

# Multiple Forms of Amylase Induced by Gibberellic Acid in Isolated Barley Aleurone Layers<sup>1</sup>

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## ABSTRACT

The addition of gibberellic acid to isolated aleurone layers of barley (*Hordeum vulgare* L.) causes the production and secretion of four  $\alpha$ -amylases. Two of these are stable at pH 3.7 and are not inactivated by ethylenediaminetetraacetate. The other two represent the classical barley  $\alpha$ -amylases; i.e., they are inactivated at pH 3.7 and by reagents which form complexes with divalent metal ions. All four forms are synthesized *de novo* in response to the addition of gibberellic acid.

Gibberellic acid controls the appearance of  $\alpha$ - and  $\beta$ -amylases in the endosperm of barley (16, 17, 29).  $\alpha$ -Amylase arises in the aleurone layer and has been shown to be made *de novo* (2-4, 28).  $\beta$ -Amylase exists in ungerminated barley seed as a zymogen and can be activated by treatment with papain and with reducing agents (14, 30). Similarly, in wheat seed,  $\beta$ -amylase exists in the starchy part of the endosperm in a protein-bound form (20-23). The liberation of  $\beta$ -amylase by proteolysis suggests that during germination the  $\beta$ -amylase is released by protease secreted from the aleurone tissue (9).

Nine amylases have been found in germinating barley seed (7); on the basis of response to inhibitors, two have been identified as  $\beta$ -amylase and five as  $\alpha$ -amylase while the character of two remains unknown. Probably all of these amylases arise in the endosperm of the seed in response to gibberellic acid (12). A complete understanding of the response of the barley endosperm to gibberellic acid requires a complete characterization of all forms of barley amylase and an identification of their tissues of origin. In this paper we report progress in this direction.

## MATERIALS AND METHODS

Barley (*Hordeum vulgare* L.) seed, cultivar Himalaya, was used as the experimental material. The procedures of seed imbibition, isolation and incubation of aleurone layers, and the amylase assays were performed as described by Chrispeels and Varner (3). The only modification was that extracts were made in  $10^{-3}$  M acetate buffer, pH 4.8. Salt was not used because it is not necessary for complete amylase extraction from isolated

aleurone, and because it caused cracking and distortion of the agar gel during electrophoresis. Unless otherwise stated, incubations continued for 24 hr.<sup>2</sup>

**Electrophoresis.** The technique used to separate amylase forms was essentially that of Sick (25). The gel consisted of 1% Difco purified agar in 0.004 M phosphate buffer, pH 7.55. The agar was dissolved by heating the solution almost to boiling. A portion (110 ml) of the agar solution was poured onto a Plexiglass tray of inside dimensions 17.5  $\times$  19.5  $\times$  0.3 cm, and the surface was flamed to remove air bubbles. After cooling at room temperature for 10 min, the tray was refrigerated for a further 5 min. Thin slits were then made in the gel at the origin (6 cm from one end of the plate) by pressing into the gel a piece of Whatman No. 2 filter paper about 0.7 mm wide. The paper was left in place for 30 sec and withdrawn. Each slit was filled with 8  $\mu$ l of enzyme solution by means of a hypodermic syringe. Up to 14 samples were loaded across the gel. Amylase solutions were diluted to about 10 units (about 30  $\mu$ g) of amylase per ml for electrophoresis. Where visual quantitative comparisons of activity between samples were desirable, the samples were diluted by the same factor.

The buffer used in the gel was also used in the electrode compartments. The compartments and the gel were connected by sheets of duPont cellulose sponge cloth cut to the width of the gel. One sheet (from the anode) was placed along the gel almost up to the origin, just behind the sample slits. The other (from the cathode compartment) was brought up to a line 7 cm from the origin. The whole tray was then covered with a glass plate, and a stabilized current of 25 ma was passed through the gel for 2 hr at 4 C. The quality of the zymogram was improved if, after 20-min electrophoresis when the amylases had moved from the origin, the slits were closed by compressing the gel from both ends.

For visualization of the amylase bands, the tray was immersed in 0.5% soluble potato starch (J. T. Baker Chemical Co.) and incubated at 37 C for 30 min. The excess starch solution was then washed off, and the gel was flooded with an I<sub>2</sub>-KI solution for about 1 min. If the amylase activity was low, the bands could be accentuated by allowing the plate to stand at room temperature for 10 to 15 min before development with I<sub>2</sub>-KI.

**Amylose Hydrolysis.** For examination of the products of amylase action, a suitable amount of amylase was added to an amylose solution (about 0.4%) (Nutritional Biochemicals Corporation) in 0.05 M acetate buffer, pH 4.8. At different time

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<sup>2</sup> It should be pointed out that it is undesirable to use a soft glass mortar and pestle when grinding with sand because sufficient glass is solubilized to raise the pH of the homogenate to as high as pH 11.4 and the zymogram of extracted amylase becomes aberrant. Some change in pH occurs even if 0.1 M buffer is used.

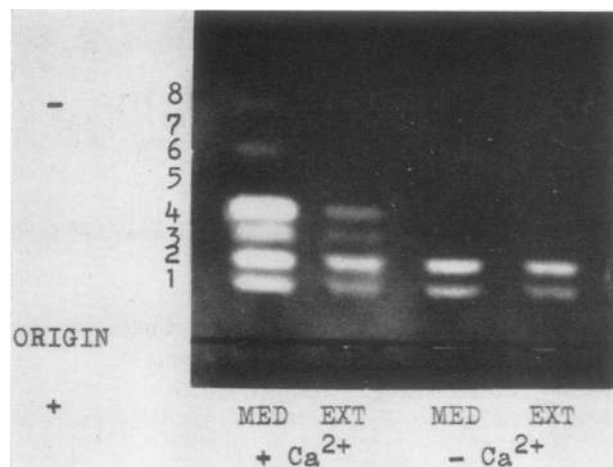


FIG. 1. Zymogram of amylases appearing in the medium (secreted) and extract (not secreted) of aleurone layers incubated for 24 hr in the presence of gibberellic acid plus and minus calcium chloride. Forms 5 to 8 occur in the media  $\pm$  calcium but produce bands which are very weak and which, at least in the case of bands 5 and 7, are not visible in the photograph. They do not occur in extracts.

intervals, samples of the reaction mixture were spotted onto Whatman No. 1 chromatography paper. The chromatogram was developed with water-absolute ethanol-nitromethane (23:41:35) for 12 hr by descending flow, and the spots were developed by the method of Robyt and French (19).

**Preparation of Radioactive Amylase.** The incorporation of radioactive amino acid into individual amylases was performed as follows. Twenty aleurone layers were incubated for 24 hr in a medium containing buffer, GA, and calcium (3) with the addition of 30  $\mu$ c of L-leucine-T(G) (Amersham, specific radioactivity 249 mc/mmmole). Amylase was then isolated from the medium by the glycogen precipitation method of Loyer and Schramm (11). The crude medium was made 40% in ethanol at 0 C. The solution was centrifuged and to it were added 2 mg of rabbit liver glycogen (Nutritional Biochemicals Corporation) which had been purified (11). The glycogen precipitate was recovered by centrifugation and dissolved in 1 ml of  $10^{-3}$  M acetate buffer, pH 4.8. This solution was allowed to stand at room temperature for 2 hr to allow glycogen hydrolysis, and then dialyzed exhaustively against  $10^{-3}$  M calcium chloride to remove the products of hydrolysis.

**Electrophoresis of Radioactive Amylase.** An agar gel was prepared as already described, and a slit 3.0 cm long was made at the origin. Fifty microliters of the radioactive amylase solution (about 8  $\mu$ g of amylase protein) were loaded into the slit, and the gel was subjected to electrophoresis. Then two parallel cuts were made in the agar at right angles to the origin, thus isolating a strip 0.9 cm wide in the center of the 3.0-cm wide amylase zone. The strip was then cut into 1.0-mm segments, beginning at the origin, and each segment was placed into 7.0 ml of a scintillation fluid consisting of 2 volumes of toluene containing 0.4% 2,5-diphenyloxazole (PPO) to 1 volume of Triton X-100 (18). The agar segments dissolve in this fluid, and counting efficiency is probably maximized although no comparisons were made. The counting data are derived from about 2.7  $\mu$ g of amylase protein. The remaining portion of the gel was developed for amylase activity for the purpose of matching enzyme activity and counting data.

## RESULTS

The amylases induced in isolated barley aleurone tissue by GA consist of eight different forms separable by agar gel electro-

phoresis. A zymogram of the amylases in the incubation medium (*i.e.*, secreted amylases) after 24 hr and in an extract of the tissue (not secreted) is shown in Fig. 1. All forms migrate toward the cathode at pH 7.55. Amylases 1 to 4 account for most of the activity of the medium and all of the activity of the extract. Amylases 5 to 8 occur only in the medium and give rise to very faint bands on the zymogram. Bands 6 and 8 are the relatively strongest ones and are visible in Fig. 1, but bands 5 and 7 are usually barely visible on the zymogram.

**General Characteristics.** If small blocks of agar containing each of the four major forms removed from one zymogram are transplanted into another gel at the origin and the gel is subjected to re-electrophoresis, each form migrates as a single band of activity with the expected mobility. Amylases 1 to 4 are thus not artifacts of electrophoresis but genuine, individual enzymes. This test was not made for amylases 5 to 8.

Differences between amylases can be detected by use of inhibitors of activity. After electrophoresis, one gel was flooded with  $4 \times 10^{-4}$  M *p*-chloromercuribenzoate *p*CMB<sup>3</sup> for 15 min and another with  $5 \times 10^{-3}$  M EDTA (disodium salt) for 20 min. The gels were washed and the zones of amylase activity developed. After treatment, only bands 3 and 4 were visible; after EDTA treatment, these bands had disappeared while 1 and 2 and 5 to 8 were somewhat enhanced in intensity. Incubation of medium from the aleurone layers with  $10^{-3}$  M *N*-ethylmaleimide (which has no effect of its own) and  $10^{-6}$  M HgCl<sub>2</sub> (which on its own caused erratic inhibitions of activity) together consistently destroyed the activity of amylases 1 and 2 and 5 to 8 but had no effect on amylases 3 and 4 over a 4-hr period. These data indicate that amylases 3 and 4 require an easily removable metal cofactor for activity, and that all other amylases may require free sulfhydryl groups for activity.

Although the major amylases responded to inhibitions in pairs (1 and 2, 3 and 4) one difference between forms 3 and 4 was found. After 4-hr exposure of crude medium to  $2.5 \times 10^{-6}$  M *p*CMB, only amylase 3 remained. Amylase 4 is not inhibited by *p*CMB over a short exposure time (see above).

The relative intensities of bands 1 and 2 and bands 3 and 4 could be changed by varying the pH of the starch solution in which the zymograms were incubated. This indicated that the two pairs of amylases had different pH optima for activity. This was confirmed by determinations of pH optima of amylase solutions containing either forms 1 and 2 or 3 and 4 (for details of preparation, see later). In 0.05 M acetate buffer, the optimum of bands 1 and 2 was broad, extending from pH 4.5 to pH 6.0, and that of bands 3 and 4 was relatively sharp at pH 5.8. Finally, forms 1 to 4 were much more heat stable than 5 to 8.

The preceding experiments were performed essentially for the purpose of comparing the amylases described here with those of Frydenberg and Nielsen (7). These authors described their two lowest mobility amylases (A and B) as being heat-resistant, inhibited by Hg<sup>2+</sup> and Cu<sup>2+</sup>, not requiring Ca<sup>2+</sup> for activity, and not readily identifiable as  $\alpha$ - or  $\beta$ -amylases. Because our electrophoretic procedures were similar to those of Frydenberg and Nielsen (7) and because amylases 1 and 2 were inactivated by *p*CMB and by Hg<sup>2+</sup> plus ethylmaleimide but not by EDTA, it is very probable that these two amylases are the amylases A and B of Frydenberg and Nielsen.

The next highest mobility group of Frydenberg and Nielsen, comprising five amylases (C-G), was described as heat-resistant, not inhibited by Hg<sup>2+</sup> or Cu<sup>2+</sup>, and requiring Ca<sup>2+</sup> for activity, and identified as  $\alpha$ -amylases. However, forms E to G were labile and reverted to the stable forms D and C. Hence we believe that our amylases 3 and 4 are the same as Frydenberg and Nielsen's C and D. Amylases 5 to 8 fall into the fastest moving group

<sup>3</sup> Abbreviation: *p*CMB: *p*-chloromercuribenzoate.

of Frydenberg and Nielsen, comprising their amylases H and I and described as heat-labile, inhibited by  $Hg^{2+}$  and  $Cu^{2+}$ , having no  $Ca^{2+}$  requirement, and identified as  $\beta$ -amylase.

Because the existing identifications rely solely on inhibitor data, we have re-examined the identity of the eight amylases of the barley endosperm.

**Amylases 5 to 8.** The occurrence of amylases 5 to 8 in the medium only was indicative that they did not arise from within the aleurone layer. A possible source of these forms was the starchy endosperms, some of which remains attached to the aleurone layers during the dissection procedure. To test this, starchy endosperm was obtained from seeds which had imbibed water for 3 days and was incubated overnight with papain solu-

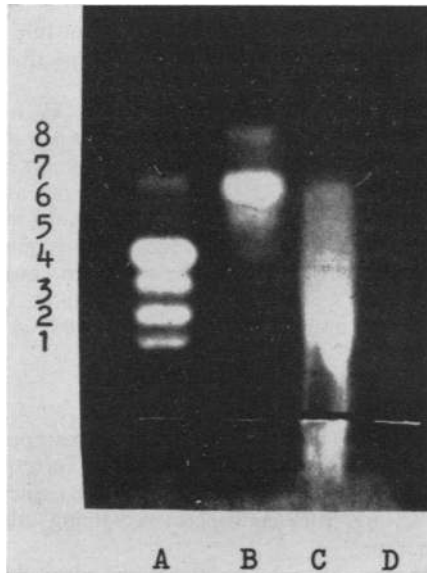


FIG. 2. The amylases released from starchy endosperm by proteolysis. A: The amylases released from isolated aleurone in response to GA and calcium (as in Fig. 1, media +  $Ca^{2+}$ ). B: Amylases 5 to 8 solubilized during incubation of starchy endosperm with papain. C: Control for B, starchy endosperm without papain. A diffuse zone of activity was obtained. D: Papain only.

tion or cysteine, or both. Papain caused solubilization of four amylases which matched forms 5 to 8 in electrophoretic mobility and in proportion, as judged from band intensities on a zymogram (Fig. 2). It appears that these amylases are probably bound to other proteins and are released by proteolysis.

A solution of solubilized endosperm amylases was exhaustively dialyzed and then used for amylose hydrolysis. Maltose was produced almost exclusively (Fig. 3A). Traces of glucose, maltotriose, and maltotetraose were also present; but these were probably produced by the very small amount of amylase 4 which can be found in starchy endosperm of seed which has imbibed water for 3 days (see Fig. 2B). Amylases 5 to 8 are therefore probably  $\beta$ -amylases. This conclusion is supported by the fact that they are not precipitated in the glycogen purification procedure (see "Materials and Methods") which specifically precipitates  $\alpha$ -amylases. It is presumed that during normal germination these amylases would be solubilized in endosperm during germination by protease synthesized in the aleurone layer (9).

**Amylases 3 and 4.** If a solution containing all eight barley endosperm amylases was dialyzed overnight against 0.02 M acetate buffer, pH 3.70, forms 3 and 4 were inactivated completely while all other forms were stable. If calcium was deleted from the incubation medium (*i.e.*, with GA in a buffer only), amylases 3 and 4 were not present in the medium (figure 1) but all other amylases were. Presumably, this reflects a requirement by these amylases for calcium and is not due to lack of production because they are inactivated by EDTA and require calcium ions for activity. Instability at low pH and a requirement for calcium are characteristic of barley  $\alpha$ -amylase (10, 15, 24).

That these amylases are  $\alpha$ -amylases is supported by a determination of the amylose hydrolysis products. A preparation of forms 3 and 4 was made as follows. A solution containing all amylases was made  $10^{-5}$  M in  $HgCl_2$  and  $10^{-3}$  M in *N*-ethylmaleimide and incubated at 50 C for 20 min. The remaining amylase (about 60%) was purified by glycogen precipitation. It consisted of only forms 3 and 4. Incubation of this solution with amylose yielded a pattern of products which was identical to that produced by *Bacillus subtilis*  $\alpha$ -amylase (Fig. 3C). Thus, it appears that amylases 3 and 4 are the classical barley  $\alpha$ -amylases.

**Amylases 1 and 2.** A solution containing only amylases 1 and

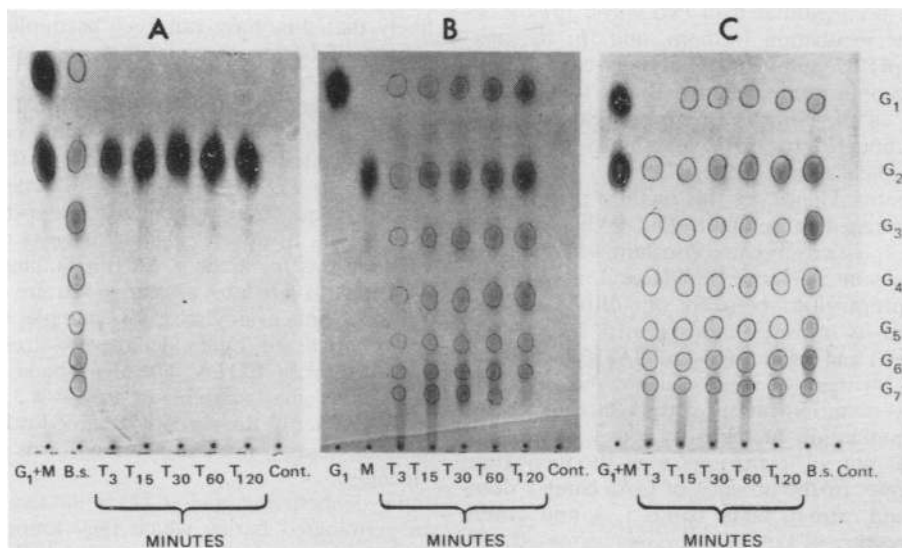


FIG. 3. Products of hydrolysis of amylose by amylases. Samples (25  $\mu$ l) of reaction mixtures were taken at the times indicated (in minutes) and spotted onto chromatography paper. A: Amylases solubilized from starch endosperm by papain (forms 5-8); B: Amylases 1 and 2; C: Amylases 3 and 4. Control: Heat-killed enzyme and substrate incubated together for 1 hr; B.s.: amylose hydrolyzed by *Bacillus subtilis*  $\alpha$ -amylase. The identities of hydrolysis products were taken from Robyt and French (19). G<sub>1</sub>: Glucose; G<sub>2</sub> and M: maltose; G<sub>3</sub>: maltotriose, etc.

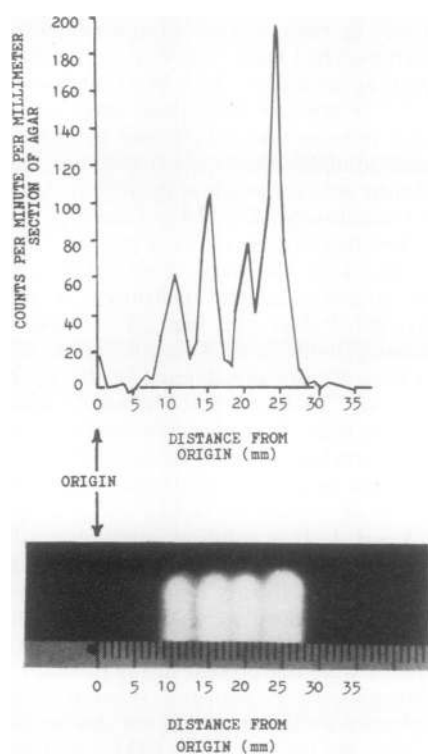


FIG. 4. Incorporation of tritiated leucine into the amylases originating in aleurone layers of barley (forms 1-4) in response to GA and calcium.

2 was prepared as follows. Aleurone layers were incubated with GA in the absence of calcium, and the amylase was precipitated with glycogen. The precipitate was dissolved in water and dialyzed for 48 hr against 0.01 M acetate buffer, pH 3.70. A zymogram heavily loaded with this preparation showed only bands 1 and 2. This preparation hydrolyzed amylose to give a spectrum of products identical to that produced by the action of *B. subtilis*  $\alpha$ -amylase on amylose, and therefore both amylases are probably  $\alpha$ -amylases (Fig. 3B).

**Contribution to Total Activity.** The contributions of the two groups of  $\alpha$ -amylases to total amylase activity in media from 24-hr incubations have been estimated in two ways: (a) by deleting calcium from the incubation medium, and (b) by incubating the medium at pH 3.7 until loss of activity ceases. Both procedures result in loss of amylases 3 and 4. By the first method it was found that 16% of the control activity (+ calcium) was retained.<sup>4</sup> However, although amylases 1 and 2 are retained in the absence of calcium, their amounts are reduced by about 40% as judged from zymograms. Hence, by this method their contribution to activity is estimated to be about 25%. When medium was incubated at pH 3.7, activity became constant when 20% of the original activity remained. Hence, amylases 1 and 2 contribute a significant proportion, between one-fifth and one-fourth, of  $\alpha$ -amylase activity in barley endosperm.

**Responses of Amylases 1 and 2 and 3 and 4 to GA.** If the amount of enzyme loaded onto the agar gel was kept low, the intensities of the amylase bands were an indication of the relative amounts of activity present. Examination of the time course of amylase production showed that not all forms appear in the incubation medium at the same time. In the absence of GA, band 2 dominated in both media and extracts while bands 1, 3, and 4 were barely visible. In the presence of GA, all amylases increased with

time, and after 25 hr the medium contained more activity attributable to amylases 3 and 4 than to 1 and 2, indicating that the response of amylases 3 and 4 to GA was much greater than that of 1 and 2. By eliminating calcium from the medium, one can estimate the effect of GA on the production of amylases 1 and 2 and 3 and 4. From the data of Chrispeels and Varner (3) (their Table 1), it can be calculated that enzymes 1 and 2 together are enhanced 2.6-fold by GA whereas enzymes 3 and 4 are increased 12.1-fold. This agrees with the estimate made by a visual examination of the zymograms.

A comparison of the distributions of bands 1 and 2 and 3 and 4 between extract and medium after 25 hr in the presence of GA showed that a greater proportion of forms 3 and 4 than of 1 and 2 was released from the tissue into the medium. Thus the two pairs of amylases differ in several respects with regard to their response to GA, and it was considered worthwhile to determine whether all four forms were synthesized *de novo* during the incubation with GA.

**<sup>3</sup>H-Leucine Labeling of Amylases 1 to 4.** All four bands of amylase forms 1 to 4 induced in the presence of radioactive leucine contained radioactivity (Fig. 4). The amount of radioactivity in each form is parallel to the amount of enzyme activity contributed by it, as judged from the zymograms and is not too different from the contributions to total activity given above for amylases 1 and 2 and 3 and 4. These data in conjunction with those of Filner and Varner (4) make it very likely that all forms of GA-inducible  $\alpha$ -amylase are made completely *de novo*.

## DISCUSSION

Our data indicate that of the eight amylases appearing in the medium surrounding isolated aleurone tissue in response to GA, four are  $\alpha$ -amylases produced in the aleurone tissue and four are  $\beta$ -amylases arising from endosperm remaining attached to the dissected aleurone.

The  $\alpha$ -amylases arise by synthesis *de novo*; the  $\beta$ -amylases seem to arise by activation of pre-existing zymogen. However, we have not attempted to show incorporation of labeled precursors into the  $\beta$ -amylases, and although similar forms exist in the endosperm as bound enzyme, the possibility that the aleurone layer has the capacity to produce these amylases cannot be entirely dismissed.

Because of the characteristics of amylases 1 and 2, it is very likely that they have not been accounted for in many previous studies of barley  $\alpha$ -amylase. A component of barley amylases stable toward low pH was found by Paleg (16) and described as being similar to  $\alpha$ -amylase in activity. The finding that amylases 1 and 2 are  $\alpha$ -amylases is interesting, considering that they are not inactivated by low pH (3.7) or by EDTA. Resistance of these enzymes to EDTA and sodium hexametaphosphate (7) as well as their production, although in somewhat reduced amounts, in the absence of added calcium suggests that they do not require calcium ions for activity. Such a finding would be surprising as all known  $\alpha$ -amylases appear to require calcium ions for activity (8, 26). Some  $\alpha$ -amylases, for example, the amylase from *Aspergillus oryzae* (6), bind calcium very strongly and are resistant to inactivation by EDTA. The aleurone layer clearly contains some calcium as small amounts of amylases 3 and 4 can be found in a tissue extract in the absence of added calcium. Thus it is possible that amylases 1 and 2 are simply forms which bind calcium very strongly.

Frydenberg and Nielsen (7) found five forms of amylase (C-G) in germinated barley which they found to be  $\alpha$ -amylases. We find only two in this group, 3 and 4; these probably correspond to C and D. One possible explanation for the discrepancy is a difference in the agar used for gel electrophoresis and hence a difference in resolution of amylases. Secondly, we have used iso-

<sup>4</sup> The contribution of the  $\beta$ -amylases to total activity is negligible when the starch assay is used.

lated aleurone tissue whereas previous studies have involved aleurone plus starchy endosperm. Could amylases E to G arise by interaction of aleurone and endosperm? We find no evidence of amylases corresponding to E to G in isolated aleurone or in starchy endosperm, but the amylase from GA-treated embryoless half-seeds contains a new form corresponding to position 5 on the zymogram. Amylases E to G are heat-labile and revert to the stable forms C and D (7) so that the possibilities are: (a) they are forms of C and D changed by some factor(s) in the starchy endosperm, and (b) they are hybrids of  $\alpha$ - and  $\beta$ -amylases. Although such hybrids have not been reported,  $\alpha$ - and  $\beta$ -amylases are similar in both amino acid composition and molecular weight. The molecular weights of  $\alpha$ -amylases are about 50,000 (5), although a weight of 60,000 has been reported for barley  $\alpha$ -amylase (24). Sweet potato  $\beta$ -amylase is either a trimer or a tetramer of subunits each with a molecular weight of 50,000 (27).

Even though the recoveries of amylase activity in the density labeling experiments published (4) were 60 to 90% (Filner and Varner, unpublished data), it is now almost certain that these losses represent a selective loss of amylase 3 or amylase 4, or of both amylases 3 and 4. Correct identification of those amylase forms which were density-labeled would have required monitoring by gel electrophoresis of the density-labeled samples before and after the equilibrium sedimentation run. For completely unequivocal results the four amylase forms should be separated from one another after incubation of the aleurone layers in the density label and each amylase form banded separately in the equilibrium sedimentation run, as has been done for some of the peroxidase enzymes from barley embryos (1).

The question of whether or not all four forms of  $\alpha$ -amylase are synthesized *de novo* after the addition of GA is nonetheless resolved in this report by the demonstration that all four forms become labeled to approximately the same extent (counts per minute per unit of amylase activity) when aleurone layers are incubated in  $^3\text{H}$ -labeled leucine.

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