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CHARACTERIZATION OF MULTIPLE FORMS OF α-GLUCAN PHOSPHORYLASE FROM *MUSA PARADISIACA* FRUITS

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Abstract—Three forms of α-glucan phosphorylase from mature banana fruit pulp separated by ammonium sulfate fractionation and DEAE-cellulose chromatography were anodic at pH 8·6 on starch gel electrophoresis. The three forms differed in sensitivity to the phenolics extracted from immature and mature banana fruit pulp. Only two forms of the enzyme were detected in immature banana fruit pulp.

INTRODUCTION

Multiple forms of α-glucan phosphorylase have been demonstrated in spinach [1], potato [2–4], *Phaseolus vulgaris* [3], *Vicia faba* [1] and blue-green algae [5,6]. Tsai and Nelson [7] found different phosphorylase isoenzymes at various stages of development of maize endosperm and suggested that two of these might have a synthetic role. The present communication reports the occurrence of multiple forms of α-glucan phosphorylase in banana pulp at two different stages of fruit development.

RESULTS

Purification of α-glucan phosphorylase

A typical purification of the enzyme from the pulp of mature banana is summarized in Table 1. The recovery of 10-fold the original activity in the 0–60% $(NH_4)_2SO_4$ fraction and about 3-fold in the 60–90% $(NH_4)_2SO_4$ fraction indicated the removal of enzyme inhibitor(s) present in the initial extract. The 0–60% $(NH_4)_2SO_4$ fraction could be resolved into two fractions by DEAE-cellulose chromatography. A component of the enzyme was weakly adsorbed and was eluted by washing the column with 5 mM Tris–HCl buffer, pH 7·2. Fractions 3 to 5 were pooled and designated phosphorylase A. The adsorbed proteins were fractionated using a linear gradient of Tris–HCl, pH 7·2. Fractions 18 to 20 eluted between 0·32 to 0·35 M Tris–HCl, were pooled and designated phosphorylase B. Of the enzyme applied to the column 66% was recovered as phosphorylase A and 10% as phos-

Table 1. Purification of α-1,4-*d*-glucan phosphorylase from mature banana fruits

Fraction	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)	Recovery of activity (%)
Initial extract	200	340	244	1·4	(100)
$(NH_4)_2SO_4$ fraction (0–60%) and Sephadex G-25 filtration	100	3450	50	69	1015
$(NH_4)_2SO_4$ fraction (60–90%) and Sephadex G-25 filtration (Fraction C)	80	920	3·2	288	271
DEAE-cellulose					
Fraction A	100	2300	1·6	1438	676
Fraction B	100	360	0·8	450	106

Only 30 ml of 0–60% $(NH_4)_2SO_4$ fraction was applied to DEAE-cellulose column; the values, however, are calculated for 100 ml of $(NH_4)_2SO_4$ fraction.

Table 2. Purification of α-1,4-*d*-glucan phosphorylase from immature banana fruits

Fraction	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)	Recovery of activity (%)
Initial extract	200	160	176	0·9	(100)
$(NH_4)_2SO_4$ fraction (0–60%) and Sephadex G-25 filtration	100	349	38	9·2	218
$(NH_4)_2SO_4$ fraction (60–90%) and Sephadex G-25 filtration (Fraction C)	80	368	2·4	153·3	230
DEAE-cellulose (Fraction B)	100	230	1·0	230	144

Only 30 ml of 0–60% $(NH_4)_2SO_4$ fraction was applied to DEAE-cellulose column; the values, however, are calculated for 100 ml of $(NH_4)_2SO_4$ fraction.

phorylase B. The chromatography step alone led to the enrichment of phosphorylase A by 21-fold and phosphorylase B by 7-fold. The fraction precipitated between 60–90% $(NH_4)_2SO_4$, designated phosphorylase C, was enriched 206-fold with 271% recovery from the initial extract. The enrichment of phosphorylases A, B and C was only apparent in view of the inhibition which occurred in the crude extract. The purification obtained in four experiments was 1180 ± 260, 525 ± 200 and 250 ± 100 units/mg protein, for phosphorylases A, B and C respectively. The specific activity of phosphorylase A was about 1·5 times higher than that of potato phosphorylase [8]. Phosphorylases B and C, however, had specific activities only about 0·45 and 0·3 of potato phosphorylase [8]. The purified fractions did not exhibit phosphatase activity towards glucose-1-phosphate and glucose-6-phosphate.

A typical purification of the enzyme from the 1600 *g* supernatant of the homogenate from the pulp tissues of immature banana is given in Table 2. Only one peak of enzyme activity was obtained when the 0–60% $(NH_4)_2SO_4$ fraction was chromatographed on DEAE-cellulose. Fractions 20 to 22 eluted between 0·35 to 0·37 M Tris–HCl were pooled and designated phosphorylase B, by analogy with the designation adopted for the enzyme forms from mature banana.

The recovery of over 2-fold the original activity in 0–60% $(NH_4)_2SO_4$ fraction and over 2-fold in 60–90% $(NH_4)_2SO_4$ fraction indicated the removal of enzyme inhibitor(s) present in the initial extract. Phosphorylase B was enriched 255-fold from the initial extract. The enzyme precipitated between 60–90% $(NH_4)_2SO_4$, designated as phosphorylase C by analogy with the enzyme from mature banana, was enriched 170-fold from the initial extract. As mentioned earlier the purification achieved was apparent, in view of the inhibition in the crude extract. The average specific activities of phosphorylases B and C in three experiments were 208 ± 30 and 140 ± 35 units/mg protein.

Nature of phosphorylase inhibitor

The increase in enzyme activity by $(NH_4)_2SO_4$ precipitation followed by Sephadex G-25 filtration indicated that the inhibitor was not protein in nature. In a separate experiment with mature banana fruit pulp about a 6-fold increase in phosphorylase activity was observed when the initial extract was subjected to Sephadex G-25 filtration. The gel-filtration resolved phenolics in two peaks, a minor one containing high MW components (fractions 10 to 13) and a major one containing low MW phenolics (fractions 27 to 31). Inhibitors of phosphorylase also emerged in two peaks, coinciding with the above peaks. This indicated that the inhibitors of phosphorylase were phenolic in nature. The fact that no bound phenolics were detected in fractions containing protein indicated that the composition of the extraction medium was such that no phenol-protein complex was formed.

Rechromatography of separated fractions

The three forms of α-glucan phosphorylase could be isolated from the 0–90% $(NH_4)_2SO_4$ pre-

Table 3. Isolation of multiple forms of α-glucan phosphorylase from mature banana fruit pulp and rechromatography on DEAE-cellulose

Fraction	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)	Recovery of activity (%)
Initial extract	75	127	108	1·2	(100)
$(NH_4)_2SO_4$ fraction (0–90%) and Sephadex G-25 filtration	30	1242	24·9	49·9	978
DEAE-cellulose					
Fraction A	30	552	0·54	1022	435
Fraction B	30	123	0·27	456	97
Fraction C	30	267	0·33	809	210
$(NH_4)_2SO_4$ precipitation of combined A, B and C fractions	30	828	0·90	920	651
Rechromatography on DEAE-cellulose					
Fraction A	40	460	0·32	1438	362
Fraction B	40	120	0·20	600	94
Fraction C	40	220	0·22	1000	173

cipitate of the initial extract of mature pulp (Table 3).

The separated fractions containing phosphorylases A, B and C on mixing and rechromatographing on DEAE-cellulose again showed three peaks of activity; eluted in washing (A), 0·33 to 0·35 M Tris–HCl pH 7·2 (B) and 0·60 to 0·62 M Tris–HCl, pH 7·2 (C). The reproducibility of the elution patterns suggested that the enzyme fractions were not artificially formed on column, but existed as natural components.

Characterization of multiple forms of the enzyme by starch gel electrophoresis

Phosphorylases A, B and C, obtained from mature banana by DEAE-cellulose chromatography, revealed one band each when subjected to starch gel electrophoresis at pH 8·6. Three bands were found when phosphorylases A, B and C were mixed together and applied to starch gel. Electrophoresis of the 0–60% $(NH_4)_2SO_4$ fraction revealed two bands corresponding to phosphorylases A and B, whereas 60–90% $(NH_4)_2SO_4$ fraction showed one band, corresponding to phosphorylase C. The 0–90% $(NH_4)_2SO_4$ fraction revealed three bands of glucan phosphorylase activity corresponding to phosphorylases A, B and C. All the bands were anodic at pH 8·6.

The 0–60% $(NH_4)_2SO_4$ fraction from immature banana gave one band only, consistent with the observation from DEAE-cellulose chromatography. The 0–90% $(NH_4)_2SO_4$ fraction showed two bands corresponding to phosphorylases B and C. Comparison of electrophoretograms of 0–90% $(NH_4)_2SO_4$ fraction from mature and immature banana show that immature banana pulp lacks phosphorylase A, an observation also consistent with DEAE-cellulose chromatography.

α-Glucan phosphorylase, starch, orthophosphate and total phenolics in pulp of developing banana

Starch and orthophosphate (P_i) contents of banana pulp from immature and mature fruits and α-glucan phosphorylase activity of homogenates are recorded in Table 4. A correlation between starch content and α-glucan phosphorylase activity could be seen, expressing the data in terms of g fr. wt of tissue. The phosphorylase activity in crude homogenates is under estimated because of the presence of endogenous inhibitors but comparison of phosphorylase activity in $(NH_4)_2SO_4$ fractions after Sephadex G-25 filtration again show higher phosphorylase activity in mature compared to immature banana fruit pulp (Tables 1 and 2). An increase in starch content and decrease in P_i level occurred in mature tissue. The decrease in P_i level in mature tissue may shift the equilibrium of α-glucan phosphorylase towards synthesis. However, the P_i content of the tissue may not represent the concentration of P_i at the

Table 4. α-Glucan phosphorylase activity, starch, orthophosphate and total phenolics content at two developmental stages of banana pulp

Stage of development	Ratio: pulp/peel (fr. wt)	Ratio: pulp/peel (dry wt)	Activity of α-glucan phosphorylase (units/g fr. wt)	Activity of α-glucan phosphorylase (units/mg protein)	Starch (mg/g fr. wt)	Orthophosphate (μg/g fr. wt)	Total phenolics (mg tannic acid equivalent/g fr. wt)
Immature	0·25	0·33	4·3	0·51	32·0	280	4·8
Mature	1·31	2·25	12·9	0·71	88·0	93	2·6

location of α-glucan phosphorylase. It was also found that total phenolics decreased with the development of the banana fruit.

Effect of phenolics extracted from banana pulp on isolated fractions

Quantitative differences were observed in response to phenolic extracts from banana pulp amongst the three fractions. Phosphorylase B was very sensitive to phenolics extracted both from immature and mature banana. Almost complete inhibition occurred at a concentration of 26 μg. Phosphorylase C was least sensitive. At 26 μg of phenolics from immature and mature banana the enzyme was inhibited 40 and 20% respectively (Table 5). Phosphorylase A was inhibited 67 and 42% respectively by the same concentration of phenolics from immature and mature banana. It also appeared that phenolics extracted from immature banana inhibited more powerfully than from mature banana.

DISCUSSION

In earlier work maximum α-glucan phosphorylase activity in banana pulp could be demonstrated only when phenol fixing agents or detergents were used, indicating the presence of endogenous phenolics as inhibitors of the enzyme [9]. Even though the supplementation prevented inactivation of enzyme, it remained in an inhibited state in the homogenates and extracts, as is evident by the increase in enzyme activity on $(NH_4)_2SO_4$ fractionation, followed by Sephadex G-25 filtration or by Sephadex G-25 filtration alone. The close correlation between inhibition and phenolic content of fractions obtained by Sephadex G-25 filtration supports the suggestion that the inhibition is due to phenolics. A number of other inhibitors of phosphorylase have been reported to occur in biological system [10-15].

Mature banana fruit pulp contains three types of α-glucan phosphorylase, which are separable by $(NH_4)_2SO_4$ fractionation and DEAE-cellulose chromatography. The different electrophoretic mobilities on starch gel, separation on DEAE-cellulose column, reproducibility of elution pattern on rechromatography, and the different properties provide good evidence that the three forms represent multiple forms of α-glucan phosphorylase.

Whereas mature banana fruit pulp contains three forms of phosphorylase (A, B and C), the immature banana fruit pulp contains only two forms, B and C. It may be argued that the failure to detect phosphorylase A in immature banana *in vitro* may be due to the inactivation of the enzyme by endogenous phenolics during the preparation

Table 5. Effect of phenolics extracted from banana fruits on banana phosphorylases

Source of phenolics	Amount of phenolics (μg tannic acid equivalent)	Phosphorylase activity (units per ml) A	B	C
	Nil	22·5	3·9	11·5
Immature banana	26	7·4	Nil	6·9
	52	3·2	Nil	3·4
Mature banana	13	19·8	0·5	10·3
	26	13·1	0·5	9·2

The protein concentration in the assay system for phosphorylase forms A, B and C were adjusted to 1·6 μg by externally added bovine serum albumin.

of cell-free extract since the phenolics derived from immature tissue are more inhibitory than those from mature tissue, and the content of total phenolics in the pulp of immature finger is higher than in the mature. In apparent support of this view was the finding that the recovery of enzyme following $(NH_4)_2SO_4$ fractionation and Sephadex filtration was 13-fold higher starting with mature tissue, whereas it was only 4-fold from immature tissue. While this possibility could not be excluded, this hypothesis appeared unlikely since phosphorylase B, which is more sensitive than A (as tested in mature banana), could be isolated from immature banana. The fact that phosphorylase A could be demonstrated only in actively starch synthesizing mature banana points to its likely participation in starch synthesis. Recently Gerbrandy and Verleur [3] found nine phosphorylase isozymes during the period of starch synthesis in potato tuber but only two during the period of starch breakdown.

EXPERIMENTAL

Plant. Banana fingers (*Musa paradisiaca*) were collected immediately before use from plants grown in the departmental garden. Fingers from two developmental stages from the same bunch were selected for the experiment. Banana fingers, 8–12 days old, having pulp to peel ratio of 0·25–0·30 (fr. wt basis), are referred to as "immature banana". The amount of pulp increased faster than peel with the development of the fruit. Banana fingers, 60–65 days old, having a pulp to peel ratio of 1·3–1·5, are referred to as "mature" banana.

α-Glucan phosphorylase activity was assayed in the direction of polysaccharide synthesis according to the method of Green and Stumpf [16] as described by Baijal *et al.* [9]. NaF was added to inhibit the phosphatase activity. One unit of the enzyme was equivalent to the liberation of 1 μmol of orthophosphate (P_i) in 30 min at 30°C under the experimental conditions. Sp. act. of the enzyme was expressed as units per mg protein.

Tests for phosphatase action towards glucose-1-phosphate and glucose-6-phosphate were carried out in the purified enzyme fractions under the assay conditions for phosphorylase.

Protein estimation was based on the method of Lowry *et al.* [17] as modified by Khanna *et al.* [18]. Bovine serum albumin was used as a standard. For protein profiles of the eluted fractions from DEAE-cellulose column, A at 260 and 280 nm was measured and protein calculated according to Kalckar [19].

Extraction and determination of total phenolics and starch. 10 g banana fruit pulp was ground with 100 ml of 95% EtOH (final concentration about 80%) in a Waring blender for 2 min and refluxed at 100° for 8 hr. The suspension was filtered and the residue re-extracted with 100 ml of 80% EtOH for 4 hr. The suspension was filtered again and both filtrates mixed. Total phenolics in the combined filtrate was determined in aliquots using the reagent of Folin and Denis [20], modified as in refs 21 and 22. For the Sephadex G-25 fraction, protein was precipitated with TCA and removed by centrifugation prior to determination of free phenolics. Bound phenolics were determined in the TCA ppt. after extraction with 80% EtOH at 100°. The EtOH extracted residue was dried as suggested by Davies and Cocking [23] and weighed samples taken for starch determination according to the method of Pucher *et al.* [24].

Inhibitor activity was determined by incorporating the inhibitor in the phosphorylase assay system. A unit of inhibitor was defined as the amount which inhibited phosphorylase activity by half a unit.

Orthophosphate analysis. The protein was precipitated with chilled TCA (10%) and P_i determined immediately in the supernatant by the method of Fiske and Subbarow [25], as modified by Khanna *et al.* [18].

Starch gel electrophoresis was carried out in a horizontal electrophoresis apparatus by the method of Smithies [26], using 13% starch. The electrode buffer consisted of 0·3 M boric acid and 0·05 M NaOH (pH 8·6). The enzyme preparation (10 to 25 units) was applied in a slot of starch gel plate. Electrophoresis was for 3–4 hr at 200 V and 15–18 mA (0–5°). The gel was then removed and sliced longitudinally by a wire. The sliced gel was incubated for 1 hr at room temp. (*ca* 25°) in the incubation mixture (3 ml of 0·5 M citrate buffer, pH 6; 6 ml of 10 mg per ml freshly-prepared starch, 4·5 ml of 0·2 M NaF; 1·5 ml of 0·1 M glucose-1-phosphate, pH 6; 30 ml H_2O), and phosphorylase bands located from the position of P_i which was detected by the reagent of Fiske and Subbarow [25].

Purification of enzyme. The following operations were carried out between 0–4°, unless otherwise stated.

Mature banana fingers were freed from peel, sliced longitudinally and the central core carrying the seeds cut out and discarded. The residual pulp was cut into small pieces and processed immediately. 40 g tissue was homogenized in a Waring blender for 1 min with 160 ml of cold 0·05 M Tris–HCl buffer, pH 7·2 containing 0·02 M freshly neutralized cysteine-HCl, 0·02 M neutralized EDTA and 1% (v/v) Triton X-100. After straining through 2 layers of muslin the homogenate was made up to 200 ml with the medium and centrifuged at 1600 *g* for 30 min. The supernatant (faint milky color) containing about 90% of glucan phosphorylase activity of the homogenate was taken for purification in the following steps. $(NH_4)_2SO_4$ (78 g) was added gradually with constant stirring to 200 ml of the extract. After 4 hr the suspension was spun at 15000 *g* for 45 min and the supernatant kept for further fractionation with $(NH_4)_2SO_4$. The ppt. (white) was ground in a chilled mortar with 40 ml of 0·05 M Tris–HCl buffer, pH 7·2 and the resulting suspension centrifuged at 15000 *g* for 45 min and the supernatant collected. The ppt. was extracted again with 10 ml of the buffer and the supernatant added to the first extract. The combined supernatant (colorless) was desalted by filtration through a column of Sephadex G-25 (3·6 × 16 cm) equilibrated with 5 mM Tris–HCl buffer, pH 7·2. An additional 52·2 g $(NH_4)_2SO_4$ was added to the supernatant obtained after removal of 0–60% $(NH_4)_2SO_4$ fraction. After 18 hr the suspension was spun at 15000 *g* for 45 min. The ppt. was ground in a mortar with 30 ml of 0·05 M Tris–HCl buffer, pH 7·2. The suspension was centrifuged at 15000 *g* for 45 min and the supernatant collected. The ppt. was once more extracted with 10 ml of the buffer and the supernatant added to the first extract. The salt from the combined supernatant (colorless) was removed by filtration through a column of Sephadex G-25 (3·6 × 16 cm) equilibrated with 5 mM Tris–HCl buffer, pH 7·2. 30 ml of the extract from the 0–60% $(NH_4)_2SO_4$ fraction (15·6 mg protein) was adsorbed onto a DEAE-cellulose column (1·4 × 21 cm), which had been equilibrated with 5 mM Tris–HCl buffer, pH 7·2. The column was washed with 50 ml of 5 mM Tris–HCl buffer, pH 7·2 (10 ml

fraction). The adsorbed protein was eluted by a linear gradient of Tris–HCl, pH 7·2. The reservoir contained 200 ml of 1 M Tris–HCl buffer, pH 7·2 and the mixing chamber 200 ml of 5 mM Tris–HCl buffer, pH 7·2. The flow rate was 2 ml per min.

The purification procedure for α-glucan phosphorylase from immature fruit involved similar steps as those for mature banana.

Isolation of multiple forms of α-glucan phosphorylase from total protein of mature pulp. $(NH_4)_2SO_4$ (49·7 g) was added to 75 ml of the initial extract to obtain 90% $(NH_4)_2SO_4$ saturation. The ppt. was ground in a chilled mortar with 15 ml of 0·05 M Tris–HCl buffer, pH 7·2 and the resulting suspension centrifuged at 15000 *g* for 45 min. The ppt. was once more extracted with 3 ml of the buffer and the supernatant added to the first extract. The residue left after re-extraction contained less than 10% of phosphorylase activity and was discarded. The combined supernatant was desalted by passing through a column of Sephadex G-25, previously equilibrated with 5 mM Tris–HCl buffer, pH 7·2. This extract (24·9 mg protein) was adsorbed onto a DEAE-cellulose column (1·4 × 30 cm) which had been equilibrated with 5 mM Tris–HCl buffer, pH 7·2. The column was washed with 50 ml of 5 mM Tris–HCl buffer, pH 7·2 and 10 ml fractions were collected. A part of the activity was eluted in washing. Fractions 3–5 (phosphorylase A). The adsorbed protein was eluted by a linear gradient of Tris–HCl buffer, pH 7·2, 5 mM–1 M. Activity was eluted between 0·28 to 0·33 M Tris–HCl in Fractions 16–19 (phosphorylase B) and between 0·55 to 0·60 M Tris–HCl in fractions 28 to 30 (phosphorylase C).

Rechromatography of separated fractions. The fractions separated by DEAE-cellulose chromatography and containing phosphorylase A, B and C activities were mixed and treated with solid $(NH_4)_2SO_4$ to 90% saturation. After 24 hr in the cold the ppt. was collected by centrifugation at 15000 *g* for 45 min and dissolved in 10 ml of 0·05 M Tris–HCl buffer pH 7·2. The salt was removed by filtration through a column of Sephadex G-25 equilibrated with 5 mM Tris–HCl buffer, pH 7·2 and rechromatographed on DEAE-cellulose column (1·4 × 30 cm) as described above.

Sephadex G-25 filtration. For experiments on phosphorylase inhibitor, 20 ml of the initial extract was subjected to filtration through a column of Sephadex G-25, coarse (3·6 × 16 cm) equilibrated with 5 mM Tris–HCl buffer, pH 7·2 containing 0·02 M neutralized cysteine-HCl and 0·02 *M* neutralized EDTA. 10 ml fractions collected.

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