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GLUCOSYLTRANSFERASE ISOZYMES IN ALGAE—II.

PROPERTIES OF BRANCHING ENZYMES*

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Abstract—The branching isozymes of a Cyanophyte, *Oscillatoria princeps*, a Rhodophyte, *Rhodymenia pertusa* and a Chlorophyte, *Spirogyra setiformis*, when isolated by two-dimensional polyacrylamide gel electrophoresis, show an evolutionary progression. The isozymes were tested with various polyglucan substrates. Those of *Oscillatoria* were able to branch amylopectin and amylose, forming a phytoglycogen-like polymer which exhibited maximum absorption of its iodine complex at 550 nm. One of the three isozymes of *Rhodymenia* synthesized the identical polysaccharide, but the other two isozymes of this alga were without effect on amylopectin. They did branch amylose, however, forming a polyglucan which showed an absorption peak of its iodine complex at 580 nm. The green alga, *Spirogyra*, had three isozymes which were without effect on amylopectin, but which did branch amylose. The branched sugar formed by these algal enzymes showed an absorption peak of the iodine complex at 580 nm.

INTRODUCTION

ONE OF the most consistent features of living organisms is the utilization of carbohydrates as respirable substrates. The enzymatic polymerization of carbohydrates into a form capable of being *stored* (such as the polyglucans), and the establishment of their use as potential sources for energizing life processes must have evolved very early during the emergence of organic life.[1] These biopolymers were probably among the first chemicals synthesized in nature and, therefore, a study of the catalytic proteins responsible for their synthesis might yield information as to how these enzymes and their products have fared during evolution.

Mounting evidence indicates that the close association found among the three groups of enzymes involved in polyglucan synthesis, may be due to their derivation from either a primordial type, or possibly from each other. Each group of enzymes has been shown to exist in two or more forms (*isozymes*).

Phosphorylase (α-1,4-glucan:orthophosphate glucosyltransferase, E.C. 2.4.1.1), at one time thought to be the enzyme exclusively responsible for the synthesis of amylose-like polyglucans, has lately become involved in some dispute as to its major function: whether it be in the direction of synthesis or degradation.[2,3] This enzyme has been shown to exist in two forms in algae[4,5] and in three forms in many animal tissues.[6,7]

Synthetase (UDP-glucose:α-1,4-glucan α-4-glucosyltransferase, E.C. 2.4.1.11), has displaced phosphorylase as the former of α-1,4-glucosyl bonds.[8] It has been reported to

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[1] R. M. KLEIN and A. CRONQUIST, *Quart. Rev. Biol.* **42**, 105 (1967).
[2] R. B. FRYDMAN, B. C. DESOUZA and C. E. CARDINI, *Biochim. Biophys. Acta* **113**, 620 (1966).
[3] N. P. BADENHUIZEN and K. R. CHANDORKAR, *Cereal Chem.* **42**, 44 (1965).
[4] J. F. FREDRICK, *Phytochem.* **2**, 413 (1963).
[5] J. F. FREDRICK, *Phytochem.* **6**, 1041 (1967).
[6] A. A. YUNIS and G. K. ARIMURA, *Biochim. Biophys. Acta* **118**, 335 (1966).
[7] E. H. FISCHER and E. G. KREBS, *Federation Proc.* **25**, 1511 (1966).
[8] L. F. LELOIR and C. E. CARDINI, *J. Am. Chem. Soc.* **79**, 6340 (1957).

exist in multiple forms in algae,[5] in molds,[9] in higher plants[10] and in animals.[11,12] Evidence suggests that the UDPG-active form may be derived from the ADPG-active one.[13]

The third group of enzymes involved in the formation of storage polyglucans is the *branching enzyme* (α-1,4-glucan:α-1,4-glucan 6-glycosyltransferase, E.C. 2.4.1.18). This group of enzymes "branches" linear maltodextrins whether these α-1,4-linked chains have originally been formed by phosphorylases or by the synthetases. Multiple forms of this enzyme have been reported in plants.[14,15] The branching enzymes determine the characteristics of the final polyglucan formed, and may also be responsible for inducing morphogenesis and changing the substrate-specificities of those enzymes forming the linear chains upon which the branching enzymes act.[9]

The relationships among these enzymes seem to be more than casual. The three groups of enzymes bind very firmly to the polysaccharides they form or act upon.[2,16,17] Analyses of the hexapeptides of the phosphorylated sites of phosphorylase and synthetase show that both enzymes contain identical amino acid sequences.[18,19] Immunological studies indicate that pure preparations of *synthetase* give precipitin lines with antibody derived against *phosphorylase*.[20] Precipitin lines, though faint, were detected when purified *branching enzyme* (Q-enzyme) preparations were run in Ouchterlony gels against antibody to algal *phosphorylases*.[21]

The distribution of all three types of enzymes appears to be almost identical in tissues.[22] Highly purified preparations prove to contain all three groups even after electrophoresis.[23,24] An evolutionary tie has been shown to exist between the α-1,4-glucosyl bond-synthesizing enzymes,[25] and perhaps such a tie exists also in so far as the 1,6-bond-forming enzymes are concerned.

Amylose, the exlusively α-1,4-glucosyl linked polymer is converted to amylopectin, an α-1,6-glucosyl branched sugar by Q enzyme.[26,27] This enzyme was thought to be *the* branching enzyme, but there appear to be others. Recent reports of two branching enzymes in maize, able to branch originally *branched* sugars such as amylopectin, have appeared.[14,28] One of the enzymes acts like a conventional Q enzyme, converting amylose into amylopectin, while the other is capable of further branching amylopectin, converting this sugar to phytoglycogens.[14]

In algae, two branching isozymes have been detected in the primitive Cyanophyceae, and

[9] B. WRIGHT, C. WARD and D. DAHLBERG, *Biochem. Biophys. Res. Commun.* **22**, 352 (1966).
[10] R. B. FRYDMAN and C. E. CARDINI, *Biochim. Biophys. Acta* **96**, 294 (1965).
[11] L. F. LELOIR and S. H. GOLDEMBERG, *J. Biol. Chem.* **235**, 919 (1960).
[12] A VARDANIS, *J. Biol. Chem.* **242**, 2306 (1967).
[13] R. B. FRYDMAN and C. E. CARDINI, *Arch. Biochem. Biophys.* **116**, 9 (1966).
[14] N. LAVINTMAN, *Arch. Biochem. Biophys.* **116**, 1 (1966).
[15] J. F. FREDRICK, *Physiol. Plantarum* **21**, 176 (1968).
[16] Z. SELINGER and M. SCHRAMM, *Biochem. Biophys. Res. Commun.* **12**, 208 (1963).
[17] R. B. FRYDMAN and C. E. CARDINI, *J. Biol. Chem.* **242**, 312 (1967).
[18] J. LARNER and F. SANGER, *J. Mol. Biol.* **11**, 491 (1965).
[19] E. H. FISCHER, D. J. GRAVES, E. R. CRITTENDEN and E. G. KREBS, *J. Biol. Chem.* **231**, 65 (1959).
[20] L. SCHLISELFELD and E. G. KREBS, A.C.S. 154th Meet. Abstracts C197 (1967).
[21] J. F. FREDRICK, *Phyton* **16**, 21 (1961).
[22] K. HALL, *J. Endocrinol.* **32**, 245 (1965).
[23] D. F. STEINER, L. YOUNGER and J. KING, *Biochemistry* **4**, 740 (1965).
[24] J. F. FREDRICK, *Phytochem.* **1**, 153 (1962).
[25] J. LARNER, *Trans. N.Y. Acad. Sci.* Ser. II **29**, 192 (1966).
[26] S. PEAT, W. J. WHELAN and J. M. BAILEY, *J. Chem. Soc.* 1422 (1953).
[27] S. A. BARKER, E. J. BOURNE, S. PEAT and I. WILKINSON, *J. Chem. Soc.* 3022 (1950).
[28] D. J. MANNERS and J. J. M. ROWE, *Chem. & Ind.* 1834 (1964).

three isozymes were found in the more evolutionary advanced Chlorophyceae.[15] No data was obtained as to the substrate requirements of the isozymes. Such data might be a source of information in so far as detecting changes in the enzymes of these life forms during their phylogenetic progression.

RESULTS

When the branching isozymes of the blue-green alga, *Oscillatoria princeps*, are separated on polyacrylamide gels, two isozymes are found. Both of these enzymes are active in converting amylose and amylopectin into more highly branched polyglucans. Figure 1 shows

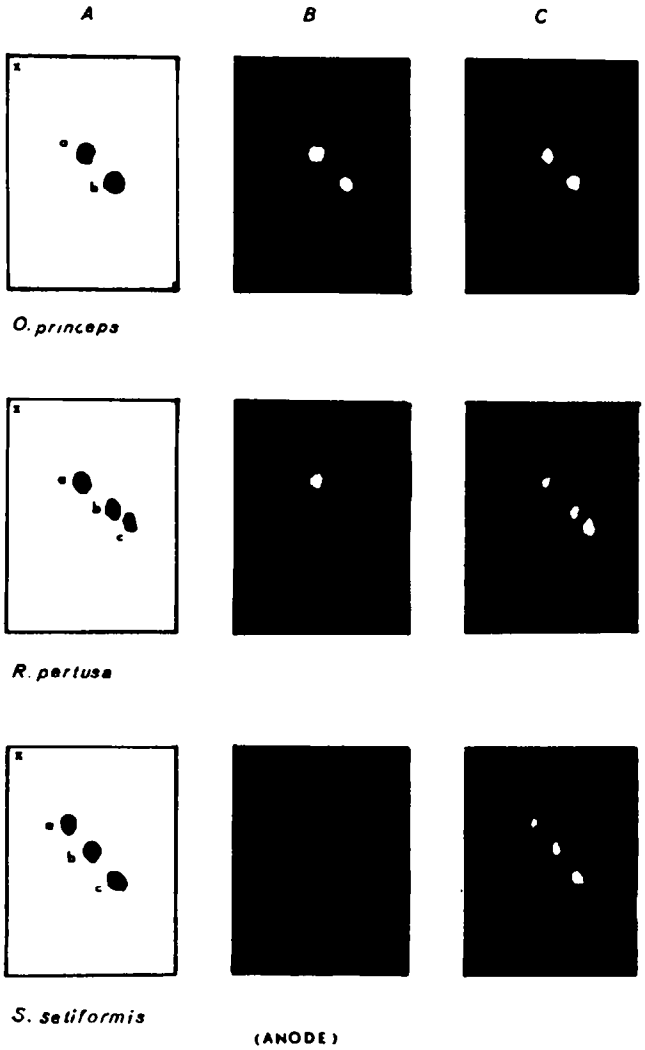

FIG. 1. BRANCHING ISOZYMES OF ALGAE.

A, orthacryl gel stained with amido-schwarz: the isozymes are labeled *a*, *b* and *c*, with *c* being the fastest anodic-moving isozyme. "x" indicates the origin of the isozymes. B, "staining" plate where amylopectin was used as substrate. C, same as B, but with amylose as substrate. Plates B and C were all stained after incubation (see text). The areas where branching enzymes are active stain a light violet or red after iodine is applied.

the results of the tests on different polyglucan substrates. Note that the branching isozymes of the red alga, *Rhodymenia pertusa*, are all active in converting amylose to an amylopectin-like polymer, but that only one of the three isozymes (Fig. 1, middle row), *a*, can convert amylopectin to a more highly branched sugar.

The more highly evolved green alga, *Spirogyra setiformis*, has three branching enzymes. All three are inactive on amylopectin, but they all branch amylose (Fig. 2, bottom row).

The absorption spectra of the iodine complexes of the polyglucans formed by the different isozymes in the three algae are shown in Fig. 2. In all cases, isozymes *a* and *b* of *Oscillatoria*

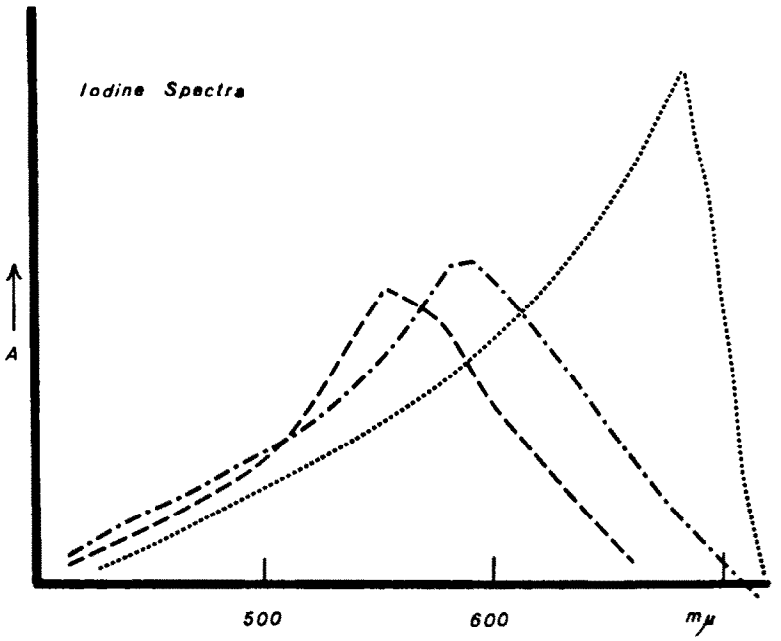

FIG. 2. SPECTRA OF IODINE COMPLEXES OF ALGAL POLYGLUCANS.

(——), absorption spectrum of polyglucan formed from both amylose and amylopectin by isozymes of *O. princeps*, and by isozyme *a* of *R. pertusa*. (–·–·–), spectrum of polyglucan formed by isozymes *b* and *c* of *R. pertusa* and all isozymes of *S. setiformis*. (.....*), spectrum of amylose for comparison. (*) "Superlose".

formed a branched sugar which showed a red iodine color with maximum absorption at 550 nm. This polyglucan was formed from either amylose or amylopectin, and it appears that this type of branched polyglucan is the end-result of the action of the branching enzymes in this particular alga (Figs. 1 and 2). The identical polyglucan was formed by isozyme *a* of *R. pertusa*.

Isozymes *b* and *c* of this red alga, however, acted only on amylose. These enzymes formed a polysaccharide with an absorption maximum of its iodine complex of 580 nm. The polysaccharide formed was exactly like that of *Spirogyra* (cf. Figs. 1 and 2). In this green alga, all three branching isozymes act only on amylose, forming the moderately branched sugar described.

DISCUSSION

Of the three closely related enzyme groups, the branching enzymes are thought to be responsible for the type of polyglucan which the plant ultimately stores.[13] In the algae used in this study, the branching isozymes seem to be of two types: those that form an amylopectin sugar from amylose, and those that branch amylopectin further, forming a phytoglycogen-like sugar (Fig. 2). The more *primitive* the alga, the *more highly branched* the polyglucan formed by the branching isozymes of that alga. It is of interest that in higher plant forms, when "primitives" appear via the mutation route, the polyglucans formed are inevitably more highly branched than the wild-type.[29]

Evidence indicates that the properties of the synthetases involved in forming linear maltosaccharides may be altered by the action of the branching enzymes.[9,13] For example, the *ADP*-glucosyltransferase is converted to the *UDP*-glucosyltransferase by combining with the polyglucan it forms and which is branched by the branching enzymes. This mechanism operates during morphogenesis in molds.[9]

The green alga, *Spirogyra*, has three branching isozymes, but none are capable of branching amylose beyond the amylopectin-like polyglucan (cf. Figs. 1 and 2). The Chlorophyta synthesize a type of starch which compares with that of higher plants in being a mixture of amylose and amylopectin.[15,30] The Cyanophyta synthesize a type of starch completely devoid of any linear component, which is pure phytoglycogen.[31,32] Both green algae, such as *Spirogyra*[5] and *Chlorella*,[33] and blue–green algae, such as *Oscillatoria* and *Nostoc*,[5,21] have been shown to contain synthetases and phosphorylases capable of forming linear polyglucans. However, the inability of the branching enzymes in *Spirogyra* to form highly branched sugars, may be the limiting factor in determining the final storage "starch" composition.

In this connexion, it is of interest that the red alga used in this study, synthesizes a "starch" which is practically all amylopectin, but none the less, has some more highly branched components (Figs. 1 and 2). This alga has a branching isozyme (Fig. 1, middle row) which is capable of forming a phytoglycogen-like sugar similar to that formed by the Cyanophyta.

Since most schemes of algal evolution place the Rhodophyta in a "blind-alley", and hence away from the main stream leading from the Cyanophyta to the Chlorophyta,[1,30] perhaps we should re-evaluate these schemes to include the Rhodophyta as the intermediate group between the lower blue–green algae and the more evolutionary advanced green algae. Such a new schema seems warranted on the basis of the branching enzymes present in these algae.

EXPERIMENTAL

Ammonium sulfate fractionated extracts of mature cultures of the three algae, *Oscillatoria princeps*, *Rhodymenia pertusa* and *Spirogyra setiformis* were subjected to preparatory polyacrylamide gel electrophoresis.[5] The isolation of the branching enzymes by orthacryl electrophoresis was accomplished as described.[5,15]

The gel slab was immediately wedged between two "staining" plates, one of which incorporated 1 per cent amylose (as "Superlose", Stein-Hall Corp., New York City) in a "small pore" polyacrylamide gel as described by Davis,[34] and the other of which contained 1 per cent amylopectin (as "Ramalin", Stein-Hall Corp.)

[29] T. J. SCHOCH, *Baker's Dig.* **21**, 1 (1947).
[30] J. LOVE, W. MACKIE, J. P. MCKINNELL and E. PERCIVAL, *J. Chem. Soc.* 4177 (1963).
[31] J. F. FREDRICK, *Physiol. Plantarum* **4**, 621 (1951).
[32] J. F. FREDRICK, *Physiol. Plantarum* **6**, 100 (1953).
[33] J. PREISS and E. GREENBERG, *Arch. Biochem. Biophys.* **118**, 702 (1967).
[34] B. J. DAVIS, in *Gel Electrophoresis* (edited by J. F. FREDRICK), p. 404. New York Academy of Sciences, New York City (1964).

instead of amylose. The "staining" plates and techniques used were those described by Doane,[35] except for a modification whereby Tris–EDTA–Borate (TEB) buffer of pH 8·6 was used instead of the buffer stipulated in her technique and with the omission of calcium chloride in the gel formula. The TEB formula has been described.[5,15]

The gel "sandwich" was incubated in a water-saturated atmosphere for 2 hr at 25°, under moderate pressure so that the sample-containing gel was flattened against both "staining" plates.

The sandwich was separated and the "staining" plates were placed in Krisman's iodine staining solution.[36] The sample-containing gel was either stained with Naphthol Blue-Black,[5] or the areas containing the isozymes were excised. The excised areas of gels were eluted with TEB in a Waring blendor and the resulting filtered eluate used in reaction mixtures of amylose or amylopectin as substrates.[15] The resulting polysaccharides were isolated with 2 vol. of 95 per cent ethanol. The precipitates were collected, washed twice with ethanol and dissolved in water. They were stained with the Krisman iodine–potassium iodide–calcium chloride reagent[36] and the absorption peaks measured on a Coleman spectrophotometer.

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[36] C. R. KRISMAN, *Anal. Biochem.* **4**, 17 (1962).
[35] W. W. DOANE, *J. Exp. Zool.* **164**, 363 (1967).