A Cell-Free System for the Study of α -Amylase Synthesis in Barley Aleurone Layers

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1. A cell-free system capable of α -amylase synthesis has been obtained from the aleurone layers of germinating barley. 2. This system requires potassium chloride, sucrose and an amino acid mixture in order to function. The crude preparation does not require calcium chloride. Chloramphenicol inhibits α -amylase synthesis as indicated both by increase in measurable enzyme activity and incorporation of L-[U- 14 C]glutamic acid.

Germinating seeds produce hydrolytic enzymes that digest the storage materials in the starchy endosperm. One of these enzymes is α -amylase and it seems certain that this enzyme is synthesized in the aleurone layers of the endosperm (Yomo, 1960a; MacLeod & Millar, 1962; Briggs, 1963; Paleg, 1964; Varner & Ram Chandra, 1964). This synthesis is apparently controlled by gibberellic acid or a related compound released from the embryo (Radley, 1959; Yomo, 1960a,b; Paleg, 1960; MacLeod & Millar, 1962; MacLeod, Duffus & Johnston, 1964).

Barley aleurone comprises a homogeneous population of non-dividing cells that can be isolated fairly readily by a number of techniques (Briggs, 1963; MacLeod, Johnston & Duffus, 1964; Paleg, 1964; Varner, 1964; Yomo & Iinuma, 1964). Hence it is a suitable tissue for studying hormonal control at the molecular level in plants. However, the aleurone cells are protected by thick walls and, though this facilitates their isolation, it hinders their homogenization. Further, the isolation of aleurone in quantity, free from starchy endosperm, is a very time-consuming task and changes are taking place in the aleurone throughout this period. The present paper describes how these problems have been partially overcome and a cell-free system has been obtained that can synthesize α -amylase when supplied with a suitable medium. Some of the properties of this system are evaluated.

MATERIALS AND METHODS

Materials. The barley used throughout was a sample of Maris Badger from the 1965 harvest. The grain was dehusked by treatment with 50% (v/v) H_2SO_4 and stored at 4°.

β -Amylase, bovine serum albumin, oyster glycogen, chloramphenicol, unlabelled amino acids, NADH, ATP, CTP, GTP and UTP were commercial samples.

Other chemicals used were as far as possible of A.R. grade.

L-[U- 14 C]Glutamic acid was obtained from The Radiochemical Centre, Amersham, Bucks.

Double-glass-distilled water was used throughout.

Germination. Dehusked barley was placed on Whatman no. 1 chromatography paper saturated with distilled water in a large plastic developing dish and left to grow at 8–12°. The low rate of growth at this temperature enabled the period of optimum α -amylase production to be determined more accurately than was possible at higher temperatures. From time to time, ten grain samples were taken and the extractable α -amylase activity was determined by the iodine-dextrin colour method of Briggs (1961). When it appeared that the rate of α -amylase production in the endosperm was near its maximum, incubation was stopped and the aleurone layers were isolated from the grain.

Isolation and fractionation of aleurone. The germinated barley grains were de-embryonated by hand and the residual endosperm was ground in a mortar with a small amount of water until as much of the starchy endosperm as possible was suspended in the water. The aleurone plus residual starchy endosperm was separated from the suspended material by passing the mixture through a Buchner funnel. As much as possible of the starchy endosperm was washed from the aleurone, which remained in the funnel. This washed preparation was thus primarily aleurone with pericarp-testa, varying amounts of starchy endosperm and possibly traces of scutellum left behind during the de-embryonation procedure.

The washed preparation was transferred to a polythene measuring cylinder with a small amount of water and frozen solid at –15°. The maximum volume of the mixture was 20 ml. The mixture was left overnight. Chilled homogenizing medium was added to a final volume of 75 ml. and the frozen preparation was homogenized with an Ultra-Turrax homogenizer. The homogenizing medium consisted of sucrose (0.6 M), EDTA (1 mM), $MgSO_4$ (2.5 mM), phosphate buffer, pH 7.4 (25 mM), and bovine serum albumin (0.1%).

Centrifugation of the homogenate was carried out in a Beckman L2 ultracentrifuge at 1°. The homogenate was first centrifuged at 6000g for 5 min. and the pellet discarded. The resultant supernatant was further centrifuged at

85000g for 5 min. and the 'mitochondrial' pellet obtained set aside. The supernatant from this centrifuging was centrifuged for 1 hr. at 109000g and the 'high-speed' supernatant poured off, leaving a microsomal pellet. This pellet and the 'mitochondrial' pellet were used without further purification.

Incubation conditions for α -amylase synthesis. The microsomal pellet was suspended in 0.85 ml. of the following basal medium: tris-HCl buffer, pH 7.4 (50 mM), sucrose (0.2 M), CaCl_2 (3 mM) and KCl (0.1 M). To this was added 0.1 ml. of an amino acid mixture; this mixture consisted of (per ml.) 7.5 μ moles of alanine, 5.8 μ moles of arginine, 6.5 μ moles of asparagine, 11.3 μ moles of aspartic acid, 1.95 μ moles of cysteine, 10.31 μ moles of glutamic acid, 6.5 μ moles of glutamine, 14.5 μ moles of glycine, 2.5 μ moles of histidine, 4.2 μ moles of phenylalanine, 6.0 μ moles of proline, 9.2 μ moles of serine, 11.6 μ moles of threonine, 4.07 μ moles of tryptophan, 2.2 μ moles of tyrosine, 6.5 μ moles of valine and 100 μ moles of tris buffer, pH 7.4 (Bishop, Roche & Nisman, 1964).

Samples (0.1 ml.) of the incubation mixture were pipetted into 10 ml. tubes and the tubes incubated at 25° for various times. It was found that the increase in α -amylase activity ceased after 15–30 min. incubation. Accordingly, a 30 min. incubation period was adopted. After this period 1 ml. of 0.1 M-NaCl solution was added to each tube and the α -amylase content assayed by the iodine-dextrin colour method. Except where otherwise indicated, experiments were performed in duplicate.

Amino acid-incorporation studies. The incubation mixture described above was used with the addition of 2 μ C of L-[U- ^{14}C]glutamic acid, representing an additional 0.267 μ mole. The total volume of the mixture and the concentrations of the other components were kept constant. The mixture was incubated at 25° for 30 min. and the α -amylase was then isolated and purified by the method of Loyter & Schramm (1962). The glycogen- α -amylase complex was redissolved in distilled water and dialysed for 2 days against unlabelled 10 mM-L-glutamic acid. Finally, 1 ml. of 0.8% glycogen was added to the solution, followed by ethanol to a final concentration of 40% (v/v). The resultant precipitate was washed with ethanol and dried *in vacuo*. The dry precipitate was added to 10 ml. of a solution containing 5 g. of 2,5-diphenyloxazole and 0.2 g. of 1,4-bis-(5-phenyloxazol-2-yl)benzene in 1 l. of toluene. The radioactivity of the sample was then determined with a Packard Tri-Carb liquid-scintillation counter, counting at 60% efficiency. Non-specific adsorption of the glutamic acid was checked by carrying out the incubation in the presence of chloramphenicol (5 mg./ml.) and then repeating the experiment as above.

RESULTS

The properties of the system are summarized in Table 1. For increased α -amylase activity to be produced, the amino acid mixture, potassium chloride and sucrose are essential. Calcium chloride does not appear to be required, though this probably reflects the crudity of the microsomal preparation, since calcium is an essential component of barley-malt α -amylase (Fischer & Haselbach, 1951). In other experiments it was shown that added nucleo-

Table 1. Requirement for each component of the incubation mixture for the production of increased α -amylase activity

Experimental conditions, determination of α -amylase and content of the complete system are given in the text. The results quoted are all for the same microsomal preparation, but similar results were obtained with other preparations. Mixtures were made up to the standard volume (0.95 ml.) with distilled water. After incubation, the α -amylase activity was determined by adding 0.8 ml. samples to the assay mixture. This raised the pH of the assay mixture from 5 to 6.7. IDC units, iodine-dextrin colour units.

	α -Amylase activity (IDC units/1000 grains of barley)
Before incubation:	
Complete system	28.9
After incubation for 30 min. at 4°:	
Complete system	24.5
After incubation for 30 min. at 25°:	
Complete system	45.4
Complete system + chloramphenicol (5 mg./ml.)	25.8
System—KCl	23.4
System— CaCl_2	52.3
System—sucrose	28.2
System—amino acids	26.2

side triphosphates (ATP, CTP, GTP and UTP; 0.2 μ mole of each) had no effect. Chloramphenicol (5 mg./ml.) completely inhibited any increase in enzyme activity. Ethanol, in which the chloramphenicol was dissolved, had no effect in the amounts used (50 μ l./ml.). The mitochondrial fraction showed no ability to produce α -amylase.

Incorporation of [U- ^{14}C]glutamic acid into α -amylase was observed. The amount of α -amylase produced was calculated from the increase in enzyme activity obtained in a comparable preparation. It was assumed that 1 μ g. of α -amylase was equivalent to 4 iodine-dextrin colour units. This assumption is based on values quoted by Briggs (1961) and Schwimmer & Balls (1948, 1949). Assuming that all of the enzyme was recovered after purification, it was calculated that the specific activity of the newly produced α -amylase was 635 counts/min./ μ g. Addition of chloramphenicol (5 mg./ml.) to the system completely inhibited incorporation of the labelled amino acid.

Fig. 1 shows the relation between the activity of the preparation and the degree of germination of the barley as indicated by the amount of extractable α -amylase. The activity is related not to the total extractable α -amylase but to the rate of increase in extractable α -amylase.

The normal incubation medium showed a fairly high bacterial count (10^4 – 10^5 bacteria/ml.) after

Fig. 1. Relationship between the ability of the aleurone microsomal preparation to produce α -amylase and the degree of germination of the barley from which it is isolated. Experimental conditions are given in the text. O, Activity of α -amylase extracted from the starchy endosperm during germination (IDC units, iodine-dextrin colour units); $\blacktriangle$, amount of α -amylase produced in 30 min. incubation in the complete medium by aleurone microsomal fractions isolated from the endosperm during germination.

plating out on nutrient agar. However, these bacteria did not appear to be responsible for the observed production of α -amylase. α -Amylase was not produced when these bacteria, after culture on nutrient agar, were incubated in the normal incubation medium together with aleurone microsomal fraction from 80 grains. The microsomal fraction used had been preincubated at 25° for 30 min. to eliminate any intrinsic ability to produce α -amylase.

DISCUSSION

It has been demonstrated previously that α -amylase is synthesized in barley aleurone after treatment with gibberellic acid (Varner & Ram Chandra, 1964; Varner, Ram Chandra & Chrispeels, 1965) and it is reasonable to suppose that this occurs during normal germination also. The system described above may well be responsible. Its activity, as shown in Fig. 1, reflects the α -amylase-synthesizing activity of the aleurone from which it is isolated. Production of increased α -amylase activity is dependent on a supply of amino acids and is inhibited by chloramphenicol, as is incorporation of [^{14}C]glutamic acid into the enzyme. This supports the view that the system is carrying out protein synthesis. On the other hand, increase in α -amylase activity does not appear to be affected by added nucleoside tri-

phosphates, which might be taken to indicate that the increase is not energy-dependent. However, work in progress by the author has indicated the presence of 'high-energy' phosphate compounds in the preparation and that these are hydrolysed completely during the 30 min. incubation.

Clearly the characteristics of this system must be further clarified by separation of the various components and, in particular, by identification of the source of energy for synthesis. It should now be possible, however, to verify directly the hypothesis that gibberellic acid or some equivalent compound controls α -amylase synthesis in aleurone by controlling the synthesis of a specific messenger RNA (Varner *et al.* 1965).

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