

# Changes in the Amylases of Hard Red Spring Wheat during Germination<sup>1</sup>

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## ABSTRACT

Amylases of Canadian hard red spring wheat were studied during germination by polyacrylamide-slab electrophoresis at pH 9.0. Two sets of  $\alpha$ -amylase isozymes form during germination. One set is composed of three isozymes, and has identical electrophoretic mobility with the  $\alpha$ -amylases found in immature kernels of wheat. The other set is composed of at least four electrophoretically slower isozymes. The electrophoretic mobility of this set of isozymes and their intensities change upon heat-treatment during the earlier stages of germination. Analysis of  $\alpha$ -amylase, formerly purified, indicated that it was the electrophoretically slower set of isozymes which had been isolated. With  $\beta$ -amylase, the original components seem to disappear at the same time that a new electrophoretically slower  $\beta$ -amylase component is formed. The electrophoretic technique can be made more sensitive to ascertain the nature of amylases in varieties of wheat with lowered amylograph viscosities.

A previous paper (1) has described a sensitive method for separating and detecting the multiple forms of amylases found in wheat, using polyacrylamide-slab electrophoresis with a constant pulsed power supply. Changes in the multiple forms of  $\alpha$ - and  $\beta$ -amylases during the growth of hard red spring (HRS) wheat varieties were studied. This paper describes the use of this method to study the changes in amylases from HRS wheat during germination.

## MATERIALS

The HRS wheats used in this study were from 1969 Canadian plant breeders' varieties.

## METHODS

Germination was carried out by placing 100 kernels of wheat in a 13 × 13.5-cm. covered sample dish containing two 13 × 13.5-cm. sheets of germination paper which had been moistened with 16 ml. of water. The sample dishes were placed in a moisture cabinet in the dark at 23°C., and samples removed after germination times ranging from 1 to 7 days. The germinated samples were air-dried at room temperature prior to analysis.

Samples were extracted, submitted to electrophoresis on polyacrylamide gels, and amylases detected as described previously (1). The amylase activity of wheat increases enormously upon germination, and 3-, 4-, and 7-day germinated wheats were diluted 1:4 with 0.01M calcium chloride solution prior to electrophoresis. The incubation time of the polyacrylamide gel with the starch or  $\beta$ -limit dextrin gel, following electrophoresis, was also reduced from 45 to 15 min.

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## RESULTS

Wheat kernels from a particular variety of wheat, germinated for 0, 1, 2, 3, 4, and 7 days, were analyzed on the same polyacrylamide slab so that a direct comparison of changes in the amylases could readily be made. Heat-treated extracts of the same samples were also run simultaneously in order to confirm location of  $\alpha$ -amylase isozymes. As the emphasis was on changes occurring during germination, some loss of resolution did occur—as it was difficult to obtain optimum conditions on the same gel for detection of all isozymes, owing to the widely varying amylase activities from samples of different germination times. The changes that occurred in Manitou wheat for amylases as detected on starch gel are shown in Fig. 1.

In the sound and 1-day-germinated samples (zymograms 1 and 3, respectively), only the major  $\beta$ -amylase components,  $\beta$ -2 and  $\beta$ -3, are visible. Four minor  $\beta$ -amylase components, which are also known to be present in mature kernels of wheat (1), were not rendered visible by the short 15-min. incubation of the polyacrylamide slab with the starch plate. At 2 days' germination (zymogram 5), a new electrophoretically slower  $\beta$ -amylase component forms, and the original major  $\beta$ -amylase components decrease in activity. The new  $\beta$ -amylase component increases in activity with time of germination, whereas the original components disappear with time and are barely visible after 4 days' germination (zymograms 7 and 9).

Looking at the formation of  $\alpha$ -amylases during germination, it was found that only after 2 days' germination are these components first visible (zymograms 5 and 6). Two sets of amylase isozymes are formed. One set is composed of three isozymes (Fig. 1, components  $\alpha$ -1,  $\alpha$ -2, and  $\alpha$ -3), and appears to have reached its maximum activity after 4 days. These three forms were found to have identical electrophoretic mobilities with the three  $\alpha$ -amylases formed during the growth of wheat. The three components did not appear to change in mobility during the entire germination period (i.e., comparison of zymograms 5 and 7). Heat-treatment

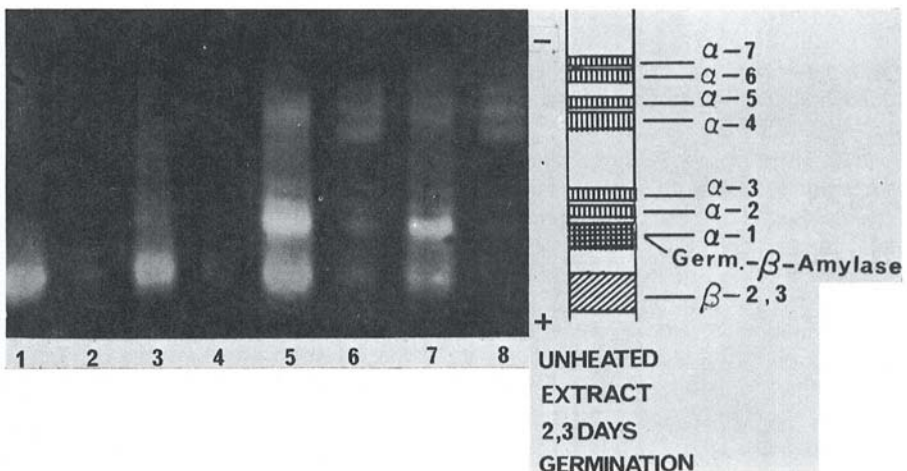


Fig. 1. Zymograms on starch gel indicating changes in amylases from Manitou wheat during germination. 1, 3, 5, 7, 9, 11: Amylases present in unheated extracts at 0, 1, 2, 3, 4, and 7 days' germination. 2, 4, 6, 8, 10, 12: Amylases present in heat-treated extracts at 0, 1, 2, 3, 4, and 7 days' germination.

did not alter the electrophoretic mobilities of these components, and only the intensity of the  $\alpha$ -1 component was diminished because of the inactivation of the newly formed germinated  $\beta$ -amylase with coincident electrophoretic mobility. The second set of amylase isozymes is electrophoretically slower. At 2 days' germination, bands appear as two closely spaced doublets (zymograms 5 and 6, components  $\alpha$ -4,  $\alpha$ -5,  $\alpha$ -6, and  $\alpha$ -7). With germination time, however, the bands change their electrophoretic mobilities and become five equally spaced  $\alpha$ -amylase components (zymograms 9 and 11), composed of three major components ( $\alpha$ -4,  $\alpha$ -5, and  $\alpha$ -6), and two very minor components ( $\alpha$ -7 and  $\alpha$ -8). As the amounts of components  $\alpha$ -7 and  $\alpha$ -8 are very small, it is difficult to say with certainty that both are present, and in certain varieties only one is clearly visible.

The zymograms for extracts of the slower moving set of isozymes upon heat-treatment were very surprising. The electrophoretic pattern found in the earlier germination times of 2 and 3 days (zymograms 5 and 7) was converted to the pattern present in the later stages of germination, consisting of the five equispaced  $\alpha$ -amylase components (zymograms 6 and 8). In addition, the apparent activity of these components had increased greatly. In samples germinated for 4 days or longer, however, zymograms from both unheated and heat-treated extracts (comparison of zymograms 9 and 10) became identical.

Zymograms on  $\beta$ -limit-dextrin gel (not shown) confirmed the results found on the starch gel.  $\beta$ -Amylase components  $\beta$ -2 and  $\beta$ -3 were absent and the intensity of  $\alpha$ -amylase component  $\alpha$ -1 was diminished, indicating that overlapping with a germinated  $\beta$ -amylase component had probably been responsible for the apparent increased activity on the starch gel.

The varieties Marquis and "Prairie Pride" were also analyzed in the same manner and found to behave almost identically during germination. With Marquis wheat, however, the amount of  $\alpha$ -8 was so small that it was difficult to be certain that this component was present (Fig. 2). A sample of Eastern white winter wheat also was found to behave identically (Fig. 3).

#### Nature of Pure $\alpha$ -Amylase Components

Previous results from this laboratory have indicated that there are four  $\alpha$ -amylase components in malted wheat (2). The present results suggest that twice this number should be present. In order to resolve this anomaly, malted wheat was purified as before by heat-treatment, acetone fractionation, glycogen-complex formation, and DEAE chromatography (2). Four peaks were again found. Samples from each peak were diluted to appropriate concentrations and analyzed by the present electrophoretic technique. As the shape of peak 1 had indicated heterogeneity, possibly by contamination with the through peak on DEAE chromatography, portions of the frontal and tail ends of this peak were analyzed. The results (Fig. 4) indicated that each peak consisted of one main isozyme, with the exception of peak 1, which had two forms present. These forms were identical in mobility and accounted for the electrophoretically slower set of  $\alpha$ -amylase isozymes (components  $\alpha$ -4,  $\alpha$ -5,  $\alpha$ -6,  $\alpha$ -7, and  $\alpha$ -8) found in germinated wheat. Reexamination of an extract purified to the glycogen-complex stage indicated that all eight isozymes were present, and suggested therefore that the electrophoretically faster set of three isozymes was not eluted from the DEAE-cellulose ion-exchange chromatography column.

#### Nature of Amylases in Kernels of Sound Wheat

It has been found in immature kernels of wheat that a set of three  $\alpha$ -amylase isozymes is present (1). In germinated wheat, this set of amylases is present, and in addition, a set of five electrophoretically slower  $\alpha$ -amylases. It should be possible, therefore, to analyze sound varieties of wheat with low amylograph viscosities and determine whether the decrease can be attributed to: a) presence of green kernels, b) presence of germinated kernels, or c) peculiar starch characteristics of the grain. To this end, the electrophoretic method was modified to make it more sensitive by:

a) Extending the incubation time to 1.5 hr. from 15 or 45 min.

b) Increasing the sample size applied on the gel from 0.05 to 0.1 ml.

c) Using only  $\beta$ -limit-dextrin slabs and making the slabs extremely thin. To ensure a uniformly thin film, the  $\beta$ -limit dextrin in agar slab was formed between two plates, with small chips of cover-glass at the corners to separate the two plates. The upper glass plate was removed, once the gel had set, by gently forcing it loose with a spatula.

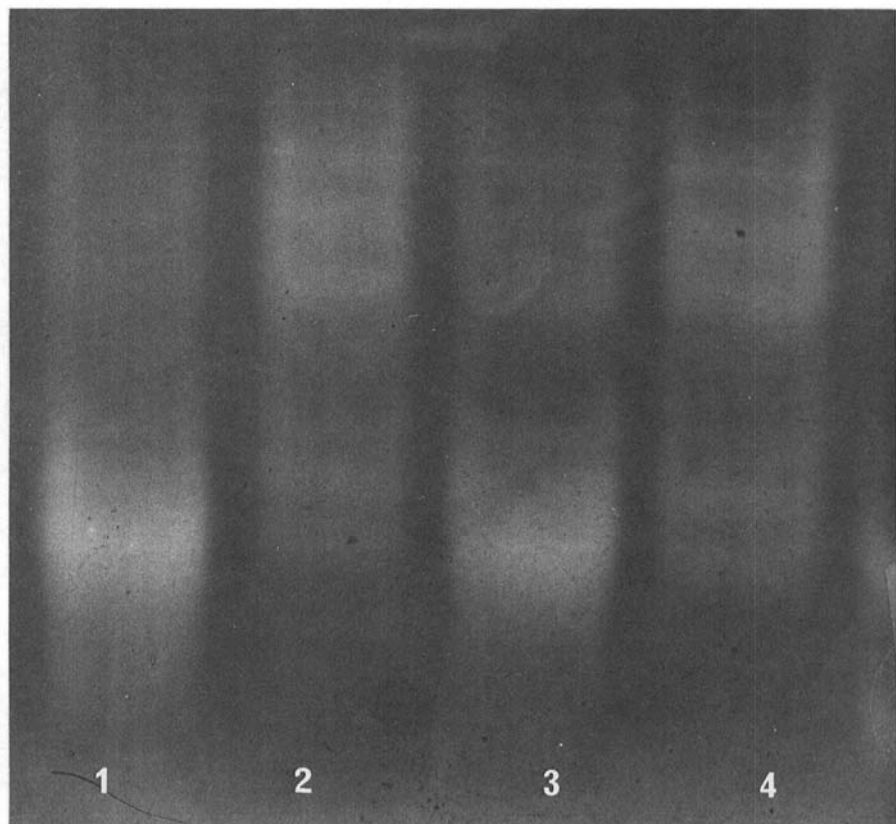


Fig. 2. Zymograms on starch gel indicating amylases present in Marquis wheat after 4 and 7 days' germination. 1, 3: Amylases present in unheated extracts at 4 and 7 days' germination. 2, 4: Amylases present in heat-treated extracts at 4 and 7 days' germination.

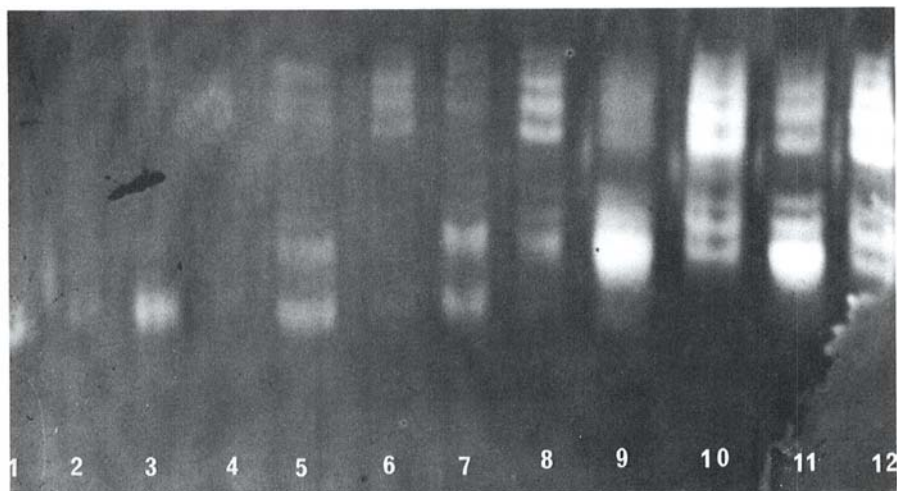


Fig. 3. Zymograms on starch gels indicating changes in amylases from a sample of Eastern white winter wheat during germination. 1, 3, 5, 7, 9, 11: Amylases present in unheated extracts at 0, 1, 2, 3, 4, and 7 days' germination. 2, 4, 6, 8, 10, 12: Amylases present in heat-treated extracts at 0, 1, 2, 3, 4, and 7 days' germination.

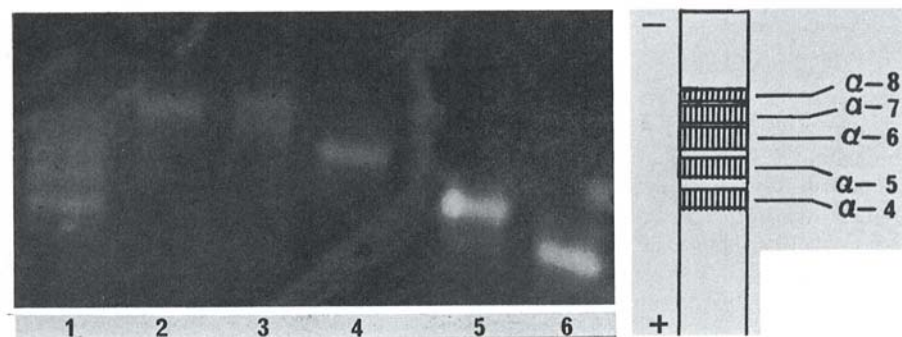


Fig. 4. Zymograms on starch gel of pure  $\alpha$ -amylase components resulting from DEAE ion-exchange chromatography. 1, Mixture of all components; 2, frontal portion of peak I; 3, tail portion of peak I; 4, peak II; 5, peak III; 6, peak IV.

d) Heat-treating the extracts prior to electrophoresis. As has been demonstrated in Fig. 1, this has the apparent effect of increasing the activity of the electrophoretically slower set of germinated  $\alpha$ -amylase components.

Samples (2 g.) from 21 Canadian plant breeders' varieties of spring wheat (Table I) with widely varying amylograph viscosities were extracted, heat-treated, and analyzed. The presence of  $\alpha$ -amylase due to germinated kernels was very evident in varieties CT-773 and CT-775, with low amylograph viscosities of 330 and 350 B.U.

TABLE I. 1969 PLANT BREEDERS' VARIETIES OF SPRING WHEAT ANALYZED BY POLYACRYLAMIDE-SLAB ELECTROPHORESIS

CT No.	Variety	Amylograph Viscosity B.U.	Grade <sup>a</sup>
773	Thatcher X CT-736	330	2 Nor.
329	CT-319 selection	340	3 Nor.
775	Centana-CT-733 X CT-738 CT-736	350	2 Nor.
296	Pembina <sup>2</sup> X Magnif Entrerriano	420	2 Nor.
740	Cypress	460	2 Nor.
932 <sup>b</sup>	Exchange X Kenya-Lemhi <sup>4</sup>	490	3 Nor.
616	Pembina <sup>2</sup> -Bage X Klein Petiso-Sonora 64	535	3 Nor.
817	Waldron	535	3 Nor.
774	Canthatch X 4351-331	570	2 Nor.
443	Manitou bx (A43, Ech, Wst, Ctr)	685	2 Nor.
1	Marquis	745	2 Nor.
282	Neepawa	795	2 Nor.
263	Manitou	800	2 Nor.
332	Ftn-Te <sup>7</sup> X Na101, K58-Tc <sup>10</sup>	800	2 Nor.
934	Parentage unknown	810	2 Nor.
503	Thatcher	820	2 Nor.
432	Manitou <sup>2</sup> X RL 4124.1	820	2 Nor.
930	Ciano 67	870	2 Nor.
615	Sonora 64 X Tezanos Pintos Precos	905	3 Nor.
427	Park	965	2 Nor.
614	CT-244 X Tezanos Pintos Precos	970	2 Nor.

<sup>a</sup>Designated grades under the Canada Grain Act as of 1969. No. 2 Nor. contains a minimum percentage by weight of 50% hard vitreous kernels. It is reasonably well matured and free from damaged kernels. No. 3 Nor. contains a minimum percentage by weight of 35% hard vitreous kernels. Although reasonably well matured, it may contain a small amount of frosted, immature kernels or other light damage.

<sup>b</sup>Soft wheat.

Similarly, varieties CT-329 (340 B.U.), CT-817 (535 B.U.), and CT-616 (535 B.U.), indicated that germinated kernels were present, although the amounts appeared slightly less; whereas varieties CT-932 (490 B.U.) and CT-296 (420 B.U.) contained faintly discernible signs of germinated  $\alpha$ -amylase. The only other variety found to contain discernible  $\alpha$ -amylase was CT-740 (Cypress), with an amylograph viscosity of 460 B.U. Quite surprising was the finding that the  $\alpha$ -amylase in this variety appeared to be predominantly of the type found in immature wheat.

## DISCUSSION

Pronounced changes occur in the amylases of HRS wheat during germination. The main  $\beta$ -amylase components of wheat begin to disappear at 2 days' germination, and a new electrophoretically slower  $\beta$ -amylase is formed. After 4 days of germination, the original  $\beta$ -amylase components have almost disappeared. The coincident disappearance of the sound  $\beta$ -amylase with the appearance of the germinated  $\beta$ -amylase may indicate that at least part of the formation of the new  $\beta$ -amylase is by transformation of one form into the other. Other factors, such as release of latent  $\beta$ -amylase and its subsequent modification, would presumably also occur.

Germinated  $\alpha$ -amylase appears after 2 days' germination, and steadily increases in amount with time. Two sets of amylases have been found. One set of three

isozymes is electrophoretically identical to those found in immature kernels, whereas the second set is found only in germinated wheat. It is well known that amylase formation in germinated seeds occurs by *de novo* synthesis (3,4). The electrophoretic identity between the faster set of germinated  $\alpha$ -amylases and the  $\alpha$ -amylases in immature kernels suggests, however, that they may be related. As the wheat kernels approach maturity, the amylases found in green kernels fall to a very low value. It may be that these same amylases are regenerated during germination, forming the electrophoretically faster set of germinated  $\alpha$ -amylases, while *de novo* synthesis also occurs and is responsible for the electrophoretically slower set of  $\alpha$ -amylases. The finding of two bands of  $\alpha$ -amylase, one of which was similar to that found in green  $\alpha$ -amylase, has also been reported recently by Olered and Jonsson (5), using agar-gel electrophoresis. The resolution on agar gel, however, was not sensitive enough to separate each band into its component isozymes.

The transition in electrophoretic mobility of the slower moving  $\alpha$ -amylases present after 2 and 3 days' germination to that found in 4- or 7-day germinated samples is difficult to explain. One possibility is that compounds are present which bind to the  $\alpha$ -amylases and change their electrophoretic mobility, and that these compounds are broken down by heat or additional germination. Also surprising is the fact that these isozymes increase in activity upon heating. This again could be explained on the basis that an inhibitor is present and is removed by heating. However, when heated and unheated extracts were compared by incubation with  $\beta$ -limit dextrin, the breakdown of this substrate, as judged by reduction in iodine-dextrin color at 540 nm., appeared very similar for both extracts.

The zymograms from Manitou, Marquis, "Prairie Pride", and a sample of Eastern white winter wheat were all very similar, and indicate that the  $\alpha$ -amylase isozymes formed with different varieties of hexaploid wheats upon germination are closely related.

The procedure that has been described in a previous paper (2) for isolating  $\alpha$ -amylase from malted wheat, of which the final step consists of DEAE-cellulose ion-exchange chromatography, appears to isolate and separate only the electrophoretically slower set of  $\alpha$ -amylase isozymes. The individual isozymes of the electrophoretically slower isozymes, when separated by the ion-exchange-chromatography step and submitted to electrophoresis, moved with their own particular mobility and indicated that the isozymes were not just artifacts which had been formed from one component because of the electrophoretic conditions.

By an increase in sensitivity of present electrophoretic technique, a method has been developed which can detect the presence of  $\alpha$ -amylase in "sound" varieties of wheat and also give some information concerning the nature of the amylases found. Thus, analyses of 21 plant breeders' samples of red spring wheat indicated that  $\alpha$ -amylase was present in all samples with amylograph viscosities lower than 550 B.U. The electrophoretic pattern indicated that in most cases the  $\alpha$ -amylase present was due to germinated kernels. However, in the case of one variety (Cypress), the electrophoretic pattern indicated that the  $\alpha$ -amylase was primarily that found in immature wheat. It is not known whether a small amount of immature kernels was responsible for this  $\alpha$ -amylase activity, or whether Cypress has varietal characteristics such that the  $\alpha$ -amylase present in the growing kernel fails to decrease to a low level at maturity, as would normally occur with most varieties.

The finding that this variety has excellent milling and baking characteristics, such as might not be expected if substantial germination had occurred, also lends credence to the fact that "green"  $\alpha$ -amylase may be responsible for lowered amylograph viscosity. The particularly small sample size required in this technique should make it particularly attractive, especially to plant breeders, for analyses of even single kernels of wheat are possible. However, if quantitative comparisons are to be made, such as between the amount of  $\alpha$ -amylase visualized on the  $\beta$ -limit-dextrin gel and that detectable by other methods, such as the amylograph and falling number, it would be necessary to select an adequate sample size for grinding, as the presence of only a few germinated kernels in the presence of a large amount of nongerminated kernels is known to exert a large effect on such methods (6).

#### Acknowledgment

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