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Gibberellic Acid-Induced Synthesis of Protease by Isolated Aleurone Layers of Barley[1]

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Summary. The production of protease by isolated aleurone layers of barley in response to gibberellic acid has been examined. The protease arises in the aleurone layer and is mostly released from the aleurone cells. The courses of release of amylase and protease from aleurone layers, the dose responses to gibberellic acid and the effects of inhibitors on the production of both enzymes are parallel. As is the case for amylase, protease is made de novo in response to the hormone. These data give some credence to the hypothesis that the effect of gibberellic acid is to promote the simultaneous synthesis and secretion of a group of hydrolases.

The amylase produced by de-embryonated endosperm of barley in response to GA is produced primarily by the aleurone layer (3, 9, 14) and the bulk of the amylase is liberated by the aleurone cells. It has also been shown that all of the amylase is made de novo (4). In addition to amylase, endosperm tissue (aleurone layers plus starchy endosperm) produces a variety of hydrolases following GA treatment (2, 8). If, as seems to be the case at this time, these also arise from and are liberated by the aleurone layers, it is of interest, for the purpose of further defining the action of GA, to determine whether or not the hydrolases are all synthesized de novo at the same time under the same control.

In order to approach the answer to this question, we have made a comparison of amylase and protease production following GA treatment. GA causes a several-fold increase in protease in the wort of malted barley (1, 5, 7), in de-embryonated barley seeds (2, 8, 17) and in isolated barley aleurone tissue (8, 18). In the latter 2 reports, however, very little of the protease was secreted. Hence, although it has been shown that aleurone tissue has the capacity to produce protease in response to GA, it has not been convincingly demonstrated that the aleurone layer is the site of production of a sufficient quantity of protease to account for endosperm hydrolysis.

Materials and Methods

Tissue Incubation and Preparative Procedures. Barley seeds (*Hordeum vulgare* cv. Himalaya) were used for the experimental material. The entire procedure of preparation of isolated aleurone layers, incubation of the tissue and preparation of the media and tissue extracts has been described in detail by Chrispeels and Varner (3). The only modification is that both media and extracts were centrifuged at 12,000 × *g* for 10 minutes. In some cases (indicated in the text) half seeds (aleurone layer plus starchy endosperm) were used instead of aleurone layers.

Enzyme Assays. The media were assayed for amylase using the method of Shuster and Gifford (13) as modified by Chrispeels and Varner (3), and the remainder of each solution was used for protease assay. Cold ethanol was added to the media and extracts with swirling until they were 80% in ethanol at which strength protease precipitation was maximal. The protein precipitates were centrifuged immediately at 12,000 × *g* for 15 minutes. No greater yield of protease was obtained by allowing the ethanolic solution to stand. The supernatants were discarded and the pellets were dissolved in 1.6 ml of 1 mM acetate buffer (pH 4.8). These solutions were then made 10 mM in β-mercaptoethanol because this procedure enhanced proteolytic activity 2 to 3 fold.

Gliadin (Nutritional Biochemicals Corporation) was used as the substrate for protease assay on the basis of its similarity in amino acid composition to hordein, the storage protein of barley endosperm (15, 16). A saturated solution of gliadin in 50 mM acetate buffer pH 4.8 was prepared at room temperature. By using the method of Lowry et al. (6), it was determined that this contained 2.8 mg/ml of protein as compared to bovine serum albumin as a standard. The solution was filtered before use.

Each reaction mixture contained 1 ml of substrate and 0.5 ml of enzyme. Reactions were run at 30°. Proteolysis was measured by determination of the release of ninhydrin positive material using the method of Moore and Stein (10). Immediately after

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adding enzyme to substrate, a 0.3 ml aliquot of reaction mixture was added to 2.0 ml ninhydrin solution. Color was developed and 5.0 ml of propanol-water diluent was added before reading optical density at 580 mμ. After 1 hour a second aliquot of the reaction mixture was assayed and the change in OD at 580 mμ used as a measure of proteolysis. One unit of protease activity was defined as a change of 1 OD unit. Optical densities were measured in a Coleman Model 6A Junior Spectrophotometer using a path length of solution of 1.4 cm. Under these conditions, optical density change was proportional to the amount of protease present over the range of activities encountered.

When the crude medium or extract was used as the enzyme solution, only a low activity could be detected. The background of free amino acids was very high and whether the failure to detect activity was due to inadequate amounts of ninhydrin reagent for color development or whether the free amino acids were inhibitory to proteolysis as shown by Oaks (11) was not determined.

Demonstration of de novo Synthesis. The density-labeling technique of Filner and Varner (4) was used to demonstrate de novo synthesis of protease. This technique involves "labeling" of newly synthesized protein with ^{18}O from $H_2{}^{18}O$ and subsequent determination of the degree of heaviness by cesium chloride density gradient centrifugation. To accomplish this, it was necessary to establish the position of normal protease (from an $H_2{}^{16}O$ incubation) on the gradient in relation to a marker and to repeat the same procedure for heavy protease (from an $H_2{}^{18}O$ incubation). Demonstration of de novo synthesis relies on a change in relative positions. Use of a marker is necessary because of small differences in gradient composition from tube to tube which makes fraction number an unreliable index of gradient density. Amylase labeled with lysine-^3H and purified from isolated aleurone layers, was used as the marker.

For normal protease, 2 gradient tubes were prepared, each containing 0.5 unit of protease. Crude centrifuged medium was used as the source of enzyme. Fractions from 1 tube (i.e. those not used for refractive index measurements or radioactive counting) were used for protease assay and corresponding fractions from the other for determination of background amino acids. This was necessary because the amino acids (from the medium) were not distributed evenly over the cesium chloride gradient and it was necessary to subtract background optical density values from protease assay values to observe the correct (and near Gaussian) distribution of protease activity.

The gradient tubes contained 10 mM sodium acetate (pH 4.8), 1 mM $CaCl_2$ and enough cesium chloride to make the mean density of the gradient 1.30. These were mixed thoroughly and then the enzyme and the marker were layered on top of the cesium chloride solution. The final volume of the gradient was 3.0 ml. The tubes were centrifuged for 65 hours at 100,000 × g to establish the gradient. Fractions were collected by puncturing the bottom of the tube with a needle. Each fraction consisted of 3 drops. Usually each tenth fraction was used for determination of refractive index and other fractions were used alternately for radioactive counting and protease (or amino acid) assay.

The assay for protease in the gradient fractions was performed as follows. The gliadin substrate solution was dialyzed to reduce amino acid content and made 10 mM in β-mercaptoethanol. Reactions were begun by adding 0.2 ml of substrate to each fraction. After 1 hour and 15 minutes at 30°, 1.0 ml of ninhydrin reagent was added to each tube and the color developed. The diluent volume was reduced to 0.5 ml. The ninhydrin positive background was determined on the fractions of the duplicate gradient by adding the ninhydrin before the substrate and developing the color immediately. In order to minimize variability in all determinations, all glassware was acid washed.

$H_2{}^{18}O$ incubations were made essentially as described by Filner and Varner. Two aleurone layers were incubated in 0.1 ml of $H_2{}^{18}O$ medium for 24 hours at 25°. The medium contained 1 μM gibberellic acid, 1 μM sodium acetate (pH 4.8), 10 mM $CaCl_2$ and 5 μg/ml of each of streptomycin, mysteclin and penicillin. At the end of the 24 hours, 0.3 ml of water was added to the incubation vessel and the total medium drawn off. The 2 aleurone layers were washed with 0.3 ml more water. The diluted medium (about 0.65 ml) was centrifuged and 0.30 ml of the clear supernatant used in each of the 2 gradient tubes (1 for amino acid background and 1 for protease assay as before). Determinations to find the distribution of protease were made as described above except proteolysis was allowed to proceed for 3 hours because of the very small amount of protease present. The reaction proceeded at a linear rate over this period of time.

Results

Protease Characteristics and Conditions for Production. If the enzyme solution is frozen at pH 4.8, there is no loss of activity for at least 2 weeks; however even at 2°, activity is lost appreciably. Samples incubated at various pH values for 24 hours at 2° and then titrated to pH 4.8 for assay, lost two-thirds of the activity at pH 4.8 and even more at pH values above and below pH 4.8. In the presence of β-mercaptoethanol, the loss at pH 4.8 was reduced by half. Apparently inactivation is not due to oxidation of the enzyme because bubbling oxygen through the solution for up to 6 hours had very little effect. On the basis of enzyme stability and the fact that release of amino acids from gliadin proceeded at a constant rate over the reaction times used, reactions were run at pH 4.8 although this was not determined to be the pH optimum.

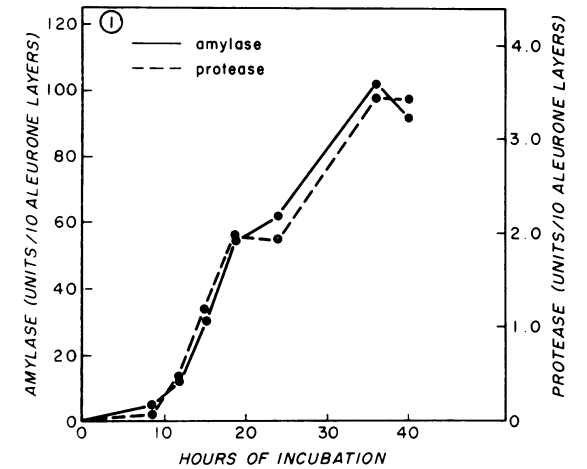
Whereas amylase production is strongly dependent on the presence of calcium during incubation (3),

Table I. *The Production of Protease by Half Seeds, Aleurone Layers and Starchy Endosperm in Response to Gibberellic Acid and Starchy Endosperm*

Ten half seeds or aleurone layers were incubated for 24 hours in buffer containing 10 mM $CaCl_2$ with or without gibberellic acid. Values are units of protease per 10 half seeds or aleurone layers.

	No GA			1 μM GA		
	Medium	Extract	Total	Medium	Extract	Total
Half seeds	0.05	0.23	0.28	1.60	1.25	2.85
Aleurone layers	0.04	0.19	0.23	2.30	0.60	2.90
Starchy endosperm	0.10	0.03	0.13	0.10	0.00	0.10

protease showed relatively small and variable responses to metal ions. At 10 mM, Ca^{2+} enhanced protease production to a variable degree (10–90 %)



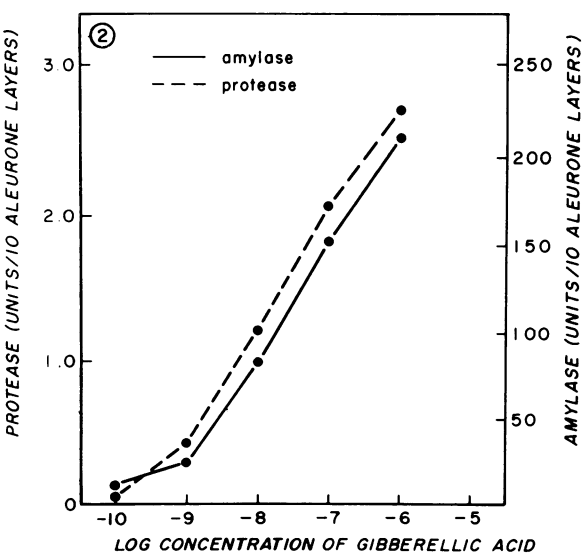


FIG. 1. The time courses of release of amylase and protease by aleurone layers in the presence of 1 μM gibberellic acid.

FIG. 2. The release of amylase and protease by aleurone layers in response to various concentrations of gibberellic acid.

and 1 mM Mn^{2+} enhanced protease to the extent of about 40 %. The effect of the latter was the same on amylase production. Only calcium, however, was used routinely in the incubation vessels.

The inhibition of barley protease is a problem of practical importance to the brewing industry. Bromate has been reported to be an effective inhibitor (7) and similar results have been obtained here. Bromate (1 mM), added to the medium surrounding the aleurone layers during incubation, reduced by 85 % the final protease level. This presumably is an effect on protease itself because amylase activity was only reduced by 17 %. This effect is similar to that found in endosperm slices (9) where bromate inhibits the GA stimulated release of nitrogenous materials but has very little effect on sugar release or endo-β-glucanase production. P-CMB and iodoacetamide are effective inhibitors in vitro but N-ethyl maleimide had no effect at pH 4.8.

Protease Production. Aleurone layers produce as much protease as half seeds (table I) in the presence or absence of GA and starchy endosperm produces virtually none. Hence, the aleurone layer is the site of production of all or most of the protease produced by the half seed. Most (79 %) of the protease is released into the medium surrounding the aleurone layers. Thus, there is no doubt that this is the enzyme responsible for hydrolysis of the reserve proteins of the endosperm. Total protease production is increased almost 12-fold by GA whereas released protease is increased by about 70-fold.

The time courses of appearance of amylase and protease in the medium are very similar (fig 1). From these data it cannot be said that the production of the 2 enzymes was initiated at exactly the same time but it is clear that there is a very close association in release.

The production of protease in response to varying concentrations of GA is similar to that of amylase (fig 2). Both are linear between 1 μμM and 1 μM GA.

These data indicate that amylase and protease are produced as a unit in which case inhibition of the production of 1 enzyme should be paralleled by the inhibition of the other. This is verified by the similarity of the effects of cycloheximide, actinomycin D and abscisin II (table II). The unit appears to be exclusively under GA control for as in the case of amylase, kinetin and IAA have no effect on protease production.

Density Labeling of Protease. Results of the density labeling experiments are shown in figure 3. Protease induced by GA in the presence of $H_2{}^{16}O$ has a mean band position 3.8 fractions heavier than amylase (about 0.64 % greater buoyant density), whereas in the presence of $H_2{}^{18}O$, the protease is 8.3 fractions heavier than amylase[3]. The shift in mean position of the heavy protease from normal protease is 4.5 fractions which corresponds to a buoyant density change of 0.76 %. Also, using the width of the curve at half height as a measure of enzyme distribution[4], there is no band broadening in the presence of $H_2{}^{18}O$. These parameters (4) are sufficient to show that the protease released by the aleurone layers is made completely de novo in response to GA.

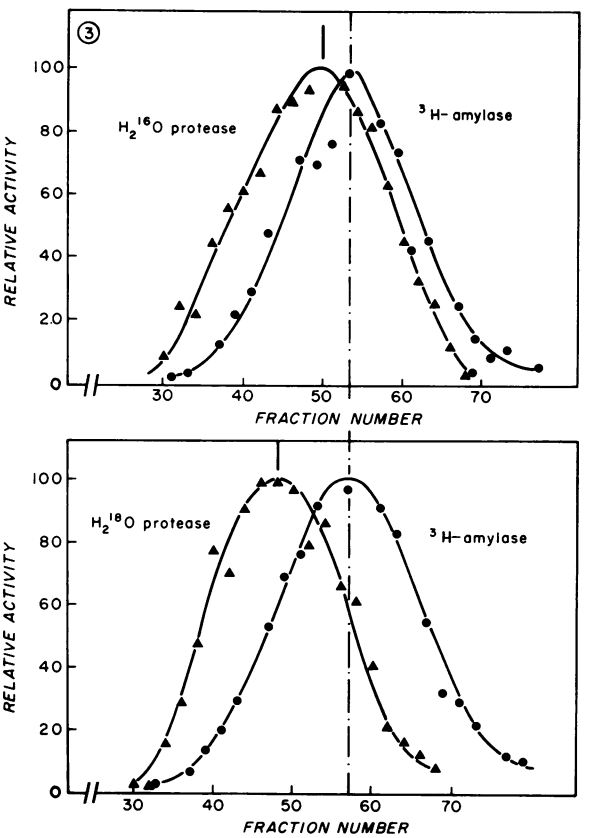


FIG. 3. The distributions of protease released by aleurone layers in the presence of $H_2{}^{16}O$ and $H_2{}^{18}O$ on cesium chloride gradients. In either case, tritiated amylase has been used as the reference.

Discussion

Because of the selection of gliadin as substrate, the protease measured is undoubtedly that protease which is responsible for the hydrolysis of endosperm protein during germination. All of the protease of the endosperm is made in the aleurone layer and most of it is released from the cells. In these aspects, protease and amylase are similar and the activities measured truly reflect their physiological function. Furthermore, the parallelisms between protease and amylase production suggest that control of the synthesis of these 2 hydrolases by GA is exerted via the same mechanism. Admittedly there are several other enzymes which increase in barley endosperm in response to GA and which have not yet been examined closely but the similarities of the production of protease and amylase indicate that the effect of GA is to promote the synthesis of a group of hydrolases which are destined to be released from the aleurone cells.

Ribonuclease production, however, although conforming to the same general principle, has some aspects which are different from the production of amylase and protease (3). Total ribonuclease production follows the same general trend as amylase and protease but most of the increased ribonuclease is at first retained in the cells, and appears in the medium only after about 24 hours of incubation with GA. The

[3] The widths of the 2 amylase curves are different. Even though the amylase solution is stored frozen, it undergoes a change which causes its band width to broaden. However, the buoyant density of the enzyme does not change and its use as a marker is therefore justified.

[4] By the use of Gaussian paper (12) it can be shown that the protease distribution deviates somewhat from normality. This is probably due to heterogeneity of the protease. It is very likely a mixture of proteases with slightly different buoyant densities. The amylase distribution is Gaussian.

Table II. *Effects of Inhibitors on Amylase and Protease Production by Isolated Aleurone Layers*

Ten aleurone layers were incubated for 24 hours in buffer containing 10 mM $CaCl_2$, 1 μM gibberellic acid and the inhibitor given.

	Amylase		Protease	
	Units per 10 aleurone layers	% Inhib.	Units per 10 aleurone layers	% Inhib.
Control	109	...	2.53	...
Cycloheximide 5 μg/ml	7.1	93	0.13	95
Cycloheximide 1 μg/ml	7.0	94	0.15	94
Actinomycin D 100 μg/ml	20.2	81	0.53	79
Actinomycin D 50 μg/ml	40.7	63	1.04	59
Abscisin II 10 μM	28.6	74	0.92	64
Abscisin II 1 μM	45.5	58	1.26	50

significance of this difference is not known but also it remains to be seen whether ribonuclease is made de novo and whether the extracted enzyme is in fact intracellular.

There is a close similarity in distribution of protease and amylase after 24 hours of incubation, four-fifths of the total activity being found in the medium and one-fifth in the tissue extract. MacLeod et al. (8) and Yomo and Iinuma (18) have demonstrated similar parallelisms between protease and amylase in isolated aleurone tissue, although in both cases the ability of the tissue to release the enzymes was impaired and most of the activity was found in aleurone extracts. Hence, it seems that amylase and protease are made and released by similar mechanisms. Furthermore, it appears that the synthesis and release processes are independent of each other.

In contrast to our findings, MacLeod et al. (8) found that initiation of amylase production preceeded protease production by at least 8 hours in isolated aleurone layers, and by 2 hours in endosperm slices. In addition, at 48 hours of incubation, amylase production was still active while protease production had essentially stopped. Production of endo-β-glucanase was initiated even earlier than that of amylase. Although the ability of the aleurone tissue to release the enzymes was impaired, the data still imply that the times of production of different hydrolases are not linked in an obligatory manner. There is no obvious explanation for the difference in results.

Throughout this paper, we have been speaking of amylase rather than α-amylase. This has been done because we have obtained indications that not all of the amylase made by aleurone layers in response to GA is α-amylase. As shown by agar gel electrophoresis, amylase from barley aleurone layers consists of 4 molecular forms, 2 of which have been tentatively identified as α-amylase and the other 2 as β-amylase. All forms increase following GA treatment. This will be the topic of a future report.

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