Changes in the Levels of Proteolytic Enzymes from Hard Red Spring Wheat during Growth and Maturation[1]

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ABSTRACT

The levels of the proteolytic enzymes hydrolyzing azocasein and α-benzoyl-L-arginine-*p*-nitroanilide (BAPA) have been followed throughout kernel development for two hard red spring wheat varieties. Azocaseinase activity increased during early kernel development until approximately 16 to 18 days after flowering, after which time it decreased. Similarly, BAPA-ase activity formed early in kernel development and remained at a constant level until near maturity, at which time the activity in one of the two varieties decreased. Azocaseinase was found predominantly in the outer branny layers, whereas the greatest amount of BAPA-ase was found in the endosperm. Levels of nitrogen and moisture were also followed throughout the development of these kernels.

The proteolytic enzymes from wheat and their role in affecting the quality of doughs by alteration of gluten proteins has been the subject of considerable research and controversy. It is now generally believed that the proteolytic activity found in sound wheats is very low and, as such, is of little significance in altering the breadmaking potential of flours made from such wheats (1). Little attention has been given, however, to the variations in the levels of proteolytic enzymes during kernel development and the possible influence such variations might have on the synthesis of wheat storage proteins. A recent study by Bushuk et al. (2) has indicated that the proteolytic activity in immature wheat kernels, as measured by the breakdown of hemoglobin at pH 3.8, was at least 3.4 times as great as the activity found in sound wheats. In the present study, the levels of the proteolytic enzymes hydrolyzing azocasein at pH 6.0 and α-benzoyl-L-arginine-*p*-nitroanilide (BAPA) at pH 8.6 were examined throughout kernel development and maturation. Azocasein was chosen as substrate for measuring proteolytic activity in preference to hemoglobin because of the recent finding that the latter substrate does not satisfactorily measure the "gluten-softening" enzyme found in wheat (3). Azocasein, on the other hand, has been used as substrate by Redman (4) to give some indication of the wheat proteases responsible for the softening of gluten.

MATERIALS AND METHODS

The hard red spring wheat variety Marquis, and a variety not licensed in Canada but referred to commonly as Prairie Pride, were planted on May 18, 1971, at the Canada Dept. of Agriculture experimental plots, Glenlea, Man. Both varieties flowered on July 19, 1971, and were sampled at a number of intervals, thereafter, throughout kernel development and up to full maturity. Excised heads at each sampling date were stored intact in a deep freezer prior to kernel analysis for proteolytic activity.

The azocasein and BAPA used in this study were purchased from Mann Research Laboratories, Orangeburg, N.Y.

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Moisture

Duplicate 1-g. samples of kernels from freshly harvested wheat were analyzed for moisture by the AACC vacuum oven method (5).

Extraction of Wheat Kernels

Fifty wheat kernels were homogenized in a "Virtis 45" homogenizer with 20 ml. of cold 0.14M acetate buffer, pH 6.0 for 30 sec. at 27,000 r.p.m. and 30 sec. at 14,000 r.p.m. Kernels of near maturity, with very low moisture, were first ground in an electric coffee mill. Following homogenization, the suspension was allowed to stand for 1 hr. at room temperature with occasional shaking. It was then divided into two portions. One portion was used directly for total azocaseinase, while the other was centrifuged 20 min. at 17,000 × g and the filtrate used for "free" or unbound azocaseinase and BAPA-ase activity determinations.

Proteolytic Activity Determination

The proteolytic activity of a wheat-flour suspension is higher than an extract made from such flour (6) when hemoglobin is used as substrate. As a consequence, both suspensions (total azocaseinase) and extracts (free azocaseinase) were tested for their ability to degrade azocasein to see if a similar behavior was found in the developing wheat kernel.

Azocaseinase Assay

Azocasein, because of its sensitivity (7), was a very suitable substrate for protease activity determinations in this study. An additional advantage of the method is that the problem of large blank values due to trichloroacetic acid (TCA)-soluble nitrogen products is obviated. The reaction was carried out similar to that described previously (8). Two milliliters of enzyme suspension or solution in a 10-ml. Erlenmeyer flask was added to 2 ml. of 2.4% azocasein dissolved in 0.05M McIlvaines citric acid-disodium phosphate buffer, pH 6.0. The reaction was then shaken gently for 2 hr. in a shaking bath at 35°C. The reaction was terminated by addition of 5ml. of 10% TCA, and the mixture filtered. Five milliliters of 0.5N sodium hydroxide was added to 5 ml. of the filtered solution and, after 20 min., the absorbance of the solution was read at 440 nm. Determinations were carried out in duplicate, as were blanks in which the sample alone was incubated for 2 hr., followed by addition of TCA and then azocasein. One unit of azocaseinase was arbitrarily defined as a change in absorbance at 440 nm. of 0.01 after 2 hr., at pH 6.0 and 35°C. Figure 1 shows the hydrolysis of azocasein after 2 hr. of reaction by varying amounts of immature and germinated Marquis wheat suspensions. A linearity exists only up to about 0.1 absorbance. This will correspond in subsequent figures to a value of 2 units per immature kernel. Azocaseinase values higher than this will be, therefore, less than their actual value, and this should be taken into account in the interpretation of results. A suspension of immature Prairie Pride wheat also behaved similarly to that of the immature Marquis wheat with linearity only up to about 0.1 absorbance.

BAPA-ase Assay

The BAPA-ase assay was carried out by the method of Erlanger et al. (9) as modified by Burger (10). One unit of BAPA-ase activity was defined as that amount

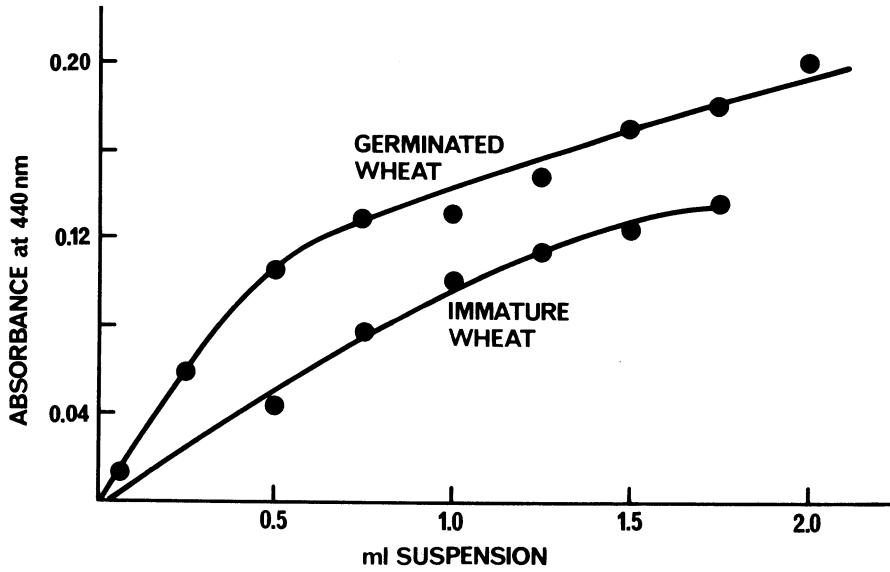


Fig. 1. Hydrolysis of azocasein by immature and germinated Marquis wheat suspensions.

which catalyzed the hydrolysis of 1 nM. of BAPA per min. at 35°C. and pH 8.6. The activity-concentration curve for the BAPA-ase assay was linear over the range of enzyme concentrations used in this study.

Nitrogen Determination

Nitrogen was determined by the micro-Kjeldahl method of Mitcheson and Stowell (11). In some cases, results were also checked by the automated cyanurate method of Crooke and Simpson (12).

RESULTS AND DISCUSSION

Two varieties of hard red spring wheat with different breadmaking quality were selected for a study of the changes in proteolytic activity of wheat that occur during kernel development. One variety, Marquis, was known to be of excellent milling and baking quality, whereas Prairie Pride was of decidedly inferior quality. Changes in moisture during kernel development were determined for both varieties (Figs. 2A and 3A), and could be used as a rough guide to estimate kernel maturity. Kernel moisture from both varieties began to decrease 15 days after flowering, with the decrease being slower in Prairie Pride. Dry weight changes are not shown, but there was a marked increase in dry weight with decrease in moisture. This was roughly parallel in both varieties, with final dry weights in both cases being about 35 mg. per kernel.

Changes in Azocaseinase and Nitrogen during Kernel Development of Marquis Wheat

Changes that occurred in the protease activity of Marquis wheat during growth

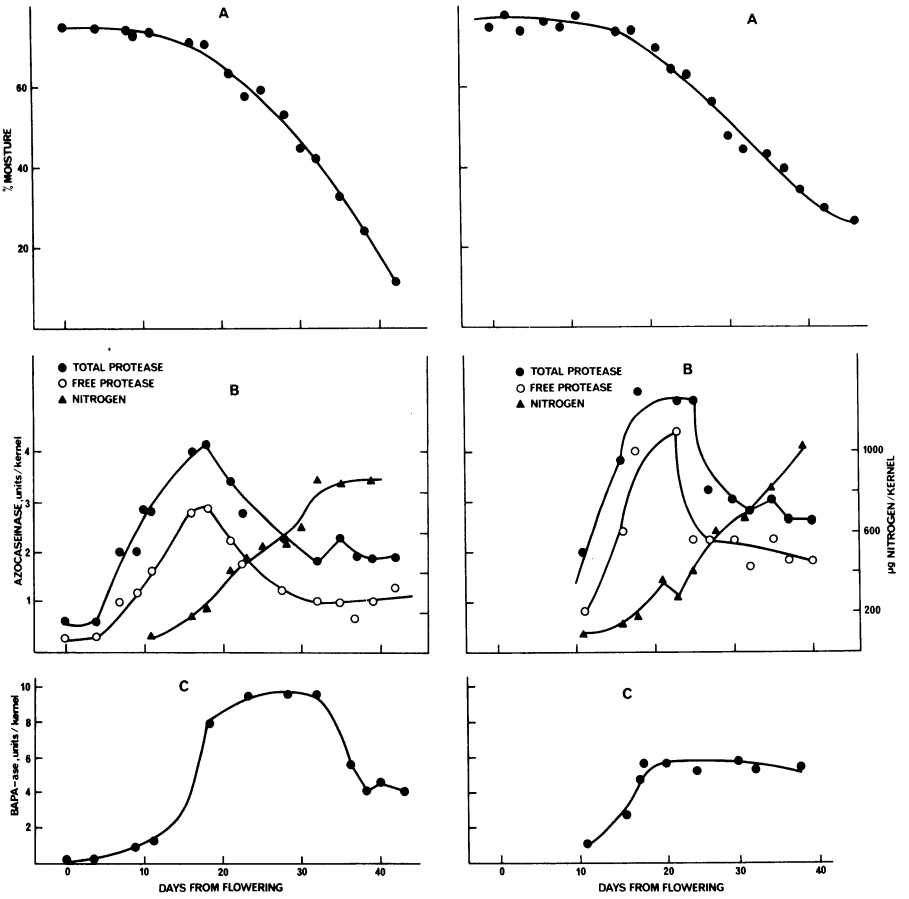


Fig. 2 (left). Changes in: A, moisture; B, total and free azocaseinase and nitrogen; and C, BAPA-ase, during growth and maturation of Marquis wheat.

Fig. 3 (right). Changes in: A, moisture; B, total and free azocaseinase and nitrogen; and C, BAPA-ase, during growth and maturation of Prairie Pride wheat.

and maturation are shown in Fig. 2B. The protease activity, as measured by the breakdown of azocasein by both suspensions of immature kernels (total protease) and extracts prepared from such suspensions (free protease) were determined. Of particular interest was that protease enzymes were formed very soon after flowering and that the level of these enzymes reached a maximum between 16 and 19 days after flowering. Thereafter, the level of protease activity decreased with increasing maturity and concomitant with decreasing moisture. From about 27 days after flowering onward, the protease activity remained at a lower but relatively constant level. Similar activity profiles were obtained with both the total and free protease activity, and at no stage in kernel development was the protease completely soluble in buffer alone. The amount of protease soluble in buffer appeared to be quite

substantial, however, and remained at a reasonably fixed amount relative to the total protease throughout kernel growth.

Nitrogen uptake during kernel development was also plotted on the same graph (Fig. 2B). Nitrogen was being rapidly incorporated at 20 to 30 days from flowering, at a time when the protease activity, although falling, was still at a high level compared to the final activity of the fully mature kernel. Jennings and Morton (13) have shown with an Australian wheat variety that nonprotein nitrogen decreased from 15% at day 19 after flowering to 3% at full maturity. The nitrogen content at 20 to 30 days from flowering, in the present study, would similarly be expected to consist largely of protein nitrogen.

Changes in Azocaseinase and Nitrogen during Kernel Development of Prairie Pride Wheat

The total and free protease for Prairie Pride is shown in Fig. 3B, and the behavior observed was similar to that of Marquis. A large peak of protease activity formed between the 18th to 25th day after flowering. Protease activity then decreased with decrease in kernel moisture until a lower but constant level was reached. As with Marquis wheat, the protease activity of a homogenized kernel suspension was greater than an extract prepared from the suspension at any stage of kernel development. Nitrogen incorporation is also shown in Fig. 3B. It occurred steadily from early kernel growth and again during a period in which the protease activity was at a substantially higher level than in the mature kernel.

The protease activity of Prairie Pride on a per-kernel basis was higher than that of Marquis wheat, both at maximum protease level found during growth and at the lower level at full maturity as indicated by comparison of Figs. 2B and 3B. The differences are even greater than shown at the time of maximum protease activity because of the nonlinearity above 2 units per kernel mentioned previously in the Methods section. Except for the very early stages of kernel development, the specific activity for the total protease activity of Prairie Pride was higher than that of Marquis wheat (Fig. 4).

Changes in Levels of the Enzyme, BAPA-ase, during Kernel Development

Both wheat and malted wheat contain an enzyme hydrolyzing the artificial substrate BAPA (8,14,15,16). This enzyme, BAPA-ase, is believed to be a peptide hydrolase, and the partially purified enzyme from wheat embryo was able to hydrolyze a number of simple peptides (15). Examination of Marquis wheat indicated that the enzyme formed very rapidly at about 17 days after flowering (Fig. 2C). Unlike protease, however, the activity of the BAPA-ase enzyme remained constant until about day 32 after flowering, after which time it decreased. With Prairie Pride, the BAPA-ase activity also rose rapidly at day 17 and remained at a constant level (Fig. 3C). The activity, however, had not fallen substantially by the last sampling date and was lower throughout development than that of Marquis. The BAPA-ase enzyme was formed at the time in the kernel development in which the protease enzyme had just reached its maximum activity.

Distribution of Protease in the Growing Wheat Kernel

To gain an insight into the anatomical distribution of the total protease and how this distribution changed during kernel development, kernels were dissected into pericarp, seed coat and aleurone, embryo and endosperm. Total protease was then determined on each part. Kernel dissections could not be carried out satisfactorily

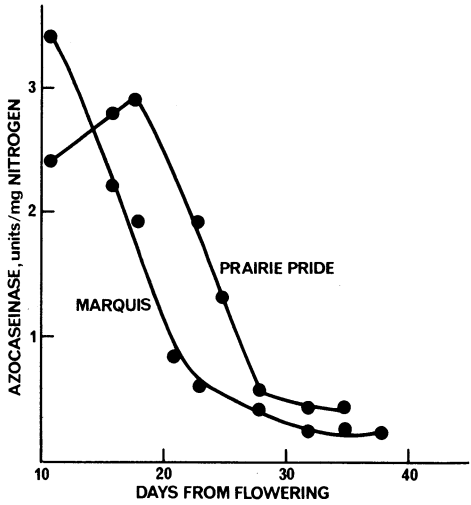


Fig. 4. Changes in the specific activity of Marquis and Prairie Pride wheat during growth and maturation.

on very immature kernels with very high moisture, nor on near-ripe samples of very low moisture, but the time interval during which the protease activity decreased from its maximum value to its lower, but constant, value at near maturity was covered. The protease activity of suspensions was least in the endosperm and greatest in the branny layers of the kernel in Marquis wheat (Fig. 5). The protease activity in the endosperm varied only slightly over the time period studied. The protease activity in the pericarp and seed coat and aleurone decreased greatly, however, with the enzymes in these tissues probably responsible for the large changes in protease activity of whole kernels between day 18 and day 30 after flowering, as shown in Fig. 2B. In the embryo, there was an increase in protease activity toward the end of kernel development, synchronous with its increase in size. Although only two dates were selected for analysis, similar changes occurred for Prairie Pride wheat. Noteworthy, perhaps, is the fact that the protease activity found in both the endosperm and seed coat and aleurone layers appeared higher for Prairie Pride than for Marquis.

Nitrogen Distribution in the Growing Wheat Kernel

To see what relation this widely varying distribution of protease in the wheat kernel might have to nitrogen content, dissected kernels were also analyzed for nitrogen. The results (Fig. 6) for both Marquis and Prairie Pride were similar. There was a slight decrease in nitrogen in the pericarp from day 17 onward, whereas the amount in the seed coat and aleurone increased slightly. Nitrogen in the endosperm rose substantially and was undoubtedly the factor responsible for the bulk of the nitrogen content during later kernel development (Fig. 2B). Nitrogen incorporation into this tissue was found to be primarily protein nitrogen by Jennings and Morton (13), who indicated that nonprotein nitrogen remained at a constant and low level.

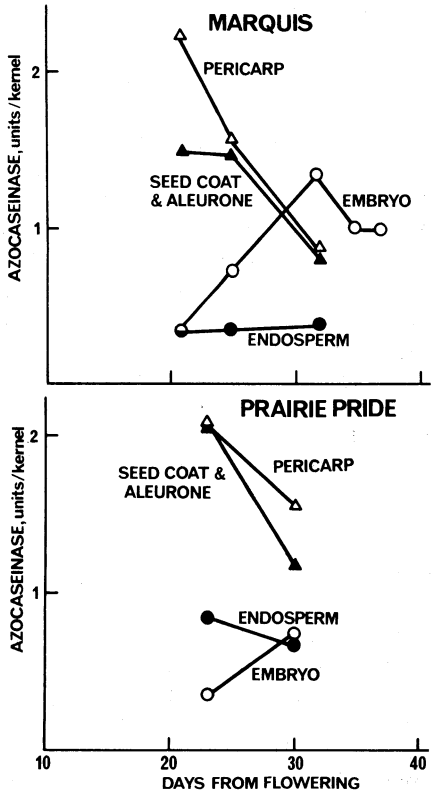
BAPA-ase Distribution in the Growing Wheat Kernel

The anatomical distribution of the peptidase, BAPA-ase, was also determined

for Marquis wheat on the 25th and 30th days after flowering. The results for the two dates were similar with about 57% of the activity in the endosperm, 21% in the seed coat and aleurone layer, 8% in the pericarp, and 14% in the embryo. This is in complete reversal to the anatomical distribution of the total protease in immature wheat kernels.

Changes in Azocaseinase during Germination

The edestin-degrading proteases of wheat are known to increase about tenfold during germination (17). To see if such were the case for the casein-degrading enzymes in the present study, germination of Marquis wheat kernels was carried out for 6 days at 23°C. on moist filter paper in a dark humidity cabinet. Kernels were withdrawn at various periods of germination and analyzed for total and free protease, using azocasein as substrate. Suspensions and extracts were diluted to ensure that the absorbance change fell within the linear portion of the enzyme concentration-absorbance curve of Fig. 1. As shown in Fig. 7, the total protease



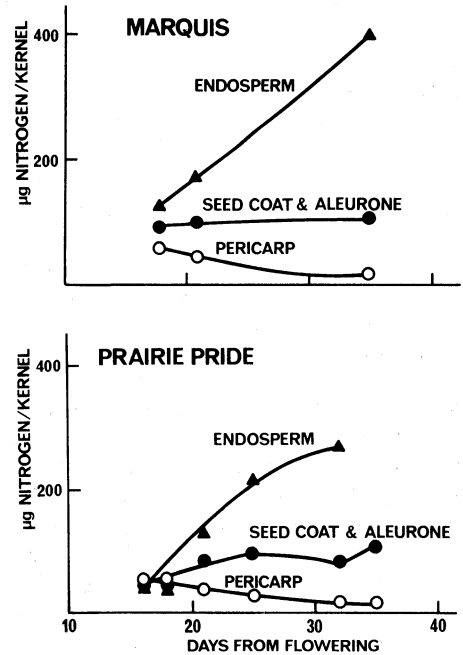


Fig. 5 (left). Anatomical distribution of azocaseinase activity in Marquis (top) and Prairie Pride (bottom) wheat kernels during growth and maturation.

Fig. 6 (right). Anatomical distribution of nitrogen in Marquis (top) and Prairie Pride (bottom) wheat kernels during growth and maturation.

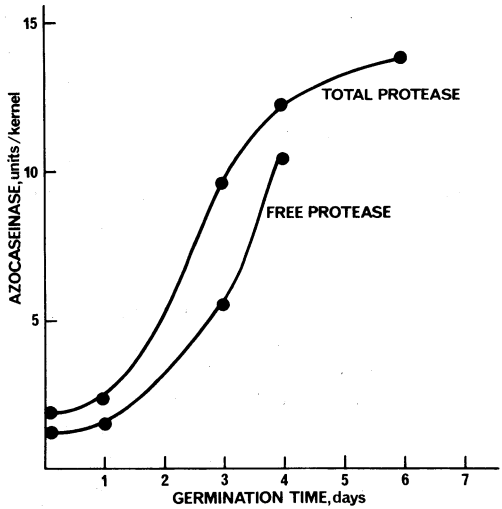


Fig. 7. Changes in total and free azocaseinase upon germination.

activity increased about eightfold, and even after 4 days of germination, part of the proteolytic activity remained insoluble in buffer. The total amount of protease activity formed after 6 days was about three times as large as the maximum amount present during the kernel development of Marquis wheat.

GENERAL DISCUSSION AND CONCLUSIONS

Two varieties of hard red spring wheat, Marquis and Prairie Pride, have been examined for changes in levels of proteolytic enzymes during kernel growth and maturation. The protease activity increased in both varieties during early kernel development and reached its maximum at about 16 to 18 days after flowering. The protease activity then lessened with decreasing moisture content until it reached a constant level at which it remained until full maturation. The largest part of the protease activity was in the outer branny layers, and it was the decrease in protease activity in these tissues, with decreasing moisture, that was responsible for the large fall in protease activity seen in the whole kernel during maturation. Work is presently under way on the changes in protease activity during kernel development for four other wheat varieties, including two durum wheats and a soft wheat variety, but results so far indicate similar behavior for all. Engel and Heins (18) and Pett (19) have studied the distribution of protease in sound wheat, using edestin as substrate. They also found that the proteolytic activity of the endosperm was much less than that of the branny layers.

Peptidase (BAPA-ase) activity appeared rapidly in the growing kernel just at the time when the protease had reached its maximum activity. In contrast to the protease, however, the BAPA-ase was found largely in the endosperm. With the variety Marquis, the level of the BAPA-ase dropped in the final maturation stages of the kernel.

Nitrogen content from the 18th day after flowering to near maturity increases

rapidly at a time when the protease activity is still large compared to its final level. The greatest nitrogen uptake with time from flowering is in the endosperm and would be largely protein nitrogen. Nitrogen decreased slightly in the pericarp as the kernel matured. One might therefore speculate that the reason for the occurrence of the high level of protease in the pericarp is to break down protein in this tissue prior to subsequent translocation of the resulting amino acids to the endosperm for subsequent protein synthesis. No conjecture can be given, however, for early occurrence of the BAPA-ase enzyme in the endosperm. Jennings and Morton (13) found that endosperm proteins under went marked changes in amino acid composition during their early formation. If this involved hydrolysis as well as synthesis of such proteins, peptidases such as BAPA-ase might play a part.

Prairie Pride, having poor breadmaking quality, also had a higher protease activity compared to Marquis with excellent breadmaking quality. By contrast, BAPA-ase activity at some stages was higher in Marquis than in Prairie Pride wheat. These findings, and that such enzymes are present during the period in which storage proteins are being laid down, suggest that interrelationships of proteases and peptidases might alter the nature of these proteins and, as a consequence, the ultimate quality of such wheats. To prove or disprove that this can occur is certainly worthy of further research and consideration.

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