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Changes in Levels of α-Amylase Components in Barley Tissues during Germination and Early Seedling Growth[1]

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ABSTRACT

Kernels of Klages barley (*Hordeum vulgare* L.) were germinated for 1 to 4 days on moist sand at 18°C. Representative kernels from each time period were dissected to give the following fractions: scutellum, subscutellar endosperm, aleurone-scutellum interface, remaining aleurone, sub-aleurone endosperm, and core endosperm. These tissues were analyzed for α-amylase components by isoelectric focusing and rocket-line immunoelectrophoresis. Although aleurone and scutellar tissues appeared to synthesize the same α-amylase components, enzyme was detected first in the scutellum. A larger proportion of scutellar α-amylase was excreted into the endosperm compared to aleurone synthesized α-amylase. Aleurone cells appeared to synthesize appreciably more α-amylase than did scutellar tissue.

There has been much discussion recently on the relative importance of aleurone and scutellar tissues in the production of hydrolytic enzymes during germination and early seedling growth of cereal grains (3, 4, 11, 23, 24). α-Amylase synthesis in barley has received particular attention because of the technological importance of this enzyme in malting and brewing and also because synthesis of the enzyme in aleurone cells from barley kernels has been used as a model system to study hormonal action on protein synthesis (7, 13). Estimates on the contribution made by the scutellum to total α-amylase synthesis in barley seedlings have varied from little or none (24), to a relatively small proportion of 14% (4) and, finally, to a high value of 50% (12).

Some studies were based on enzyme histological (23) or immunohistological (11, 12) techniques that were not quantitative although they gave convincing qualitative data. In others (24), isolated, incubated barley embryos were shown to contain little or no α-amylase but possible secretion of the enzyme was not investigated nor was the effect of barley cultivar.

Little quantitative information is available on the relative amounts of α-amylase isoenzymes in tissues of barley kernels at different stages of germination and early seedling growth. In the present study an attempt has been made to obtain such data. Barley kernels, germinated for different periods of time, were dissected into a number of sections. Immune sera specific for each α-amylase group (I and II) were used in conjunction with the sensitive technique of line immunoelectrophoresis (16) and direct tissue line immunoelectrophoresis (9) to determine quantitatively changes in the levels of α-amylases I and II in these different sections. These results provide information on the sites of synthesis of the two groups of α-amylase and the rates at which the enzymes move into the endosperm.

MATERIALS AND METHODS

Germination Conditions. The two-rowed barley (*Hordeum vulgare* L. cv Klages) used in this study was grown in test plots in Brandon, Manitoba in 1976. Barley kernels were dehusked by a 3-h steep in 50% (v/v) H_2SO_4 (8) followed by thorough washing with sterile, distilled H_2O. Petri dishes, each containing 25 dehusked kernels, 50 g sterile sand, and 10 ml sterile H_2O, were incubated in a germination chamber at 18°C and high humidity. After 24, 48, 72, and 96 h, Petri dishes were removed and frozen at −20°C until analyzed.

Dissection. Frozen kernels were dissected on a cold plate using an Olympus dissecting microscope. Tissues were frozen immediately after they were separated from the kernel. Ten kernels from each sample were completely dissected to give the following six fractions (Fig. 1): center portion of the scutellum, crushed cell layer and subscutellar starch, strip of aleurone and adhering scutellum along the aleurone-scutellum interface (Fig. 1A), residual aleurone, sub-aleurone starch, and core starch. Care was taken to ensure that each fraction was clean and not contaminated by material from another fraction. Frozen tissues were freeze-dried, weighed, and ground to a fine powder. α-Amylase levels did not appear to be affected by the dissection technique.

α-Amylase Activity. This was carried out as described previously using amylopectin β-limit dextrin as substrate (2, 21).

Isoelectric Focusing. This was performed as described previously (17, 22). Tissues from 10 kernels were extracted with 4 ml of mM $CaCl_2$ in a mortar and centrifuged (10,000g, 10 min). Approximately 50 μl of tissue extracts from 24- and 48-h germinated kernels and 30 μl of the 72- and 96-h samples were applied to the gel on small pads of filter paper.

Rocket-Line Immunoelectrophoresis. This was carried out essentially as described previously for the direct analysis of tissues (9) or, when the α-amylase content in the tissues was too high, for the analysis of their extracts (20). A description of the technique with the anti α-amylase II immune serum is given in Figure 2. With the anti α-amylase I immune serum the conditions were identical except that an extract of developing barley seeds (1 g/6 ml extraction buffer and dialysis against the buffer without NaCl) was used in a 30% concentration in the strip gel. Extraction buffer: 50 mM veronal buffer (pH 8.60 containing 200 mM NaCl and 1 mM $CaCl_2$.

For direct analysis of tissues, weighed amounts of finely ground tissues were deposited directly into the wells and two portions of

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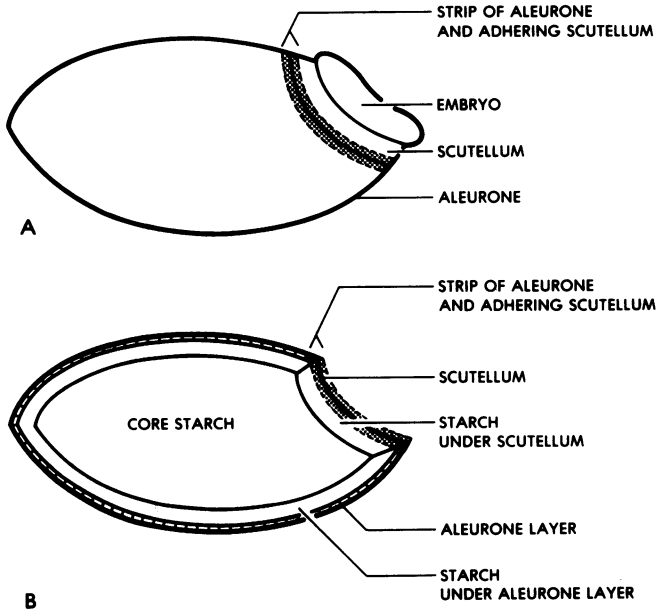

FIG. 1. Origin of dissected tissues. A, Dehusked kernel showing position of narrow strip of tissue containing the aleurone-scutellum interface. B, Longitudinal section of embryo-less kernel showing source of tissues analyzed.

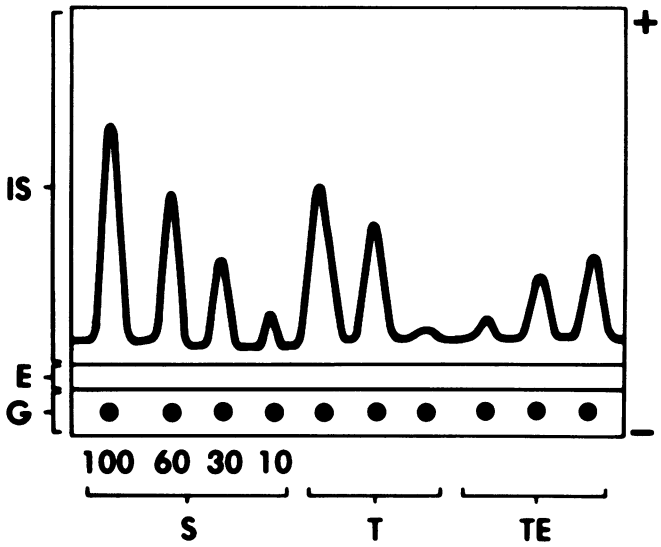

FIG. 2. Comparison of α-amylase II antigen content in different barley tissues or barley tissue extracts. IS, 1.2% agarose gel containing 0.1% anti α-amylase II immune serum. E, 0.5-cm agarose gel strip containing 0.3% malt extract (extraction 1 g/6 ml extraction buffer). G, Agarose gel. Wells were filled before analysis with serial concentrations of the standard extract (S) or with known amounts of finely ground tissues (T) or with 20 μl of tissue extracts (TE). For details on tissue extracts and direct application of tissues to the gel, see "Materials and Methods." Electrophoresis was run at 4° C and 5 v/cm for 19 h. After electrophoresis the gel was soaked for 1 h at room temperature in a 2% starch solution prepared in 200 mM sodium acetate buffer (pH 5.5) containing 1 mM $CaCl_2$ previously incubated for 15 h at 37°C with 25 mg β-amylase (Wallerstein Laboratories no. 1018.3) for 1 g starch. The gels were stained with iodine.

veronal buffer (25 mM, pH 8.6) were poured into the wells at 30-min intervals. Then, 30 min later, the wells were covered with agarose and electrophoresis was started. When the α-amylase content of tissues was too high, 2 to 20 mg (depending on preliminary experiments) were extracted with 200 μl of the extraction buffer. Twenty μl of the extract were deposited in the wells before starting electrophoresis.

The technique was standardized using an extract of freeze-dried Klages green malt (0.5 g malt + 3 ml of the extraction buffer). Various dilutions of this extract were subjected to rocket-line immunoelectrophoresis and, after electrophoresis, peaks obtained were traced on paper, cut out and weighed.

Relationships between peak weights and amount of malt extract used were determined. A value of 100 was arbitrarily assigned to a 10-fold dilution of the extract. Standard lines were constructed for α-amylases I and II. These scales were used to determine the amount of α-amylase in known weights of the different tissues or in their extracts.

RESULTS AND DISCUSSION

The sample of Klages barley used in this study contained no detectable α-amylase (Table I) indicating that it was a sound sample. Only a trace of α-amylase was detected after 24 h of germination but, thereafter, enzyme activity increased rapidly. This is typical of the rate of α-amylase synthesis during germination and early seedling growth of a good quality malting barley (18).

Kernels were dissected as shown in Figure 1. Differentiation of sub-aleurone and sub-scutellar starch was somewhat arbitrary but an attempt was made to include only the central core of endosperm tissue above the scutellum in the sub-scutellar fraction. In this way, information was obtained on the relative amounts of α-amylase secreted by the scutellum and aleurone. By removing the layer of aleurone associated with the embryo (24) along with the edges of the scutellum, an attempt was made to obtain aleurone-free scutella.

Previous studies using isoelectric focusing (18, 20) showed that α-amylases from malted or germinated barley were heterogeneous and appeared to consist of three main groups of α-amylase designated α-amylases I, II, and III. More recent results (25) have shown that α-amylase III is a mixture of α-amylase II and a low mol wt protein. This may explain the immunochemical identity observed previously between α-amylases II and III (20). Therefore, only α-amylases I and II are distinct groups of α-amylase with different immunochemical properties (1, 14) and coded for by different genes (6, 13). Because the isoelectric focusing technique used in this study is able to separate α-amylase II from the so-called α-amylase III these two groups are shown in Figure 3.

Focusing patterns for α-amylases from germinated whole kernels are not shown because they have been documented previously (18). All tissues appeared to contain α-amylase groups I, II, and III but only results for scutella and aleurones are shown in this report (Figs. 3 and 4). This is a qualitative technique, and visual examination of these patterns does not give quantitative information about the amount of each α-amylase group present (20). However, these qualitative patterns are in general agreement with the quantitative data shown in Figures 5 and 6. For example, a trace of enzyme activity was detected in the scutellum after 24 h of germination (Fig. 4) but none was detected in the aleurone (Fig. 3). At all subsequent stages, there appeared to be significantly more enzyme in aleurone layers compared to scutellar tissue.

Table I. *α-Amylase Activity in Germinated Kernels of Klages Barley*

	Germination Time (Days)				
	0	1	2	3	4
α-Amylase activity (IDC[a] units/kernel)	Not detectable (<1)	12	138	1976	5645

[a] Iodine Dextrin Colour (2).

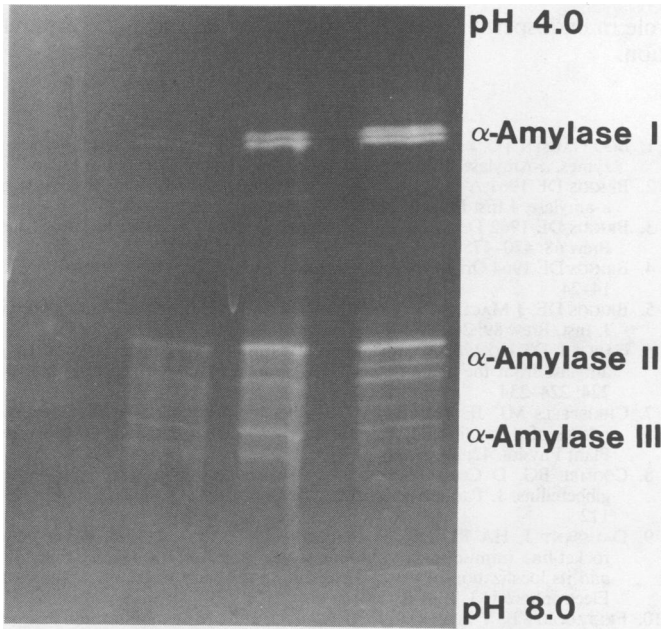

FIG. 3. Isoelectric focusing of α-amylase components in aleurone layers at different stages of germination. From left to right: tissues dissected from seeds germinated for 1, 2, 3, 4 d.

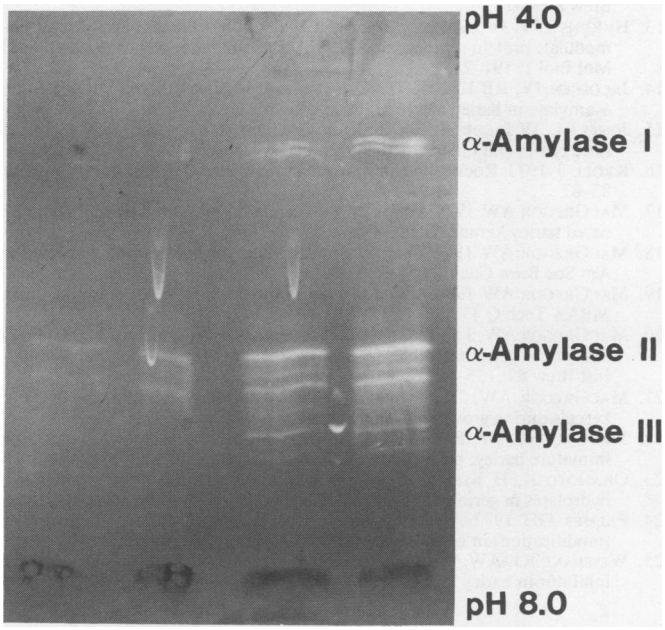

FIG. 4. Isoelectric focusing of α-amylase components in scutella at different stages of germination. From left to right: tissues dissected from seeds germinated for 1, 2, 3, 4 d. Rocket-like streaks are artifacts on gel surface.

Since α-amylases II and III provided a reaction of identity with the anti α-amylase II antibodies, the antibodies can be used to determine quantitatively α-amylases II plus III; the combined groups are referred to as α-amylase II. Antibodies specific for α-amylase I were used to determine this enzyme in the different tissues studied. The technique of line immunoelectrophoresis (Fig. 2) was used to follow, quantitatively, changes in the α-amylase I and II contents of dissected tissues during germination.

Results for α-amylase I are shown in Figure 5. Little or no α-amylase I was detected in any kernel fraction after only 1 d of germination. However, all fractions, except core starch, contained α-amylase I after 2 d. In general, the amount of antigen present in each tissue increased with time of germination. Scutella contained only small amounts of antigen but significantly more antigen was found in the underlying endosperm, indicating that enzyme had been actively secreted from the scutellum. Much more enzyme was found in the aleurone and aleurone-scutellum interface tissues. However, a much smaller proportion of this antigen was excreted into the endosperm and was not detected there until after 3 d of germination. Only during days 3 and 4 was a relatively small amount of enzyme detected in central endosperm tissue.

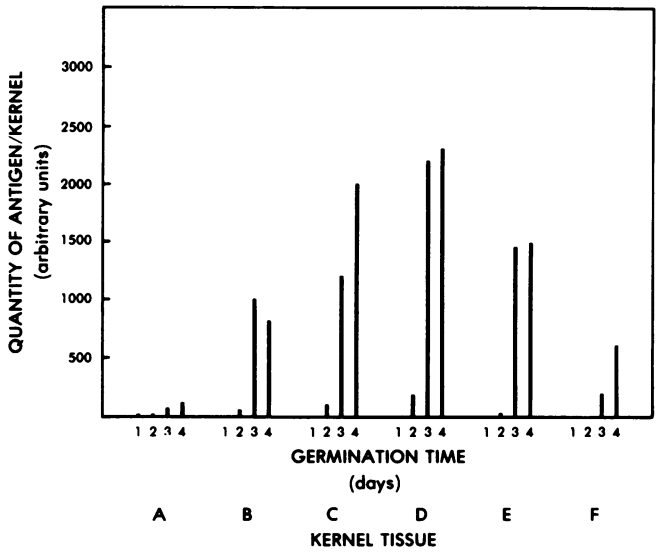

FIG. 5. Changes in α-amylase I levels in barley tissues during germination. Tissues analyzed were scutellum (A), sub-scutellar starch (B), aleurone-scutellum interface (C), remaining aleurone (D), sub-aleurone starch (E), and core starch (F).

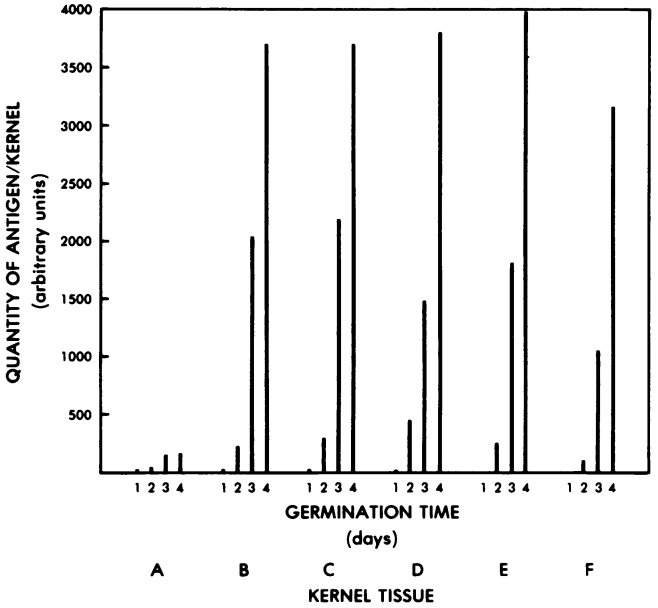

FIG. 6. Changes in α-amylase II levels in barley tissues during germination. Tissues analyzed were the same as in Figure 5.

Similar results for α-amylase II are shown in Figure 6. After 1 d, enzyme was detected in all kernel fractions except in starch immediately under the aleurone layer and in central endosperm samples. Enzyme amount increased in all fractions during germination. Earlier detection of α-amylase II over α-amylase I is

due, probably, to the relatively small amount of α-amylase I present. In germinated or malted barley kernels, α-amylase I accounts for only 5 to 10% of total α-amylase activity (20). As was the case for α-amylase I, a much higher proportion of scutellar α-amylase II was secreted into the endosperm compared to aleurone α-amylase II. Since aleurone-scutellum interface material was mainly aleurone then approximately half of the aleurone-produced α-amylase II remained within the tissue after 4 d of germination (Fig. 6, C and D). The remainder was found in the endosperm (Fig. 6, E and F). Scutellar tissue contained only about 5% of the enzyme amount found in the sub-scutellar endosperm. Because it was difficult to differentiate precisely between sub-scutellar, sub-aleurone, and core starch areas, quantitative results shown in Figures 5 and 6 should be treated with a degree of caution. However, the trends are consistent and obvious. A large proportion of scutellum-synthesized enzyme is excreted into the endosperm whereas only about 50% of aleurone-synthesized enzyme is found in the endosperm after 4 d. The rate of secretion of α-amylase II, the major α-amylase component, from aleurone cells may well depend on the amount of available Ca^{2+} present (15). These results support previous observations (3, 5, 10, 11, 19, 23) that the scutellum synthesizes α-amylase during early stages of germination. In addition, scutellum and aleurone synthesized both main groups of α-amylase. Rapid secretion of α-amylase from the scutellum may explain the apparent absence of α-amylase in incubated, dissected embryos reported previously (24).

Care must be exercised when trying to compare the reported times of appearance of α-amylase isoenzymes in different tissues in germinating cereal grains. Because α-amylase I is usually only a small proportion of total α-amylase, there can be problems with sensitivity of detection techniques at very early stages of germination. In addition, there is wide variation in the germination conditions used for such studies. For example, germination temperatures of 15°C (11) to 28°C (23) have been used; kernels have been germinated on moist sand (11) on wet filter paper (23) or malted (4); different barley cultivars have been used and there can be wide cultivar variation in the rate of α-amylase synthesis (18). However, the difference found in this study concerning the *in vivo* export of the enzyme from the scutellum or from the aleurone layer into the endosperm may not be an exceptional phenomenon in cereal seeds since it has also been suggested in a study on wheat (9). It should not be surprising that some controversy has arisen over the sequence of biochemical events that occurs during early stages of barley germination! There seems to be little doubt that aleurone cells are the major source of α-amylase in early seedling stages of barley, but enzymes from the scutellum may play an important role in endosperm degradation during initial stages of germination.

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