Protease and Nitrate Reductase Seasonal Patterns and Their Relation to Grain Protein Production of "High" *vs.* "Low" Protein Wheat Varieties

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Nitrate reductase was higher in three wheat varieties having "Atlas 66" germ plasm for high grain protein potential than "Triumph 64," a hard red winter wheat (*Triticum aestivum* L.) with a low grain protein potential. Leaf protease was found to have two pH optima, pH 4 and 7. Protease (pH 4.0) levels were higher for the low protein variety prior to flowering; however, the high protein varieties exhibited higher protease levels after flowering. High nitrate reductase before flowering and high leaf protease activities after flowering appear to be related to high grain protein production.

Crop physiologists are seeking to obtain biochemical criteria that will be useful to the plant breeders in the selection of superior varieties. One possible approach is the analyses of regulating enzyme systems. Enzymes are the direct product of the gene, and their level of activity should be related to the metabolic potential of the genotype. We should be able to screen plant material for activity levels of important enzymes and produce genotypes having desirable enzyme levels.

Significant genetic differences in the protein content of wheat (*Triticum aestivum* L.) were obtained by Middleton *et al.* (1954), who reported that Atlas 66, a soft winter wheat, produced significantly more protein in its grain than other soft winter wheat varieties. Johnson *et al.* (1968) have shown a 15 to 25% higher grain protein in new varieties having Atlas 66 as a parent than in the varieties previously used.

Increased nitrogen applications have been shown to increase grain yield and grain protein percent (Schlehuber and Tucker, 1959; Welch *et al.*, 1966). Soil fertility and moisture levels are major environmental factors which may significantly influence the quantity of protein synthesized in the wheat grain. Haunold *et al.* (1962) stated that protein formation in wheat grain is a function of the genetic factors interacting with factors of soil and climate.

Nitrate is the primary soil nitrogen form available to plants, and nitrate reductase (NR) initiates the reduction process from nitrate to amino acids and protein. The rate-limiting enzyme in this reduction sequence is NR (Beevers and Hageman, 1969). Increased protein formation and decreased nitrate content of the plant are associated with increased NR activity (Hageman *et al.*, 1961). Croy and Hageman (1970) reported that supplemental nitrogen applications increased NR activity and grain protein in wheat. Duffield (1971) also found increases in NR activity in high protein wheat varieties.

Johnson *et al.* (1968) studied the physiological aspects of high protein in wheat and concluded that high protein content in the grain of certain varieties was the result of more efficient and complete translocation of nitrogenous compounds from the vegetative parts of the plant to the grain. Seth *et al.* (1960) observed that the differences in grain protein were associated with differences in the rate of protein degradation in the vegetative parts of the plant or protein synthesis in the developing kernel.

Most of the nitrogen in green plants is in the form of high molecular weight compounds which presumably must be hydrolyzed to amino acids before translocation. Beevers (1968) and Mounfield (1936) observed the presence of protease enzymes in pea cotyledons and wheat seeds during germination and pointed out that these enzymes break down the reserve proteins into amino acids and other low molecular weight compounds which are translocated to the growing parts.

The objectives of this study were to determine the influence of two fertility levels on the seasonal activities of NR and protease in the leaves of wheat varieties having different grain protein contents and to determine the relationship of these enzymes to grain protein production and grain yield.

MATERIALS AND METHODS

Four wheat varieties were grown in field nurseries on the Agronomy Research Station, Oklahoma State University, Stillwater, in 1969–1970: NB 65317, NB 65679, B 4930, and Triumph 64.

The first two varieties were obtained from the Nebraska Agricultural Experiment Station, Lincoln, Neb., and the third was from Indiana Agricultural Experiment Station, Lafayette, Ind. The three high protein genotypes have Atlas 66 in their parentage, and Triumph 64 is a variety widely grown in Oklahoma which has lower protein potential than the other varieties. Plot size consisted of four rows of 3.1 m with 30-cm spacing between rows, and plots were arranged in a randomized complete block design with four replications. A uniform fertilizer application of 20–22–0 kg/ha of N–P–K (18–45–0 lb/acre of N, P_2O_5, K_2O) was applied to all plots as a preplant application. The nursery was planted on Oct 28, 1969. Two fertilizer levels of 67 and 134 kg/ha of nitrogen (as NH_4NO_3) were applied on April 5, 1970, to two replications selected at random.

Fresh leaf blade samples were collected at random from the two outside rows of the plots for the measurement of enzyme levels, and the two center rows were used to determine the grain yield and grain protein percent. Plant samples were placed in a plastic bag immediately after sampling and were covered with ice to prevent any loss of enzyme activity. Samples were obtained on Apr 7, 14, 21, and 28 and May 5 and 12 for analyses of NR, protease, water-soluble protein, Kjeldahl protein, and nitrate contents. All the green leaf blades were composited on these dates. Samples were analyzed separately as middle and top leaves on May 21 and only top leaves were analyzed on May 28 for proteases, water-soluble protein, and Kjeldahl protein. The NR levels were very low on April 28 and thereafter.

Crude enzyme preparations for NR were prepared and

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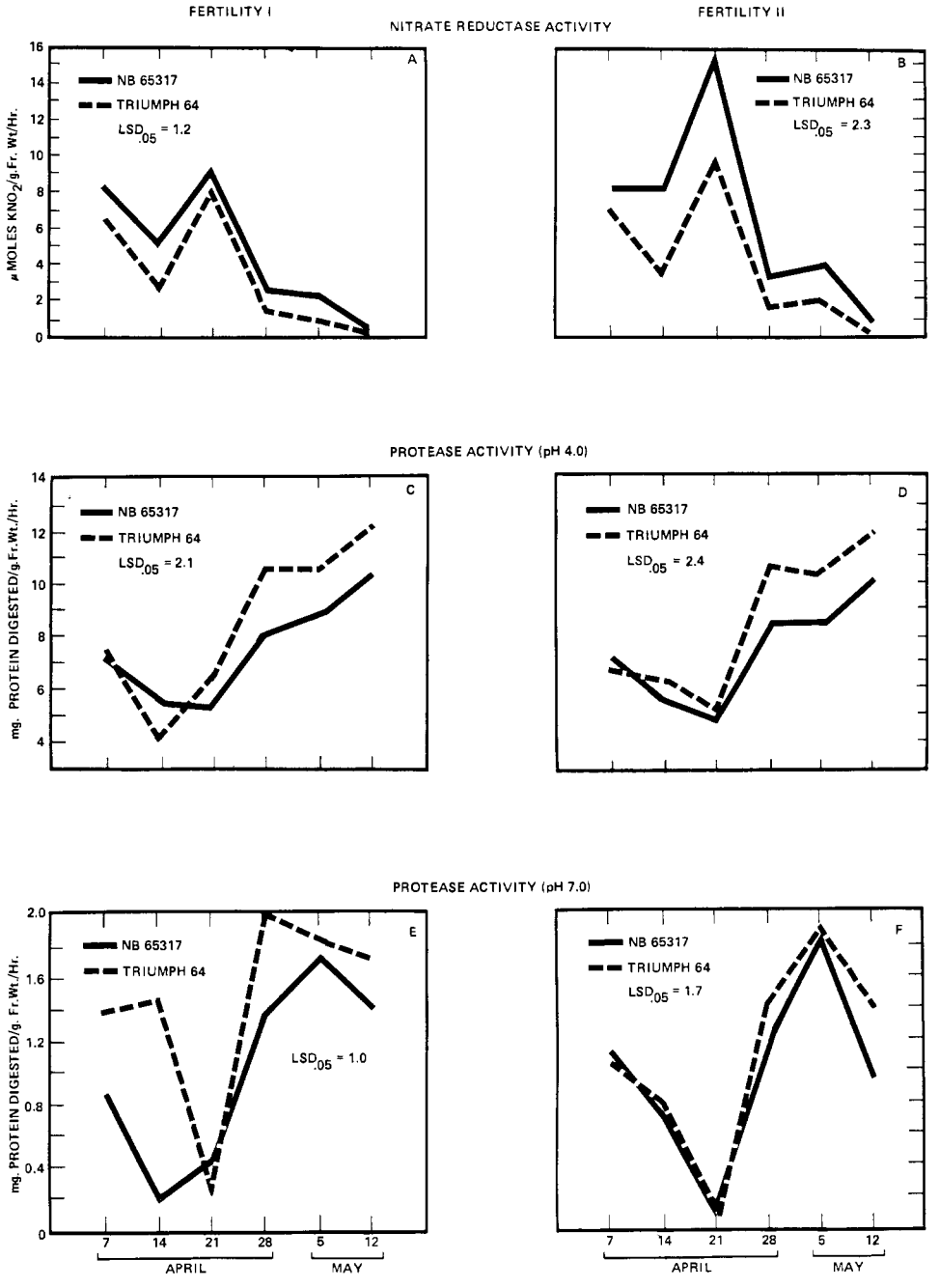

Figure 1. Nitrate reductase activity, protease activity (pH 4), and protease activity (pH 7) for NB 65317 and Triumph 64 wheat varieties during early spring

assayed as described by Croy and Hageman (1970). The same procedure was used for protease extraction except that the pH of the extraction solution was adjusted to 7.0 before plant samples were homogenized. Assay procedures were carried out as described by Rao and Croy (1971), except that the substrate pH was adjusted to 4.0 and 7.0. Water-soluble protein content of the crude enzyme extract was estimated (Lowry *et al.*, 1951) using 5% TCA-precipitable material. Nitrate contents were estimated by the method of Woolley *et al.* (1960), and protein percent was analyzed by the micro-Kjeldahl method.

RESULTS AND DISCUSSION

The activity levels of NR and protease were not found to be significantly different among the high grain protein wheat varieties; therefore, NB 65317 was selected as representative of a high grain protein wheat for comparison with Triumph 64, a lower grain protein wheat.

Seasonal patterns for NR before the flowering stage were significantly different between the varieties NB 65317 and Triumph 64 and among sampling dates. The variety X sampling date interaction was significant (Figure 1, A and B). NR levels were highest on Apr 21, when plants were in the jointing stage. While NR levels were increased in response to fertility, these increases were not significant because of the small number of treatments and reps.

The seasonal patterns for protease (pH 4.0) activity before the flowering stage were significant for sampling dates and the variety X sampling date interaction, but not between the fertility levels nor varieties (Figure 1, C and D). Protease activity levels were lowest on Apr 14 and 21 and increased after Apr 21, the date on which NR activity started declining. Triumph 64 had higher protease (pH 4) levels than NB 65317

Table I. Pattern of Leaf Water-Soluble Protein, Nitrate Content, and Percent Kjeldahl Protein before the Flowering Stage for Wheat

Sampling dates	Fertility I		Fertility II	
	NB 65317	Triumph 64	NB 65317	Triumph 64
	Water-soluble protein, mg/g of fresh weight			
April 7	27.9 a[a]	24.0 ab	29.6 a	18.5 b
April 14	29.1 a	28.4 a	31.3 a	25.1 a
April 21	23.8 ab	26.4 a	27.1 a	25.1 a
April 28	17.4 b	17.0 b	19.6 b	15.4 b
May 5	22.1 ab	21.9 ab	23.8 ab	21.0 ab
May 12	14.8 b	13.8 b	17.7 b	11.9 b
	Nitrate N, μg/g of fresh weight			
April 7	53 c	60 c	53 c	37 c
April 14	57 c	59 c	158 bc	100 c
April 21	494 ab	350 b	556 abc	293 b
April 28	477 ab	344 b	1002 a	504 a
May 5	843 a	408 b	930 a	523 a
May 12	478 ab	980 a	764 a	1073 a
	Leaf Kjeldahl protein, percent (oven-dry tissue)			
April 7	27.5 a	27.2 a	27.7 a	25.7 a
April 14	25.3 a	25.8 a	27.1 a	25.2 a
April 21	23.7 b	23.8 b	24.9 ab	23.7 b
April 28	25.1 a	23.7 b	25.4 a	22.2 b
May 5	23.5 b	22.7 b	23.6 b	22.0 b
May 12	19.7 c	17.9 c	20.2 bc	21.1 b

[a] Means having the same letter are not significantly different using $LSD_{0.05}$.

up to May, probably as a result of a week earlier maturity than NB 65317. The protease activity (pH 7.0) was not influenced significantly either by fertility levels or varieties (Figure 1, E and F).

Water-soluble protein, Kjeldahl protein, and nitrate concentrations in the leaf blades before flowering were not influenced by either fertility or varieties; however, NB 65317 in general exhibited higher water-soluble protein and leaf Kjeldahl protein than Triumph 64 (Table I). Differences were observed in all these characters among sampling dates. In general, water-soluble protein and leaf Kjeldahl protein decreased and nitrate content increased with time.

On May 21, protease activity, water-soluble protein, and leaf protein were higher in the top leaves than the middle leaves of both varieties (Table II). Between May 21 and 28 protease activity (pH 4.0) of the top leaves of NB 65317 decreased rapidly for the 67 kg/ha treatment, while the activity levels declined more slowly for the 134 kg/ha. Protease activity (pH 4) was much higher on May 21 in Triumph 64 and although activity had declined in NB 65317 on May 28, the protease on this date was still higher than in Triumph 64. Protease activity (pH 7.0) was significantly higher in the top leaves of NB 65317 than in those of Triumph 64 in the 134 kg N treatment. Protease (pH 7) declined from May 21 to May 28. Additional nitrogen enhanced protease (pH 7) in the top leaves of NB 65317 but not in the top leaves of Triumph 64. The earlier maturity of Triumph 64 may have affected its protease activity.

Water-soluble protein concentrations were different for varieties, position of leaves, and among sampling dates after flowering (Table II). The higher fertility level did not influence the water-soluble protein concentrations in the middle leaves, but water-soluble protein was higher in the top leaves with a higher fertility level. NB 65317 had higher water-soluble protein concentrations than Triumph 64. A rapid decline in water-soluble protein content was observed in the top leaves of NB 65317 between May 21 and 28.

No significant differences in plant protein percent (Kjeldahl) were observed between fertilizer levels; however, varieties exhibited significant differences, especially in the top leaves (Table II). Declines in protein percent in the top leaves of NB 65317 between the last two sampling dates were significant, whereas protein in Triumph 64 also declined but not significantly. This significant decline in protein percent in the top leaf blades of NB 65317 could be the reflection of protease activity at this time, which was still higher in NB 65317 than Triumph 64. On May 21, NB 65317 had 3.85% higher protein in the top leaves in response to the high fertility levels, while Triumph 64 had only a 2.4% greater protein, suggesting a superior response of NB 65317 to the higher nitrogen level.

Grain yield, grain protein percent, and protein per unit area at both fertility levels are shown in Table III. The increased fertility level significantly increased the grain yield of NB 65317 but not of Triumph 64. Triumph 64 had an increase in grain protein percent with the higher fertility level, while NB 65317 showed a slight decline in grain protein percent. Both varieties exhibited higher protein production per unit area with a higher fertility level, but NB 65317 had a much greater increase in protein production than Triumph 64.

The observations presented above indicate that the high grain protein wheat variety generally possessed higher NR prior to heading and higher protease after heading than did the lower grain protein wheat. It was not possible to show differences in leaf Kjeldahl protein or water-soluble protein between varieties (Table I), which would have given support

Table II. Leaf Protease, Water-Soluble Protein, and Percent Protein after Flowering For NB 65317 and Triumph 64

Varieties and dates	Position of leaves	Protease (4.0)[a] kg/ha N		Protease (7.0)[a] kg/ha N		Water-soluble protein[b] kg/ha N		Percent Kjeldahl protein[c] kg/ha N	
		67	134	67	134	67	134	67	134
NB 65317									
May 21	Top	12.7	13.5	1.6	2.1	12.3	19.6	13.0	16.8
	Middle	3.3	3.5	0.4	0.8	2.5	2.7	7.3	8.9
May 28	Top	7.5	11.9	1.3	1.5	6.4	8.0	9.5	11.7
Triumph 64									
May 21	Top	6.2	8.7	1.7	1.4	3.6	5.9	12.4	14.8
	Middle	1.6	1.5	0.2	0.9	2.5	2.8	11.3	13.7
May 28	Top	5.4	6.1	1.2	0.6	5.5	5.2	11.7	11.5
$LSD_{0.05}$		1.1		0.2		1.6		1.9	

[a] mg of protein digested/g of fresh weight. [b] mg/g of fresh weight. [c] Dry weight basis.

Table III. Average Grain Yield, Grain Protein Percent, and Grain Protein Production of NB 65317 and Triumph 64

	NB 65317	Triumph 64	$LSD_{0.05}$
Yield, kg/ha			
67 kg N/ha	1851	2661	
134 kg N/ha	3171	2751	
Average	2511	2706	311
Grain protein percent			
67 kg N/ha	19.5	12.8	
134 kg N/ha	19.0	15.6	
Average	19.3	14.2	1.8
Grain protein, kg/ha			
67 kg N/ha	361	341	
134 kg N/ha	602	429	
Average	481	385	58

for differences in nitrogen reduction. In contrast, the grain protein was greater in the high protein line, which is consistent with the higher protease after heading in the high protein line.

LITERATURE CITED

Beevers, L., *Phytochemistry* **7,** 1837 (1968).
Beevers, L., Hageman, R. H., *Annu. Rev. Plant Physiol.* **19,** 495 (1969).
Croy, L. I., Hageman, R. H., *Crop Sci.* **10,** 280 (1970).
Duffield, R. D., Master's Thesis, Oklahoma State University, Stillwater, Okla., 1971.
Hageman, R. H., Flesher, D., Gitter, A., *Crop Sci.* **1,** 201 (1961).
Haunold, A., Johnson, V. A., Schmidt, J. W., *Agron. J.* **54,** 121 (1962).
Johnson, V. A., Schmidt, J. W., Mattern, P. J., *Econ. Bot.* **22,** 16 25 (1968).
Lowry, O. H., Roseborough, N. J., Farr, A. L., Randall, R. J., *J. Biol. Chem.* **193,** 257 (1951).
Middleton, G. K., Bode, E. C., Bales, B. B., *Agron. J.* **46,** 500 (1954).
Mounfield, J. D., *Biochem. J.* **30,** 1778 (1936).
Rao, S. C., Croy, L. I., *Crop Sci.* **11,** 790 (1971).
Schlehuber, A. M., Tucker, B. B., *Cereal Sci. Today* **4,** 240 (1959).
Seth, J., Hebert, T. T., Middleton, G. K., *Agron. J.* **52,** 207 (1960).
Welch, L. F., Johnson, R. F., Pendleton, J. W., Miller, L. B., *Agron. J.* **58,** 271 (1966).
Woolley, J. T., Hicks, G. P., Hageman, R. H., J. AGR. FOOD CHEM. **8,** 481 (1960).

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Reactions of Heated Milk and Amino Sugars with *p*-Dimethylaminobenzaldehyde

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Milk heated at sterilizing temperatures developed chromogens producing a red color upon reaction with *p*-dimethylaminobenzaldehyde (*p*-DMAB) in a reaction mixture of formic acid and chloroform. Results for heated milk indicated that the *p*-DMAB reactivity was proportional to the heating time for temperatures in the range 100–120°. Precursors for the reaction were identified as predominantly *N*-acetylhexosamines on the basis of the specificity of the reaction and by the absorption spectra of the chromophores. Products formed by nonenzymatic browning were also reactive, but their contribution to the total *p*-DMAB reactivity was small. The principal chromogens formed in heated milk may be the mono- and dianhydro derivatives of *N*-acetylhexosamines. A secondary reaction with *p*-DMAB occurred when milk was kept in contact with the reaction mixture for several hours, but this reaction was not heat induced and did not appear to involve amino sugars.

Heat processing of milk and other fluid food products leads to complex changes and interactions among the constituents. During a study of heat effects on milk at elevated temperatures (Hansen, 1967), the observation was made that the product, after heat sterilization, developed a capacity for reacting with *p*-dimethylaminobenzaldehyde (*p*-DMAB). The reaction resulted in a pink coloration similar to the color produced by certain amino sugars following heating under alkaline conditions (Morgan and Elson, 1934). Although *p*-DMAB is commonly used for detecting amino sugars, the reagent is nonspecific since it produces color with a number of components, including pyrroles, phenols, many indole derivatives, and sialic acids. However, the solvent for *p*-DMAB and the conditions of reaction may be selected to permit the use of *p*-DMAB as a diagnostic tool (Kent and Whitehouse, 1955).

The purpose of this paper is to present a method for measuring the *p*-DMAB reactivity of heated milk and to identify the components responsible for this heat-induced change.

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EXPERIMENTAL SECTION

Materials. *N*-Acetylglucosamine, *N*-acetylgalactosamine, and *N*-acetylmannosamine were obtained from Calbiochem and formic acid and chloroform (A. R.) were obtained from Baker Chemical Company. The buffer (pH 6.8) was the lactose-free, synthetic milk salt system of Jenness and Koops (1962). This buffer was selected due to its similarity with the salt system of milk. Sterile milk dialysate was prepared by the method of Koka and Mikolajcik (1967) with frequent transfers over several days of the dialysis bags into fresh milk in order to approach equilibrium of dialyzable components with the intact milk system. This system was considered to be approximately equal in composition to the native, protein-free milk system described by Murphy and Whitney (1956). Milk ultrafiltrate was prepared using the LKB suction-ultrafiltration support, Model 6301 A, equipped with Visking No 32 dialysis tubing.

p-DMAB REAGENT. *p*-Dimethylaminobenzaldehyde was obtained from Matheson Coleman and Bell, and a 4% (w/v) solution was prepared in chloroform and stored in amber bottles.

Preparation of Samples. Reconstituted skim milk (9.1%)