Carbohydrate Research, 26 (1973) 385–392

(1→4)-α-D-GLUCAN PHOSPHORYLASE [(1→4)-α-D-GLUCAN:ORTHOPHOSPHATE GLUCOSYLTRANSFERASE] ISOENZYMES FROM SWEET CORN SEED EMBRYO*

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(Received September 25th, 1972; accepted in revised form, November 15th, 1972)

ABSTRACT

The presence of multiple forms of phosphorylase [(1→4)-α-D-glucan:orthophosphate glucosyltransferase] in sugary maize seeds was demonstrated by polyacrylamide-gel-disc electrophoresis. The patterns of phosphorylase isoenzymes from immature and from germinating seeds were different. Most of the isoenzymes from embryo of germinated seeds precipitated at an ammonium sulfate concentration above 45% of saturation. The most cathodic band of the zymograms appeared on the third day of germination, then disappeared. This form of phosphorylase occurred only in the embryo of germinating seeds and it was absent both in the immature embryo and in the endosperm at any stage of development. The slow-moving embryo isoenzyme was purified through chromatography on DEAE-cellulose. Its kinetic properties and enlargement mechanism were studied.

INTRODUCTION

Certain varieties of sweet-corn seeds (sugary) have the unique property of containing two branched polysaccharides in the endosperm: insoluble amylopectin in the starch grain and phytoglycogen, a soluble polysaccharide which resembles animal glycogen, in its structure. The occurrence of phytoglycogen in these seeds suggests the possibility of the existence of enzymic systems different from those of other plants which only contain amylopectin.

Various enzymes related to the metabolism of both polysaccharides have been studied in the endosperm of sweet corn seeds. Two starch synthetases have been described, one in a soluble state and the other in a particulate state, attached to the grain. These two synthetases require different sugar nucleotides[1]. Two branching enzymes, one for amylose and one for amylopectin have been described by Lavintman[2]. Different debranching enzymes for amylopectin and for phytoglycogen could also be deduced from the results of Lee *et al.*[3]. Tsai and Nelson[4] have found different isoenzymes of phosphorylase in normal nonsugary maize seeds. In a previous report[5],

*Dedicated to Professor V. Deulofeu, in honor of his 70th birthday.

one of the phosphorylase [EC 2.4.1.1, (1→4)-α-D-glucan:orthophosphate glucosyltransferase] isoenzymes from immature endosperm of sugary varieties of maize seeds has been studied and it was demonstrated that the main difference between this isoenzyme and the potato phosphorylase was in their mechanism of chain elongation of phytoglycogen.

The embryo and the endosperm have a different genetic origin, the former being of the sporophytic generation and the latter of the gametophytic one. Akatsuka and Nelson[6] showed that the starch granule bound-starch synthetase from embryo tissues is different from that of endosperm. Preiss *et al.*[7] reported the isolation from embryo of starchy maize seeds of an ADP-D-glucose pyrophosphorylase distinct from the enzyme isolated from endosperm. The present work was undertaken in order to study phosphorylase isoenzymes in embryos of germinating sweet corn seeds during the first steps of germination.

RESULTS AND DISCUSSION

The presence of multiple forms of phosphorylase in sugary maize seeds could be demonstrated by polyacrylamide-gel-disc electrophoresis. As shown in Fig. 1, the patterns of phosphorylase isoenzymes from immature and from germinating seeds were different, but all enzyme forms could use amylose, amylopectin, or phytoglycogen as a primer. When mature or germinating seeds were dissected, the activity was only found in embryos. The results obtained with ammonium sulfate fractionation of extracts of embryos at different intervals of germination are shown in Fig. 1 B and 1 D. Only one phosphorylase band, whose migration rate varied slightly throughout

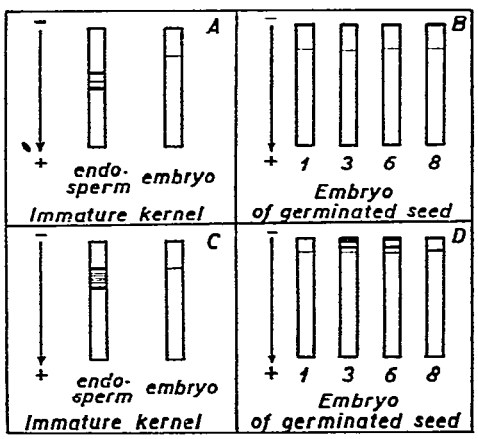

Fig. 1. Diagrammatic zymograms of phosphorylase obtained from embryo and endosperm of immature kernel and from embryo of germinated mature seeds. Experimental details for the enzyme preparation and disk electrophoresis are described in Experimental. Concentration of ammonium sulfate at 25–45% (A and B) and 45–80% (C and D) of saturation. The days of germination are indicated below each diagram in B and D. In all cases, amylose, amylopectin, or phytoglycogen were used as primers.

germination, was found in fractions precipitated at ammonium sulfate concentrations of 25–45% of saturation. (Fig. 1B). The isoenzyme pattern of fractions precipitated at ammonium sulfate concentrations of 45–80% of saturation (Fig. 1D) changed significantly during germination. It reached a maximum of three bands on the third day after the beginning of germination, and then diminished. Two new, intense deep-blue bands, located near the cathodic end, appeared on the third day, when amylose, amylopectin, or phytoglycogen were used as a primer (Fig. 1D). The color intensity of the new bands decreased as the embryo developed, and the bands did not appear at all on the eighth day (Fig. 1D). No phosphorylase activity could be detected in any of the enzymic preparations that contained pure maltose as a primer or were without any primer.

It is noteworthy that the most cathodic band was absent in zymograms from either embryos or endosperms of immature seeds (Fig. 1A and 1C). This isoenzyme band only appeared in embryos during germination. This observation prompted an investigation of its properties after partial purification, when the zymogram showed only the most cathodic band.

Kinetic properties. — The reaction rates were linear with regard to protein concentration and time in the range used to obtain the present data. The optimum activity was at pH 6.1 with the buffer 2-morpholinoethanesulfonic acid (MES). The K_m value for α-D-glucopyranosyl phosphate was 1.77×10^{-3}M. This value was lower than those reported for phosphorylases obtained from immature endosperm[5], potato[8], liver[9], and muscle[9].

At the same weight concentration, phytoglycogen was the best acceptor. When activity with phytoglycogen was considered as 100%, the activity with amylopectin was equal to 76% and that with amylose to 42%. The ϕ-limit dextrin was the most efficient primer among the phytoglycogen dextrins tested. Its activity was equal to 78%, whereas the β-limit and the ϕ,β-limit dextrins had lower rates.

As observed with other phosphorylases[5], pure maltose did not serve as a primer, but generally commercial samples of maltose contain enough maltotriose to be active[10]. However, when the external branches of phytoglycogen were reduced to two D-glucose residues (ϕ,β-limit dextrin of phytoglycogen), they could act as substrates. Maltotriose was the shortest malto-oligosaccharide that served as a primer. The rate of oligosaccharide activity increased as the chain length increased from maltotriose to maltoheptaose.

K_m values obtained with several primers are shown in Table I. It appears that the enzyme displays a high affinity for amylopectin and its β-limit dextrin, a lower one for phytoglycogen, and even a lower one for the ϕ-limit or the β-limit dextrin of phytoglycogen. It was of interest to compare the affinities for malto-oligosaccharides with those for phytoglycogen, amylopectin, and polysaccharide dextrins having external branches of similar length. The K_m for maltotriose was about 6 times lower than that for the β-limit dextrin of phytoglycogen (outer chain-length of 2–3 D-glucose residues). Similarly, the K_m for maltotetraose was about 6 times lower than that for the ϕ-limit dextrin of phytoglycogen (outer chain-length of 4 D-glucose residues).

TABLE I

K_m VALUES FOR DIFFERENT ACCEPTORS

Acceptor	$K_m \times 10^{5a}$	$V_{max}{}^b$
Amylopectin	0.22 (0.85)[c]	18
Amylopectin β-limit dextrin	0.05	4.7
Phytoglycogen	14.9 (21.0)[c]	94
Phytoglycogen β-limit dextrin	31.2 (20.0)[c]	7.1
Phytoglycogen ϕ-limit dextrin	39.5	
Maltotriose	5.5	6.5
Maltotetraose	6.7	4.9
Maltoheptaose	0.04	5.3
Isomaltopentaose	0.76	4.3

[a]The molarity of the K_m values of the polysaccharides is expressed in end-groups, assuming about 9% for phytoglycogen and its dextrins, and 4.5% for amylopectin and its dextrin. [b]In nmoles of inorganic phosphate released per min. [c]The values reported for immature endosperm phosphorylase[5] are indicated in parentheses.

The affinity of the enzyme for chains of seven D-glucose moieties (maltoheptaose) was much higher than that observed for phytoglycogen (outer chain-length of 7–9 residues). The affinity for oligosaccharides raised as the chain-length increased from maltotriose to maltoheptaose. In the same way, when comparing phytoglycogen with its β-limit dextrin or its ϕ-limit dextrin, the affinity increased with the increase in the chain-length of their outer branches (Table I). The effect of the external branches on the affinity appeared to be quite different in the case of amylopectin and its β-limit dextrin. The K_m value for the latter compound was lower than that of the former compound and of the same order as that for maltoheptaose. It is likely that the less compact structure of the amylopectin dextrin allows all outer branches to act as primers in spite of branching. The high affinity for isomaltopentaose would support this suggestion. It is evident from these results that the polysaccharide core has a definite influence on the elongation of the external branches by the enzyme.

As shown in Table I, the affinities of the germinated embryo phosphorylase towards amylopectin, phytoglycogen, and β-limit dextrin of phytoglycogen were different from those reported for the immature endosperm phosphorylase[5]. On the other hand, it is noteworthy that the K_m (amylopectin) and the K_m (maltoheptaose) of the embryo enzyme were 0.22×10^{-5}M and 0.04×10^{-5}M, respectively (Table I), whereas the K_m (amylopectin) and the K_m (maltoheptaose) of the potato phosphorylase were in reverse order, at 4×10^{-5}M and 19×10^{-5}M, respectively[9].

Inhibitors. — ADP-D-glucose and UDP-D-glucose were found to inhibit the phosphorylase activity from the embryos of germinating seeds. The extent of inhibition of the enzyme by 5mM ADP-D-glucose was 58% and by 5mM UDP-D-glucose 40%. These sugar nucleotides behaved kinetically as inhibitors of the noncompetitive type. The immature endosperm enzyme was also inhibited by ADP-D-glucose, but it was less sensitive to UDP-D-glucose inhibition[5].

TABLE II

EFFECT OF CONCENTRATION OF CITRATE IONS ON SWEET-CORN EMBRYO PHOSPHORYLASE AND STARCH SYNTHETASE ACTIVITIES[a]

Citrate ion concentration (mM)	*Activity*[b]		
	Sweet corn embryo phosphorylase	*Sweet corn starch synthetase*[c]	
		Particulate	*Soluble*
0	100	100	100
5	92		110
10	85		120
20		200	
50	32	250	187
100	24	310	255
150	5		

[a]The conditions of the enzymic assay are described in Experimental. [b]The activity in the absence of citrate ions is taken as 100. [c]Soluble and particulate sweet-corn starch synthetases were prepared and assayed by the methods previously described[1].

The activity of the embryo preparation was measured in the presence of increasing amounts of sodium citrate. Table II shows that the activity was greatly decreased by this addition (20 times at 0.15M concentration). This result is in disagreement with that observed for the soluble or particulate sweet-corn starch synthetase, where the citrate ion produced an activation of more than 250% (Table II). The concentrations of citrate ions used in the present work were much lower than those found by Ozbun *et al.*[11] to give stimulation of the starch synthetases obtained from developing kernels of waxy maize. We used a concentration of citrate ions lying within the physiological level in the cell[12]. It has also been shown that the citrate ion activates the sweet-corn branching enzyme that acts on amylose (5 times the activation by a 0.15M concentration) (Ref. 2). The opposite effect of the citrate ion acting, on one side, upon the starch synthetase and the branching enzyme and, on the other side, upon the phosphorylase and the inhibition of the latter enzyme by ADP-D-glucose (the main substrate for both soluble and particulate starch transglucosylases) would suggest that these metabolites are involved in a control mechanism of starch synthesis.

Mechanism of action on different primers. — The mechanism by which D-glucose residues were incorporated into different primers was studied according to the technique of Whelan and Bailey[13], by the use of the method of Krisman[14] for determination of the iodine spectrum. As shown in Fig. 2, all substrates tested gave schemes of chain elongation that could be considered of the "multi-chain" type. The typical "single-chain" mechanism reported for potato phosphorylase in the presence of inefficient primers[13,15] was not observed in any case. As has been indicated for animal glycogen[16], not all the external branches appeared to be elongated. From the calculated number of D-glucose residues incorporated and the spectrum obtained, we could conclude that there was no preferential chain-length for the activity of the

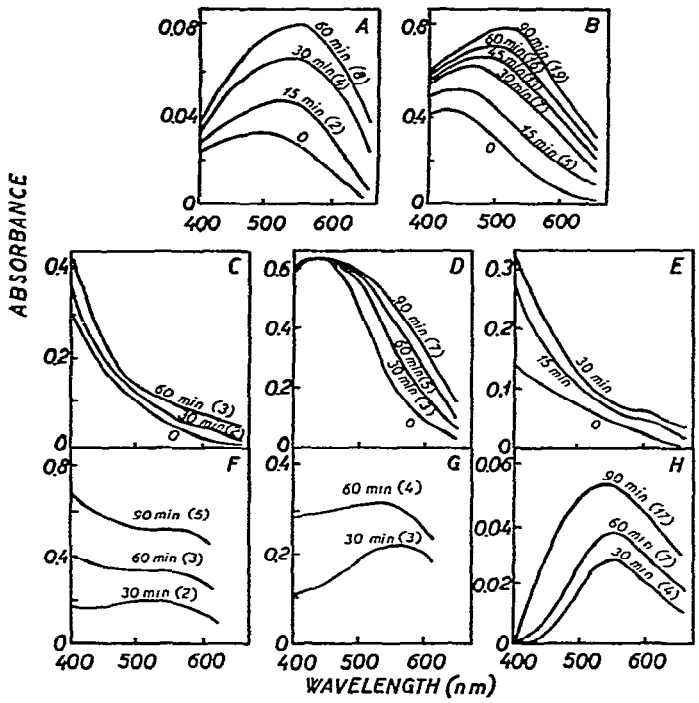

Fig. 2. Mechanism of chain elongation. Light-absorption curves of iodine-stained products during synthesis in presence of various primers. The standard reaction mixture contained: (A) Amylopectin (5 μg), (B) phytoglycogen (25 μg), (C) β-limit dextrin of phytoglycogen (3.2 μg), (D) ϕ-limit dextrin of phytoglycogen (41 μg), (E) ϕ,β-limit dextrin of phytoglycogen (1.7 μg), (F) maltotriose (0.04μ mole), (G) maltotetraose (0.03 μmole), and (H) maltoheptaose (7 nmoles). At the time of incubation indicated on each curve, the inorganic phosphate liberated[17] and the light-absorption curves were determined[14]. The number of α-D-glucosyl residues incorporated in each case was calculated according to Whelan and Bailey[13], and it is reported in parenthesis.

enzyme. This mechanism resembles the one described for immature endosperm phosphorylase[5], and it differs markedly from those demonstrated for potato[15] and other phosphorylases.

EXPERIMENTAL

Analytical methods. — Inorganic phosphate content was measured by the method of Fiske and SubbaRow[17] and protein content according to Lowry *et al.*[18].

Substrates. — The phosphorylase ϕ-limit dextrin of phytoglycogen was prepared as described by Lee *et al.*[19]. The ϕ,β-limit dextrin of phytoglycogen was obtained by treatment of the corresponding muscle-phosphorylase-limite dextrin with sweet-potato β-amylase, and the maltose was removed by dialysis against water[20].

Determination of phosphorylase activity. — The incubation mixture contained 2-morpholinoethanesulfonic acid (MES) buffer (10 μmoles), pH 6.1, amylopectin

(0.05 mg), α-D-glucopyranosyl phosphate (1 μmole), and 40 μl of enzyme (0.4 mg of protein) in a total volume of 100 μl. Incubation was carried out during 30 min at 37°. The reaction was stopped by addition of 5% trichloroacetic acid (0.9 ml). The mixture was centrifuged and the inorganic phosphate liberated was measured in the supernatant fluid. One unit of enzymic activity was considered equal to the amount of enzyme that catalyzes the release of one μmole of inorganic phosphate in 1 min. All other materials and methods were described previously[5].

Isolation of isoenzymes and examination by disk electrophoresis. — Kernels of sweet corn (*Zea mays*, var. Golden Bantam) were steeped in water for 4 h and then allowed to germinate on wet filter paper at 25° in the dark, over periods of 1, 3, 6, and 8 days. The embryo (scutellum with the plumule and the radicle) was separated, suspended in 2 vol. of 10mM 2-amino-2-hydroxymethyl-1,3-propanediol (Tris) maleate buffer, pH 7.4, containing 0.5mM 1,4-dithiothreitol, and homogenized during 2 min with a Waring blendor. The suspension was filtered through cheese-cloth and centrifuged at 25,000 × *g* during 20 min. The supernatant fluid was fractionated with ammonium sulfate and the fractions precipitating between the concentrations of 25 and 45% of saturation, and between 45 and 80% of saturation were collected and dissolved in buffer (0.05 vol. of the initial suspension). These fractions were submitted to gel electrophoresis by the procedures of Ornstein[21] and Davis[22], slightly modified by Siepman[23]. The large-pore gel contained 7% acrylamide. The amount of phosphorylase in the sample applied to the gel ranged from 1 to 2 milliunits. The reservoir buffer (42mM Tris, 1.7mM ethylenediaminetetraacetic acid (EDTA) and 50mM boric acid, pH 8.2) was carefully layered over the sample, filling the rest of the tube. Electrophoresis was performed in a cold-room (4°) at a constant current of 3mA per tube for approximately 3 h. The gels were then removed from the glass tubes and placed in an appropriate solution for protein staining or activity detection.

The phosphorylase activity in the gels was detected as follows: after electrophoresis, each gel was incubated overnight at room temperature in a mixture containing 0.1M citrate buffer, pH 5.1, 19mM α-D-glucopyranosyl phosphate, and as primers 0.8% phytoglycogen, 0.05% amylopectin, or 0.1% amylose. Control incubations were carried out without α-D-glucopyranosyl phosphate or without primer. After incubation, the gels were washed with a few changes of distilled water, stained with a dilute iodine–potassium iodine solution in 0.2M acetate buffer, pH 4.8, during a few min, and finally washed several times with a dilute solution of acetic acid.

Partial purification of a slow-moving isoenzyme from seeds after 3 days of germination. — Phosphorylase was isolated from embryos obtained from seeds after 3 days of germination, according to the method just described. In this case, the fraction precipitating at concentrations of ammonium sulfate between 60 and 85% of saturation was collected and dissolved in 10mM Tris maleate buffer, pH 7.4, containing 0.5mM 1,4-dithiothreitol and 15% glycerol. The suspension was passed through a column (10 × 0.9 cm) of Sephadex G-25 which had been equilibrated with the same buffer. The enzymically active fractions were applied to a column (15 × 1.5 cm) of DEAE-cellulose or DEAE-Sephadex A-50, previously equilibrated with the same

buffer. The enzymic activity was found in the fractions first to be eluted. This treatment resulted in a 10-fold increase of the specific activity, as compared to that of the starting material. The active fractions were tested for amylases, phosphatases, branching, and D-enzyme activities with negative results, and were then stored at 4°.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Luis F. Leloir and other members of the Instituto de Investigaciones Bioquímicas for discussions and criticisms, and to Dr. Carlos Lantos for his help in the English version of the manuscript. This investigation was supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina) and the University of Buenos Aires.

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