a continuation of this research, using ten cultivars of wheat differing in grain protein. A major objective of this study was to find the sampling period when the best correlations between

nitrate reductase, protease and grain protein occur. In the fourth chapter the characteristics of leaf protease are examined. These enzymes are involved in degradation of protein in the vegetative portion of the plant; thus, proteases would be associated with potential levels of nitrogenous material available to the grain. A characterization study would provide a better understanding of the nature of these enzymes. If differences in protein genotypes could be detected in young seedlings, screening for high protein genotypes could be done more rapidly. The fifth chapter deals with seedling testing for enzyme levels as well as the responses of nitrate reductase and protease activities to different nitrogen sources and temperature.

CHAPTER II

INHERITANCE OF PROTEASES IN VEGETATIVE PARTS OF WINTER WHEAT PLANTS AND THEIR RELATIONSHIP TO GRAIN PROTEIN PRODUCTION[1]

Abstract

Heritability estimates were calculated for protease 4 and 7, alpha amino nitrogen, water soluble protein, grain and forage nitrogen and grain yield using F_2 generation from a cross of a high and low protein parents.[2] Protease 7 and forage nitrogen had high heritability estimates and could possibly be used as tools in breeding for high grain protein.

Correlation coefficients were calculated and numerous correlations between protease 7 and other factors of nitrogen metabolism were found.

The F_2 plants were classified according to protein content as high, medium or low. Protein heritability based on regression of F_3 progeny and F_2 plants was low, however, the high protein class did contain the F_3 lines with the highest protein content and suggests that selection in F_2 for protein content might be successful.

The correlation coefficients based on F_3 lines revealed a negative

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[1]Article coauthored with L. I. Croy for submission to Crop Science for publication.

[2]Abbreviations used in this paper: NR-nitrate reductase, WSP-water soluble proteins.

relationship between nitrate reductase and protease. Additional Key Words for Indexing: Nitrate reductase, Heritability coefficients, Grain nitrogen.

Introduction and Literature Review

One of the major problems facing the world is a shortage of protein. Since wheat is a staple in the diet of a large proportion of the world's population, an increase in wheat protein would be significant. Early research on breeding for high grain protein in wheat indicated that grain protein was negatively correlated with grain yield (Clark, 1926). The data indicated that inheritance of grain protein was as complex as the inheritance for yield. In a study on protein content of 13 soft red winter wheats, large varietal differences were found (Middleton, et al., 1954). Cultivars having 'Frondosa' or 'Frontiera' in their parentage were significantly higher in both protein and yield than the standard cultivars. Considerable variation in protein content was found in populations from crosses of 'Wichita' and 'Atlas 66' in the F_2 generation when compared to parental populations (Stuber, et al., 1962). Several F_2 plants exceeded the protein value found in the high protein parent, suggesting that the low protein parent may have contributed a gene or genes for high protein potential absent in Atlas 66. Consequently polygenic control of protein was suggested with no indications of a preponderance of dominant genes for either high or low grain protein. Grain protein heritability estimates of .678 to .827 were obtained depending on the method of calculation.

Other workers have calculated heritabilities for grain protein. Heritability estimates of .54 to .69 for grain protein were calculated

for soft winter wheat crosses derived from Atlas 66 parentage (Davis, et al., 1961). The ratio of population means to parental means indicated partial dominance for low protein content. Lofgen, et al., (1968) using crosses of Atlas 66 with 'Kaw' and 'Triumph' estimated a three or four gene difference for protein content in the parents and intergeneration regression heritability estimates ranging from .374 to .636 were calculated. Heritability estimates ranging from .37 to .70 were obtained by Lebsock, et al., (1964). Frequency distribution of F_3 lines from high and low protein crosses were constructed and protein content was found to be distributed normally over the range of protein present in the parents. Broad sense and narrow sense estimates of heritability of .56 and .28 respectively for Atlas 66 x 'Comanche' populations grown under greenhouse conditions were found by Haunold, et al., (1962).

Plant growth and yield are the result of a series of biochemical reactions present in the plant each of which is catalyzed by specific enzymes (Hageman, et al., 1968). If these enzymes can be isolated and their control mechanism determined, then selection for genotypes superior for a particular enzyme would hasten development of superior genotypes for complex traits such as grain protein.

Nitrate reductase (NR) (E.C.1.6.6.1.) is an enzyme whose study could produce valuable insight into the complex metabolism involved in grain protein production. Nitrate reductase is appropriate because it is known to be (A) the first enzyme in the pathway of nitrate reduction (Kessler, 1964); (B) substrate inducible (Afridi, et al., 1965, Beevers, et al., 1965); (C) labile <u>in vivo</u> under environmental stress (Mattas and Pauli, 1965); (D) variable in activity levels both diurally and seasonally (Hageman, et al., 1961); (E) related to total reduced nitrogen

accumulated in the plant (Schrader, et al., 1968); (F) associated with increased protein formation and decreased nitrate content (Hageman, et al., 1961); and (G) linearly related to total grain protein production within a genotype (Croy and Hageman, 1970).

In a study of nitrate reductase, Hageman, et al., (1961) found that the diurnal variation in NR was correlated positively with water soluble protein (WSP) content and negatively with nitrate content. However, Zieserl, et al., (1963) found no overall correlation between NR and WSP content, although seasonal protein content paralleled NR activity with a 7 to 10 day lag period.

The relationship between NR and grain protein was studied by several workers. Deckard, et al., (1973) working with corn found that NR activity of the total leaf canopy, expressed as seasonal average activity or converted into seasonal input of reduced nitrogen, showed a significant positive correlation with grain protein. The highest correlation between NR activity and yield of grain protein was obtained during the ear initiation and development stage.

A correlation coefficient of .856 between NR activity and WSP content in winter wheat was found by Harper and Paulsen (1967); and, NR activity decreased as the plants approached maturity. This decrease was associated with a decrease in nitrate uptake; although, tissue aging and high temperature could have been causal agents.

Zieserl and Hageman (1962) evaluated 47 inbred lines of corn for NR activity and discovered that some inbreds had enzyme levels up to five times higher than that found in other inbreds. Also, significant differences in nitrate and WSP contents were found; however, no positive correlation was found between NR activity and WSP or negative

correlation between NR and nitrate content. Differences were noted in the seasonal pattern of NR which could be associated with different genetic backgrounds.

Zieserl, et al., (1963) working with four corn hybrids and their parents found that seasonal mean NR levels conformed to a generally additive mode of inheritance. Schrader, et al., (1966) ranked corn inbred lines as low or high with regard to seasonal mean levels of NR. Crosses were made among the high x high, high x low, and low x low lines. None of the high x high F_1 hybrids showed higher enzyme activity than the midparent value. The high x low F_1 hybrids had activities intermediate to the parental inbreds. Within the low x low F_1 hybrids two exhibited heterosis while others were not significantly different from the midparent value. A two-locus system with dominance was suggested by Warner, et al., (1969) as the control of NR inheritance in corn.

Croy and Hageman (1970) found that there was a significant positive correlation between input of reduced nitrogen and the accumulation of grain protein in two wheat cultivars differing in grain protein content. The high protein cultivar, Ponca, had higher WSP content and NR activity than the low grain protein cultivar, Monon.

Estimates of heritability up to .717 for NR on a single date were found by Duffield, et al., (1972). However, NR activity was found to be greatly influenced by physiological stage of development and environmental factors. Pooled heritability estimates of .290 were obtained when data were pooled across dates.

Johnson, et al., (1968) stated that high grain protein was not associated with differential nitrogen uptake or nitrogen accumulation in the plant. Evidence points to more efficient and complete translocation

of nitrogen from the other parts of the plant to the grain as the physiological basis of high grain protein. Plants of 'Warrior,' a low grain protein cultivar, contained significantly more nitrogen in the forage than the high protein selection, NE 65305, but this nitrogen was not translocated to the grain, implicating translocation as a probable major factor in grain protein accumulation.

Seth, et al., (1960) working with wheat found no differences in the nitrogen content of the vegetative parts of high and low protein cultivars. However, the data indicated a more rapid transfer of nitrogenous materials from the vegetative parts to the heads of the high protein cultivars. Protease, the enzyme responsible for the breakdown of proteinaceous materials, was found to have higher activity in high protein wheat than in low protein wheat after flowering (Rao and Croy, 1972). Low protein cultivars had higher leaf protease activity before flowering but were surpassed by the high protein cultivar after flowering.

Among three rice cultivars with similar grain yield, the cultivar with highest protein content tended to translocate more leaf nitrogen to the developing grains than the rice with average grain protein content (Perez, et al., 1973). This high protein cultivar had higher protease activity in the leaves than the lower protein cultivar.

The objectives of this study were to determine (A) the heritability of protease, NR, WSP, Alpha Amino Nitrogen, and grain and forage nitrogen; (B) the relationship among these variables; (C) the relationship of these variables to grain protein in wheat.

Materials and Methods

Experiment One

This study was conducted on Kirkland silt loam soil at the Agronomy Research Station, Stillwater, Oklahoma. The parents, NE 65679 and D145B4, and F_2 seeds were provided by Dr. E. L. Smith from greenhouse crosses. NE 65679, a selection from a cross between Atlas 66 and Comanche, was obtained from Dr. V. A. Johnson, U. S. D. A. A. R. S., Agricultural Experiment Station, Lincoln, Nebraska. D145B4 is a 'Triumph-type' hard red winter wheat tracing to breeder's samples bequeathed to Oklahoma State University by Joseph E. Danne. D145B4 is early in maturity, well adapted to Oklahoma and exhibits intermediate protein content. NE 65679 is later in maturity, less adapted to Oklahoma conditions and has higher protein content than D145B4. These seeds were planted on November 1, 1971, and blocked by sampling date. On January 18, 1972, two additional blocks were planted.

Within the blocks, an area of 3.35 m^2 was plotted at 0.305 meter intervals in a checkerboard manner. One seed was planted at each 0.305 meter mark in a randomized pattern with regard to genotype. Each block contained 24 F_2 plants, and 4 of the high and low protein parent randomly placed. Border plants were planted around the blocks in an attempt to equalize competition between test plants. Extra rows of space planted plants of each genotype were planted and these plants were used when a plant was missing within a block.

The plots were fertilized with the equivalent of 20.0-21.8-0 Kg/ha N-P-K preplant and topdressed with 67.2 Kg/ha of ammonium nitrate on March 16th. One gram samples of leafy material were sampled between

8-9 A.M. January 12, April 14, May 19, May 25, and June 1, 1972, and packed in ice.

The January 12th date represents a period of winter dormancy with little growth; April 14th, a period of rapid growth just preceding inflorescence initiation, May 19th and 25th, periods of rapid enlargement of kernels and rapid translocation of materials to developing kernels; and June 1st, a period of senescence of the vegetative parts of the plant and terminal stage of grain development. The May 25th and June 1st dates were sampled from blocks planted on January 18th.

Seven ml of a 25mM K_2HPO_4, 5mM EDTA, 2mM cysteine solution were added per gram of plant tissue and homogenized for two minutes in a motorized Thomas homogenizer. The homogenate was strained through a double layer of cheesecloth and the suspension cleared by centrifugation at 0°C. The cleared solution was decanted and used as a crude extract for all tests.

Protease activity was measured by the method of Kuo and Yang (1966) with some modifications. Assay tubes contained 2.0 mls of 0.5% bovine hemoglobin (Sigma Chem. Co.) dissolved in the citrate phosphate buffers. The pH 4 solution contained 15.4 mM citric acid and 16.5 mM sodium phosphate; the pH 7 solution contained 3.3 mM citric acid and 21.8 mM sodium phosphate. pH levels of 4 and 7 (protease 4 and 7, respectively) were based on protease pH optima determined previously by Rao (1971). Crude enzyme extract (0.2 ml) was added to the buffered hemoglobin solution in two tubes. Immediately 2.2 ml of 10% trichloracetic acid (TCA) were added to one tube to precipitate protein and inactivate the protease enzymes. The assay tube (NO TCA) and the inactivated tube (blank) were incubated at 40 C for two hours and then the reaction was

terminated in the assay tube by the addition of 2.2 ml of 10% TCA. Both the tubes were then centrifuged at 1000 x g for 10 minutes to sediment the undigested hemoglobin. The supernatant was decanted and saved for protein determination by the method of Lowry, et al., (1951). Protease activity per hour was measured as the difference in digested nitrogenous materials between the assay and blank. Bovine serum albumin was used to standardize the protein test.

WSP of the crude enzyme preparation was determined on 5% TCA precipitable material by Lowry, et al., (1951) procedure. Nitrate content was determined by the method of Woolley et al., (1960). Alpha amino nitrogen was estimated by the procedure of Yemm and Cocking (1955) on the crude extract.

Forage and grain nitrogen were determined on oven dry mature tissue by micro-kjeldahl procedure. NR activity was determined on the last three sampling dates by the method of Croy and Hageman (1970).

Estimates of heritability were calculated according to formula used by Burton (1951):

$$H = \frac{VF_2 - \frac{(Vp_1 + Vp_2 + VF_1)}{3}}{VF_2}$$

H--broad sense heritability

Vp_1--variance of one parent

Vp_2--variance of other parent

VF_1--variance of F_1 population

VF_2--Phenotypic variance of F_2 (estimate of total variance)

$\frac{(Vp_1 + Vp_2 + VF_1)}{3}$ mean phenotypic variance of nonsegregating population. Since F_1 generation was not present in this experiment, its variance term was deleted and the sum of Vp_1, Vp_2 was divided by two.

Multivariant analyses of variance were performed on data pooled across dates as well as on a single date basis and correlation coefficients adjusted for genotype were determined. The multivariate analysis of variance program contained in the statistical analysis system designed by Barr and Goodnight (1972) was used to determine the adjusted correlation coefficients. All correlation coefficients used in this study are adjusted for genotype and the pooled coefficients were adjusted for genotype and genotype by date. Since the adjusted correlation coefficients were expected to be rather low due to the multiplicity of factors which can affect the protein character and due to the preliminary nature of the research it was decided the ten percent level of probability would be used in evaluating adjusted correlation coefficients.

Experiment Two

The F_3 seeds were obtained from F_2 plants of the previous experiment. The F_2 individuals were selected first on the basis of grain yield per plant and then on the basis of protein content as determined by the method described by Udy (1956). After protein analysis, seeds from F_2 plants were grouped into three classes based on protein content. The high protein group had protein percentages ranging from 17.13 to 18.99, the medium group ranged from 15.56 to 16.64, and the low group ranged from 13.84 to 15.23. Each group contained ten F_3 lines. The entries within each group were planted in two row plots in a randomized pattern in three replications on September 6th. A seeding rate of 6.4

gm per 3.05 m row was used. The soil type and fertilization was the same as for the first experiment except 100.8 Kg/ha ammonium nitrate were applied on March 15th.

Replication one was sampled on April 11 and May 11, replication two on April 13 and May 14, and replication three on April 18 and May 16. Sampling techniques were similar to the previous experiment except that samples were taken from five to six locations within the left row of the plot in order to sample genetic diversity within the selections. These subsamples were combined for enzyme extraction. Grinding and extraction techniques were the same as in the previous experiment except that the samples were ground in a Virtis 45 homogenizer at medium speed for one minute. Analytical techniques for protease 4, 7, WSP, NR, nitrate and forage protein were the same as in the previous experiment, however, alpha amino nitrogen was measured by the method of Moore and Stein (1948).

Weather conditions were quite different in the two years in which this study was conducted. The first year was extremely dry with high temperatures in mid-April which caused sterility of many florets. The second year was extremely wet particularly in March, and cool temperatures prevailed throughout the spring. Precipitation data for the two years are shown in Table I.

TABLE I

PRECIPITATION FOR CROP YEARS 1971-1972 AND 1972-1973 AT AGRONOMY RESEARCH STATION, STILLWATER, OKLAHOMA

Month	1971-1972	1972-1973
	Millimeters	
September	184.87	64.36
October	57.18	126.41
November	14.36	96.41
December	73.07	34.61
January	2.05	83.08
February	3.59	30.77
March	26.92	198.20
April	59.49	88.20
May	65.13	82.05
Total	486.66	824.09

Results and Discussion

Experiment One

The estimates of heritability for seven variables are shown in Table II. Pooled heritabilities for protease 7 (.638) and forage nitrogen (.673) were high enough to be important criterion in a breeding program. However, it must be remembered that the parents did not differ significantly for these variables so higher heritabilities would be expected than if significant differences occurred between the parents. The heritability coefficients for yield and grain nitrogen were both negative and best estimated as zero. These values were extremely low compared to values calculated by Davis, et al., (1961). Sunderman, et al., (1965) found low heritability values for grain protein content and stated that selection for this characteristic in the F_2 would be ineffective.

On a pooled basis, heritability estimates for protease 4, alpha amino nitrogen, and WSP were low. However, on an individual date basis, heritability estimates were high for protease 4 on April 14; for alpha amino nitrogen on Jan. 12, May 19, and May 25; and for WSP on Jan. 12 and May 25. The extreme variation in heritability coefficients was a reflection of the large environmental influence, small sample size, and possibly growth patterns of the parents. D145B4 selection matured earlier than NE 65679. An interesting feature of these data was the fact that for most variables a large proportion of the variance within the parents could be associated with the high protein parent. This would suggest that the high protein parent was not as homogeneous as the low protein parent. If this were the case then the estimates of

TABLE II

BROAD SENSE HERITABILITY COEFFICIENTS FOR PROTEASE 4 AND 7, ALPHA AMINO NITROGEN, WATER SOLUBLE PROTEIN, GRAIN NITROGEN, FORAGE NITROGEN, AND YIELD FOR CROP YEARS 1971-1972

Variable	Date					
	Jan. 12	April 14	May 19	May 25	June 1	Pooled
Protease 4	-539.00*	57.61	40.81	-38.46*	-18.74*	12.62
Protease 7	36.56	60.72	74.72	74.78	63.12	63.82
Alpha Amino Nitrogen	72.49	-----[1]	74.77	79.28	47.29	3.59
Water Soluble Protein	85.39	0.0	14.61	68.68	-51.33*	27.54
Grain Nitrogen						-31.09*
Forage Nitrogen						66.47
Yield						- 2.75*

*Value is negative and best estimated as zero.

[1]Datum lost.

heritability would be reduced by the increased variance associated with the parents.

The mean of all F_2 plants was practically the same as the mid-parent value for grain protein (16.20, 16.18% respectively). However, when the percentage grain protein was plotted against frequency of occurrence (Figure 1), F_2 population was shown to be skewed toward the low protein value which might suggest a slight dominance effect for low protein percentage. Lebsock, et al., (1964) also found low protein percentage to be partially dominant over high.

Protease 4 activity was not significantly different among genotypes on any sampling date (Table III). However, as physiological maturity was approached a tendency for F_2 plants to have higher protease 4 activity than that of the parents was evident (Figure 2). This tendency was most pronounced on the May 25 sampling date.

Although protease 7 did not reach significantly different levels on any sampling date (Table III), the levels of activity measured on the last four dates were substantially higher for the high grain protein parent and the F_2 genotypes than for the low grain protein parent (Figure 2). This suggests that high protease 7 activity was associated with higher protein contents present in these genotypes.

Rao and Croy (1972) found that protease 4 activity was higher in a low protein cultivar than in a high protein cultivar prior to flowering but lower after flowering. In this study, similar data was found before flowering; however, the reversal noted previously after flowering was not found. This inconsistency may be the result of differences in environmental conditions over the two years studied.

Alpha amino nitrogen was not significantly different on any

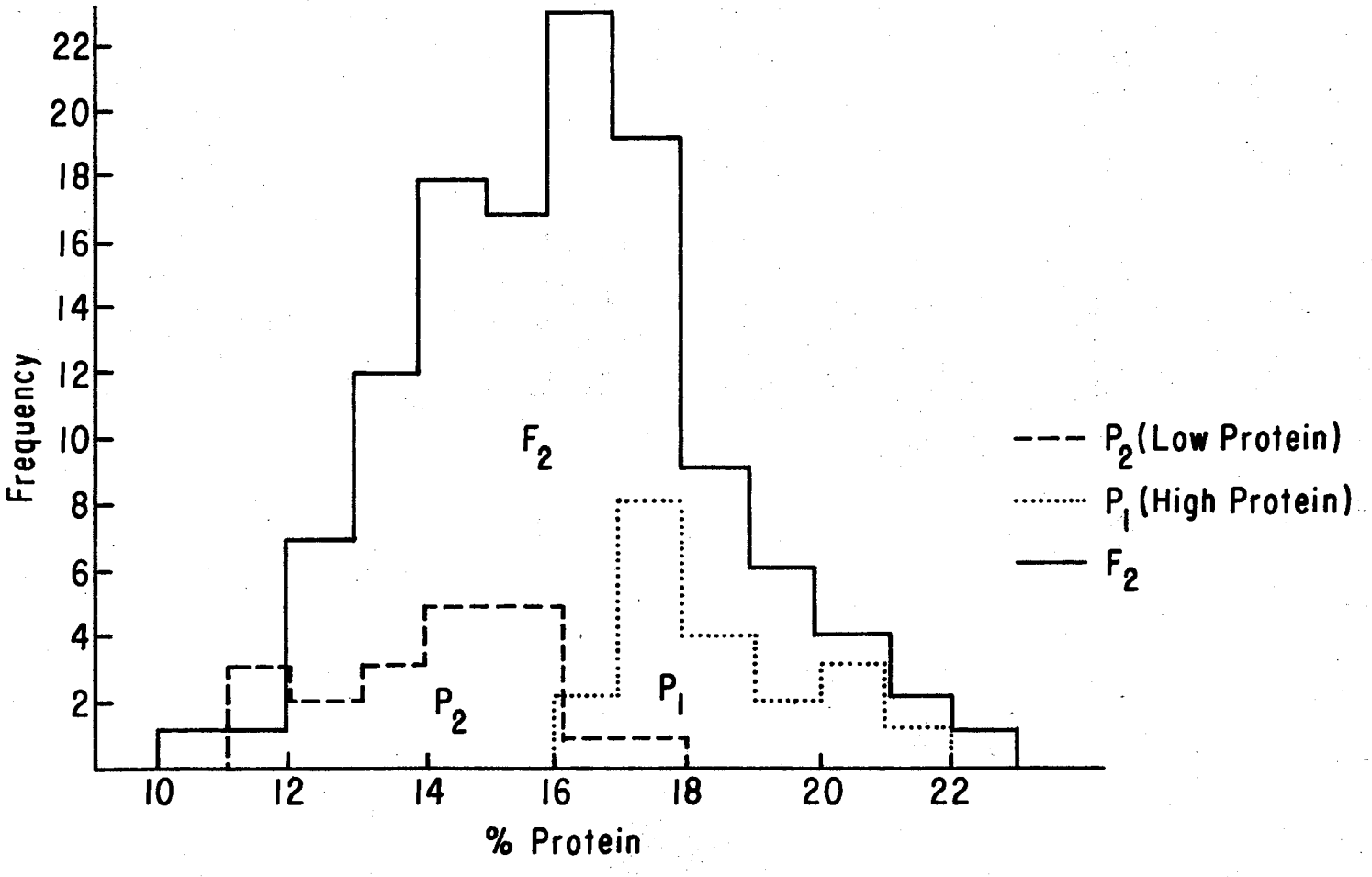

Figure 1. Frequency of Protein Content for High, Low Protein Parents and Their F_2 Population for Crop Year 1971-1972

TABLE III

SIGNIFICANCE OF PROTEASE 4 AND 7, ALPHA AMINO NITROGEN, AND WATER SOLUBLE PROTEIN FOR FIVE SAMPLING DATES, JANUARY 12, APRIL 14, MAY 19 AND 25 AND JUNE 1, 1972

Factor	Date				
	Jan. 12	April 14	May 19	May 25	June 1
Protease 4	NS	NS	NS	*	NS
Protease 7	NS	NS	NS	NS	NS
Alpha Amino Nitrogen	NS	1	NS	NS	NS
Water Soluble Protein	NS	*	NS	NS	NS

NS - Denotes nonsignificance.

*Significance by F-test but invalidated by unequal variances.

[1]Datum lost.

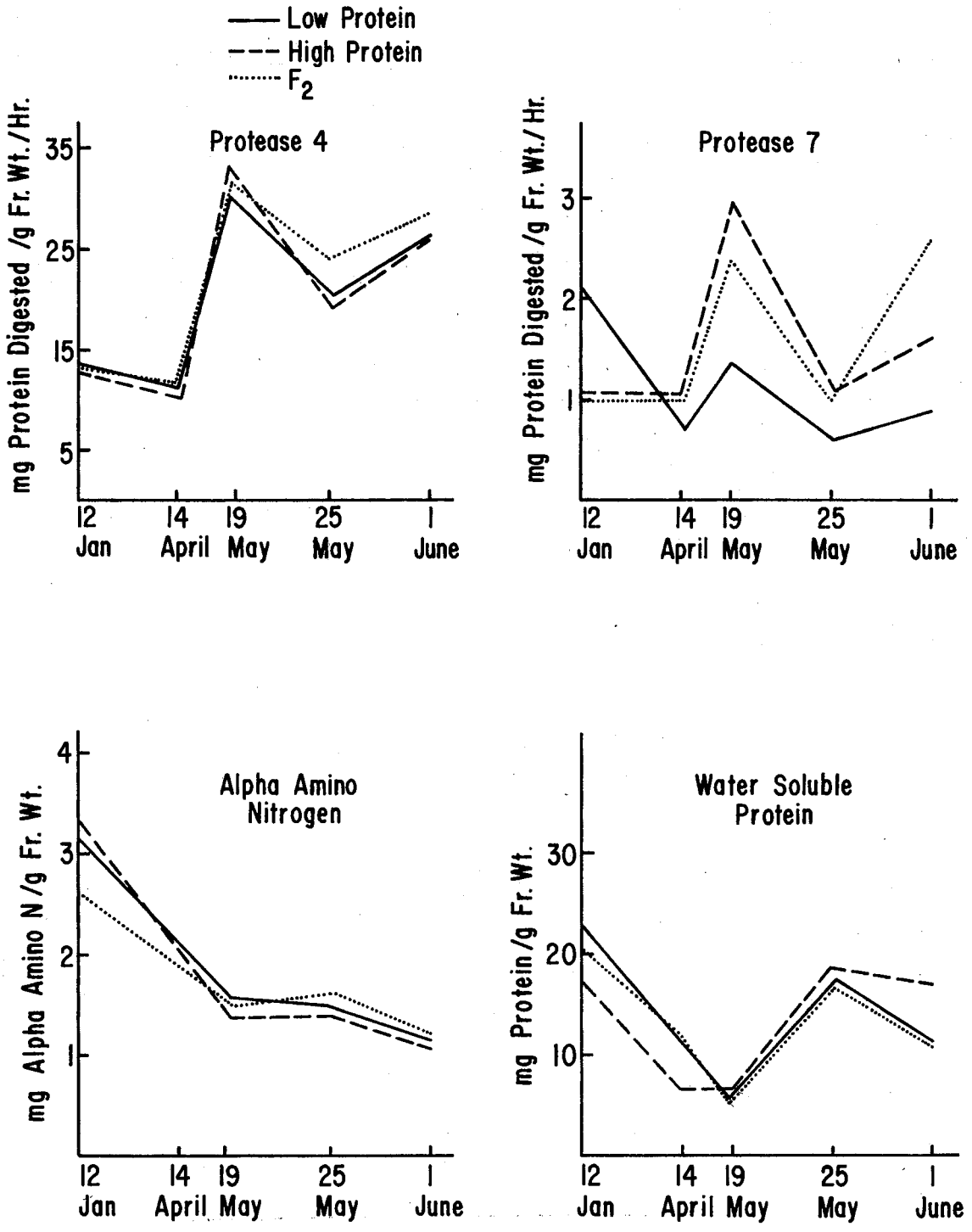

Figure 2. Seasonal Patterns of Protease 4 and 7, Alpha Amino Nitrogen, and Water Soluble Protein for High, and Low Protein Parents and Their F_2 Crosses for 1971-1972 Crop Year

sampling date among genotypes (Table III). Levels were highest on the early sampling dates and generally declined with maturity (Figure 2). The F_2 population tended to have higher amounts of alpha amino nitrogen than the parents.

Water soluble protein (WSP) was not significantly different among genotypes on any sampling date (Table III). On the first two dates the high protein parent was low in WSP content but it was higher on the last three dates (Figure 2).

The data are consistent with the hypothesis that protease 4 is degrading protein to alpha amino nitrogen. The higher activity of protease 4 in the F_2 compared to the high protein parent on the last two dates is associated with elevated amounts of alpha amino nitrogen and reduced amounts of WSP. The correlation coefficients further confirm this association particularly on June 1 (Table IV). There was a positive correlation between alpha amino nitrogen and protease 4 and a negative correlation between protease 4 and WSP on June 1. Also, on May 19, a positive correlation between protease 4 and alpha amino nitrogen was found.

Rao (1972) stated that protease 4 cleaves protein into low molecular weight compounds while protease 7 cleaves protein or peptides into amino acids. Since the low protein parent was higher in protease 4 activity, it may contain many peptides which could be detected as alpha amino nitrogen by the method of Yemm and Cocking (1955); however, these compounds could not be translocated to the grain. A trend for the high protein parent to have higher protease 7 activity was found which could result in higher contents of amino acids. These amino acids would be readily available for translocation to the grain and could be

TABLE IV

NOTEWORTHY CORRELATION COEFFICIENTS FOR FIVE DATES, JANUARY 12, APRIL 14, MAY 19 AND 25 AND JUNE 1 AND ACROSS DATES FOR CROP YEAR 1971-1972

Variables Correlated		Date	Correlation Coefficient
Protease 7	Alpha Amino Nitrogen	January 12	-0.307+
Protease 7	Forage Nitrogen	January 12	0.357+
Protease 4	Yield	January 12	-0.308+
Protease 7	Grain Nitrogen	April 14	-0.403*
Protease 4	Alpha Amino Nitrogen	May 19	0.341+
Protease 4	Yield	May 19	0.358*
Protease 4	Forage Nitrogen	May 25	-0.410*
Alpha Amino Nitrogen	Grain Nitrogen	May 25	-0.433*
Water Soluble Protein	Yield	May 25	0.471*
Protease 4	Alpha Amino Nitrogen	June 1	0.367*
Alpha Amino Nitrogen	Grain Nitrogen	June 1	-0.313+
Protease 4	Water Soluble Protein	June 1	-0.332+
Grain Nitrogen	Forage Nitrogen	Across Dates	0.355**
Grain Nitrogen	Yield	Across Dates	-0.280**
Forage Nitrogen	Yield	Across Dates	0.358**

*Denotes significance at 5% level of probability.

**Denotes significance at 1% level of probability.

+Denotes significance at 10% level of probability.

incorporated into proteins. This idea is reenforced by results obtained in this study, particularly by the occurrence of significant negative correlations between alpha amino nitrogen and grain nitrogen on the May 25 and June 1 sampling dates. These correlations should occur if protease 7 is degrading proteins to amino acids which are then being translocated to the head.

Significant correlations were found between protease 7 and forage nitrogen and significant negative correlation between protease 7 and alpha amino nitrogen were found on the January 12 sampling date (Table IV). Also a negative correlation was found between protease 7 and grain nitrogen on the April 14 sampling date. These correlations are difficult to explain physiologically.

A correlation between yield and WSP occurred on the May 25 sampling date. This suggests that high levels of vegetative protein on this date were necessary for high yield. Wallace, Ozbun and Munger (1972) state that one half of the total leaf protein is in the form of 1,5 ribulose diphosphate carboxlyase, the enzyme which catalyzes attachment of CO_2 in CO_2 fixation. Therefore, a positive correlation between WSP and yield seems likely since with higher WSP more enzymes could be available for CO_2 fixation. This additional carbohydrate production could then be translocated to the head for incorporation into grain.

When the data were pooled across dates, significant differences among genotypes occurred for forage and grain protein (Table V). The high protein parent was significantly higher in grain nitrogen than the F_2 population or the low protein parent. Also the low protein parent was significantly lower in grain nitrogen than the F_2 population. The high protein parent had significantly less forage nitrogen than the F_2

TABLE V

MEANS FOR PROTEASE 4 AND 7, ALPHA AMINO NITROGEN, WATER SOLUBLE PROTEIN, GRAIN AND FORAGE NITROGEN FOR HIGH AND LOW PROTEIN PARENTS AND F_2 POPULATIONS RESULTING FROM THEIR CROSS IN 1971-1972

	Protease 4	Protease 7	Alpha Amino Nitrogen	Water Soluble Protein	Grain Nitrogen	Forage Nitrogen
	mg Protein Digested/hr/g Fr. Wt.		ug/g Fr. Wt.	mg/g Fr. Wt.	Percent	
High	22.50	1.70	1790	15.20	3.20	0.53
Low	22.50	1.25	1810	14.20	2.50	0.55
F_2	24.60	1.84	1731	13.70	2.90	0.63
	NS	NS	NS	NS	**+	*++

NS - Denotes nonsignificance.

*Denotes significance at 5% level of probability.

**Denotes significance at 1% level of probability.

+LSD_{01} for high protein parent compared to F_2 = 0.330, LSD_{05} = 0.236.
LSD_{01} for low protein parent compared to F_2 = 0.305, LSD_{05} = 0.218.

++LSD_{05} for parents compared to F_2 = 0.0959.

population although, it was not different from the low protein parent. None of the other parameters measured were significantly different among the genotypes when the data were pooled across dates.

Significant negative correlation coefficients were obtained between grain nitrogen and yield. Forage nitrogen and grain nitrogen and yield and forage nitrogen were positively correlated (Table IV).

The negative correlation between yield and grain nitrogen has been reported by other workers (Stuber, et al., 1962). The correlation between forage nitrogen and grain nitrogen suggests that a plant must have a high potential to reduce nitrogen before high protein will result. This high level of nitrate reduced results in high levels of nitrogen in both the grain and forage.

An interesting feature of the statistical analysis was that several variables were described as statistically significant by F-tests which were not of magnitude which would be declared significantly different by t-tests. This discrepancy is the result of unequal genotype variances as determined by Bartlett's test (Appendix Table XXVI). Equal variance is an assumption for F-tests. The variance within the high protein parent was larger than within the low protein parent. Cochran and Cox (1957) stated that if variances are not equal then both the significance levels and sensitivity of the F-test are affected and too many significant results are obtained. In this case, protease 4 on May 25 and on a pooled basis as well as WSP on April 14 were declared significant but were not. These data suggest that the high protein parent was not homogeneous with regard to protease 4 activity and WSP content. This lack of homogeneity in the high protein parent is possibly the reason that significantly different levels of protease 4 and WSP were

not found between the parents.

Experiment Two

The heritability estimates for grain protein based on the regression of F_3 lines on F_2 plants was 13.15%. This value was lower than that found by most other workers (Haunold, et al., 1962) although it was similar to that of Sunderman et al., (1965). When protein classifications were plotted against protein levels and frequency of occurrence (Figure 3), the classes overlapped. However, the high protein class contained the selection with the highest protein percentage. The high class had a higher grain protein mean (15.11) than the medium and low classes (14.55 and 14.64 respectively). This suggests that although heritability for grain protein is low, selection in F_2 for high protein could lead to increased protein levels in subsequent generations.

On the April sampling date, none of the variables was significantly different (Table VI). On the April 11 sampling date there was a positive correlation between protease 4 and WSP and between protease 4 and protease 7 (Table VII). A high negative correlation coefficient was noted between protease 4 and NR.

The correlation between protease 4 and WSP suggests that protease 4 is substrate inducible since high amounts of WSP tend to produce high enzyme activity. The correlation between protease 4 and 7 suggests that both enzymes are affected to some degree by similar conditions.

The negative correlation between protease 4 and NR and between protease 7 and NR on May 14 probably reflect the seasonal pattern of activity of these enzymes. Rao (1972) noted a decline in the NR activity as protease was increasing late in the growing season. A similar

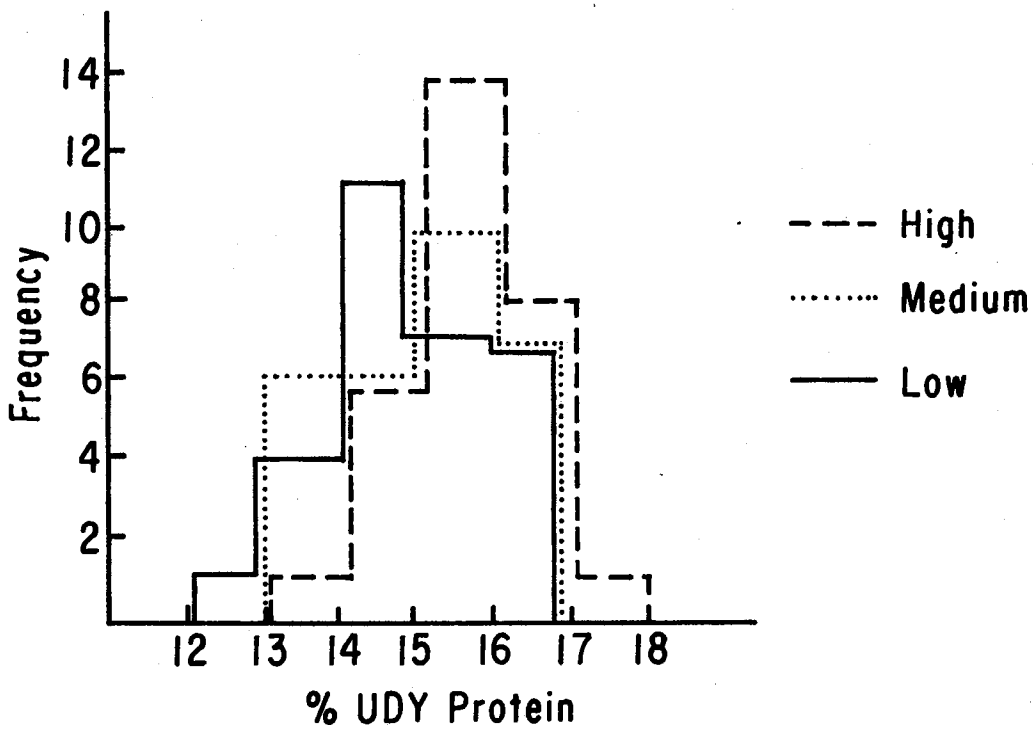

Figure 3. Frequency of Protein Content for F_3 Populations Resulting From a Cross of High and Low Protein Parents for 1972-1973 Crop Year

TABLE VI

MEANS FOR PROTEASE 4 AND 7, WATER SOLUBLE PROTEIN, NITRATE, ALPHA AMINO NITROGEN, AND NITRATE REDUCTASE FOR APRIL, 1973

F_3 Populations	Protease 4	Protease 7	Water Soluble Protein	Nitrate	Alpha Amino Nitrogen	Nitrate Reductase
	mg Protein Digested/ hr/g Fr. Wt.		mg Protein/ g Fr. Wt.	ug/g Fr. Wt.		umoles NO_2/ hr/g Fr. Wt.
1	15.0	3.69	30.8	203	2602	5.76
2	13.4	1.87	32.7	290	1728	5.43
3	17.9	3.08	34.6	196	2680	4.10
4	14.8	1.66	36.0	152	2571	3.69
5	14.8	3.80	34.3	219	1717	4.82
6	13.0	2.24	29.6	339	2777	5.40
7	17.8	1.31	37.7	229	4650	4.20
8	14.1	3.47	37.2	232	2697	4.63
9	12.5	1.90	32.8	205	2605	4.25
10	15.6	2.22	41.7	253	3656	2.74
11	13.8	1.18	30.2	207	2562	4.45
12	13.1	2.18	32.4	275	1678	5.39
13	15.1	3.21	37.4	140	2314	5.06
14	12.5	0.96	39.1	241	2428	6.08
15	15.7	1.81	40.5	181	2466	3.69
16	15.0	2.00	32.9	126	1528	3.96
17	10.7	1.61	33.9	367	2199	4.56
18	17.2	1.12	49.1	219	3190	3.69
19	14.6	1.65	36.3	304	3593	4.90
20	12.7	1.44	35.5	157	3714	5.57
21	14.6	1.64	30.8	176	1841	3.65
22	9.1	1.79	33.9	189	2781	4.89

TABLE VI (Continued)

F_3 Populations	Protease 4	Protease 7	Water Soluble Protein	Nitrate	Alpha Amino Nitrogen	Nitrate Reductase
	mg Protein Digested/ hr/g Fr. Wt.		mg Protein/ g Fr. Wt.	ug/g Fr. Wt.		umoles NO_2/ hr/g Fr. Wt.
23	11.5	1.81	35.2	220	2358	5.67
24	15.0	1.00	35.5	140	1445	4.67
25	12.9	1.87	37.6	365	2364	5.82
26	17.2	2.95	42.8	107	3731	2.38
27	16.0	1.47	39.5	357	2308	3.56
28	11.5	1.95	29.9	220	1623	4.93
29	15.3	0.83	85.5	167	2638	5.96
30	13.3	1.21	30.9	280	2755	4.93
	NS	NS	NS	NS	NS	NS

NS - Denotes nonsignificance at 5% level.

TABLE VII

NOTEWORTHY CORRELATION COEFFICIENTS FOR TWO PERIODS, APRIL AND MAY IN 1972-1973

Variables Correlated		Date	Correlation Coefficient
Nitrate Reductase	Protease 4	April 11	-0.618**
Protease 7	Protease 4	April 11	0.298*
Water Soluble Protein	Protease 4	April 11	0.251+
Water Soluble Protein	Nitrate	April 11	-0.419**
Protease 7	Water Soluble Protein	May 14	-0.328*
Protease 7	Alpha Amino Nitrogen	May 14	-0.346**
Protease 7	Nitrate Reductase	May 14	-0.376**
Protease 7	Yield	May 14	-0.251+
Protease 4	Yield	May 14	-0.217+
Water Soluble Protein	Grain Protein	May 14	-0.272*

*Denotes significance at 5% level of probability.

**Denotes significance at 1% level of probability.

+Denotes significance at 10% level of probability.

pattern was present in this study.

On the May sampling date, WSP and NR were significantly different at 5 and 1% levels respectively among the F_3 lines (Table VIII). Six of F_3 selections were higher in NR activity. Of these, all but one was substantially above the mean of the F_3 populations with regard to final grain protein content. With regard to WSP, fourteen of the F_3 populations were higher in WSP; however, high WSP seemed to have little bearing on the levels of final grain protein.

Many of the correlation coefficients were high on the May date (Table VII). The negative correlations between protease 4 and 7 and yield are quite interesting. These suggest that if protease activity is high, yield will be low. Wallace, Ozbun and Munger (1972) suggest that 1,5 ribulose diphosphate carboxylase accounts for one half the total leaf protein and if protease is actively reducing the level of this enzyme, then reduced CO_2 fixation could occur. This reduction could ultimately result in a reduction in yield. The occurrence of a negative correlation between protease 7 and WSP found in the present study tends to support this idea since it indicates that protease 7 reduces the amount of leaf protein as well as the amount of 1,5 ribulose diphosphate carboxylase.

The F_3 populations were significantly different at 1% level for yield and grain protein (Table VIII). The F_3 selection identified as line number 14 is of particular interest since it had the highest level of grain protein and also a high yield. It should be a good selection for further study.

TABLE VIII

MEANS FOR GRAIN PROTEIN, PROTEASE 4 AND 7, WATER SOLUBLE PROTEIN, NITRATE, ALPHA AMINO NITROGEN, NITRATE REDUCTASE AND YIELD FOR MAY, 1973

F_3 Populations	Grain Protein	Protease 4	Protease 7	Water Soluble Protein	Nitrate	Alpha Amino Nitrogen	Nitrate Reductase	Yield
	Percent	mg Protein Digested/ hr/g Fr. Wt.		mg Protein/ g Fr. Wt.	ug/g Fr. Wt.		umoles NO_2/ hr/g Fr. Wt.	Kg/ha
1	14.92	16.23	5.18	26.7	243	3234	0.07	3789
2	13.90	21.32	2.55	32.7	313	2855	0.67	3534
3	15.17	22.87	2.98	38.4	251	2415	0.74	3932
4	14.63	21.19	3.92	25.5	285	3145	0.15	3932
5	13.88	22.24	3.50	27.0	251	3294	0.14	5240
6	13.55	22.29	2.86	31.7	266	2812	0.29	4031
7	13.12	23.69	4.11	28.7	255	2813	0.07	4337
8	13.55	23.43	3.86	20.4	470	3007	0.17	4621
9	15.56	22.79	2.27	24.3	300	2688	0.11	4216
10	15.22	23.27	1.18	28.7	235	2884	0.18	4076
11	15.32	22.28	2.44	40.6	281	2667	0.73	3718
12	15.86	22.13	2.48	34.3	263	2813	0.80	3462
13	15.55	23.49	2.78	35.5	213	3104	0.19	3572
14	16.11	23.84	2.93	30.4	250	2816	0.33	4529
15	14.62	20.07	3.60	30.2	260	2583	0.15	4216
16	15.53	22.77	3.04	28.7	299	2688	0.19	4977
17	15.97	21.64	5.51	40.3	402	2881	0.72	3320
18	15.19	21.40	2.68	35.2	220	2770	0.22	4003
19	15.80	21.91	3.16	41.8	296	2718	0.31	4316
20	14.89	21.88	4.36	31.7	368	2859	0.02	4785
21	14.93	22.92	3.13	38.2	207	2564	0.17	4102
22	15.24	22.19	2.17	36.6	281	3108	0.67	3107

TABLE VIII (Continued)

F_3 Populations	Grain Protein	Protease 4	Protease 7	Water Soluble Protein	Nitrate	Alpha Amino Nitrogen	Nitrate Reductase	Yield
	Percent	mg Protein Digested/ hr/g Fr. Wt.		mg Protein/ g Fr. Wt.	ug/g Fr. Wt.		umoles NO_2/ hr/g Fr. Wt.	Kg/ha
23	15.20	23.29	2.76	36.3	308	3286	0.33	3982
24	13.55	23.16	2.80	28.9	237	2494	0.09	4337
25	13.68	24.07	3.09	37.0	339	3171	0.48	4216
26	14.04	22.15	2.68	38.9	281	3101	0.52	4195
27	13.92	24.37	2.94	44.9	317	2689	0.24	3555
28	14.81	20.87	2.71	35.9	301	2627	0.72	4316
29	13.86	22.36	3.05	25.2	269	3040	0.08	4479
30	15.44	22.01	2.56	42.0	277	3119	0.97	3861
LSD_{05}	1.13	NS	NS	10.92	NS	NS	0.41	982

NS - Denotes nonsignificance at 5% level.

Summary

If one examines the data of this experiment in light of the objectives, several conclusions can be made:

1. Protease 7 and forage nitrogen had sufficiently high heritabilities that their inclusion in a breeding program for increasing grain protein should be practical.

3. Heritability estimates based on individual sampling date for several variables could be of value in a breeding program i.e., protease 4, April 14, alpha amino nitrogen on January 12, May 19 and 25, and WSP on January 12 and May 25. However, more study is needed to find the ideal sampling date.

3. The pooled correlations were low for all variables which could be used to predict final grain protein.

4. Alpha amino nitrogen measured on May 25 and June 1 was correlated negatively with grain nitrogen, however, these sampling dates may be too late in the season, thus limiting their value to the breeder. On April 14, protease 7 was negatively correlated with grain nitrogen. This information could possibly aid a breeder in selection for high protein.

5. The pattern of development of protease 7, as well as the numerous correlations between it and other factors in nitrogen metabolism suggests that this enzyme would be one which should be studied more thoroughly.

6. The extreme variation in weather conditions which prevailed in these two years makes comparison of the years difficult, if not

impossible. The fact that NR and nitrate were not detectable the first year and found throughout the second year exemplifies this difference.

CHAPTER III

LEVELS OF NITRATE REDUCTASE AND PROTEASE IN VEGETATIVE PART OF WINTER WHEAT CULTIVARS[1, 2]

Abstract

Ten winter wheat cultivars ranging from high to low protein content were analyzed for protease 4, protease 7 and nitrate reductase activity. The cultivars differed in protease 4, protease 7, and nitrate reductase activity. Protease 7 and nitrate reductase were found to correlate with forage and grain protein. It is suggested that nitrate reductase could be used to predict final grain protein. This work adds evidence to the suggestion that protease enzymes and nitrate reductase provide nitrogen substrate for the plant. The nitrate reductase system was most active in the early stage of growth and protease was most active as the plant matured. Additional key words for indexing: Grain Protein.

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[1] Article coauthored with L. I. Croy for submission to Crop Science for publication.

[2] Abbreviations used in this paper: NR-nitrate reductase; NAD-nicatinamide adenine dinucleotide; NAD-nicotinamide adenine dinucleotide phosphate; WSP-water soluble proteins.

Introduction and Literature Review

Most plants require nitrate as a source of nitrogen. This nitrate once absorbed by the plant must be reduced to ammonia for the formation of amino acids by amination and transamination of keto acids. NR is the rate limiting enzyme in the reduction of nitrate to ammonia. Evans and Nason (1953) were the first to extract NR in a partially purified form from higher plants.

The induction of NR was found to be approximately proportional to the level of nitrate in the tissue (Beevers, et al., 1965). An increase in NR activity in response to an increase in the nitrate levels of the nutrient media has been observed (Hageman and Flesher, 1960).

The relationship of NR to protein production has been studied by many workers. Deckard, et al., (1973) found a significant correlation between NR activity and grain protein. A significant positive correlation between seasonal NR activity and percent grain protein in 15 wheat cultivars was found by Eilrich (1968). Croy and Hageman (1970) found a significant positive correlation between input of reduced nitrogen estimated from NR activity and the accumulation of grain protein in the wheat cultivars 'Ponca' and 'Monon'. The high protein cultivar Ponca had higher levels of NR activity than the low protein cultivar.

Johnson, et al., (1968) concluded that more efficient and complete translocation of nitrogen from the vegetative plant parts to the grain is the physiological basis of high protein grain. A low protein cultivar, 'Warrior' was found to contain more nitrogen in the vegetative plant than a high protein selection 'NE 65305'. A possible reason for the slow rate of translocation in Warrior could be a low level of

protease enzyme which would lessen degradation of proteins to amino acids for translocation to the grain.

Many workers have studied the proteases of seeds during germination. An increase in protease activity during germination of wheat seeds was found by Mounfield (1936). Two types of enzymes were present which he classified as a proteinase and dipeptidase. He noted that by the seventh day after germination a tenfold increase in activity had occurred. Irving and Fontaine (1945) found a proteolytic enzyme in peanut meal which would hydrolyze benzoyl-l arginine amide.

Working with sorghum, Garg and Virupaksha (1970) found that resting seeds had low proteolytic activity and during the first two days of germination, the activity remained low; however, by the sixth day a threefold increase was noted. This enzyme specifically cleaved the peptide linkage involving alpha-carboxyl group of either aspartic acid or glutamic acid with the release of the acyl portion of these acidic amino acids. Palmiano and Juliano (1972) found that protease activity was highest on the fifth day after germination in rice. This protease which was synthesized or liberated during germination probably has properties similar to those of the proteases of mature grain. An acid protease was found associated with protein bodies in ungerminated barley seeds (Ory and Henningsen, 1969). This protease catalyzed the initial production of amino acids from reserve protein. A protease in peanut cotyledons was found to increase in activity during the first week of germination (Mainguy, et al., 1972). However, this protease could not digest complex proteinaceous materials.

Work with proteases of vegetative parts of the plant is not as prevalent as work with seed proteases probably because the enzyme occurs

in low levels in leaves. Greenberg and Winnick (1945) listed eleven plants which had protease activity. In most of these plants the protease activity was found in the latex. Working with many plant species including wheat, Tracey (1948) found protease activity in both sap and fiber; however, this activity was about 1/10 of that found in pineapple. Kawashima, et al., (1968) examined a protease from tobacco leaves. They found that matured leaves of tobacco lost half their protein during three days flue curing. Simultaneous with this loss was an increase in protease activity.

Protease can be synthesized during the senescence of leaves (Martin and Thimann, 1972). De novo synthesis of this proteolytic enzyme may be the primary biochemical change in senescence. Two proteolytic enzymes were found, one with peak activity at pH 3 and the other with peak activity at pH 7.5. A neutral protease from etiolated oat shoots was described by Pike and Briggs (1972). It degraded phytochrome and was a endoprotease. This protease would degrade a variety of proteins including casein, phytochrome, and hemoglobin. The protease was only a protease (not peptidase, amidase or estrase) and was not specific as to bond cleavage.

In apple leaves, protease activity followed a seasonal pattern with senescence (Spencer and Titus, 1972). Protease levels were highest after the first frost in the fall. However, protease activity was not correlated with loss of leaf protein. Total protein had declined to 40% from its maximum before proteolytic activity began to increase significantly.

High protein lines of wheat have higher levels of protease than low protein lines in the seedling stage (Rao and Croy, 1971). Their

data suggest that the high protease activity of the high protein line resulted in more rapid rate of growth than that measured in the low protein line. The high protein cultivars exhibited higher levels of protease activity after the flag leaf stage than low protein cultivars. High protease levels were associated with increases in grain yield and grain protein production per acre (Rao and Croy, 1972). High protein lines of rice had higher protease activity in the grain than low protein cultivars (Cruz, et al., 1970). However, no association between protease activity and accumulation of protein in the ripened grain was found. The mean levels of protease activity in leaf blades were higher at flowering and during grain development in rice cultivars giving a high yield of grain protein (Perez, et al., 1972).

The objectives of this experiment were to measure NR and protease activities and determine the relationship of these activities to grain protein content.

Materials and Methods

This experiment included ten cultivars which varied in grain protein content. The cultivar B4930 is a Purdue selection which was previously classified as having high protein content derived from crosses of 'Atlas 66'. The cultivars NE 65305, NE 65317, NE 65318, NE 65320, and NE 65679, are Nebraska selections previously classified as having high protein derived from Atlas 66 crosses. The cultivars 'Genessee' and 'Monon' are low protein cultivars. 'Triumph 64' was included because it is a cultivar well adapted to Oklahoma conditions. It is intermediate in protein content. 'Warrior' was included because it contained high protein in the forage but low protein in the grain

(Johnson, et al., 1968).

These experiments were conducted on Stillwater Agronomy Research Station. The cultivars were planted on Kirkland silt loam in a randomized block pattern on October 6, 1971, at a seeding rate of 67.2 Kg/ha. Prior to planting 20.0-21.8-0 Kg/ha N-P-K were applied to the test area. Each plot consisted of four rows 3.965 m long which were trimmed to 3.05 m early the following spring. Four replications were planted. On March 15, 100.8 Kg/ha actual N as ammonium nitrate were applied to the plots. Samples were taken for enzyme extraction on February 23, March 14, April 26, and May 21.

Extraction and assay procedures for protease 4 and 7, alpha amino nitrogen, nitrate reductase, water soluble protein, and nitrate, were the same as the second experiment in the previous chapter. However, percent grain protein was determined by the Udy analysis method instead of micro-kjeldahl procedures as in the previous experiment. Forage protein was determined by micro-kjeldahl procedure on mature vegetative tissue. Multivariate analysis of variance was performed to obtain the correlation coefficients after adjusting for cultivar effects.

The ten cultivars were divided into protein and maturity groups based on 1973 data. The groups are listed in Table IX.

Results and Discussion

Protease 4 activity was significantly different at 1% level on February 23, and April 12 and at 5% level on March 12 (Table X). On the February 23 sampling date the cultivars Triumph 64, Genessee, and NE 65320 were lower in activity than the other cultivars. NE 65318 was lower than the other cultivars on March 12 sampling date. On April 26

TABLE IX

GRAIN PROTEIN GROUPS AND MATURITY GROUPS
FOR CROP YEAR 1972-1973

Protein Group					
Low	% Protein	Medium	% Protein	High	% Protein
NE 65318	14.5	B4930	15.6	NE 65305	16.2
Genessee	13.0	Triumph	15.6	NE 65317	16.9
Monon	14.5	Warrior	15.9	NE 65320	16.1
				NE 65679	16.4

Maturity Group					
Early	Days to Heading	Medium	Days to Heading	Late	Days to Heading
Triumph	201	Warrior	204	Genessee	209
Monon	202	NE 65317	205	NE 65305	209
		NE 65318	203	B4930	209
		NE 65320	204		
		NE 65679	205		

TABLE X

PROTEASE ACTIVITY FOR TEN WHEAT CULTIVARS FOR FOUR DATES, FEBRUARY 23, MARCH 12, APRIL 26, AND MAY 21, 1973

Cultivar	Sampling Dates			
	February 23	March 12	April 26	May 21
	mg Protein Digested/hr/g Fr. Wt.			
B4930	9.42	4.72	14.23	18.27
Genessee	7.07	5.24	15.29	17.32
Monon	9.21	5.30	19.72	21.68
NE 65305	8.55	5.43	16.61	20.85
NE 65317	7.85	5.07	17.12	18.93
NE 65318	7.92	3.30	17.98	22.62
NE 65320	6.60	5.09	17.43	19.21
NE 65679	9.72	5.23	16.18	20.03
Triumph 64	6.79	5.13	20.41	21.10
Warrior	8.08	4.70	18.67	22.10
	LSD_{01} 2.27		LSD_{01} 3.75	NS
	LSD_{05} 1.68	LSD_{05} 1.98	LSD_{05} 2.77	

sampling date Monon, Triumph 64, and Warrior were significantly higher in activity than the other cultivars. It is interesting to note that these cultivars were early or medium in maturity which suggests the higher protease 4 activity was associated with later stages of physiological development. Protease 4 activity was not significantly different on the May 21 date. There seemed to be little association between grain protein content and protease activity.

No clear cut pattern of protease 4 activity with protein groups could be found (Figure 4). When maturity group was plotted against sampling date, it was revealed that the early maturity group was substantially higher in activity on the two later sampling dates than medium or late maturity group. The medium maturity group was intermediate, and late maturity group was low in activity (Figure 5). The enhanced activity for the early maturity group was probably a reflection of earlier senescence in the leaves for this group.

A negative correlation was found between protease 4 and yield on March 12 (Table XI). Two possible explanations for this correlation are: (1) that high protease 4 activity on this date results in a loss of material which, if present later, could be incorporated into the grain; possibly this "loss" of material is incorporated into membranes within the leaf tissue; (2) that high protease 4 activity results in a degradation of enzyme responsible for CO_2 fixation which could result in a reduction of carbohydrates available for translocation to the grain.

Also a negative correlation was found between protease 4 and nitrate on the May 21 sampling date (Table XI). This implies that as nitrates decrease, protease 4 increases. These data suggest that protease 4 is related to nitrogen metabolism. It would seem that nitrogenous

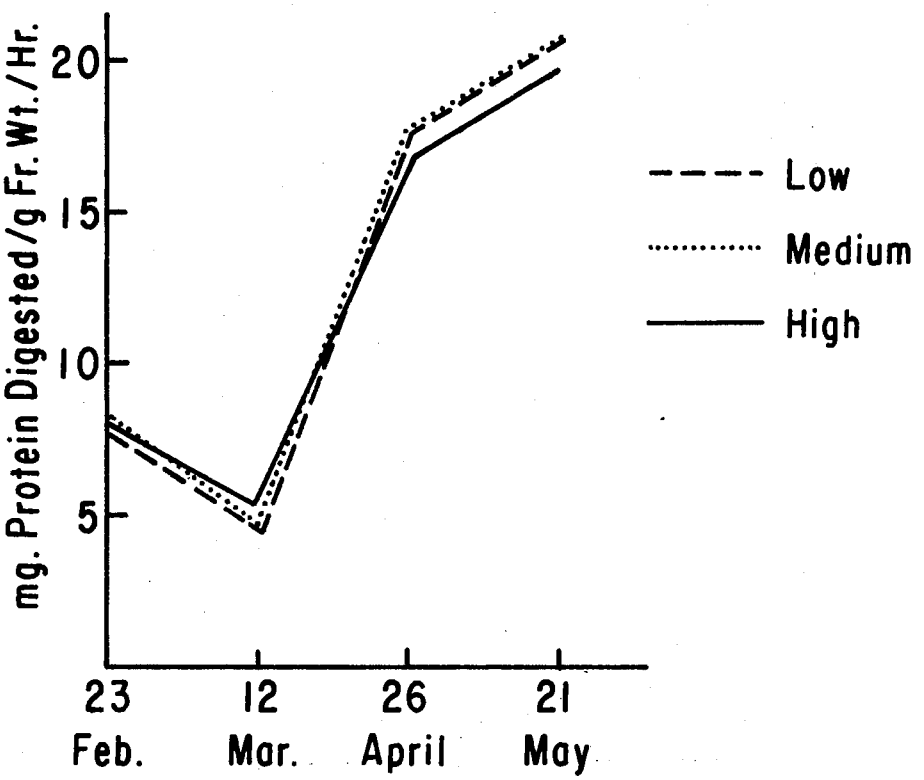

Figure 4. Protease 4 for Protein Groups for Sampling Dates, February 23, March 12, April 26, and May 21, 1973

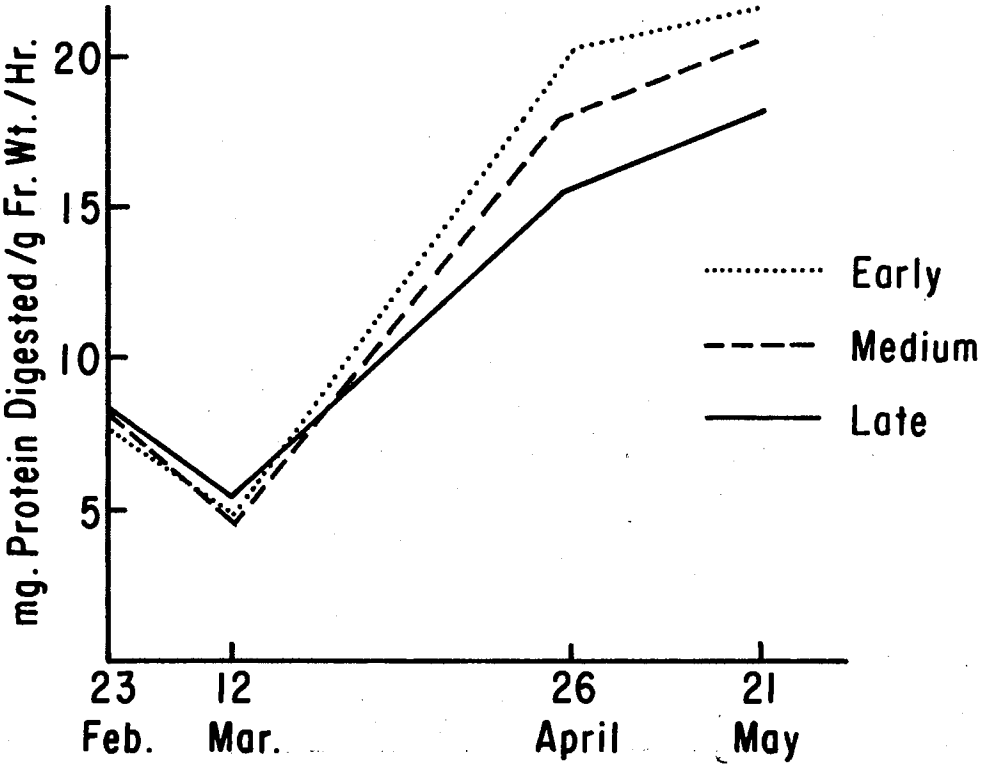

Figure 5. Protease 4 for Maturity Groups for Sampling Dates, February 23, March 12, April 26, and May 21, 1973

TABLE XI

NOTEWORTHY CORRELATION COEFFICIENTS FOR FOUR SAMPLING DATES, FEBRUARY 23, MARCH 12, APRIL 26, AND MAY 21, 1973

Variables Correlated		Date	Correlation Coefficient
Nitrate Reductase	Alpha Amino Nitrogen	Feb. 23	-0.508**
Forage Protein	Water Soluble Protein	Feb. 23	-0.331+
Protease 7	Alpha Amino Nitrogen	March 12	0.386*
Protease 7	Nitrate Reductase	March 12	0.392+
Protease 7	Forage Protein	March 12	0.372*
Protease 4	Yield	March 12	-0.343+
Nitrate	Water Soluble Protein	March 12	0.383*
Nitrate	Nitrate Reductase	March 12	0.411*
Nitrate Reductase	Forage Protein	April 26	0.351+
Nitrate Reductase	Grain Protein	April 26	0.335+
Nitrate Reductase	Yield	April 26	-0.328+
Protease 7	Forage Protein	April 26	0.389+
Protease 4	Nitrate	May 21	-0.413*
Protease 7	Forage Protein	May 21	-0.409*
Nitrate Reductase	Forage Protein	May 21	0.446+
Water Soluble Protein	Yield	May 21	-0.321+

*Denotes significance at 5% level of probability.

**Denotes significance at 1% level of probability.

+Denotes significance at 10% level of probability.

material can be supplied by either reduction through NR or by degradation by proteases. The amount of these processes would seem to be controlled by the availability of substrate. If nitrate levels were low then NR activity was reduced and then the needs for amino acids were provided by protease enzymes. The trend for NR activity to decrease with maturity while protease 4 increased, substantiates this idea.

Differences among cultivars for protease 7 activity were significant on the May 21 sampling date but not for any of the other dates (Table XII). Monon, Triumph 64, and Warrior were higher in activity than the other cultivars. These data are not consistent with the findings of Rao and Croy (1972), who found that higher levels of protease 7 were associated with the high protein cultivar in the growing season. However, it must be remembered that Triumph 64 and Monon mature earlier than the other cultivars while Warrior is medium in maturity. Therefore, the peak of activity may have not been reached in the later maturing cultivars on this date. The plot of maturity group against date shows that the early maturity group had higher activity on the last date than either the medium or late maturity group (Figure 6). Also the low protein group was higher in protease 7 activity on the last date than the medium or high group (Figure 7). Both of these patterns suggest that peak activity had not been reached on the last sampling date in the medium and late maturity groups and high and medium protein groups.

Several correlations were found with protease 7. Positive correlation was found between protease 7 and forage protein on the March 12 and April 26 sampling dates while a negative correlation was found on the May 21 sampling date (Table XI). The positive correlations may be associated with degradation products of protease 7 being incorporated

TABLE XII

PROTEASE 7 FOR TEN WHEAT CULTIVARS FOR FOUR SAMPLING DATES, FEBRUARY 23, MARCH 12, APRIL 26, AND MAY 21, 1973

Cultivar	Sampling Dates			
	February 23	March 12	April 26	May 21
	mg Protein Digested/hr/g Fr. Wt.			
B4930	0.95	0.54	2.64	1.86
Genessee	0.59	0.24	2.57	3.51
Monon	1.81	0.27	2.38	6.49
NE 65305	1.59	0.47	2.72	3.63
NE 65317	0.80	0.54	2.68	3.15
NE 65318	1.34	0.46	2.67	3.15
NE 65320	1.13	0.57	2.61	2.72
NE 65679	0.99	0.56	2.03	2.58
Triumph 64	1.70	0.55	2.38	4.08
Warrior	1.76	0.79	2.06	4.00
LSD_{05}	NS	NS	NS	2.13

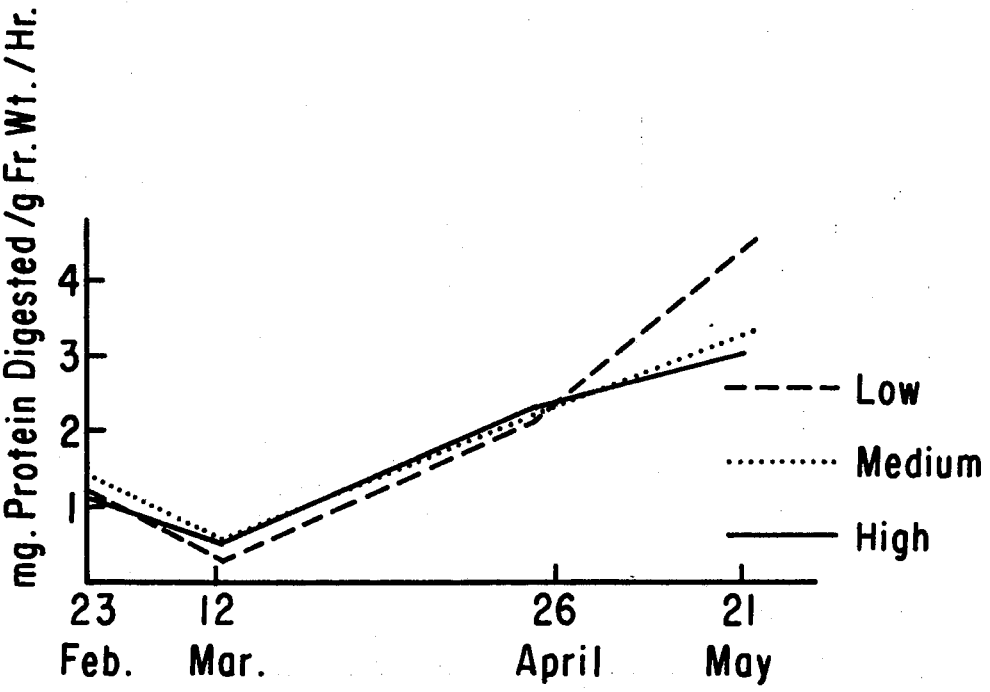

Figure 6. Protease 7 for Protein Groups for Sampling Dates, February 23, March 12, April 26, and May 21, 1973

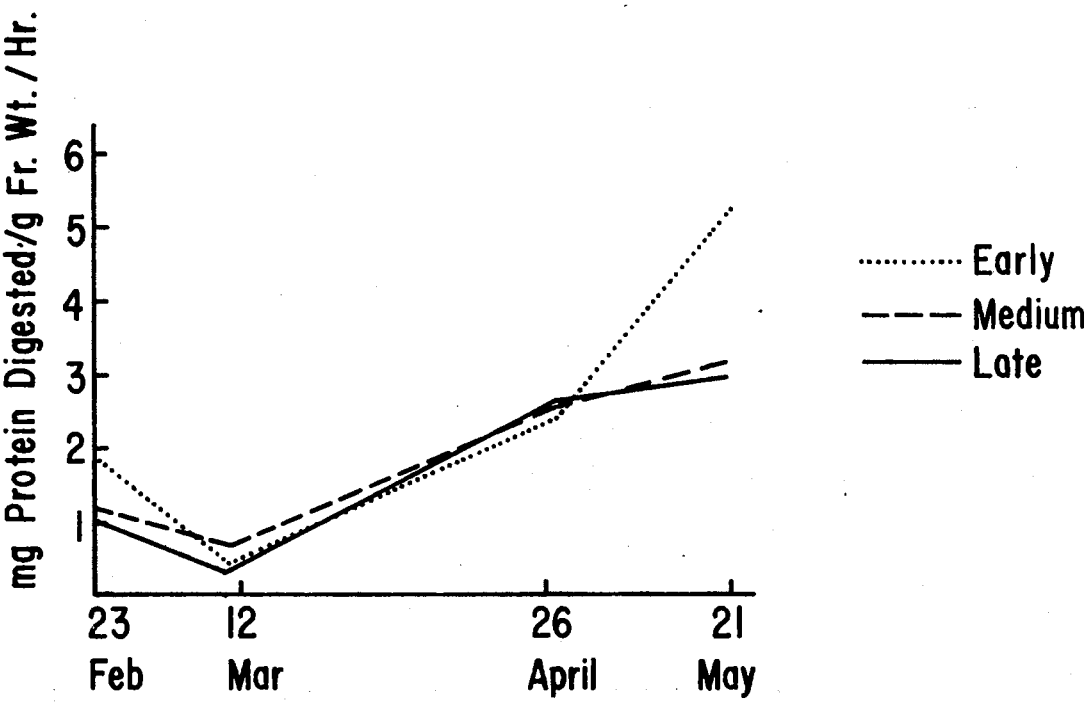

Figure 7. Protease 7 for Maturity Groups for Sampling Dates, February 23, March 12, April 26, and May 21, 1973

into protein structures which will remain in the forage as membranes. The negative correlation suggests that protease 7 was actively breaking down proteins in the forage and that these degradation products were being translocated out of the forage to the grain.

On the March 12 sampling date, protease 7 was positively correlated with alpha amino nitrogen. This correlation would be expected since protease 7 breaks proteins into amino acids; therefore, high protease 7 activity should produce high alpha amino nitrogen.

Differences among cultivars for nitrate reductase activity were significant on the April 26 sampling date (Table XIII). The B4930 and NE 65317 were higher than the other cultivars in NR activity.

The pattern of NR activity (Figure 8) was similar for the late and medium maturity groups with the late group maintaining slightly higher activity later in the season. The early maturity group lost activity earlier than the other groups and was lower in activity on all dates sampled. The pattern for protein groups was marked by high levels of NR activity for the high protein group on March 12 (Figure 9). The medium protein class was low on March 12 but regained some activity on April 26. The low group was inactive on both the March 12 and April 26 sampling dates.

Nitrate reductase activity was correlated with several factors (Table XI). One of the most interesting of these was the positive correlation between NR and forage protein on April 26 and the May 21 sampling dates and with grain protein on April 26 sampling date. These correlations indicated that NR can be related to plant protein content. These data also indicate that measures of NR at this stage of growth could possibly predict the level of protein in the grain. Deckard

TABLE XIII

NITRATE REDUCTASE ACTIVITY FOR TEN WHEAT CULTIVARS FOR FOUR SAMPLING DATES, FEBRUARY 23, MARCH 12, APRIL 26, AND MAY 21, 1973

Cultivar	Sampling Dates			
	February 23	March 12	April 26	May 21
	umoles KNO_2/hr/g Fr. Wt.			
B4930	4.02	0.62	1.09	0.02
Genessee	2.74	0.28	0.48	0.08
Monon	2.92	0.79	0.78	0.00
NE 65305	3.66	0.66	0.63	0.00
NE 65317	3.81	0.38	0.80	0.02
NE 65318	3.92	0.30	0.30	0.00
NE 65320	3.71	1.16	0.43	0.03
NE 65679	3.31	0.88	0.78	0.00
Triumph 64	3.77	0.27	0.34	0.03
Warrior	3.32	0.40	0.49	0.00
LSD_{05}	NS	NS	0.49	NS

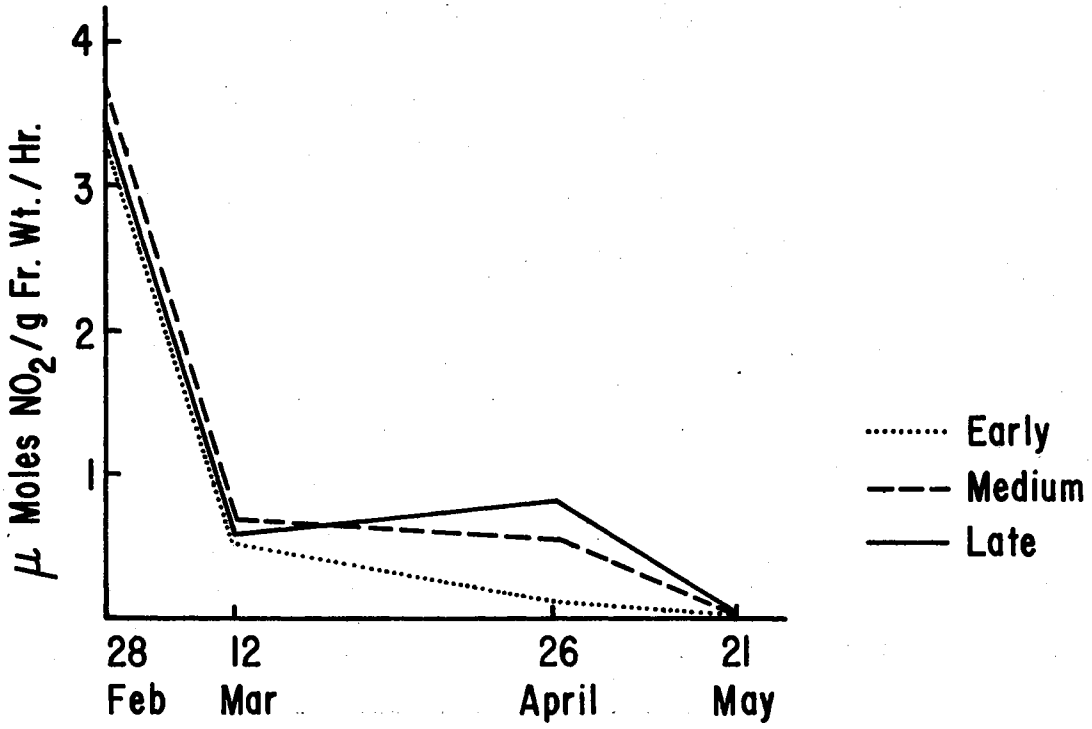

Figure 8. Nitrate Reductase for Maturity Groups for Sampling Dates, February 23, March 12, April 26, and May 21, 1973

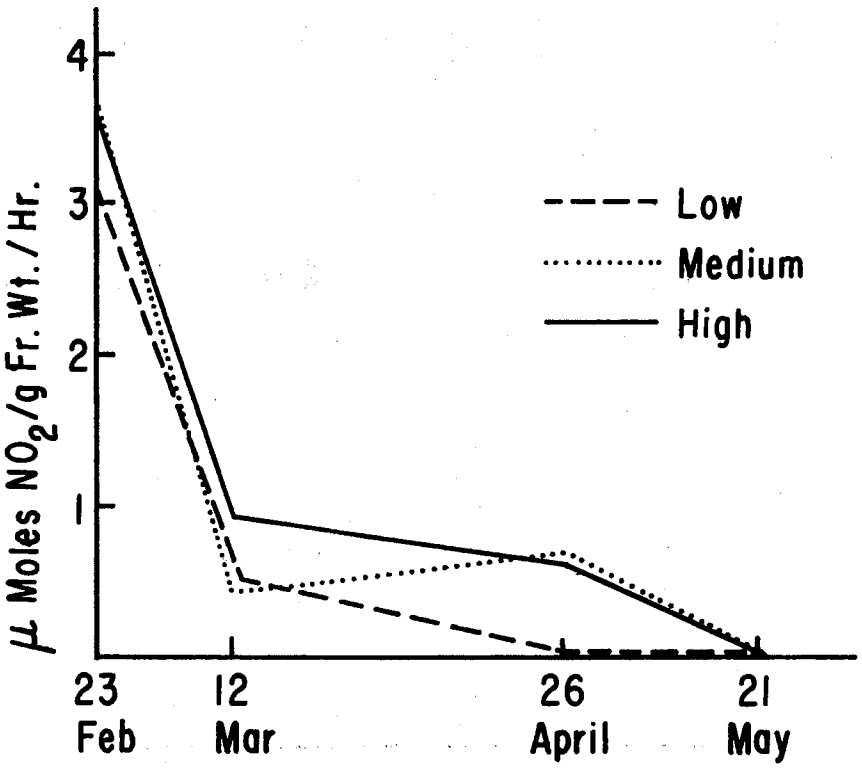

Figure 9. Nitrate Reductase for Protein Groups for Sampling Dates, February 23, March 12, April 26, and May 21, 1973

(1973) also found a strong relationship between NR activity and grain protein. These data suggest that if NR activity level can be increased in a cultivar then higher grain protein should result.

On the February 23 sampling date, a negative correlation was found between NR and levels of alpha amino nitrogen. This correlation implies that if amino acids are low, NR activity will be high. This information suggests that NR activity may be controlled by amino acid levels as proposed by Filner (1966).

On the March 12 sampling date there was a positive correlation between NR and nitrate. This should be expected since NR is known to be substrate inductible (Afridi, et al., 1965; Beevers, et al., 1965). The positive correlation between WSP and nitrate on the same date probably is related to the correlation between NR and nitrate in that high NR activity provides amino acids which can be incorporated into protein to increase WSP. Also on March 12 a positive correlation between NR and protease 7 occurred. This correlation suggests that both enzymes are controlled by the same factor possibly amino acid levels.

Differences among cultivars for WSP were significant at 1% level on the May 21 sampling date (Table XIV). The cultivars Triumph 64 and NE 65679 were considerably lower in WSP levels than the other cultivars. The WSP contents were unusually high for all cultivars for this late stage of growth. These high values were probably related to the unusually high moisture levels during the growing season which allowed for maximum forage growth.

WSP was negatively correlated with forage protein on February 23 sampling date (Table XI). This correlation is difficult to explain physiologically. WSP was also negatively correlated with yield on May

TABLE XIV

WATER SOLUBLE PROTEIN CONTENT OF TEN WHEAT CULTIVARS FOR FOUR SAMPLING DATES, FEBRUARY 23, MARCH 12, APRIL 26, AND MAY 21, 1973

Cultivar	Sampling Dates			
	February 23	March 12	April 26	May 21
	mg Protein/g Fr. Wt.			
B4930	22.5	19.4	20.0	47.8
Genessee	23.6	23.4	26.7	44.7
Monon	22.5	21.5	23.3	35.1
NE 65305	19.4	21.3	22.3	37.9
NE 65317	24.2	18.8	22.7	32.6
NE 65318	21.4	12.4	24.4	38.5
NE 65320	20.8	23.0	24.1	47.1
NE 65679	17.0	20.7	30.0	27.3
Triumph 64	17.8	19.8	26.1	13.7
Warrior	20.1	15.4	23.3	36.4
LSD_{01}	NS	NS	NS	17.4
LSD_{05}				12.9

21. This correlation possibly relates to the idea that WSP must be degraded and these degradation products translocated to the grain for maximum yield to occur. Therefore, as WSP is degraded, its level decreases while translocable products increase which increase yield.

Alpha amino nitrogen and nitrate were not significantly different on any sampling date (Tables XV, XVI).

The cultivars were significantly different at the 1% level for grain protein, but not for forage protein or yield. 'Genessee' was low in grain protein and NE 65317 was high.

Summary

Several observations can be made from this study:

1. Protease 4 activity was different among the cultivars on the early season sampling dates. However, little association between protease 4 levels and final grain protein could be found. Protease 4 levels appeared to be associated more closely with stage of maturity than with levels of grain protein.

2. The cultivars were different in protease 7 activity late in the growing season with low protein cultivars having higher activity. Also, early maturing cultivars had higher activity later in the season. However, the possibility exists that the other cultivars had not reached peak activity.

3. NR activity was significantly different among the cultivars only on the April 26 sampling date. Generally the cultivars with high NR activity were those which had high final grain protein. Also, correlations were found between NR and forage and grain protein in the later part of the growing season.

TABLE XV

ALPHA AMINO NITROGEN CONTENT FOR TEN WHEAT CULTIVARS FOR FOUR SAMPLING DATES, FEBRUARY 23, MARCH 12, APRIL 26, AND MAY 21, 1973

	Sampling Dates			
Cultivar	February 23	March 12	April 26	May 21
	ug/g Fr. Wt.			
B4930	1246	853	1918	1468
Genessee	1211	1054	1344	1613
Monon	1593	974	1727	1059
NE 65305	1284	1279	1501	1098
NE 65317	1526	856	1429	1335
NE 65318	1466	751	1796	1175
NE 65320	1435	779	1599	881
NE 65679	1200	1118	1301	1285
Triumph 64	1368	647	1535	1532
Warrior	1304	706	1639	1315
LSD_{05}	NS	NS	NS	NS

TABLE XVI

NITRATE CONTENT OF TEN WHEAT CULTIVARS FOR FOUR SAMPLING DATES, FEBRUARY 23, MARCH 12, APRIL 26, AND MAY 21, 1973

Cultivar	Sampling Dates			
	February 23	March 12	April 26	May 21
	ug/g Fr. Wt.			
B4930	1232	1684	814	780
Genessee	826	1057	661	638
Monon	752	1767	381	926
NE 65305	782	1050	272	710
NE 65317	612	1099	311	644
NE 65318	572	1540	227	526
NE 65320	639	814	343	512
NE 65679	618	1391	483	703
Triumph 64	706	1324	263	937
Warrior	565	684	292	521
LSD_{05}	NS	NS	NS	NS

4. Predictions of grain protein could be made from measurements of NR in plants at the stage of growth present between late March and April.

5. Nitrate reductase and protease systems are related in that each acts as a source of amino acids for protein synthesis. Nitrate reductase is most active early in the growing season with protease most active as the plant matures.

CHAPTER IV

PROTEASES FROM WHEAT FORAGE[1, 2]

Abstract

The effects of varied substrate, temperatures, pH, inhibitors, ionic strength and extraction buffer on protease enzymes of winter wheat forage were determined. Hemoglobin was the best substrate tested and protease activity was highest at pH's 4 and 7. The pH activity peaks are believed to be two enzymes denoted as protease 4 and 7. Protease 4 had a temperature optimum of at least 50 C while protease 7 optimum was 40 C. The Inhibitor study indicated that protease 4 contains an active sulphydryl group. Also, high ionic strength reduced protease 4 and 7 activity. Additional key words for indexing: Ionic strength, Sulphydryl groups.

Introduction and Literature Review

Mounfield (1936) described the characteristics of a proteinase and dipeptidase from aqueous extracts of germinated wheat seeds. He found

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[1]Article coauthored with L. I. Croy for submission to Crop Science for publication.

[2]Abbreviations used in this paper: DNTB-5,5[1] Dithio-bis-(2-Nitro Benzoic Acid), PMSF-Phenylmethyl sulfonylfluoride, Cleland Reagent-dithiothreitol, Km-Michaelis constant.

that wheat proteinase was relatively stable in buffer solution maintained at pH 4 or 6 but was destroyed at pH 8 in less than three days. The dipeptidase lost its activity slowly at pH 6 but was almost immediately destroyed by exposure to a medium at pH 4. Furthermore both enzymes were activated by cyanide.

Many organic substances have been shown to affect protease enzymes. A protease from tobacco leaves was found to be enhanced in activity by 0.02 M solutions of sodium sulfide, sodium hyposulphite, potassium cyanide, ascorbic acid and cysteine by Tracey (1948). Iodoacetate and copper were shown to be inhibitors. The enzyme was described by Tracey (1948) as a 'papain' type protease.

Other types of proteases have been found. Irving and Fontaine (1945) found a proteolytic enzyme in peanut meal that was capable of hydrolyzing benzoyl-l-arginine amide to yield benzoyl-l arginine and ammonia. This enzyme called arachain was trypsin-like. It was unaffected by cysteine, ascorbic acid and cystine.

Proteases seem to vary widely in pH optima. Johnson, et al., (1956) described a protease involved in bread-making. This protease had a pH optimum which varied with substrate. The pH optimum was 3 to 4 using hemoglobin as substrate or 5.5 to 6.0 with casein substrate. A protease in soybean flour was found which had a pH optimum of 5.5 and a temperature optimum of 50 C (Weil, et al., 1966).

Kawashima, et al., (1968) found a protease enzyme in leaves of curing tobacco. It had a pH optimum of 5.5 and was inactivated at temperatures higher than 40 C. They also noted that cysteine, glutathione, mercaptoethanol, and ascorbate increased the enzymes activity while copper, iron, magnesium, zinc, and cyanide ions had no effect on

it. Two protease enzymes were found in stem bromelain (Minami, et al., 1971). One had an acidic isoelectric point while the other was a basic. Cysteine was an activator for both enzymes.

Protease enzyme from germinated sorghum seeds was examined by Garg and Virupaksha (1970a). This enzyme had maximal activity at pH 3.6 and less than 20 percent of maximum at pH 5.0. It had a temperature optimum of 50 C and Michaelis constant value of 0.55 mg/ml for bovine serine albumin. The metal ions mercury, copper, zinc, iron, inhibited the enzyme activity by 30 to 50 percent. In a subsequent paper (1970) this enzyme was shown to specifically cleave the peptide linkage involving the dicarboxyl group of aspartic acid and glutamic acids with the release of the acyl portion.

Many proteases from germinated or ungerminated seeds and young seedlings have been described. An acidic protease which is associated with protein bodies in ungerminated barley was found by Ory and Hennigsen (1969). The initial production of amino acids from reserve protein is catalyzed by this acid protease.

Several proteinases from the aleurone of barley with a pH optima ranging from 3.9 to 9 were described by Sunderblom, et al., (1972). The main component of these proteinases was a SH-proteinase with pH optimum of 3.9.

Mainguy, et al., (1972) examined a protease from six-day old peanut seedling cotyledons. This enzyme was barely affected by potassium iodide or hydrogen peroxide. They classified this enzyme as a serine protease.

A protease was found in pea cotyledons which had two pH optima (Guardiola and Sutcliffe, 1971). One optimum was at pH 5 and the other

near pH 7. This enzyme was described as nonspecific endo-peptidase or mixture of exo-endo peptidases.

Horiguchi and Kilagishi (1969) showed the presence of a protease which had two pH optima (3 and 8) in rice when rice glutelin was used as substrate.

There has been some characterization of proteases from older plant tissue. A protease with pH optima of 3 and 7.5 and a minimum at 5 with hemoglobin substrate was described by Martin and Thimann (1972). L-serine enhanced the activity of this enzyme. Pike and Briggs (1972) studied a protease from oats and found that it was an endoprotease with pH optimum of 6.4. It was inhibited by high ionic strength but with small specific ion effects. Also $HgCl_2$ and DTNB were strong inhibitors. They concluded that this protease was one which had no specificity for bond cleavage location.

Rao (1971) found a protease in wheat leaves and stated that it was a complex of two enzymes.

The objective of this study was to characterize the protease enzymes isolated from wheat leaves.

Materials and Methods

All tests were performed on NE 65317, a high protein wheat derived from a cross of 'Atlas 66' and 'Comanche'. The plant material was one to two weeks of age at the time of evaluation. All extractions and assay procedures were as described in previous experiments except where indicated. As in previous experiments, proteases measured at pH 4 and 7 are designated as protease 4 and 7, respectively.

Substrate Evaluation

Bovine hemoglobin, gluten, plant protein, and blood serum albumin were tested as substrates for the protease enzymes. These substrates were tested at concentrations of 0.25 percent final volume. The plant protein was extracted from wheat plants growing in the field using the standard protease extraction procedures. The extract was combined with an equal volume of 10% trichloracetic acid and allowed to stand for two hours so that precipitation would be complete. The precipitant was then spun down by centrifugation for ten minutes at 2000 g. The supernatant was decanted and the sediment allowed to dry. After drying, the material was added to the usual pH 4 and pH 7 buffers for enzyme activity determinations.

Extraction Buffers

Extraction buffers consisting of EDTA-PO_4, Tris,(Hydroxymethyl Aminomethane) and PO_4 were examined. The EDTA: PO_4 buffer is the standard extraction buffer for protease. The phosphate buffer system was .01 M concentration adjusted to pH 7 by the combining of KH_2PO_4 and K_2HPO_4. The Tris buffer was .01 M concentration of Tris adjusted to pH 7 with .01 M HCL.

Extraction Buffer Additives

Cysteine, cystine, glutathione and sucrose were tested as additives to the extraction solution. Cysteine, cystine and glutathione were at 1mM concentration and sucrose was a 5% solution.

Buffer Strength

The effects of ionic strength on protease activity was examined, using three concentrations of EDTA and phosphate: 1mM, .01 M and 0.1 M.

Substrate Concentrations and Michaelis Constant

Substrate concentrations of 0.00391, 0.0078, 0.0156, 0.03125, 0.0625, 0.125, 0.25 and 0.5 mg of hemoglobin per milliliter were tested. The Km was calculated plotting 1/velocity against 1/substrate according to Lineweaver and Burk (1934).

Temperature Effects

The optimum temperature for protease activity was determined by measuring activity at 10, 20, 30, 40, 50 C.

pH Optima

The pH optima were determined by measuring activity at pH 3 through 9. Varied concentrations of citric acid-sodium phosphate buffer were used for pH 3 to 7. Tris buffer was used for pH 8-9.

Inhibitors

PMSF, DTNB, Cleland reagent were tested as inhibitors of protease activity. As were the metal ions FE^{+++}, Zn^{++}, Ni^{++}, Sn^{++}, Ca^{++}, Mg^{++}, K^{+}, Hg^{++}. All metal ions were at 0.25 mM concentrations. PMSF was 1.74 mg/ml, DTNB at 17.42 mg/ml and Cleland reagent at 15 mg/ml. DTNB was dissolved in methanol and PMSF was dissolved in 5% ethanol solution, this concentration of ethanol had little effect on protease activity;

however, the methanol solution did reduce protease activity, complicating the effects of DTNB. All materials tested as inhibitors were incubated with the enzyme extract for thirty minutes prior to assay procedures.

Results and Discussion

Protease 4 and 7 activity levels were highest using the hemoglobin substrate (Table XVII). The activity was almost twice as large as that using plant protein. Protease activity levels were low in blood serum albumin with almost no activity on gluten.

Extraction Buffers

The highest protease 7 activity was obtained when EDTA-PO_4 extraction buffer was used (Table XVIII). The highest protease 4 activity levels was obtained with the phosphate buffer. Protease 4 and 7 activities were low when Tris was used as the extraction buffer.

Extract Additives

The addition of cysteine enhanced protease 4 and 7 activities while cystine, glutathione, sucrose decreased activity substantially (Table XIX). These data suggest a sulfhydryl group may be involved in the active site of protease since cysteine acts as a sulfhydryl protecting agent. Kawashima, et al., (1968) found that cysteine enhanced the activity of protease enzyme in tobacco leaves.

TABLE XVII

PROTEASE ACTIVITY MEASURED AT pH 4 AND 7 ON FOUR SUBSTRATES

Substrate	pH 4	pH 7
	mg Protein Digested/hr/g Fr. Wt.	
0.5% hemoglobin	6.60	2.60
0.5% gluten	0.32	0.00
0.5% plant protein	3.50	1.00
10 mg/ml BSA	1.23	0.29

Assay conducted at 40 C for two hours.

TABLE XVIII

EXTRACTION BUFFER EFFECTS ON PROTEASE 4 AND 7 ACTIVITIES

Buffer	pH 4	pH 7
	mg Protein Digested/hr/g Fr. Wt.	
EDTA:PO_4	8.01	4.43
Tris	6.40	2.46
PO_4	8.72	3.15

Assay conducted with 0.5% hemoglobin substrate for two hours at 40 C.

TABLE XIX

EFFECTS OF EXTRACTION BUFFER ADDITIVES
ON PROTEASE 4 AND 7 ACTIVITIES

Buffer Additives	pH 4	pH 7
	mg Protein Digested/hr/g Fr. Wt.	
cysteine	13.43	3.87
cystine	7.73	3.15
glutathione	10.14	3.27
surcose	5.02	1.24
buffer only	11.77	3.40

Assay conducted with 0.5% hemoglobin as substrate for two hours at 40 C.

Buffer Strength

The high concentration of EDTA and phosphate reduced activity of the protease enzymes (Table XX) indicating that high ionic strength adversely affects protease activity. Pike and Briggs (1972) also found that high ionic strength inhibited protease activity in oats. For some unexplained reason activity was low for all treatments in this test.

Substrate Concentration

The plot of substrate concentration against protease 4 activity (Figure 10) shows a concentration of greater than .125 mg/ml allowing a reaction rate approximating zero order, while at lower concentrations first order reaction rates are approximated. The Lineweaver-Burk plot of substrate against substrate concentration by velocity (Figure 11) approaches a straight line relationship with Km of 0.0437 mg/ml. The data for protease 7 activity were very erratic in all experiments and were

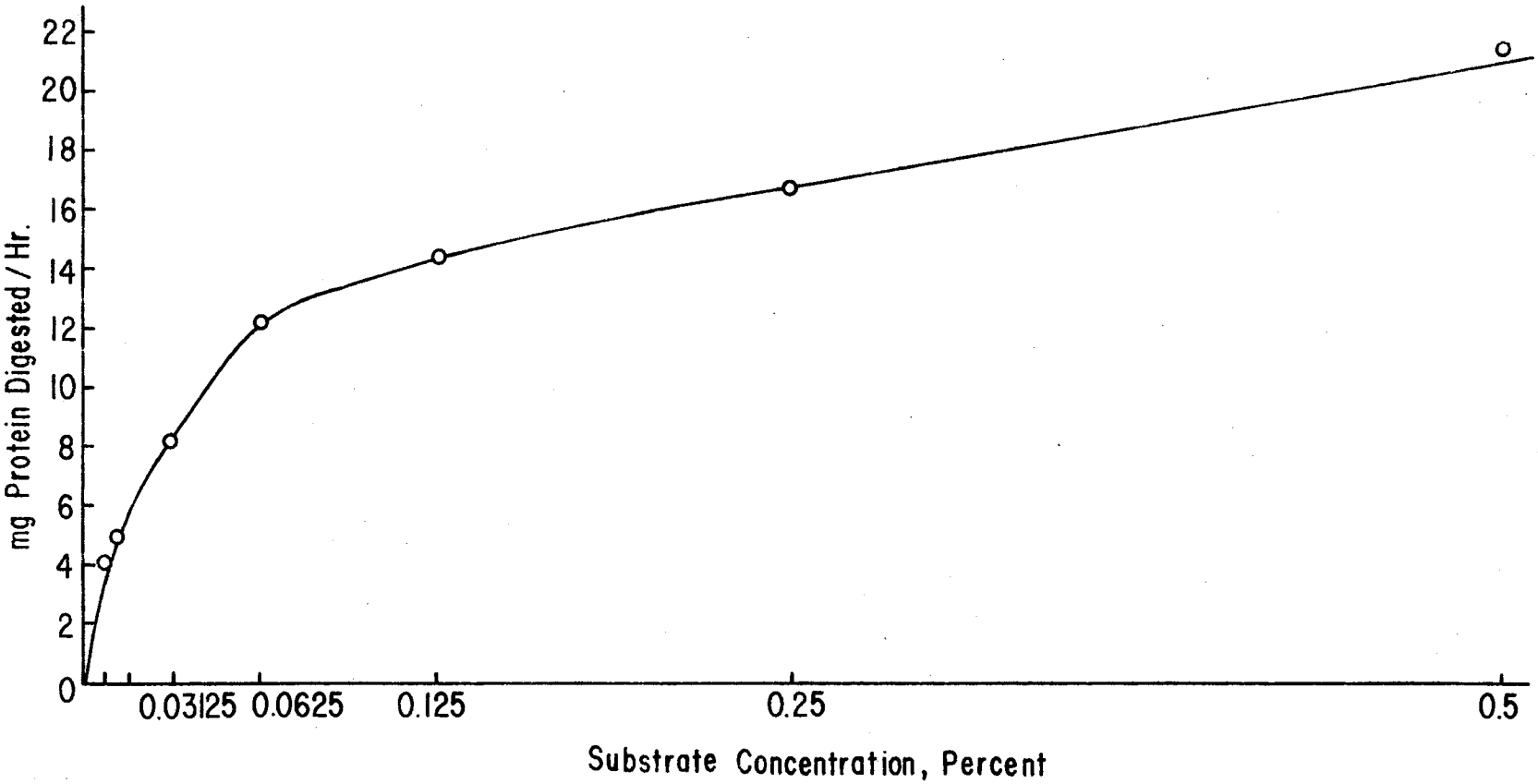

Figure 10. Activity of Protease 4 for Various Concentrations of Hemoglobin

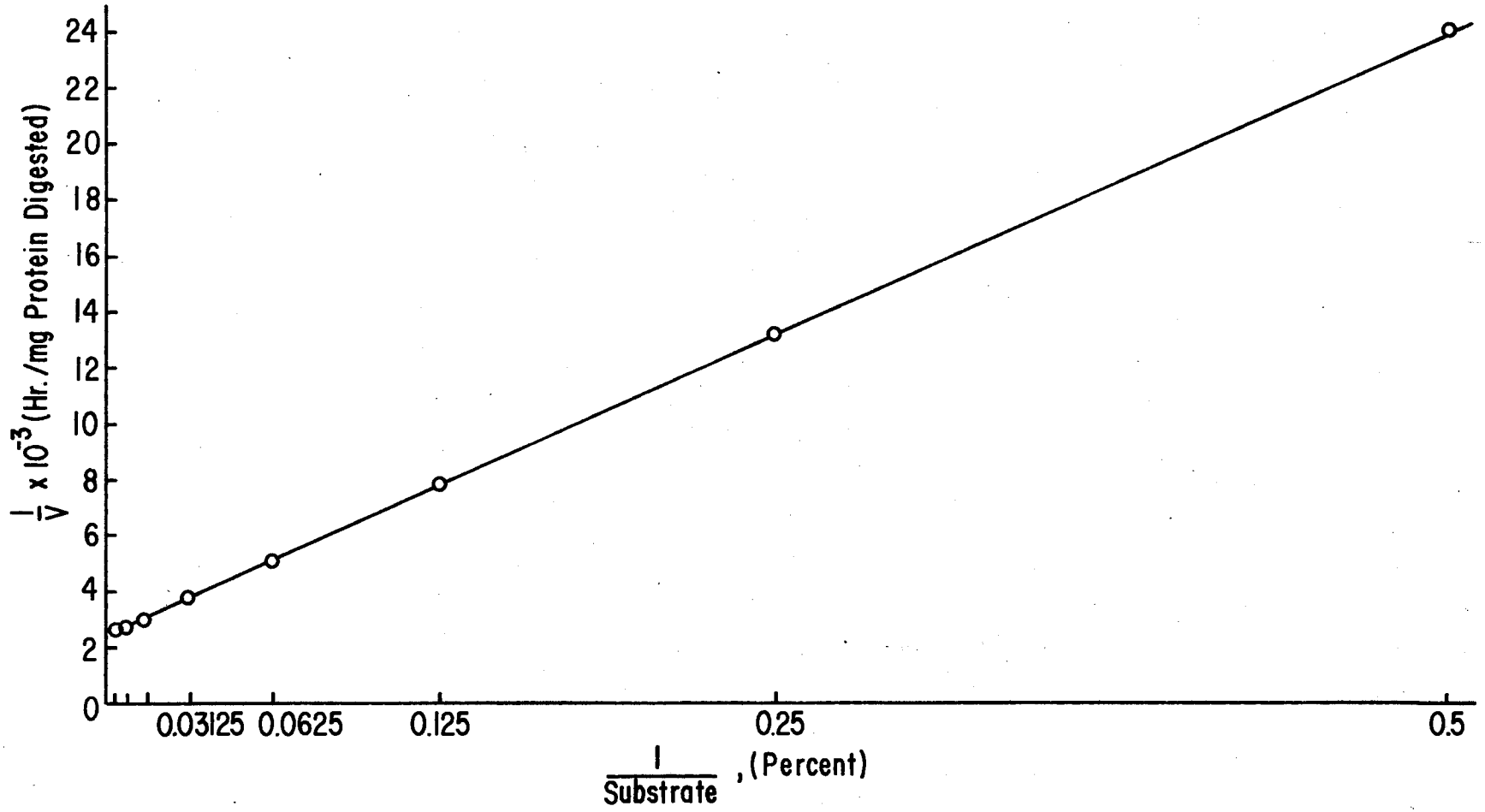

Figure 11. Hemoglobin Concentration Plotted Against the Reciprocal of Enzyme Velocity for Protease 4 of Maturing Wheat

not analyzed further. These data were obtained from wheat protease collected from plants in the maturation stage rather than on week old seedlings.

TABLE XX

EFFECTS OF BUFFER STRENGTH ON PROTEASE 4 AND 7 ACTIVITIES

Strength	pH 4	pH 7
	mg Protein Digested/hr/g Fr. Wt.	
.001 M EDTA	2.59	0.28
.01 M EDTA	3.12	0.11
.1 M EDTA	0.47	0.00
.001 M PO_4	1.13	0.115
.01 M PO_4	2.82	0.35
.1 M PO_4	2.72	0.22

.5% hemoglobin was used as substrate; incubation for two hours at 40 C.

Temperature

The temperature optima were at 40 C for protease 7 and 50 C or higher for protease 4 (Figure 12). Temperatures above 50 C were not tested. Garg and Virupaksha (1970) found a temperature optimum of 50 C for sorghum protease enzyme. Weil, et al, (1966) also found a temperature optimum for protease of 50 C. However, Rao (1971) found the optimum temperature for wheat protease was 40 C.

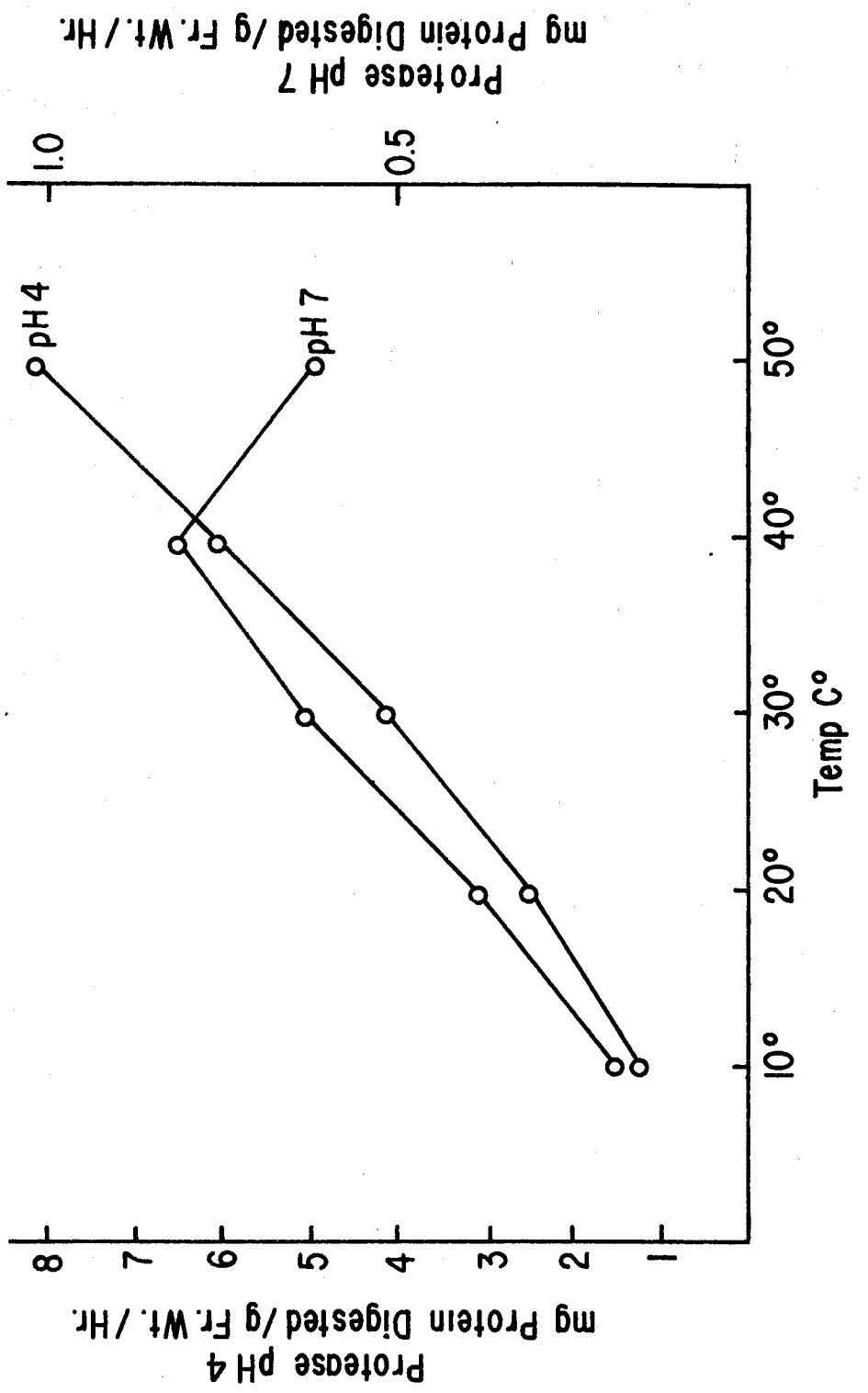

Figure 12. Temperature Responses of Protease 4 and 7 Activities

pH Optimum

The plot of protease activity against pH reveals two activity peaks, the larger at pH 4 and a smaller broader peak at pH 7 (Figure 13). Rao (1971) found similar data working with wheat. Sundblom, et al., (1972) found that in germinating barley a protease with pH optima of 3.9 and 7 existed. Martin and Thimann (1972) found pH optima at pH 3 and 7.5 with a minimum at pH 5 using hemoglobin as a substrate. Garg and Virupaksha (1970b) found a protease in sorghum with peak activity at pH 3.6 and negligible activity above pH 5. Minami, et al., (1971) found high protease activity on casein over the range from 7 to 8.5.

Inhibitors

Cleland reagent and DTNB were the most inhibitory while PMSF was slightly inhibitory to protease 4 activity (Table XXI). When protease 7 activity was measured, Cleland reagent and PMSF were most inhibitory. The data suggest the involvement of a sulfhydryl group on the enzyme since both Cleland reagent and DTNB affect sulfhydryl groups. Pike and Brigg (1972) found a stimulatory effect of DTNB on a protease enzyme of oats; however, it is not improbable that the protease enzymes in oats and wheat are of a different type.

The Hg^{++} was the most inhibitory of the metal ions and since it is a sulfhydryl antagonist this supports the results observed with DTNB and Cleland reagents (Table XXII); also, cyanide was an inhibitor. Cyanide inhibition was not found by Mounfield (1936) who found increased protease activity with cyanide. However, he found that peptidases were inhibited by cyanid; therefore, the inhibition noted in the present

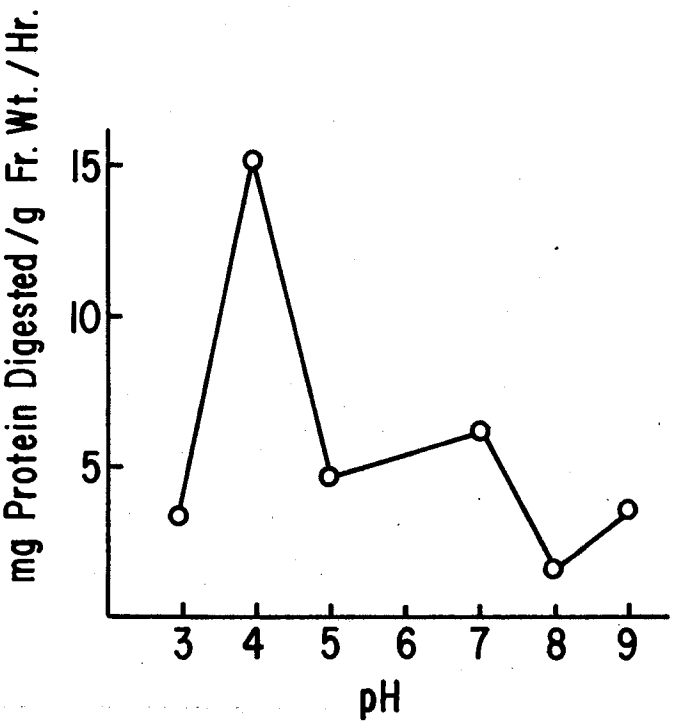

Figure 13. pH Responses of Protease Activity

study suggests that this enzyme is a peptidase.

TABLE XXI

EFFECTS OF PHENYLMETHYL SULFONYLFLUORIDE, 5,5 DITHIO-BIS (2-NITRO BENZOIC ACID) AND CLELAND REAGENT ON PROTEASE 4 AND 7 ACTIVITIES

Inhibitors	pH 4 % Inhibition	pH 7 % Inhibition
PMSF	17.2%	11.3%
Cleland Reagent	24.4%	52.5%
DTNB	3.9%	17.3%
Water	0.0%	0.0%

.5% hemoglobin substrate; incubated for two hours at 40 C.

TABLE XXII

PERCENTAGE INHIBITION OF PROTEASE 4 ACTIVITY BY METAL IONS AND CYANIDE

Metal Ions	5mM	25mM
	Percent	
Fe^{+++}	13.8	28.3
Zn^{++}	7.7	14.9
Ni^{++}	20.8	22.4
Sn^{++}	1.9	19.9
Ca^{++}	30.0	39.7
Mg^{++}	0.0	13.7
CN^{-}	28.7	50.3
K^{+}	1.7	4.9
Hg^{++}	43.4	48.6

Metal ions were incubated with enzyme extract for thirty minutes prior to activity determination. Determinations were made on .5% hemoglobin substrate incubated for two hours at 40 C.

Summary

The study of protease enzymes of wheat presented, supports several conclusions:

1. Hemoglobin substrate supports the highest levels of wheat protease activity.

2. EDTA:PO_4 buffer was a satisfactory buffer system for extraction of protease enzymes from wheat.

3. Cysteine added to the extraction buffer enhanced protease activity.

4. High ionic strength solutions reduced protease activities.

5. Km of 0.0437 mg/ml was noted for protease 4 activity on hemoglobin.

6. Temperature optima of 40 C for protease 7 and 50 C or higher for protease 4 were obtained.

7. pH optima for protease activity were at 4 and 7.

8. The inhibitor study gives evidence that wheat protease contains an active sulfhydryl group and is a peptidase.

9. Further study is needed to better characterize wheat protease active sites and degradation products.

CHAPTER V

THE EFFECTS OF NITROGEN SOURCES AND TEMPERATURE ON NITRATE REDUCTASE AND PROTEASE OF A 'HIGH' AND 'LOW' GRAIN PROTEIN WHEAT[1, 2]

Abstract

The effects of nitrate only and nitrate plus ammonium nitrogen on nitrate reductase (NR) and protease 4 and 7 of high and low grain protein cultivars were determined. The presence of ammonium ion increased protease 4 and decreased NR and protease 7 activity. The high and low grain protein cultivars were found to differ in levels of NR and protease 7. The high grain protein cultivar had higher NR activity and lower protease 7 activity than the low grain protein cultivar. Temperature regimes of 18-25 C and 25-30 night-day temperatures were found to have little effect on NR and protease activities. Additional key words for indexing: Ammonium, Nitrate, Temperature.

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[1] Article coauthored with L. I. Croy for submission to Crop Science for publication.

[2] Abbreviations used in this paper: NR-nitrate reductase, WSP-water soluble proteins, TCA-trichloroacetic acid.

Introduction and Literature Review

Seed protein content is associated with seedling vigor. When total dry matter production is used as a measure of seedling vigor, high positive correlation was found between seedling vigor and seed protein content within wheat cultivar by Lowe and Ries (1972). No differences were found between the performance of low and high protein seed at emergence of the coleoptile but after seven days the high protein seedlings were taller with larger leaf area and higher shoot dry matter. In a subsequent study, the absolute amount of endosperm protein was linearly related to seedling growth and was an important source of nutrients for the germinating embryos and young seedlings (Lowe and Ries, 1973). There are two postulates for the differential responses of high and low protein seeds:

1. Different levels of respiratory substrate and amino acids are associated with differences in endosperm protein;

2. Different levels of enzyme induction are associated with different levels of amino acids which result from hydrolysis of seed protein.

The degradation of reserve seed protein seems to be mediated by protease enzymes. Ory and Henningsen (1969) working with barley, showed that in germination the initial production of amino acids from reserve protein is catalyzed by an acid protease. Other workers have reported the presence of proteases in germinating seeds (Wiley and Ashton, 1967).

Several control mechanisms have been suggested for regulation of protease of germinating seeds. Oaks (1965) proposed that protease degradation of protein in the endosperm was a process regulated by the

demands of the embryo for amino acids. However, other workers have suggested a more complex system of degradation control. Beevers and Guernsey (1966) found that degradation of protein during germination could not be explained simply by the amount of protease present and suggested that another mechanism for controlling the rate of degradation of reserve protein must exist. Further evidence for a complex system of regulation of protein degradation was found by Guardiola and Sutcliffe (1971). They found that control of protein hydrolysis in pea cotyledons was not mediated through the level of protease enzyme as indicated by proteolytic activity of tissue extracts, but that protease activity seemed to be regulated by the shoot probably through a harmonal effect.

Differences in protease activities have been found between high and low grain protein wheat cultivars. Rao and Croy (1971) found high levels of protease in seedlings associated with the high protein cultivars suggesting that the high protease levels of the high protein line promoted higher WSP, amino acids, and indoacetic acid. This resulted in more rapid growth rate than for the low protein line.

Another enzyme important to early plant growth is NR. Schrader and Hageman (1967) have stated that NR is an important enzyme to any study of nitrogen metabolism because it is:

1. the first enzyme in the pathway of nitrate reduction;
2. inducible by substrate;
3. labile *in vivo* under environmental stress;
4. variable in level both diurnally and seasonally;
5. linearly related to grain protein in wheat within a genotype.

The activity of NR has been shown to be regulated by the presence

of nitrogenous compounds in the growth medium. The induction of NR was found to be approximately proportional to nitrate level in the tissue (Beevers, et al., 1965). Increases in nitrate in the nutrient media have been shown to increase NR activity (Hageman and Flesher, 1960). NR was shown to increase with the addition of nitrate and to decrease with addition of ammonium in Chlorella vilgaris (Syrett and Morris, 1963). Furthermore they found that ammonium chloride, urea, aspartic acid, glutamic acid, leucine, histidine, arginine, citrulline, ornithine, and lysine inhibited the induction and synthesis of NR in vivo. They concluded that in Chlorella the control of NR is by repression of enzyme synthesis and not by feedback inhibition. Filner (1966) and Beever, et al., (1965) observed that NR was repressed by ammonia in lower plants but not in higher plants. In tobacco cell cultures, NR was regulated by nitrate and end-product amino acids (Filner, 1966). Nitrate was shown to induce, while casein hydrolysate and amino acids inhibited nitrate uptake and NR activity (Ziekle and Filner, 1971). Proline repressed NR activity and accumulated in plants grown under stress conditions which resulted in loss of NR activity (Filner, 1966). However, Schrader and Hageman (1967) found enhancement of NR induction in the presence of ammonium salts, which they attributed to increased levels of amino acids and amides derived from the readily available ammonium ions. They also found that all L-amino acids, including proline, enhanced NR induction. They concluded that amino acids are not natural inhibitors of NR.

Work with apple seedlings showed maximum NR activity when nitrate was the only source of nitrogen (Frith, 1972). If only ammonium were present, little NR activity occurred. The reduction was possibly due to

the effects of ammonium ions on absorption of nitrate. Ammonium markedly inhibited nitrate absorption in nitrogen starved wheat seedlings but did not decrease the proportion of absorbed nitrate reduced (Minotti, Williams, and Jackson, 1969). It seems that ammonium or products of ammonium do not interfere with induction, stability or activity of NR. Furthermore, when ammonium and nitrate are present in equal amounts, ammonium uptake generally exceeds nitrate uptake. The addition of ammonium substantially decreased the concentration of nitrate in wheat plants even though the external concentration of nitrate remained high (Cox and Reishenhour, 1973). Ammonia fed plants had higher levels of total nitrogen and amino acids than nitrate fed plants. High levels of amino acids in maize roots were associated with ammonium absorption (Ingverson and Ivanko, 1970). These elevated levels were due to more suitable conditions for the incorporation of nitrogen into keto acids which act as precursors of various amino acids.

The absorption of nitrate and ammonium by wheat plants is temperature dependent. Minotti, Williams and Jackson (1969) showed that maximal absorption of ammonium occurred at 25 C whereas the nitrate absorption maximum occurred at 35 C. Marked changes in nitrogen constituents and enzyme activities in wheat plants were shown to be a function of temperature (Srivastava and Fowden, 1972). Growth at low temperature increased levels of glutamic acid, aspartic acid, alpha aminobutryric acid, alanine, serine and lysine. Also the activity of glutamic decarboxylase was increased by low temperatures.

The objectives of this experiment were threefold:

1. to test the effect of varietal differences for grain protein on levels of NR and protease;

2. to test the effect of nitrogen source on NR and protease activities;

3. to test the effect of temperature on NR and protease activities.

Materials and Methods

The cultivars 'Genessee' and NE 65317 were chosen for use in this experiment because of their differences in grain protein. Genessee, a low protein line, is a white wheat. The "high" protein line is a selection from a cross between 'Atlas 66' and 'Comanche'. One hundred seeds of each cultivar were planted at uniform depth in plastic trays using perlite as a support medium. Four trays of each cultivar were planted and two were placed in a controlled environment chamber at either 18-25 C or 25-30 C night-day temperatures at light intensity of 2000 foot candles with 14 hour days. At each temperature one tray was subirrigated with 0.5 strength Hoagland's solution containing only nitrate as the source of nitrogen. The other tray was subirrigated with 0.5 strength Hoagland's solution in which ammonium ions accounted for half the total nitrogen supply. Plants were collected for analysis 5, 7, 9, 11, 13, and 15 days after the experiment initiation. Plant tissue was sampled by cutting the plants at the level of the perlite and placing them on ice. The roots of the sampled plants were removed to stop regrowth. Samples were collected between 9:00 and 10:00 A.M. on all dates. The samples were immediately brought to the laboratory and homogenized in a motorized Thomas homogenizer for two minutes. Extraction procedures were those previously described by Croy and Hageman (1970) for NR. The grinding medium contained 50 mM potassium phosphate, 1.0 mM cysteine and 35 mM EDTA adjusted to pH 7.0 with KOH. The same

plant extraction was used for protease and NR activity determinations. The plant extract after grinding was filtered through two layers of cheesecloth and centrifuged for 15 minutes at 13,000 rpm (20,850 x g). After centrifugation the supernatant was decanted and used for all enzyme assays (Croy and Hageman, 1970). The protease assay procedure was that of Kuo and Yang (1966) with certain modifications. The assay tubes contained 2.0 ml of freshly prepared 0.5% bovine hemoglobin dissolved in citrate-phosphate buffer (15.4 mM citric acid and 16.5 mM sodium phosphate for pH 4 and 3.3 mM citric acid and 21.8 mM sodium phosphate for pH 7). Protease activity was determined at pH 4 and 7, and these activities are called protease 4 and 7 subsequently. A 0.2 ml sample of enzyme extract was added to duplicate tubes of 0.5% hemoglobin and 2.2 ml of 10% TCA solution was added to one tube (blank) to stop enzyme activity. The two tubes were incubated at 40 C for two hours. After two hours, 2.2 ml of 10% TCA solution was added to the assay tube to stop the reaction. Samples were stirred and centrifuged at 2,000 rpm (1,000 x g) for ten minutes to sediment all undigested hemoglobin. The supernatant was collected and assayed for digested nitrogenous material by the Lowry method (Lowry, et al., 1955) using bovine serum albumin as a standard. The difference in the assay and blank was used to estimate protease activity.

Alpha amino nitrogen was assayed by procedure of Yemm and Cocking (1955) using isoleucine as a standard. WSP content was determined for the crude enzyme extract by precipitation with 5% TCA and performing a Lowry procedure (Lowry et al., 1955). Plant total nitrogen percentage was determined by the micro-Kjeldahl procedure.

The experiment was performed three times. Each experiment was

considered as a replication for statistical purposes. The data were analyzed statistically as a split-split plot with the main plot being temperature and subplots nitrogen source and cultivar.

Results and Discussion

The levels of NR within each cultivar were significantly different (Table XXIII). The high protein cultivar had higher NR activity on all dates sampled (Figure 14). The pattern of activity was similar in both cultivars with maximum activity occurring on the seventh day and then declining. The marked decrease in activity on days 11, 13, and 15 for the nitrate only treatment suggests that some type of regulation of NR activity occurred. However, the type of regulation could not be ascertained from the experiment, and it appeared to be associated with both low nitrate and high amino acids. Increasing nitrate in the ammonium treatment (Figure 15) probably was the reason for the lack of a decline in NR activity in that treatment. Zieserl and Hageman (1962) found levels of NR activity were different between inbred lines of corn when the plants were 2-3 weeks old. Croy (1967) found that 'Ponca' wheat, which has a higher grain protein content than 'Monon', also had a higher NR activity.

The presence of ammonium ions in the nutrient medium were found to reduce NR activity compared to nitrate alone (Table XXIII). Differences occurred on days 5, 7, 9 with no significant differences on days 11, 13, and 15 (Figure 16). The data suggest that ammonium was inhibiting NR activity or nitrate uptake. If one assumes that the level of nitrate in the ammonium treated plants should be one-half that in nitrate treated plants simply due to the difference exogenous nitrate concentration,

TABLE XXIII

CULTIVARS, NITROGEN TREATMENTS, TEMPERATURE MEANS FOR NITRATE REDUCTASE, NITRATE AND ALPHA AMINO NITROGEN

Factors	Nitrate Reductase	Nitrate	Alpha Amino Nitrogen
	umoles NO_2/g Fr. Wt/hr		ug/g Fr. Wt.
Genessee	7.25	2960	3197
NE 65317	10.34**	3170	3310
Ammonium	7.95	2050	3782**
Nitrate only	9.64**	4080**	2724
18-25 C	8.58	3040	3175
25-30 C	9.01	3080	3331

** Denotes significance at 1% level

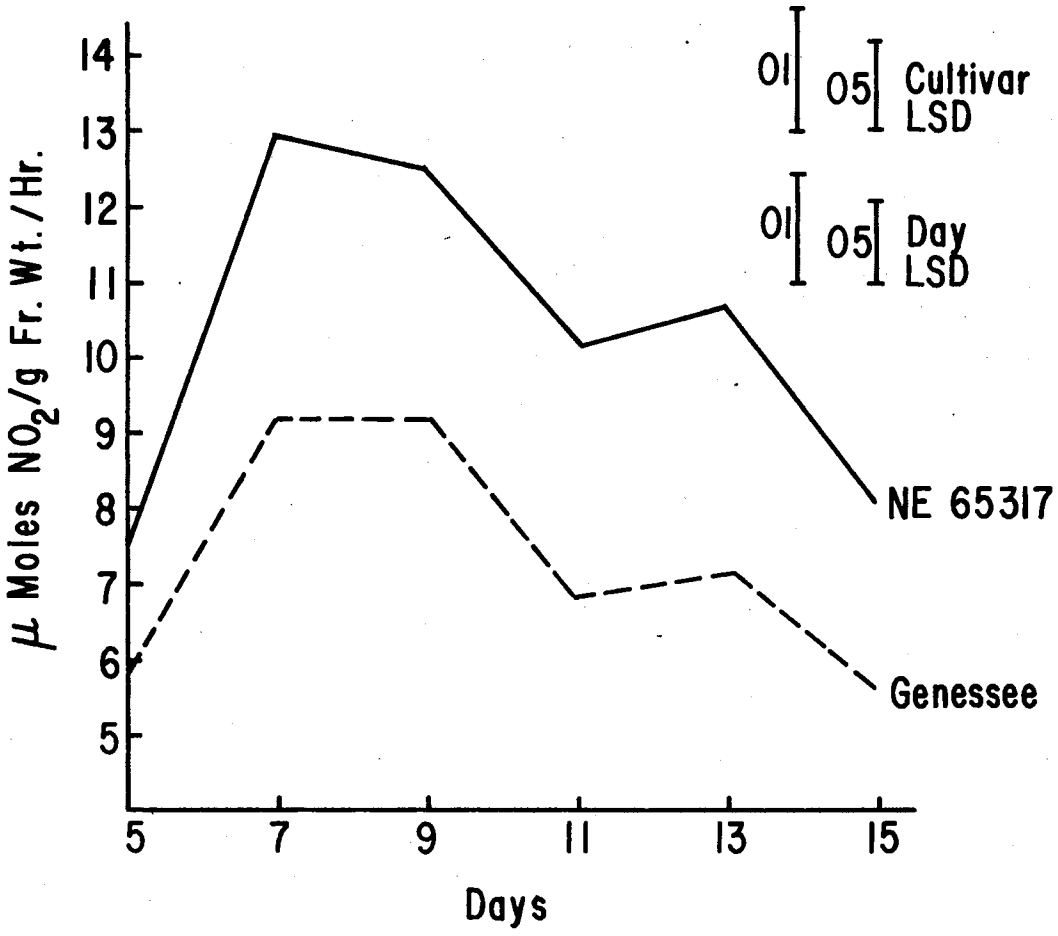

Figure 14. The Influence of Plant Age on Nitrate Reductase Activity for Two Wheat Cultivars

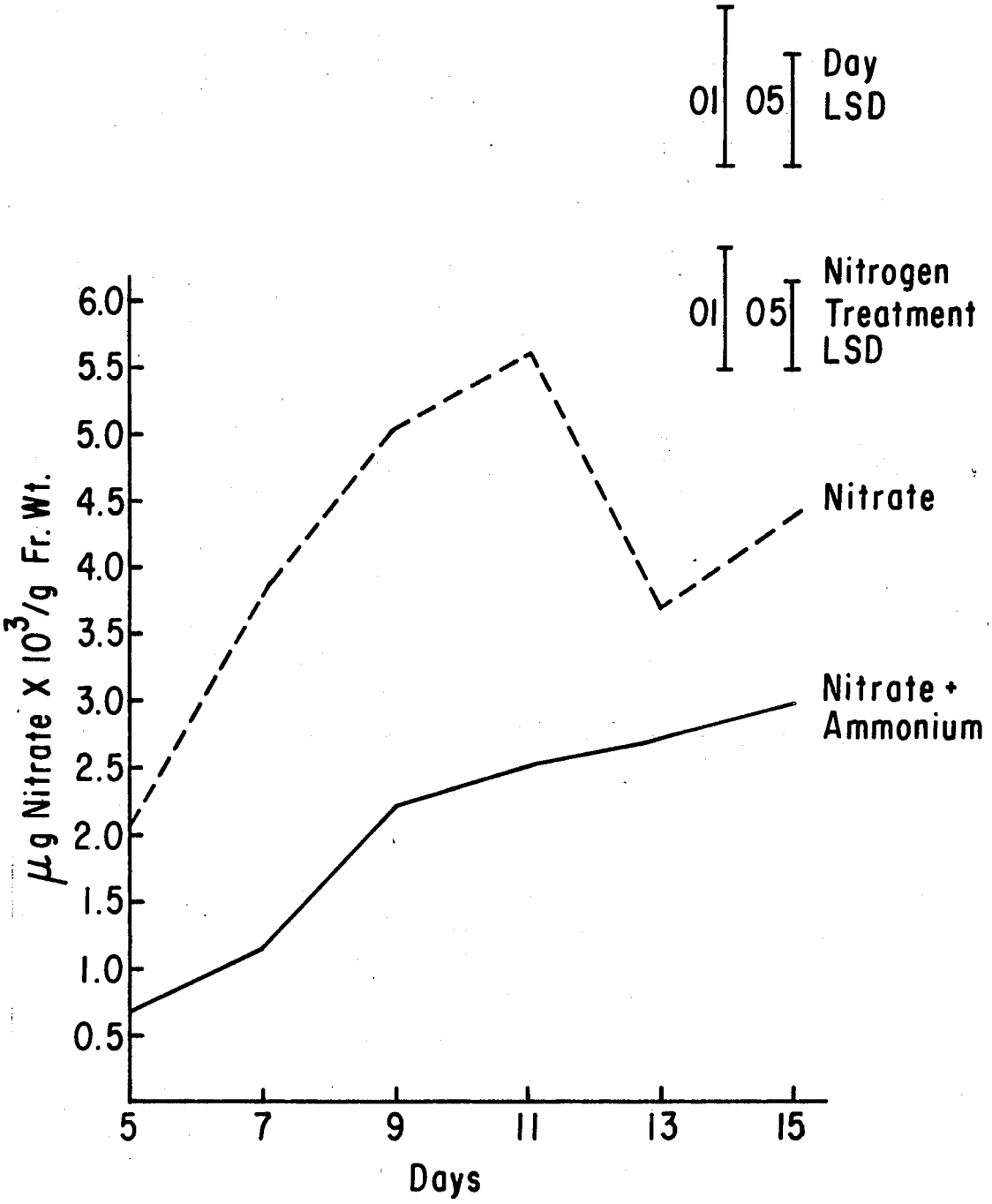

Figure 15. The Influence of Plant Age on Nitrate Levels for Two Nitrogen Treatments

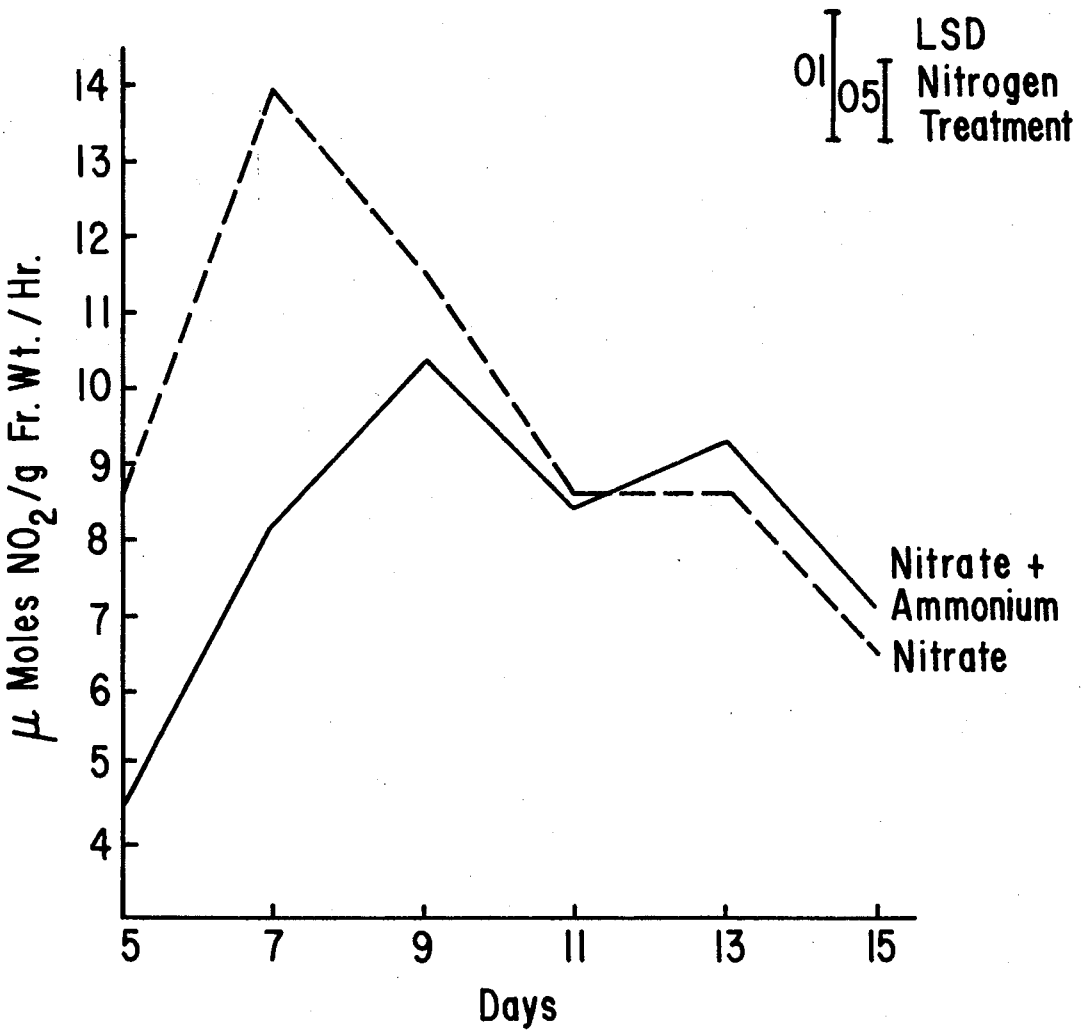

Figure 16. The Influence of Plant Age on Nitrate Reductase Activity for Two Nitrogen Treatments

then any additional reduction is related to the presence of the ammonium ion. The reduction is 36% on days 5 and 7, and 12 and 9 percentage on days 9 and 11 respectively (Table XXIV). The decline in inhibition was possibly caused by bacterial oxidation of the ammonium in the nutrient medium with time. However, it cannot be deduced conclusively from this study whether this reduction in NR activity is due solely to decreases in nitrate uptake or inhibition of NR. However, it would seem that the reduction in NR upon addition of ammonium noted was a result of reduced nitrate uptake and, consequently, reduced substrate for nitrate reduction.

Ward and Miller (1971) working with tomatoes found fertilization with ammonium nitrate resulted in accumulation of amino acids, particularly in arginine. NR in tobacco can be regulated by end-product amino acids (Filner, 1966). It is conceivable that this regulation is brought about by reduction in nitrate levels since Heimer and Filner (1971) showed that nitrate uptake in tobacco XD cells is subject to end-product control by amino acids. Casein hydrolysate and amino acids have been shown to inhibit the development of NR activity (Zielke and Filner, 1971). Therefore, if ammonium increases amino acids levels it could reduce levels of NR. From data collected in this experiment it was apparent that ammonium significantly increased the levels of alpha amino nitrogen presence in the plants (Figure 17). Therefore, it seems possible that this increased level of amino acid was indirectly reducing the level of NR present. However, in work by Schrader and Hageman (1967) they did not show reduction in NR in response to high endogenous levels of amino acids in the induction media. Consequently the reduction noted in NR cannot be attributed to either low nitrate or high

TABLE XXIV

LEAF NITRATE CONTENT AS A FUNCTION OF NITROGEN TREATMENTS

	Day					
	5	7	9	11	13	15
	Mg x 10^2					
Nitrate only	2.13	3.58	5.06	5.62	3.69	4.42
Ammonium + nitrate	0.67	1.15	2.21	2.53	2.73	2.97
Difference from 1/2 nitrate only and ammonium + nitrate	-0.39	-0.64	-0.31	-0.28	+0.89	+0.76
% reduced leaf nitrate	36%	36%	12%	9%		

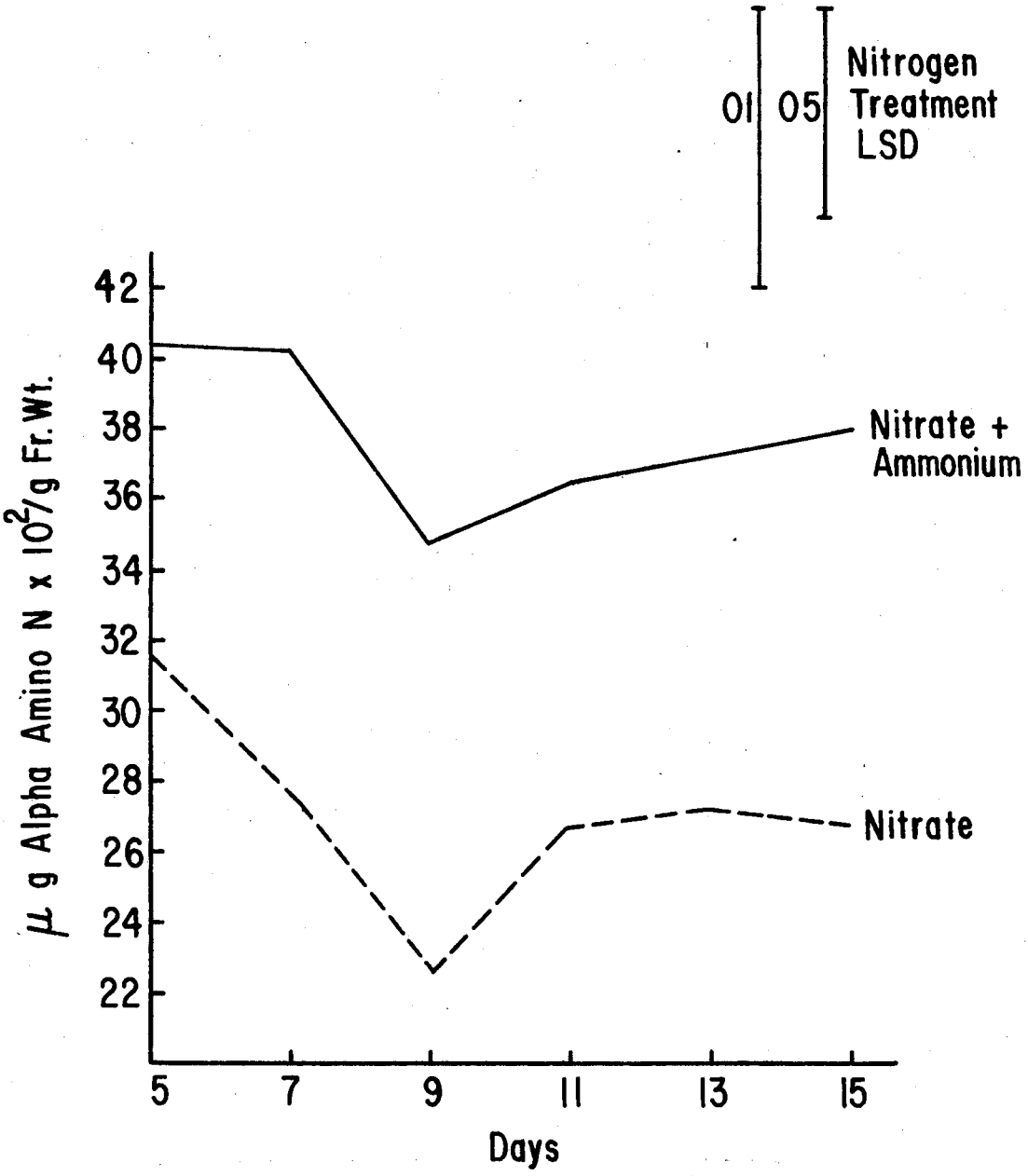

Figure 17. The Influence of Plant Age on Alpha Amino Nitrogen for Two Nitrogen Treatments

amino acids conclusively. The temperature used did not have a significant effect on NR (Table XXIII). This might be expected since both temperatures used are within the range where Croy (1967) found good expression of NR activity. It is interesting to note the ammonium caused a delay of two days in the maximal activity of NR. This delay plus the fact that nitrate within the plant increased with time suggests that the ammonium was being converted to nitrate by bacteria.

Protease 4 levels were not significantly different for cultivars (Table XXV). This is in contrast to data of Rao and Croy (1971) who showed differences in protease levels for high and low protein genotypes in wheat. The pattern of development of protease 4 activity was similar in both cultivars with peaks on days 9 and 15 (Figure 18).

The addition of ammonium to the nutrient media caused a marked increase in the activity of protease 4 (Figure 19). These data are difficult to interpret since Oakes (1965) stated that protease activity in the cotyledon is regulated by amino acid levels, low amino acids causing higher activity. However, it must be remembered that leaf and cotyledon are quite different anatomically and similarity in enzyme regulation might not be expected. Although a reduction in protease activity was found to be associated with increased amino acids by Yomo and Varner (1973) in pea cotyledons. These workers found that as amino acid content increased in cotyledons, the rate of storage protein loss and protease activity was reduced. Also the rate of storage protein loss and protease activity were retarded by the presence of casein hydrolysate.

There is a paradox in that high protease activity should reduce WSP content, since protein is the substrate for protease. However, this was not true with the ammonium treatment since it also increased WSP

TABLE XXV

PROTEASE 4 AND 7 ACTIVITIES, WATER SOLUBLE PROTEIN AND KJELDHAL NITROGEN MEANS AS A FUNCTION OF CULTIVAR, NITROGEN TREATMENT AND TEMPERATURE

	Protease 4	Protease 7	Water Soluble Protein	Kjeldahl Nitrogen
	mg Protein Digested/hr/g Fr. Wt.		mg/g Fr. Wt.	Percent
Genessee	11.05	2.09	23.87**	4.93
NE 65317	11.18	1.78**	25.72	5.36
Ammonium	12.17**	1.82	26.21	5.57
Nitrate only	10.07	2.04	23.38**	4.71**
18-25 C	11.32	1.97	23.45*	5.31
25-30 C	10.92	1.90	26.14	4.97

*Denotes significance at 5% level.

**Denotes significance at 1% level.

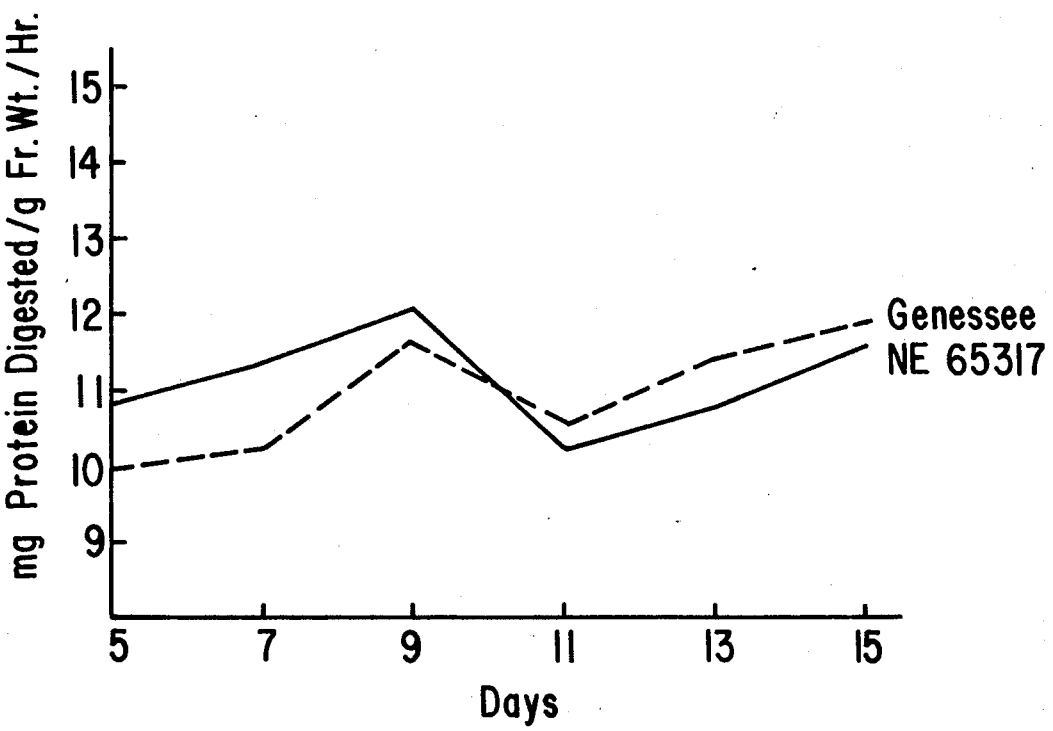

Figure 18. The Influence of Plant Age on Protease 4 Activity for Two Wheat Cultivars

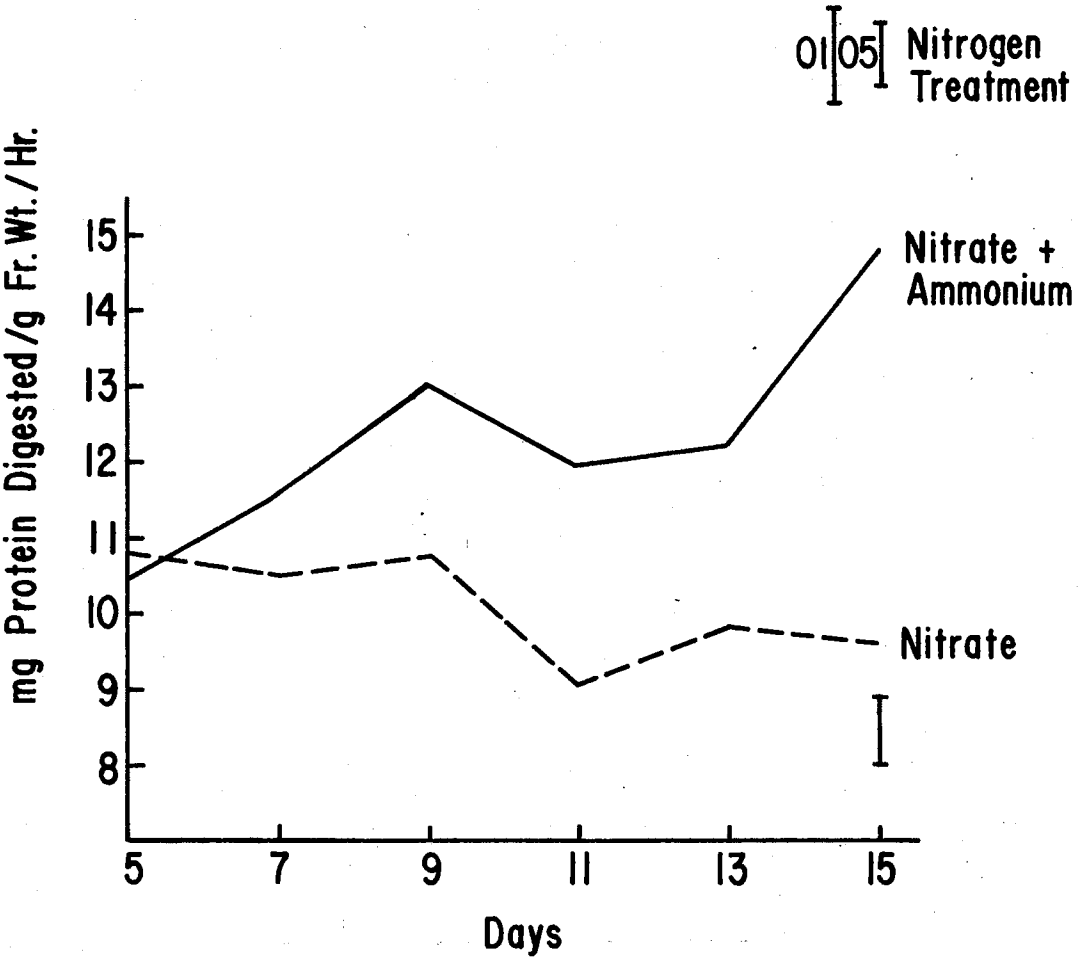

Figure 19. The Influence of Plant Age on Protease 4 Activity for Two Nitrogen Treatments

proteins (Figure 20). The possibility exists that ammonium treatment was supplying nitrogen at high enough levels that protein synthesis was surpassing protease degradation of protein. These data raise the question about the importance of protease 4 in the metabolism of a juvenile plant. The lack of correspondence between protease 4 and WSP levels suggests that only a part of the enzyme is active in vivo in protein degradation. Beevers and Guernsey (1966) suggest that degradation of protein at the early stages of germination in peas cannot be explained simply by the amount of protease present. A complex mechanism of control may be involved in protease regulation in the plant. This control could involve isolation of protease enzyme within the plant cells; possibly in the vacuole or in lysosomes. The possibility of active site inactivation by inhibitors or conformational changes exists also. Guardiola and Sutcliffe (1971) stated that protease activity seems to be closely linked to senescence of cotyledon of peas. Therefore, it is possible that protease 4 is functioning in senescence in the shoot. Support for this idea comes from the fact that protease 4 levels tend to increase with time (Figure 18). Temperature regime had no effect on protease levels measured at pH 4 (Table XXV).

Differences between the cultivars were significant for protease 7. Genessee, the low protein cultivar, was higher in activity particularly on the later days than NE 65317 (Table XXV, Figure 21). However, high protease activity was shown to be associated with high protein wheat cultivars by Rao and Croy (1971). The addition of ammonium generally lowered the levels of protease 7, particularly on the first two days (Figure 22). This information, plus the increased alpha amino nitrogen levels, suggests that alpha amino nitrogen was regulating protease 7

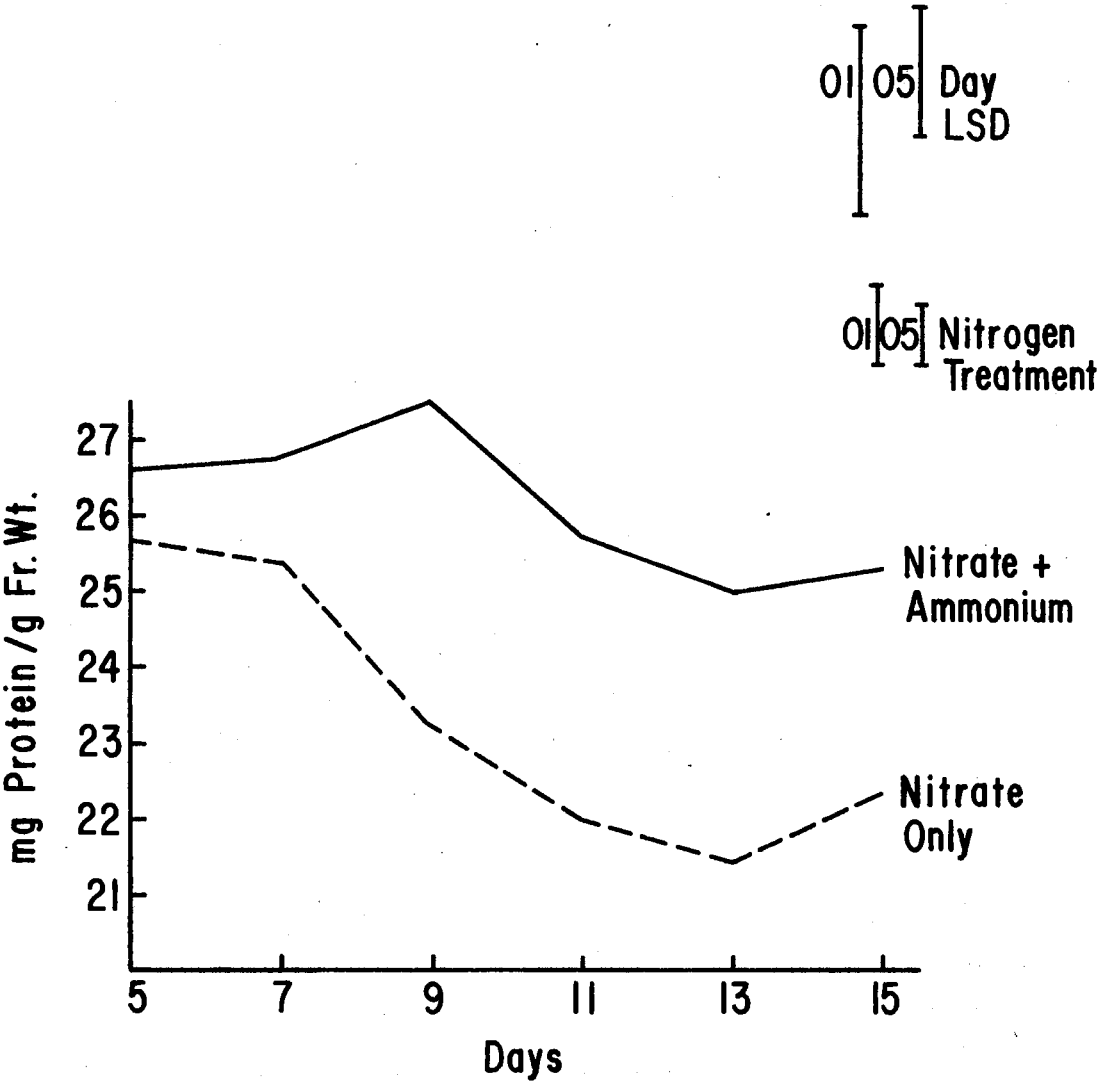

Figure 20. The Influence of Plant Age on Water Soluble Protein for Two Nitrogen Treatments

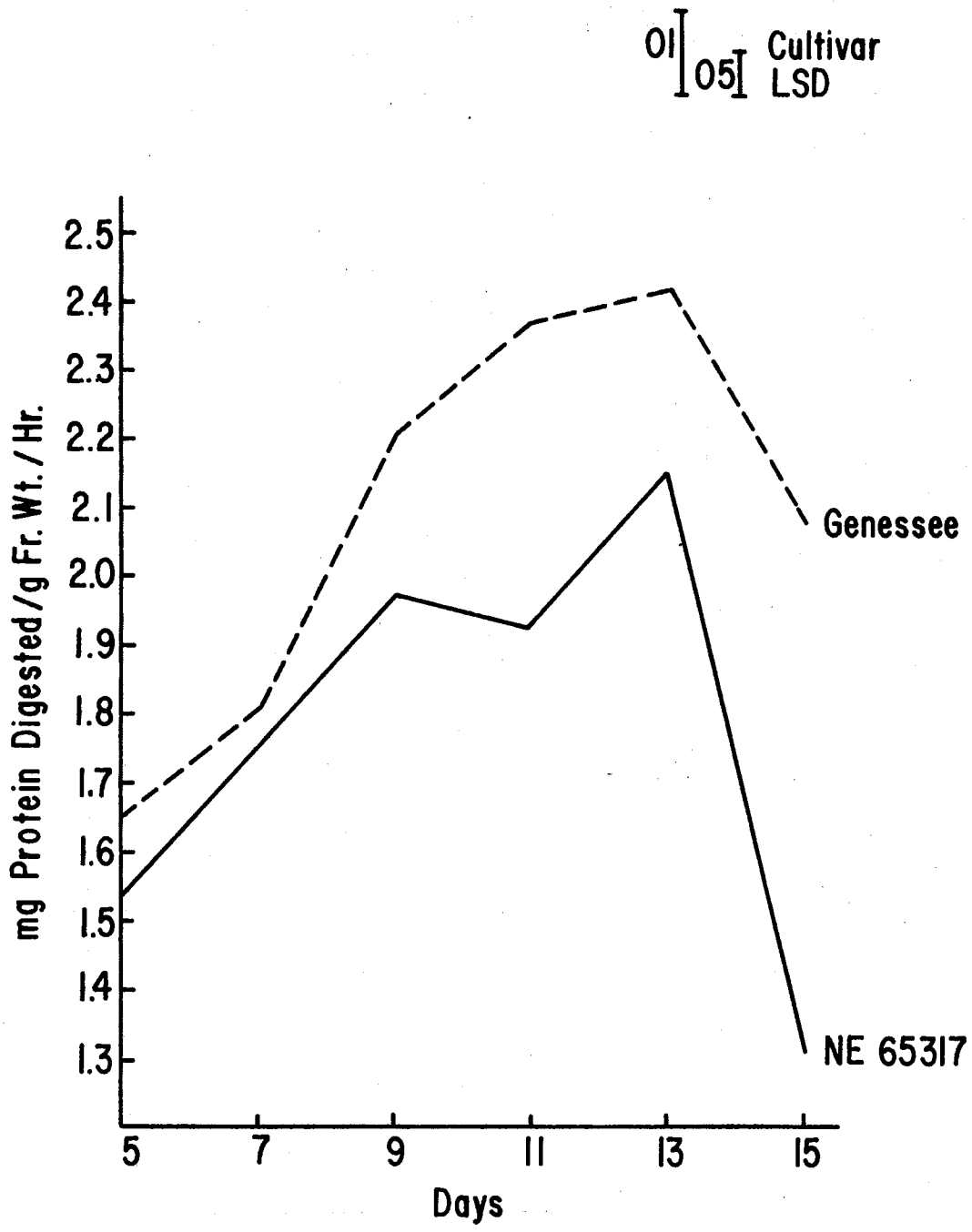

Figure 21. The Influence of Plant Age on Protease 7 Activity for Two Wheat Cultivars

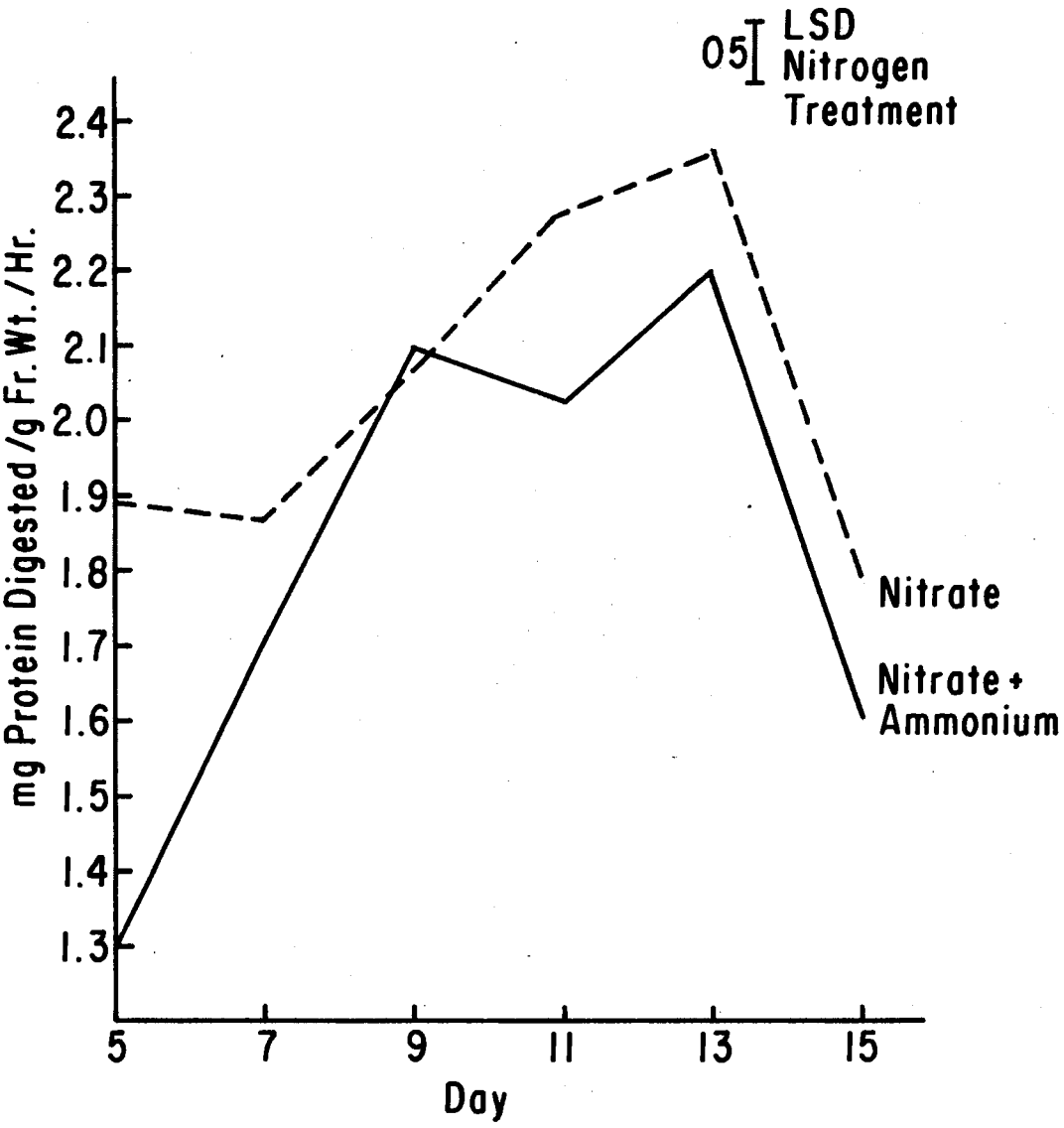

Figure 22. The Influence of Plant Age on Protease 7 Activity for Two Nitrogen Treatments

activity. Protease levels in young seedlings did not correspond well with protease activity found in plants approaching physiological maturity with regard to relationships between protease and protein levels of the grain as found by Rao and Croy (1972) and Perez, et al., (1973).

Temperature regime had no effect on protease 7 levels (Table XXV).

The levels of Kjeldahl nitrogen were significantly different between the cultivars with the high protein cultivar having the higher value (Table XXV). This is a reflection of the initial higher nitrogen levels in the seed of the high protein cultivar and the higher NR activity. Kjeldahl nitrogen levels throughout the experiment were similar for both cultivars (Figure 23). The general pattern was a reduction in Kjeldahl nitrogen with time, however, the last day showed a marked increase and was probably a reflection of the level of nitrate present. The addition of ammonium significantly increased Kjeldahl nitrogen, as would be expected in light of the increase in alpha amino nitrogen and WSP associated with ammonium. Temperature regime had no effect on levels of Kjeldahl nitrogen present (Table XXV).

WSP was higher in the high protein cultivar and addition of ammonium increased its value (Figures 20, 24). Pattern of WSP content across days was similar to Kjeldahl nitrogen. WSP levels were significantly different with temperature (Table XXV). The higher temperature regime produced more WSP than the low regime (Figure 25). This increase is probably a reflection of the increased enzyme activity or the increased nutrient absorption usually associated with high temperature; although the activity of NR and protease 4 and 7 were not significantly affected by temperature regime.

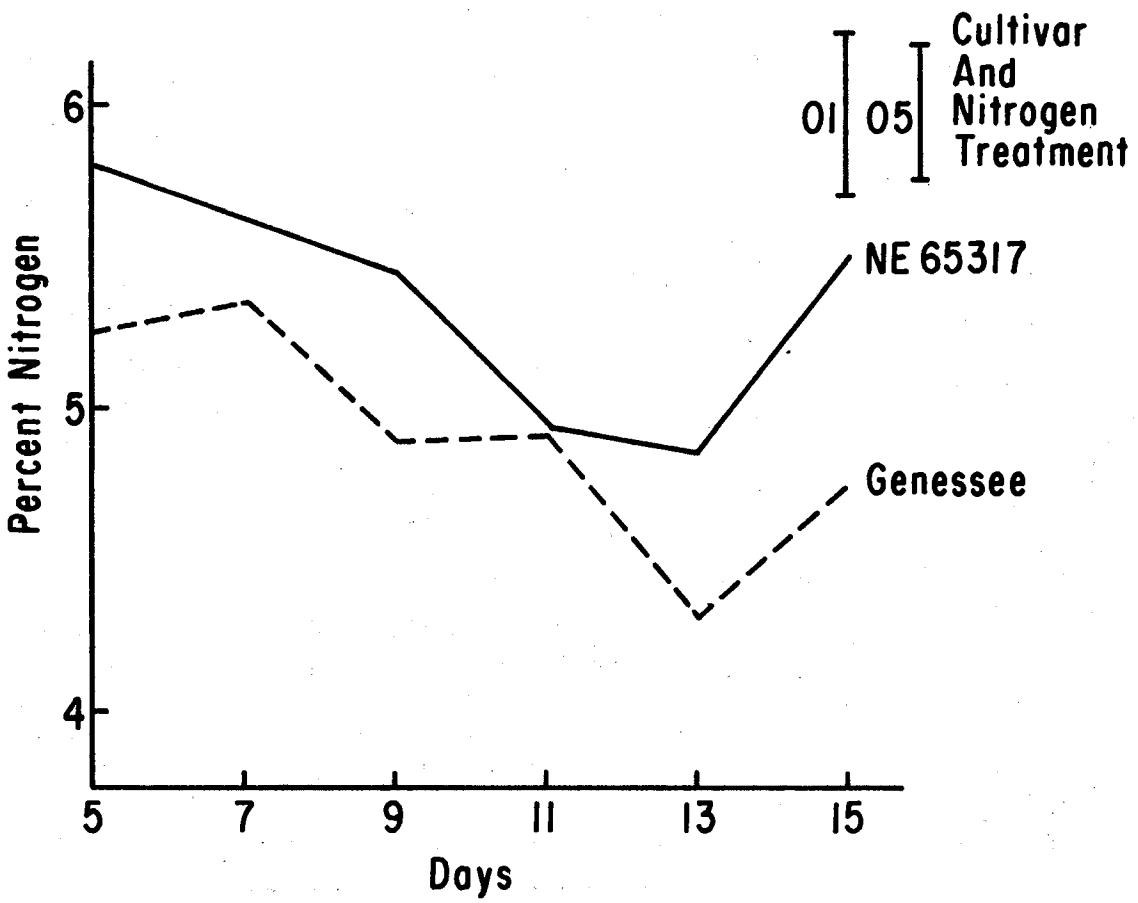

Figure 23. The Influence of Plant Age on Kjeldahl Nitrogen for Two Wheat Cultivars

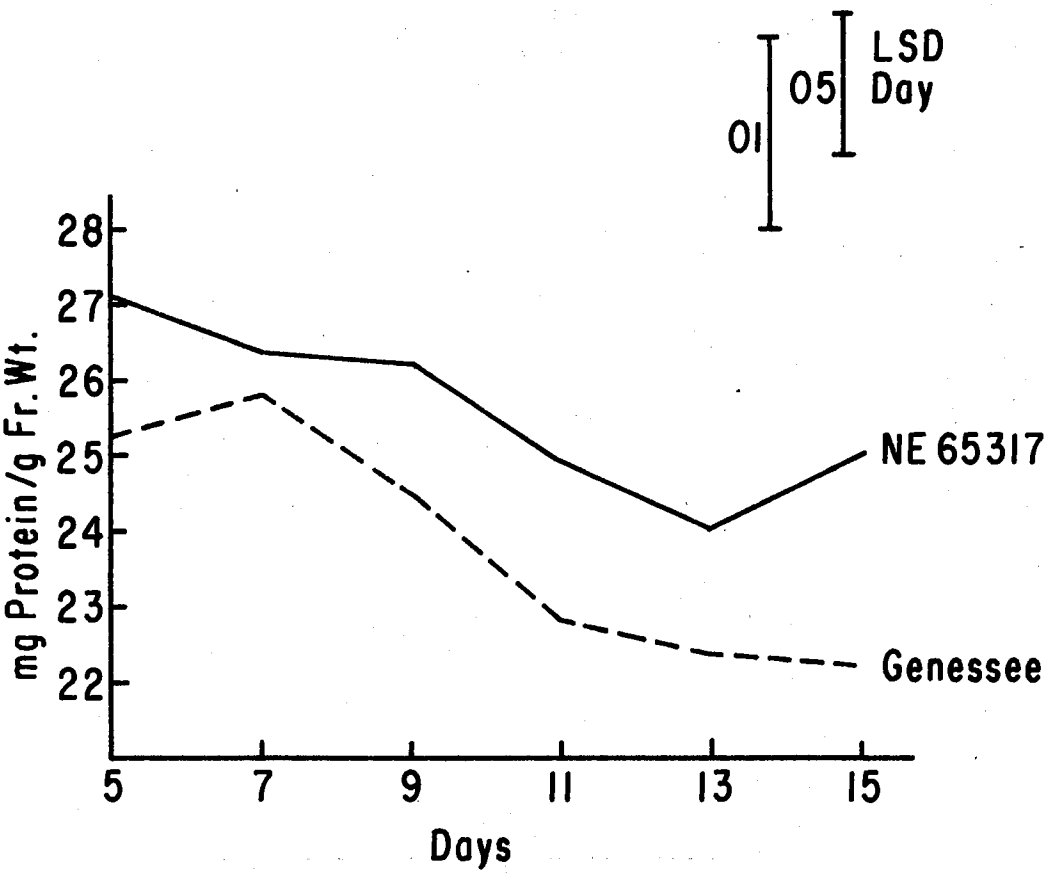

Figure 24. The Influence of Plant Age on Water Soluble Protein for Two Wheat Cultivars

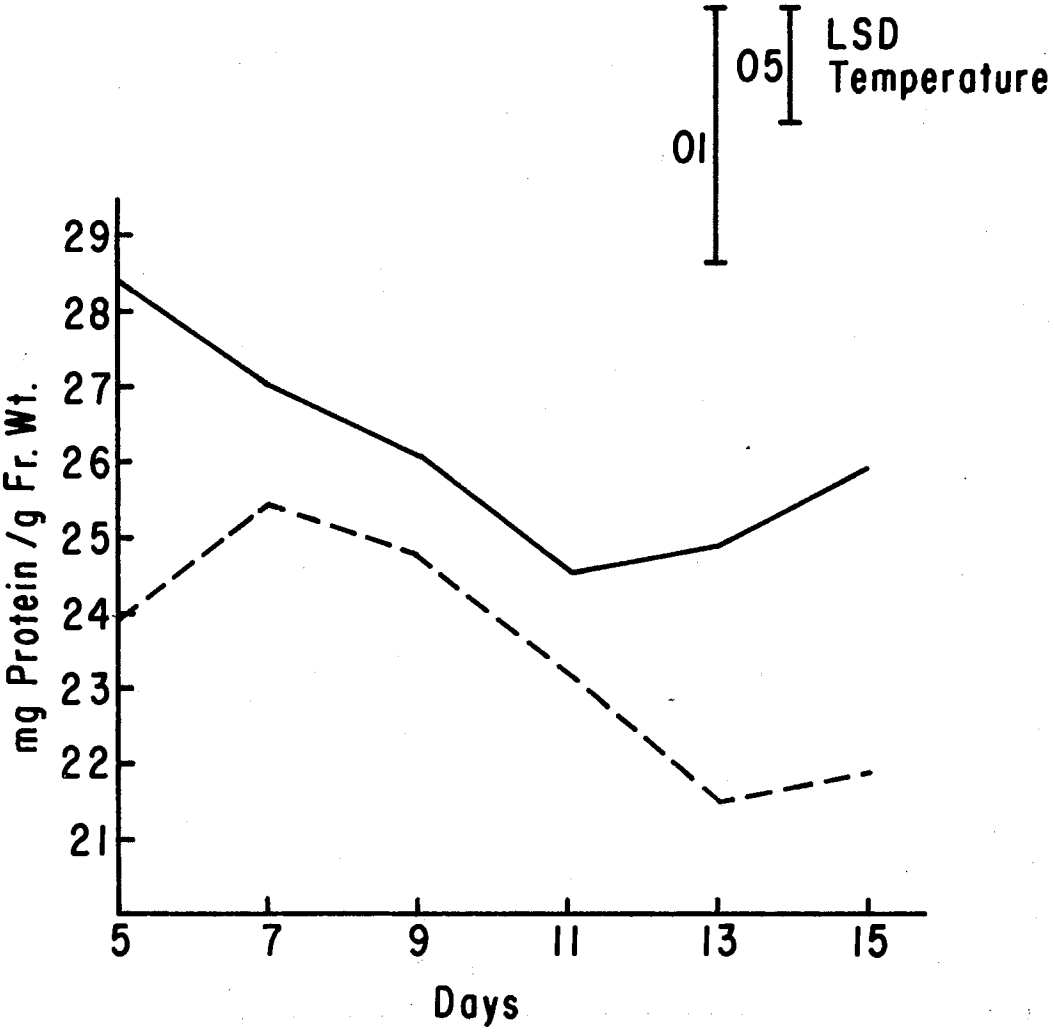

Figure 25. The Influence of Plant Age on Water Soluble Protein for Two Temperature Regimes

The temperature by ammonium interaction was significant for WSP and is probably a reflection of the large effect of temperature and ammonium additions on WSP.

Alpha amino nitrogen was not significantly different between the cultivars or temperatures; however, the addition of ammonium did significantly increase the levels of alpha amino nitrogen in the plants (Figure 17).

Nitrate levels were not significantly different between cultivars or temperatures. The addition of ammonium did cause a significant difference for the reasons discussed under ammonium effect on NR (Figure 15).

Summary

Conclusions drawn from the results of this study indicate:

1. Genetic differences in grain protein are reflected in the levels of NR and protease 7 in young seedling but not in levels of protease 4;

2. Ammonium additions reduce NR, increase protease 4 and reduce protease 7 activity;

3. The temperatures used had no signficant effects on NR or protease activities, although it did affect WSP contents.

CHAPTER VI

SUMMARY

These studies, although preliminary in nature, reveal several features of the nitrate reductase and protease systems. Nitrate reductase activity was found to correlate with both forage and grain protein. These correlations suggest that breeding for increased nitrate reductase activity could be a tool for increasing grain protein. Large variation for nitrate reductase activity was found among the cultivars tested. Therefore, genetic differences for nitrate reductase apparently exist and it should be possible to identify types with high levels.

The regulation of nitrate reductase in the plant seems to be dependent on both nitrate and alpha amino nitrogen levels. A positive correlation was found between nitrate reductase and nitrate and negative correlation with alpha amino nitrogen. These data were consistent with a nitrate induction and alpha amino nitrogen inhibition of nitrate reductase activity. Ammonium ions supplied in the nutrient media were found to reduce nitrate reductase activity possibly by reducing the amount of nitrate in the leaf and increasing the alpha amino nitrogen. Either of these effects would tend to reduce nitrate reductase activity.

Protease activities were found to differ among the cultivars tested indicating that genetic diversity does exist for these characters. Two proteases were postulated. One protease had a pH optimum of 4 with a temperature optimum of at least 50 C, while the other had a pH optimum

of 7 and a temperature optimum of 40 C. Protease measured at pH 7 seems to have correlated with plant protein on more dates than protease 4. Several positive correlations between protease 7 and forage protein were found before flowering with negative correlation after flowering. In young seedlings protease 7 is higher for the low protein genotype than for the high grain protein genotype. The occurrence of differences at early stage of development is significant in that it might allow a breeder to select for high protein character without growing out the seed; therefore, the relationship protease 7 and grain protein in seedlings should be studied further.

Also the information that protease 7 has fairly high heritability coefficients would indicate that further study of this enzyme might be advantageous to increasing grain protein.

Protease 4 was found to be negatively correlated with forage nitrogen late in the growing season.

These studies seem to have proposed more questions than they have answered. In subsequent studies more attention should be given to growth pattern of the cultivars. Physiological age seems to have a great effect on these enzymes. If future experiments were grouped according to maturity group, much of the inconsistencies of these data could be removed.

Also a more rapid and sensitive test for protease 7 is needed. The use of color releasing medium for protease 7 activity measurements might be useful in this regard. This procedure would reduce the amount of time required to run a sample; therefore, increasing the number of samples which can be run in a day. If more samples could be run within a given period then the reliability of parameters such as heritability

could be improved.

Several conclusions can be drawn from these studies:

1. Genetic diversity does exist for nitrate reductase and protease characters;

2. Protease measures at pH 7 has a high enough heritability that it could be used as a tool in a breeding program;

3. Control of nitrate reductase and protease systems was complex and seems to involve alpha amino nitrogen and nitrate;

4. More study is needed to further understand the nature and function of these enzymes in the wheat plant.

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APPENDIX

TABLE XXVI

BARTLETT'S TEST OF HOMOGENEITY OF VARIANCES FOR WATER SOLUBLE PROTEIN ON APRIL 14, AND JUNE 1 AND FOR PROTEASE 4 ON MAY 25, 1972, AND ON A POOLED BASIS

Date	Variable	df	Chi Square	1% Probability
April 14	WSP	2	32.3**	9.21
June 1	WSP	2	39.0**	9.21
Pooled	Protease 4	2	308.69**	9.21
May 25	Protease 4	2	38.269**	9.21

**Denotes significance at 1% level of probability.

VITA

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Doctor of Philosophy

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