Evolution of α-Amylase Components During Initial Stages of Barley Germination With and Without Prior Steeping[1]

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ABSTRACT

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Conquest barley kernels were germinated for five days on moist filter paper or steeped for two days and then germinated in moist air for three days. Samples of kernels were collected each day and freeze-dried. Isoelectric focusing analysis showed that three main groups of α-amylase were synthesized in both series. Rocket-line immunoelectrophoresis and combined isoelectric focusing and crossed immunoelectrophoresis showed that 1) α-amylase activity and the amount of α-amylase protein increased in parallel during germination, 2) steeping preferentially inhibited the synthesis of α-amylase I, and 3) steeping decreased the α-amylase II/α-amylase III ratio during germination.

The evolution of α-amylases during the germination of barley kernels has been widely studied (Briggs 1964, 1968; MacGregor 1978; Verbeek et al 1973). α-Amylase from germinated or malted barley is not a single entity but a mixture of enzymes. Recent advances in protein analytical techniques such as isoelectric focusing and immunochemical characterization of cereal proteins (Daussant 1978, Daussant et al 1977, MacGregor 1978), used separately or together, offer the possibility of investigating the qualitative and quantitative evolution of α-amylase components during germination. The aim of this study was to follow the evolution of α-amylase polymorphism during barley germination, with and without prior steeping, by using isoelectric focusing and to obtain quantitative data on the evolution of α-amylase proteins by using immunochemical techniques.

MATERIALS AND METHODS

Germination

Kernels of Conquest barley (*Hordeum vulgare* L., a Canadian six-row cultivar) were soaked for 20 min in 1.5% sodium hypochlorite solution and rinsed thoroughly with deionized sterile water. They were then germinated by two different methods.

Nonsteeped. Kernels were germinated at 18°C in sterile petri dishes, each containing two pieces of Whatman No. 1 filter paper, 50 kernels, and 4 ml of water. Samples (4 petri dishes each time) were removed after 24, 48, 72, 96, and 120 hr; roots and shoots were removed; and the samples were freeze-dried and ground in a Wiley mill through a 1-mm sieve.

Steeped. A 150-g sample of sterilized barley kernels was steeped in aerated distilled water at 18°C. The water was changed and the barley was air-rested after 18 and 24 hr. Samples of barley were removed after 24 and 48 hr and frozen. After 48 hr, excess water was removed from the remaining barley, which was then placed in a perforated drum in a germinator at 18°C with high humidity. Samples were removed and frozen after 24, 48, and 72 hr. All samples were freeze-dried, cleaned, and ground in a Wiley mill through a 1-mm sieve.

Extraction

Barley grist (1 g) was ground in a mortar at room temperature with 6 ml of 0.05M sodium barbital buffer containing 0.2M NaCl and 0.001M $CaCl_2$. After 30 min the extract was centrifuged (20,000 × g) and dialyzed for 1 hr against the extraction buffer diluted twice but containing no NaCl.

Immune Serum

Two immune sera were used in these studies. One was induced in rabbits by α-amylase purified from germinated barley seeds, and the other was induced by α-amylase purified from developing wheat seeds. These sera were described previously (Daussant 1978).

Isoelectric Focusing

Isoelectric focusing was performed as described previously (MacGregor 1976) except that the equipment was kept in a cold room (4°C) and water at 0°C was used as refrigerant. α-Amylase characterization was conducted as described previously (MacGregor et al 1974), but the concentration of dextrin used was increased twofold.

Immunoelectrophoresis

Rocket-line immunoelectrophoresis (Krøll 1973) combined with α-amylase detection was performed as described for wheat (Daussant 1978). The combined technique of isoelectric focusing in polyacrylamide gels and crossed electrophoresis in agarose gels containing antibodies (Söderholm et al 1975) was modified for α-amylase investigations. At the completion of isoelectric focusing, a strip of the polyacrylamide gel containing a portion of the sample being analyzed was incubated on a dextrin plate to detect the bands of α-amylase. Using the dextrin plate as a guide, the portions of the remainder of the gel containing the various groups of α-amylase were cut out and transferred upside down to the agarose gels containing α-amylase antibodies. α-Amylase characterization on agarose gels after crossed immunoelectrophoresis was done as described previously (Daussant 1978).

RESULTS AND DISCUSSION

The general time course of α-amylase evolution during barley germination has been known for many years (Lüers 1933) and it is now generally accepted that the enzyme is produced by de novo synthesis (Briggs 1963, Grabar and Daussant 1964, Varner 1964). The rate of production of the enzyme depends on many factors, such as barley variety, availability of air and moisture, temperature of germination, and the addition of plant growth hormones such as gibberellic acid (Briggs 1968, MacGregor 1978). Each barley sample will synthesize α-amylase at a rate that depends on the conditions of germination. Results of both germination series in our investigation are shown in Fig. 1. For both series, the burst of α-amylase synthesis occurred between days 3 and 5, but the level of activity was much higher for the nonsteeped sample. Although detectable amounts of α-amylase were present after one and two days of steeping, the levels were very low, and subsequent α-amylase synthesis was less rapid in this series. No α-amylase was detected in the steeping water. After five days of germination, the steeped kernels contained only 50% of the activity present in the nonsteeped sample. The moisture levels of the steeped barley were

38 and 46% after 24 and 48 hr, respectively, and exposure to this high moisture content had a detrimental effect on the synthesis of α-amylase.

The sprout lengths of the samples in the two series were quite different. In the nonsteeped series, all samples were chitted after 24 hr and the sprouts had grown to about 3 cm after five days of germination. Chitting was extensive in the other series only after 48 hr of steeping. After a further three days of germination, the sprouts had reached a length of 1.5–2 cm. Therefore, complete immersion of the barley in water reduced the rate of germination as judged by both sprout length and α-amylase synthesis. Presumably, lack of oxygen caused this reduced germination.

Isoelectric focusing patterns of α-amylases extracted from seeds germinated with and without steeping are shown in Fig. 2. Because of experimental conditions, the very small amount of α-amylase present in the mature Conquest sample was not detected by the dextrin-plate technique, although the enzyme was clearly visible in both samples after one day of germination. There were no major qualitative differences in the α-amylase II and III patterns of the two series, but secondary components of both groups appeared after two days for the nonsteeped kernels and after three days for the steeped kernels. Equal amounts of all extracts were analyzed by isoelectric focusing so that the evolution of the various α-amylase enzymes could be observed. Individual components of α-amylases II and III were not readily discernible after four and five days of germination, however, because of the very large amount of α-amylase in the extracts at this stage. To obtain clear banding in these samples, very short incubation would have been required, but then α-amylase I would not have been so readily detected.

The α-amylase I components of the two series were different. This fast moving, anodic constituent was clearly visible after one day of steeping and appeared to consist of two widely spaced bands of activity. In the nonsteeped series, this constituent originally consisted of one band of activity, and another slightly more acidic component appeared after five days of germination. However, the enzyme was not clearly visible by isoelectric focusing (Fig. 2) until the third day of germination although it had been detectable on the original dextrin plates after one and two days of germination. These results are in general agreement with those reported earlier (MacGregor 1978).

As discussed earlier (MacGregor 1978), results from isoelectric focusing analysis such as those shown in Fig. 2 are not quantitative, and other methods must be used to determine the relative amounts of α-amylase enzymes in cereal kernels at different stages of germination. One approach is to use ion exchange chromatography to separate components and then to determine the amount of each component separately. This is relatively simple with α-amylases I and II, but α-amylases II and III have similar isoelectric points and are difficult to separate. An immunochemical approach (Daussant 1978) appeared to offer a solution to this problem.

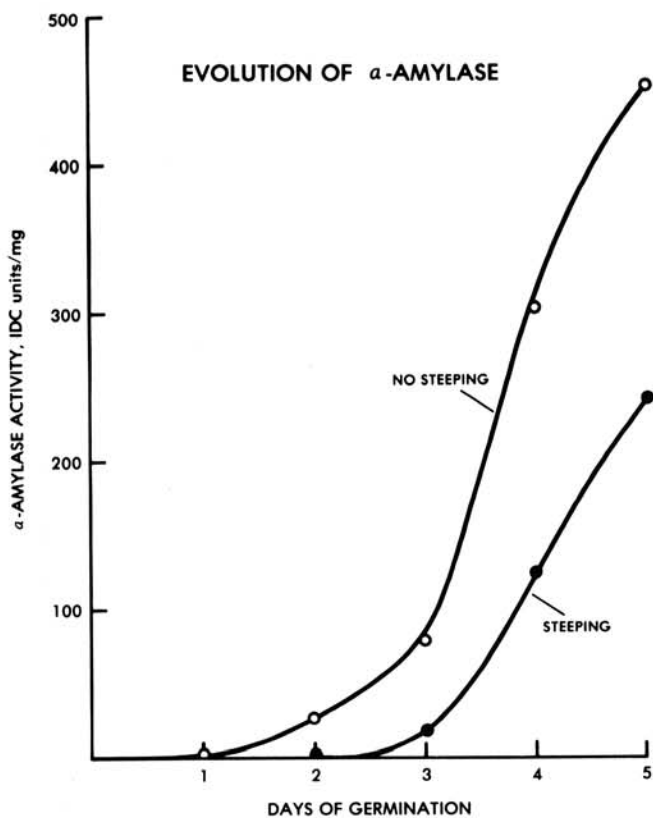Before reliable quantitative data can be obtained from such

Fig. 1. Evolution of total α-amylase in Conquest barley during germination. • = germination without steeping; o = germination in moist air after prior steeping.
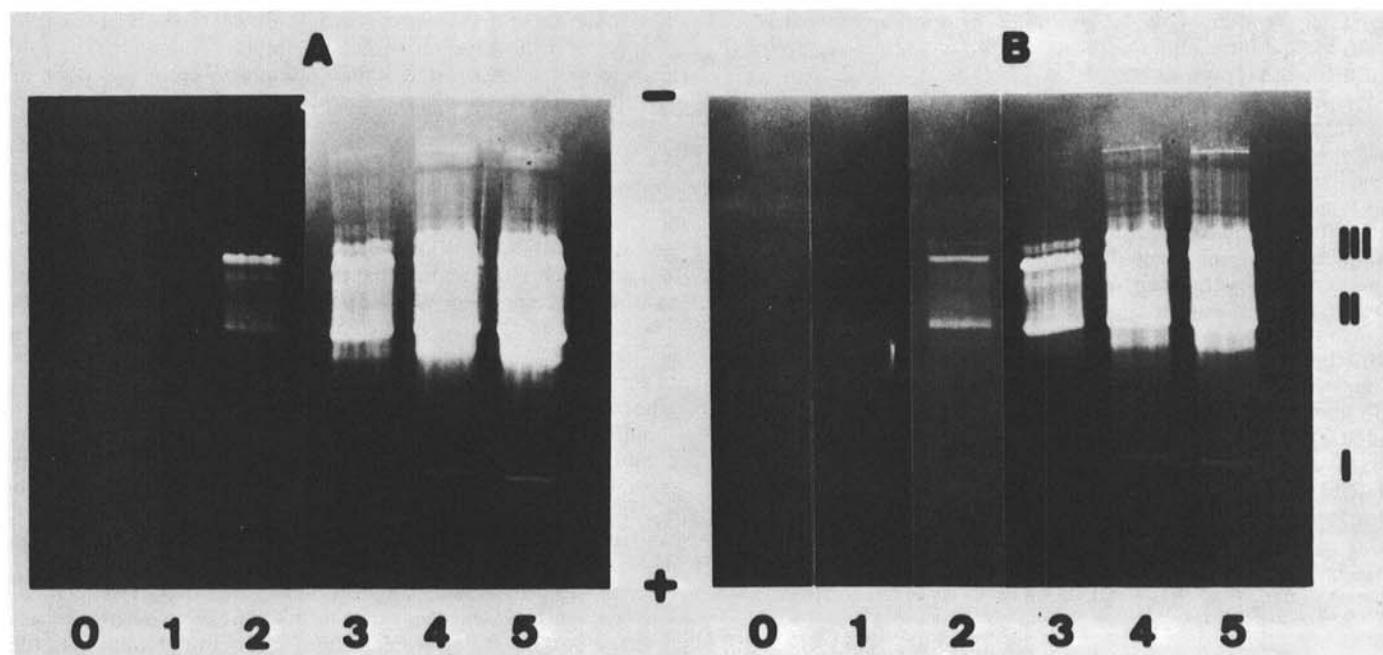

Fig. 2. Isoelectric focusing analysis of extracts of Conquest barley germinated without steeping (A) and with prior steeping (B). A pH 4–8 gradient was used.

immunochemical experiments, the antigenic relationships must be established between the components analyzed and the immune sera used . For α-amylases I, II, and III of germinated barley, these may be summarized as follows: α-Amylases II and III were antigenically identical when an immune serum induced in rabbits with an α-amylase purified from germinated barley was used (MacGregor and Daussant, *unpublished*). This serum reacts with all forms of α-amylase extracted from seven-day germinated barley kernels (Daussant et al 1974). However, α-amylases I and II displayed differences in their antigenic structures (Daussant et al 1974, Bøg-Hansen and Daussant 1974), so they are not identical proteins. This immune serum was used only to determine the quantitative evolution of α-amylases II and III in this study.

The serum reacted only weakly with antigen I (Daussant et al 1974). In preliminary studies, however, immune serum prepared by injecting rabbits with α-amylase purified from developing wheat seeds (Daussant et al 1977) had a better titer in antibodies specific for malt α-amylase I. The latter immune serum was therefore used in this study for characterizing α-amylase I. It is of interest to note that close antigenic relationships between α-amylases of certain cereals, particularly barley and wheat, have been reported (Alexandrescu and Mihailescu 1973, Daussant and Grabar 1966).

In the first series of experiments, Daussant's technique (1978) for studying wheat α-amylases was adapted to follow the quantitative evolution of two α-amylase antigens during barley germination. These two antigens were called antigen I, which corresponds to α-

TABLE I
Relative Amounts of α-Amylases I, II, and III in Germinated Barley Kernels[a]

α-Amylase	Sample	Relative Amount of Extract Used	Peak Area Obtained[b]	Calculated Peak Area of Original Extract[c]
I	4-Day (nonsteeped)	100	37.2	37.2
	4-Day (nonsteeped)	50	18.0	36.0 (37.7)
	4-Day (nonsteeped)	25	10.0	40.0
	4-Day (steeped and germinated)	600	13.0	2.2
II	4-Day (nonsteeped)	50	39.2	78.4
	4-Day (nonsteeped)	25	21.0	84.0 (77.3)
	4-Day (nonsteeped)	12.5	8.7	69.6
	2-Day (nonsteeped)	200	8.0	4.0
	4-Day (steeped and germinated)	100	16.5	16.5
III	4-Day (nonsteeped)	50	55.0	110.0
	4-Day (nonsteeped)	25	32.1	128.4 (118.9)
	4-Day (nonsteeped)	12.5	14.8	118.4
	2-Day (nonsteeped)	200	20.1	10.1
	4-Day (steeped and germinated)	100	57.2	57.2

[a]Kernels germinated for two and four days and kernels steeped for two days and then germinated for two days.
[b]Obtained by cutting out and weighing the peaks shown in Fig. 4.
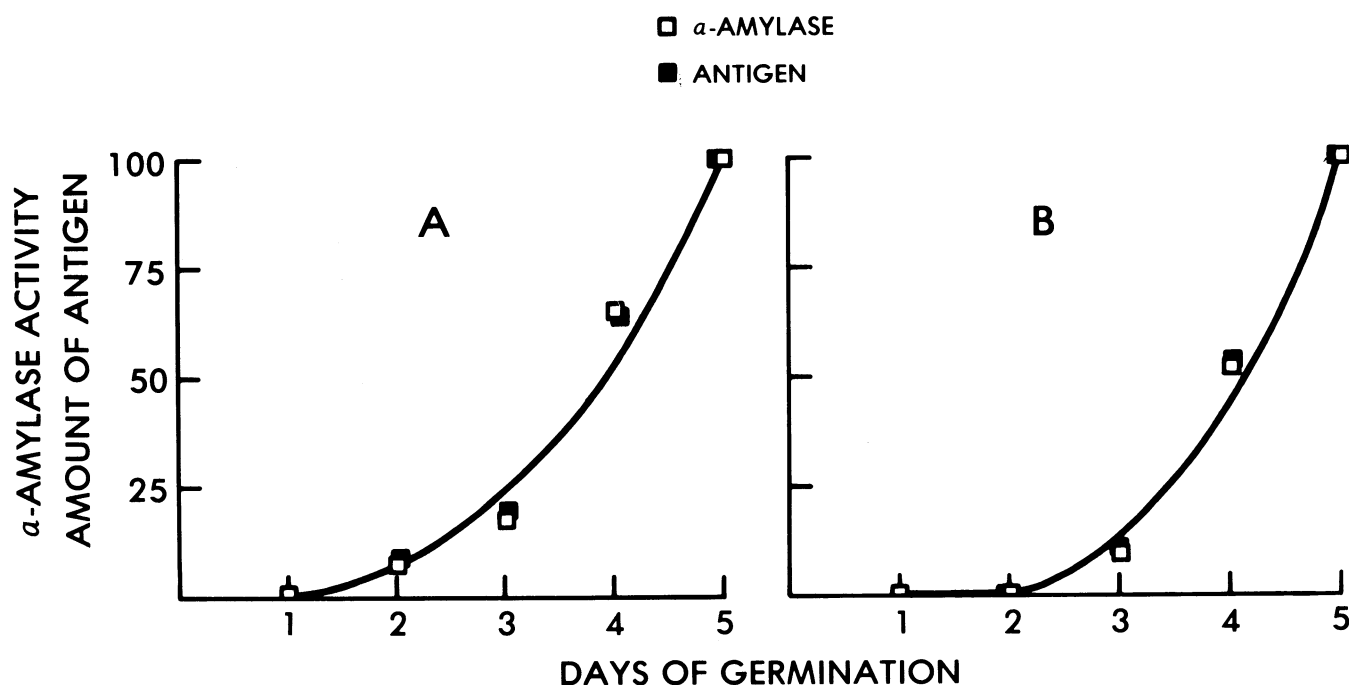[c]Peak area × dilution of extract. Numbers in parentheses are averages.

□ α-AMYLASE

■ ANTIGEN

Fig. 3. Evolution of total α-amylase activity (■) and of α-amylase antigen II (□), which corresponded to α-amylase groups II and III, in Conquest barley germinated without steeping (A) and after prior steeping (B). The α-amylase activity and the amount of antigen in each sample were expressed as a percentage of the amount of each present in the five-day germinated sample of the corresponding series.

amylase I, and antigen II, which corresponds to α-amylases II and III (Daussant et al 1974). Antigen refers to total enzyme protein (both active and inactive) whereas α-amylase refers only to the active enzyme. Therefore, these two terms are not identical in all cases. Previous results showed that the activity of α-amylase I was much lower than that of α-amylase II (MacGregor 1978). The results for antigen II, as well as the increase in total α-amylase activity, are shown in Fig. 3. Results for both series were expressed as a percentage of either the amount of antigen or of α-amylase in the corresponding five-day germinated sample. For both series, the evolution of antigen II paralleled the increase in α-amylase activity, suggesting that the bulk of the activity increase was due to the accumulation of antigen II. Antigen was not detected in the extracts of ungerminated seeds, but it was detectable in a 1,000-fold dilution of extracts of five-day germinated seeds. This further confirms that the antigen does not exist in ungerminated seeds in a soluble but inactive form (Grabar and Daussant 1964).

Results for antigen I (α-amylase I) were not clear and are not shown. Precipitin peaks of this antigen were either not detected or were hidden by peaks of antigen II. The precise reason for the technique's failure to give satisfactory results is not known but was probably because the amount of antigen I was very small compared

Fig. 4. Quantitative estimation of antigens I, II, and III. After isoelectric focusing of different amounts of the extracts, portions of the polyacrylamide gel, each containing either α-amylase I, II, or III, were transferred to the agarose gels by the "laying on" technique. **I: IS**, agarose gel containing 0.3% immune serum prepared against α-amylase from developing wheat seeds; **B**, intermediate agarose gel strip containing an extract of developing barley kernels; **A**, agarose gel to which the polyacrylamide gel pieces were transferred. **II and III: IS**, agarose gel containing 0.1% immune serum prepared against α-amylase from germinated barley kernels. Samples 2, 4, and 4S represent 2-day and 4-day samples germinated without steeping and 4-day sample germinated with steeping, respectively. Undiluted extract applied to isoelectric focusing gel on one piece of paper was given a value of 100; on two pieces, 200; and on six pieces, 600. Extracts were diluted two, four, and eight times to give concentration values of 50, 25, and 12.5.

with the amount of antigen II.

To study further the quantitative evolution of the three α-amylase groups, I, II, and III, the techniques of crossed immunoelectrophoresis and isoelectric focusing were combined. Kernels before and during the stage of rapid enzyme synthesis (two-day sample and four-day sample, respectively) were used. For each antigen, a scale of reference was established by using diluted portions of the extract of four-day germinated kernels from the nonsteeped series (Fig. 4). The concentration of the undiluted extract was denoted as 100. After isoelectric focusing, the amounts of antigens II and III present were analyzed by immunoelectrophoresis, but line immunoelectrophoresis was used for antigen I to obtain a better visual picture of the amount of this minor component (Fig. 4).

Antigen I was not detected in extracts of nonsteeped seeds after two days of germination (Fig. 4) but synthesis of the antigen must have been very rapid between the second and fourth day. These results agree with previous results (MacGregor 1978), which also showed rapid synthesis of α-amylase I during this stage of germination.

The peaks (Fig. 4) were cut out and weighed to give relative estimates of the amount of each antigen. The results are shown in Table I.

The extract from four-day germinated kernels contained about 17 times more antigen I than did the sample from the four-day steeped series. This large difference is not evident from the results shown in Fig. 2, thus emphasizing the potential danger of trying visually to quantitatively evaluate the results of isoelectric focusing experiments.

Between the second and fourth days of germination, the antigen II component of the nonsteeped series increased markedly (about 20-fold). These results agree with those shown in Fig. 1, which indicate rapid synthesis of total α-amylase during this period. In the steeped series, antigen II production during this period was much less and was about 20% of that obtained in the nonsteeped series.

Antigen III also showed a very large increase (about 12-fold) between the second and fourth days of germination. The four-day steeped kernels contained about 50% of the antigen of the nonsteeped sample. This difference was much less than that for antigens I and II. Therefore, the antigen II/antigen III ratio was not the same in both series. In the nonsteeped series, it was 0.65; in the steeped series, 0.29. Furthermore, this ratio changed during germination. For the nonsteeped series, it went from 0.40 after two days to 0.65 after four days. The steeped series cannot be compared similarly because the antigen II and III contents of the two-day sample were too small to measure. These results suggest that steeping not only repressed the rate of α-amylase synthesis but also modified the ratio of the two dominant α-amylase components.

The fact that the relative rates of synthesis of the major α-amylases differed in the two germination series raises an interesting question. Would other physiologic conditions, such as changes in the oxygen content of the germination atmosphere or changes in the moisture content of the grain during germination, also have an effect? A genetic effect exists (other studies have shown that α-amylase components are synthesized at different rates in different varieties), but the effect of environment on these syntheses has not been studied.
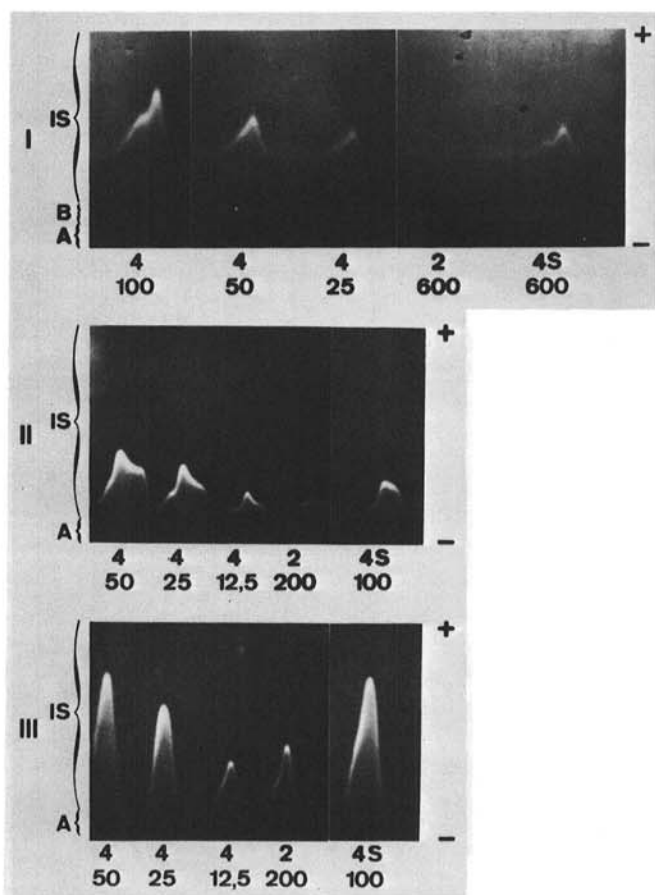
Preliminary studies have shown that the α-amylase II-to-III ratio is greatly increased during kilning, so the ratio of the two enzymes could be used as a marker for determining the severity of kilning. Studies are under way to investigate further the nature of this interconversion.

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