Arch. Biochem. Biophysics, 38, 231- 236, (1952)
Starch synthesizing enzymes of green gram (Phaseolus radiatus)
J. Sri Ram, and K. V. Giri

Starch-Synthesizing Enzymes of Green Gram (Phaseolus Radiatus)

J. Sri Ram and K. V. Giri

From the Department of Biochemistry, Indian Institute of Science, Bangalore, India

Received September 19, 1951

Introduction

During the past few years considerable information has become available concerning the synthesis of amylose and amylopectin, the two components of starch, by enzymes isolated from potato. Ever since the discovery by Hanes (1) of the existence of an enzyme, phosphorylase, in potato and peas, which synthesises amylose from glucose 1-phosphate, the isolation and characterization of the other enzyme responsible for the synthesis of amylopectin in plants, has been the subject of intensive study by Peat and co-workers (2–11). They have isolated an enzyme, designated as the Q-enzyme, from potatoes, broad beans, and wrinkled peas, which converts linear amylose into branched amylopectin. Recently, however, Beckmann and Roger (12) adduced evidence to show that the product obtained by the action of the Q-enzyme fraction of potato juice on amylose is not amylopectin, as potato preparations contain fatty acids which form complexes with amylose, the resulting complex possessing the properties of amylopectin. Thus the existence of Q-enzyme in potato has been questioned by these authors. Nussenbaum and Hassid (13) could not confirm the claim of the above authors, that the synthetic amylopectin is a combination of amylose with fatty acids, but substantiated the observations of Peat and co-workers that the Q-enzyme is capable of transforming the linear amylose to a branched polysaccharide possessing the properties of amylopectin.

In view of these conflicting results and the importance of the Q-enzyme in relation to the mechanism of starch synthesis in plants, we report our findings on the occurrence and characterization of similar enzymes in plants investigated by us. In the course of our investigations on the phosphorylases of Indian pulses, it was observed

that green gram (*Phaseolus radiatus*) contained, in addition to phosphorylase, another enzyme, which resembles, in its action on amylose, the Q-enzyme isolated by Peat and co-workers from other plant materials. By a somewhat simple procedure an active preparation could be obtained reasonably free from amylases.

In this paper we propose to give a brief account of the methods of isolation of these two enzymes, namely phosphorylase and Q-enzyme, from green gram and describe some of their properties.

Experimental

Green gram was powdered well and extracted with five times its weight of water in the presence of toluene, for 12 hr. at 0°C., and centrifuged. The clear extract thus obtained was used for the isolation of the enzymes.

Preparation of Phosphorylase

The phosphorylase was prepared from the aqueous extract by fractional precipitation with ammonium sulfate. To 100 ml. of the extract (pH adjusted to 6.0) 17.5 g. of solid ammonium sulfate was added, and the pH was readjusted to 6.0. The precipitate formed was removed on the centrifuge and rejected. To the clear supernatant, which was colored yellow, was added 20 g. of solid ammonium sulfate and centrifuged after again adjusting the pH to 6.0. The precipitate was dissolved in 100 ml. of water and again fractionated as before. The final precipitate was dissolved in 50 ml. of water, dialyzed, and used.

Active preparations of the phosphorylase were also obtained by fractional precipitation with acetone. To the aqueous extract, one-fifth the volume of acetone was added and the inert material precipitated was removed by centrifugation. To the clear supernatant solution more acetone was added bringing the total volume of acetone to three times the volume of the aqueous extract. The precipitate formed was centrifuged, dissolved in a convenient volume of water, and again centrifuged to remove the suspended impurities. All these operations were conducted at a low temperature (0–10°C.).

Preparation of Q-Enzyme

We could also isolate from green gram, an enzyme similar in its action on amylose to the Q-enzyme isolated by Peat and co-workers from potato, wrinkled peas, and broad beans.

In the first instance, the enzyme was prepared according to the procedure described by Barker, Bourne, and Peat (6), involving precipitation of the enzyme and other proteins from the aqueous extract by the addition of lead acetate, elution of the lead–protein complex with sodium hydrogen carbonate solution in the presence of CO_2, and precipitation of the enzyme from the eluate with neutral ammonium sulfate. However, in view of the fact that the amylase content of green gram is very low, we could obtain an equally active and pure Q-enzyme preparation by eliminating the precipitation of the enzyme by lead acetate. Accordingly the following method was adopted for the preparation of the enzyme.

To 100 ml. of the aqueous extract (pH 5.8), 0.2–0.3 g. of kaolin was added and centrifuged. The pH of the supernatant was adjusted to 7.2–7.5, and solid ammonium sulfate (17.5 g./100 ml.) was added and the pH was again adjusted to 7.2–7.5 immediately. The precipitate obtained was removed by centrifugation and dissolved in 10 ml. of water. It was again centrifuged to remove the suspended material and used as such in the present investigation. This preparation contained the Q-enzyme without any contamination of amylases.

Preparation of Amylose

Amylose was prepared from sweet potato starch by selective precipitation with thymol (14). The amylose solutions were prepared by dissolving it in 1 *N* NaOH by warming for a short time in a boiling water bath and neutralizing with 1 *N* HCl.

β-Amylase of Sweet Potato

β-Amylase was prepared from sweet potato by the method described by Giri (15).

β-Amylase of Rice

The enzyme was extracted overnight from rice powder with eight times its weight of 0.067 *M* (*M*/15) phosphate buffer of pH 7.0 and was precipitated out from its solution with 3 vol. of acetone. The precipitate obtained was dried, dissolved in a convenient volume of water, and used.

α-Amylase (Saliva)

The amylase was prepared from saliva as described by Bourne, Macey, and Peat (4).

Determination of Phosphorylase Activity

The phosphorylase activity was estimated by the method of Green and Stumpf (16).

The reaction mixture always contained:

1 ml. of 0.1 *M* glucose 1-phosphate solution,
+2 ml. of 0.1 *M* citrate buffer (pH 6.3),
+2 ml. of enzyme solution,

and made up to a suitable volume (7–11 ml.), depending on the addition of substances whose influence on the activity of the enzyme was to be investigated. The reaction was carried out at 35 ± 0.1°C. The inorganic phosphorus liberated in 5 min. was estimated colorimetrically by the method of Fiske and SubbaRow (17).

Characterization of the Enzyme Present in the First Fraction Obtained by Addition of 17.5 ml. of Ammonium Sulfate to 100 ml. of the Aqueous Extract

The method adopted for the characterization of the enzyme was similar to that described by Bourne, Macey, and Peat (4), by following its action on amylose and determining the blue value and the corresponding liberation of reducing sugars at known intervals of time.

The reaction mixture contained 40 ml. of amylose solution containing 50 mg. of amylose + 10 ml. of enzyme solution. The pH of the solution was always found to be 7.0. No buffer was added.

The blue value was measured using an Evans photoelectric colorimeter, and the reducing sugars were determined by the method of Shaffer and Hartmann (18).

Results

Phosphorylase

The phosphorylase was found to exert its optimum activity at pH 6.3, and the optimum temperature for its activity was found to be 55°C. At 65°C. it lost its activity completely. The activity was not affected by dialysis and ultrafiltration. Glucose, fructose, and several other sugars, even at high concentrations (0.05 *M*), had no effect on the enzyme. Metallic ions, Mn^{++}, Co^{++}, Zn^{++}, Mg^{++}, and Fe^{+++}, (0.01 *M*), did not have any influence on the activity. KCN (0.01 *M*), $HgCl_2$ (0.001 *M*), and $AgNO_3$ (0.01 *M*) completely inactivated the enzyme. Cu^{++} (0.01 *M*) inhibited only slightly. Salivary α-amylase, β-amylase of sweet potato and soyabean, and aqueous extracts of wheat and barley completely inhibited the enzyme even at very low concentrations.

Q-Enzyme

Action on Amylose. The action of the Q-enzyme on amylose was studied by measuring the blue value and reducing sugars formed during the action of the enzyme at pH 7.0 which is the optimum for this enzyme. Both the crude aqueous extract and purified enzyme solution were used as sources of the Q-enzyme. For purposes of comparison the action on amylose of β-amylase of rice and α-amylase of saliva was also investigated, the pH of the digests being adjusted to 4.8 and 7.0, respectively. The results obtained are illustrated by the curves of Fig. 1 in which the observed blue values as percentage of original are plotted against the apparent percentage conversion into maltose. The curves indicate that the mode of action of the green gram enzyme is entirely different from the action of the other amylases on amylose. The purified enzyme liberates very little reducing sugars while the crude aqueous extract liberates somewhat more. Different preparations obtained by the above method of purification differed slightly in the amount of reducing substances liberated. The highest value obtained, however, never exceeded 5% (calculated as maltose) even after 48 hr. digestion with the enzyme, while the blue-staining

amylose was converted into a pink-staining substance. Occasionally, the reducing value was as low as 1.5–2%.

The optimum temperature for the Q-enzyme action was 24°C. It was completely inactivated at 50°C.

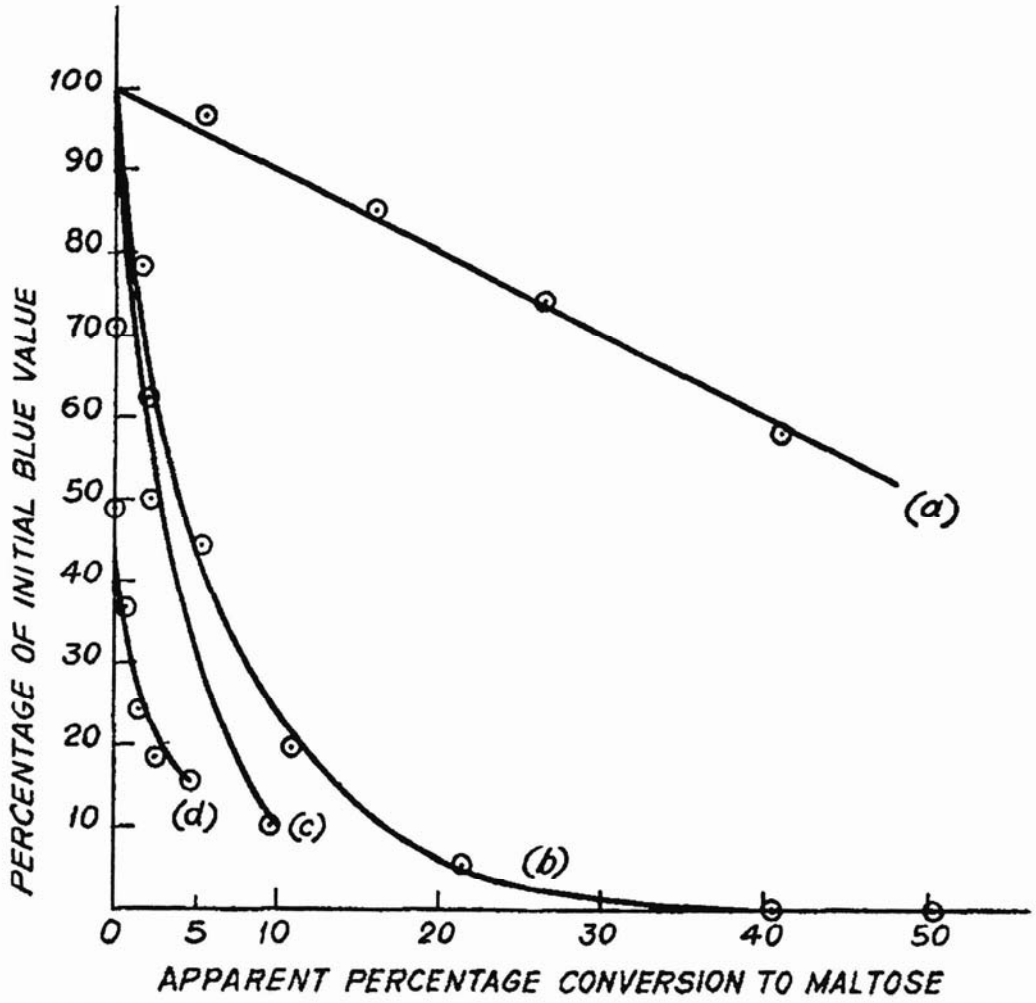

FIG. 1. Blue value, maltose conversion curves for (*a*) β-amylase, (*b*) salivary α-amylase, (*c*) crude aqueous extract of green gram, and (*d*) Q-enzyme of green gram.

β-Amylolysis of the Product Formed by the Action of Green Gram Enzyme on Amylose

Characterization of the product formed by the action of the green gram enzyme on amylose was next studied by hydrolysis with β-amylase (from sweet potato). The digest obtained after the action of the green gram enzyme on amylose was kept in a boiling water bath for 10 min. to destroy the enzyme and then subjected to β-amylolysis after adjusting the pH to 4.8. The hydrolysis limit was determined by estimating the reducing sugars formed after complete digestion

with β-amylase. Simultaneously the limit of hydrolysis of the same quantity of amylose by the action of this enzyme was also determined. The values obtained for the limiting percentage conversion into maltose from several experiments carried out on the hydrolysis of the product obtained by the action of the green gram enzyme on amylose, ranged between 62–66, while the limit of hydrolysis of the amylose with β-amylase (from sweet potato) was 96–98%.

These results show that the two enzyme preparations isolated from green gram exert the same function as the phosphorylase and Q-enzyme of potato, and the mechanism of starch synthesis in green gram appears to be the same as in potato.

Summary

Green gram (*Phaseolus radiatus*) is shown to contain phosphorylase and another enzyme resembling the Q-enzyme which converts amylose into amylopectin.

Methods are described for the isolation of these two enzymes from the aqueous extract of green gram.

Some of the properties of the two enzymes have been described.

References

1. Hanes, C. S., *Proc. Roy. Soc.* (London) **B128,** 421; **B129,** 174 (1940).
2. Haworth, W. N., Peat, S., and Bourne, E. J., *Nature* **154,** 236 (1944).
3. Bourne, E. J., and Peat, S., *J. Chem. Soc.* **1945,** 877.
4. Bourne, E. J., Macey, A., and Peat, S., *J. Chem. Soc.* **1945,** 882.
5. Barker, S. A., Bourne, E. J., and Peat, S., *J. Chem. Soc.* **1949,** 1705.
6. Barker, S. A., Bourne, E. J., and Peat, S., *J. Chem. Soc.* **1949,** 1712.
7. Barker, S. A., Bourne, E. J., Wilkinson, I. A., and Peat, S., *J. Chem. Soc.* **1950,** 84.
8. Barker, S. A., Bourne, E. J., Wilkinson, I. A., and Peat, S., *J. Chem. Soc.* **1950,** 93.
9. Barker, S. A., Bourne, E. J., Peat, S., and Wilkinson, I. A., *J. Chem. Soc.* **1950,** 3022.
10. Hobson, P. N., Whelan, W. J., and Peat, S., *J. Chem. Soc.* **1950,** 3566.
11. Hobson, P. N., Whelan, W. J., and Peat, S., *J. Chem. Soc.* **1951,** 596.
12. Beckmann, C. O., and Roger, M., *J. Biol. Chem.* **190,** 467 (1951).
13. Nussenbaum, S., and Hassid, W. Z., *J. Biol. Chem.* **190,** 673 (1951).
14. Haworth, W. N., Peat, S., and Sagrott, *Nature* **157,** 19 (1946); Lakshmanan, S., Sri Ram, J., and Giri, K. V., *Current Sci.* (India) **17,** 366 (1948).
15. Giri, K. V., *J. Indian Chem. Soc.* **11,** 339 (1934).
16. Green, D. E., and Stumpf, P. K., *J. Biol. Chem.* **142,** 355 (1942).
17. Fiske, C. H., and SubbaRow, Y., *J. Biol. Chem.* **66,** 375 (1925).
18. Shaffer, P. A., and Hartmann, A. F., *J. Biol. Chem.* **45,** 377 (1921).