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A PRIMER INDEPENDENT FORM OF POTATO TUBER PHOSPHORYLASE

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SUMMARY - Phosphorylase (EC 2.4.1.1) from potato tuber occurs in two forms: one independent and the other dependent on the addition of primer for activity. These activities could be separated in several isoenzymes by DEAE-cellulose chromatography and by electrophoresis in polyacrylamide gel. Only one of the separated isoenzymes had unprimed activity, which was found to be markedly stimulated by the presence of bovine seroalbumin.

A previous paper from this laboratory (1) demonstrated the formation of a protein bound α-1,4 glucan carried out by a soluble preparation of potato phosphorylase without primer addition, provided that appropriate concentrations of glucose-1-P were employed. We have also reported (2) that rabbit muscle phosphorylase b is capable of forming protein linked α-1,4 glucosidic chains upon incubation of the enzyme under certain conditions with no primer addition (unprimed activity).

Multiple forms of α-glucan phosphorylases (EC 2.4.1.1) have been described in potato tuber (3-5) and other plant tissues (6-10). This report describes the isolation of a phosphorylase isoenzyme from potato tuber which catalyzes the formation of a protein linked glucan in the absence of added primer. This activity could be separated from other phosphorylase activities by DEAE-cellulose chromatography and electrophoresis in polyacrylamide gels.

MATERIALS AND METHODS

The enzyme preparation was isolated from potato tuber as already described (1). An aliquot containing 40-50 mg protein was applied to a DEAE-

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cellulose column (1.8 x 15 cm) equilibrated with 50 mM Tris-HCl buffer, pH 7.2, containing 7 mM mercaptoethanol (buffer A). The protein was eluted with a linear KCl gradient (0-0.5 M) made in buffer A. Fractions of about 3 ml were collected. When indicated, column fractions containing phosphorylase activity were pooled, concentrated by ultrafiltration over an UM-2 membrane (Amicon Inc.) and stored at 4ºC.

Assay of primed phosphorylase activity - This was determined by adding 30 µl of column effluent to a medium containing 0.24 nmole of [^{14}C]glucose-1-P (75,000 cpm) and 0.3 mg of oyster glycogen (Sigma) in a final volume of 50 µl. After incubation for 60 min at 37ºC, 1 ml of methanol was added and [^{14}C]glucose incorporation in the methanol precipitate was measured as already described (1).

Assay of unprimed phosphorylase activity - This was determined by adding 30 µl of column effluent to a medium containing 0.24 nmole of [^{14}C]glucose-1-P (75,000 cpm), 1 µmole of glucose-1-P and 0.25 mg of bovine seroalbumin (Sigma) in a final volume of 50 µl. After incubation for 180 min at 37ºC, 1 ml of 5% trichloroacetic acid was added and the ^{14}C glucose incorporation in the trichloroacetic acid precipitate was measured as previously reported (1).

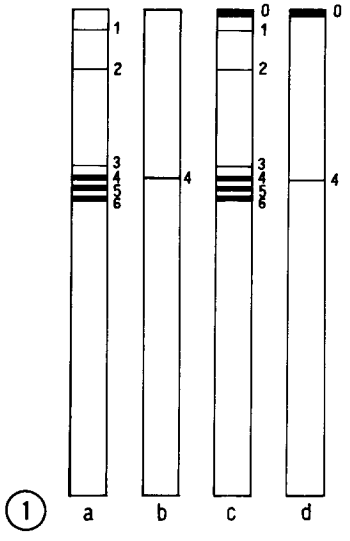
Electrophoresis - Disc gel electrophoresis under non-denaturing conditions was performed on 7% acrylamide gel according to Davis (11) with the modifications already described (10). When indicated, acrylamide was polymerized in the presence of 0.1% glycogen (12, 13). Protein bands were located by staining with Amidoblack 10B.

Location of primed and unprimed phosphorylase activities in polyacrylamide gels - The detection of both phosphorylase activities in gels was based on iodine complex formation with the glucan product. Citrate buffer (pH 5.1), 300 µmoles; glycogen, 24 mg; and glucose-1-P, 60 µmoles were mixed together in a volume of 3 ml and incubated with the gels overnight at room temperature. The reaction mixture was then removed and the gels immersed in a 0.2 M acetate buffer solution (pH 4.8) containing 0.2% KI and 0.02% I_2. Phosphorylase activity was detected by blue band formation on the gel. When glycogen was omitted in the reaction mixture and in the gel, a blue band formation was only observed with unprimed phosphorylase. For radioactive samples, 2-mm gel slices were incubated in a mixture containing 20 mM $MnCl_2$ and 0.24 nmole of [^{14}C]glucose-1-P (75,000 cpm) for 60 min. Non loaded or albumin containing gels were similarly handled as controls. After incubation, the slices were extensively rinsed with distilled water containing 20 mM unlabeled glucose-1-P, placed in vials containing 1 ml of 0.3% sodium dodecyl sulfate, maintained at 40ºC for 24 h (14), and counted in the liquid scintillator mixture described by Bray (15). Protein was measured according to Lowry et al. (16) using bovine seroalbumin as standard.

RESULTS

Location of phosphorylase activity on analytical gel

As can be seen in Fig. 1 a, gel electrophoresis of the potato tuber enzyme



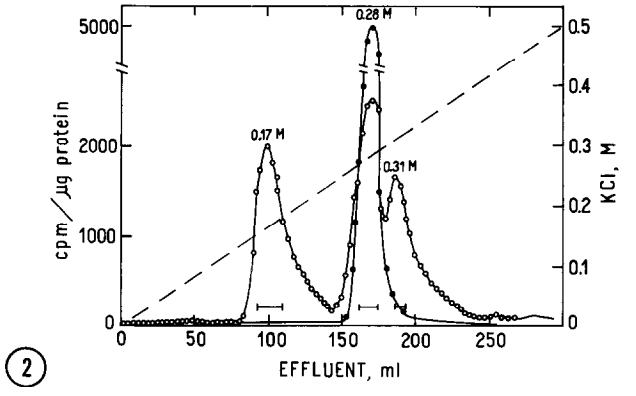


Fig. 1.- Gels stained by the iodine-iodide reagent to show primed and unprimed phosphorylase activities. The enzymatic preparation (1) (500 µg of protein) incubated (gels c and d) or not incubated (gels a and b) in the presence of 20 mM glucose-1-P was applied on polyacrylamide gels and electrophoresed. Gels a and c were stained for localization of primed activity, and gels b and d for unprimed activity, as outlined in Materials and Methods. Anodic end is shown down.

Fig. 2.- DEAE-cellulose chromatography of potato tuber enzyme preparation (1). Aliquots of the fractions were tested for primed and unprimed phosphorylase activity. For details, see the text. Fractions which were pooled are indicated by the bracket (⊢—⊣). (o-o) Primed phosphorylase; (●-●) unprimed phosphorylase.

preparation (1) led to the appearance of six bands with primed phosphorylase activity using either the primer inclusion technique (12, 13) or the addition of primer after separation. On the other hand, only one band was developed when a similarly loaded gel was incubated in the absence of glycogen (Fig. 1 b). When this enzyme preparation was incubated with 20 mM glucose-1-P before electrophoresis, a new deep blue coloured band near the gel origin could be revealed in the presence (Fig. 1 c) as well as in the absence (Fig. 1 d) of primer. This slow moving band, although less intensely coloured, can also be detected by staining the gel immediately after the electrophoretic run without requiring incubation of the gel. In order to elucidate whether glucosylation in the absence of primer oc-

TABLE I - [^{14}C]Glucose Incorporation in the Glucosylated Protein Isolated by Polyacrylamide Disc Gel Electrophoresis

Sample	[^{14}C] Glucose incorporation into the gel slice
	cpm
Gel 1	402
Gel 2	317
Gel 3	2216
Gel 4	2198

2-mm slices of gels were assayed for unprimed phosphorylase activity. For details, see text. Gel 1: non loaded gel; Gel 2: slice corresponding to albumin band in an albumin loaded gel; Gel 3 and Gel 4: duplicate gel slices corresponding to unlabeled glucosylated protein.

curs on a protein with phosphorylase activity, the following experiment was performed: triplicate samples were incubated under standard conditions (1), but with unlabeled glucose-1-P. The incubation mixtures were then applied on polyacrylamide gels and submitted to electrophoresis. After the run, one gel was stained with the iodine-iodide reagent to localize the band corresponding to glucosylated protein. Two-mm slices corresponding to this band were cut out from the other gels, in duplicate, incubated and counted as described under Materials and Methods. As shown in Table I , the protein glucosylated during the first incubation catalyzed the incorporation of [^{14}C]glucose even after its electrophoretic separation. Thus, the occurrence of enzymatic activity in this particular band was established.

Chromatography on DEAE-cellulose

In order to study this unprimed phosphorylase, a potato tuber enzyme preparation (1) was chromatographed on a DEAE-cellulose column. As shown in Fig. 2, the enzymatic preparation yielded three distinct peaks of primed phos-

TABLE II - Effect of Albumin Concentration on Unprimed Phosphorylase Activity from Peak II

Albumin	Radioactivity incorporation
mg/ml	cpm
-	3,951
0.4	6,593
1	9,940
2	9,312
3	11,791
5	11,647

Conditions were as described under Materials and Methods, except for the concentration of albumin which was varied as indicated.

phorylase activity, the first of which eluted at 0.17 M KCl (type I), the second at 0.28 M KCl (type II) and the third at 0.31 M KCl (type III). When aliquots of the column fractions were tested for unprimed phosphorylase activity, a single peak was obtained in the same position of the gradient where primed type II phosphorylase appeared. No activity of unprimed phosphorylase was detected in the zones corresponding to type I or to type III phosphorylases, even increasing incubation time or the amount of protein in the reaction mixture.

Initial attempts to determine unprimed phosphorylase type II activity were hampered by the low activity of the enzyme in fairly dilute solutions and under the conditions used for fractionation. We subsequently found that the addition of albumin to the incubation mixture markedly stimulated radioactivity incorporation in the trichloroacetic acid precipitate (unprimed activity) (Table II). As can be seen, the addition of 250 µg of albumin to the active column fraction in the absence of primer, led to a substantial promotion of unprimed phosphorylase type II activity. Albumin was also examined for a possible activating effect on phosphorylases types I, II and III in the absence and in the presence of primer. As

can be seen in Table III, albumin was ineffective in enhancing any of these activities, except that of the already mentioned unprimed phosphorylase type II.

When aliquots of pooled active column fractions from each peak were electrophoresed in glycogen containing gels, it can be seen that peak I consisted of isoenzyme 1; peak II contained isoenzyme 4 and peak III comprised isoenzymes 2, 3 and 5 (Fig. 3 A). However, when a similar electrophoretic run was performed without primer inclusion or ulterior addition, a single light blue band was visible on the gel, coincident with isoenzyme 4 (not shown). No bands corresponding to the other isoenzymes could be detected in the absence of primer.

Incubation of phosphorylase type II with 20 mM glucose-1-P in the absence of primer for 60 min prior to the electrophoretic run, led to the appearance of a deep blue staining band near the gel origin and the almost disappearance of isoenzyme 4 band, after incubation of the gel in the absence as well as in the presence of primer (Fig. 3 B).

DISCUSSION

Two kinds of phosphorylase activities were found in potato tuber: primed and unprimed (Fig. 1). Following column chromatography on DEAE-cellulose, three activity peaks could be identified in the presence of primer, while only one of them (eluting at 0.28 M KCl) had unprimed activity (Fig. 2). The fact that the unprimed activity is greatly stimulated by bovine seroalbumin is remarkable, as this protein had no effect on any of the three primed activities (Table III). A similar activating effect of albumin on the unprimed activity of ADP-glucose:starch glucosyltransferase from waxy maize was reported by Ozbun et al. (17). Although the mechanism of albumin action is still unknown, it seems likely that it might induce the proper conformation of the enzyme for unprimed activity.

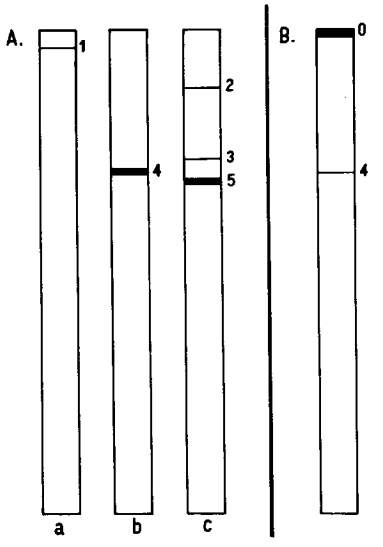


Fig. 3.- Polyacrylamide gel electrophoresis. Pooled protein fractions from each peak with phosphorylase activity (200-300 µg) from DEAE-cellulose chromatography were electrophoresed and stained for localization of enzymatic activity. Anodic end is shown down. A. Samples from: (a) peak I, (b) peak II and (c) peak III were run in glycogen containing gels. B. An aliquot from peak II was incubated in the presence of 20 mM glucose-1-P, submitted to electrophoresis without primer inclusion and stained for unprimed activity. The same electrophoretic profile was obtained when incubation of the gel was performed in the presence of glycogen.

TABLE III - Activity of Primed and Unprimed Phosphorylases from Peak I, Peak II and Peak III in the Absence and in the Presence of 5 mg/ml Albumin

Column fraction	Primed activity[a] - albumin	Primed activity[a] + albumin	Unprimed activity[a] - albumin	Unprimed activity[a] + albumin
Peak I	1991	1981	10	11
Peak II	2345	2447	3226	4795
Peak III	1628	1583	29	55

The incubation mixture was as outlined in Materials and Methods, with the modifications indicated.
[a] cpm/µg of protein

It is worth pointing out that the slow moving glucosylated protein seems to be formed at the expense of isoenzyme 4 (Fig. 3 B), preserving its catalytic

activity measured in the absence as well as in the presence of primer. Studies to identify the physiological function(s) of the phosphorylase isoenzymes are in progress.

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