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Purification and characterization of phosphorylase from tubers of *Dioscorea dumentorum*

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Abstract

Phosphorylase from tubers of *Dioscorea dumentorum* was fractionated by $(NH_4)_2SO_4$ precipitation and further purified to apparent homogeneity. Two forms of phosphorylase, Dd_1 and Dd_2 were obtained with different K_m, optimum temperatures and pH values. They obeyed Michaelis-Menten kinetics, they were activated by AMP, Mg^{2+} and Ca^{2+} but inhibited by ATP, ADP, ADP-glucose and Na_2SO_4. The molecular masses found for Dd_1 and Dd_2 were 102 000 and 195 000 respectively. SDS gel electrophoresis indicated that Dd_2 is a dimer and Dd_1 is a monomer. Both phosphorylase forms contained pyridoxal-5'-phosphate as their prosthetic group, which was essential for their activity. Dd_2 is more active in starch synthesis while Dd_1 is more active in starch degradation. The affinity of the enzyme forms for P_i is not a measure of the rate of glucan degradation. The decline in activities of the enzymes between pH 5.5 and 7.5 was due to unsuitable ionic forms of the enzymes and not due to irreversible denaturation. Both forms of phosphorylase showed double displacement reaction mechanism in direction of phosphorolysis, while in direction of synthesis, sequential mechanism was indicated. Indoleacetic acid activated the enzyme forms in direction of starch degradation but acted as an inhibitor in direction of glucan synthesis. The results of this study suggest that Dd_2 may have some synthetic function while Dd_1 has a degradative role. The effect of pH on the phosphorylase activity suggests histidine as the amino acid residue around the active site that might be involved in enzyme catalysis.

Introduction

α-Glucan phosphorylase (α-1,4-glucan orthophosphate glucantransferase, EC 2.4.1.1) from some plant tissues such as maize (Tsai and Nelson 1969) and mistletoe leaves (Khanna *et al.* 1971), potato tubers (Gerbrandy and Doorgeest 1972), banana fruits (Singh and Sanwal 1976) and pea seeds (Matheson and Richardson 1978) have

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Abbreviations: G-1-P - glucose-1-phosphate; IAA - indole-3-acetic acid; P5'P - pyridoxal-5'-phosphate; Mr - relative molecular mass.

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been isolated and multiple forms obtained. Properties of these enzyme forms have been studied and found to exhibit unique kinetics (Khanna *et al.* 1971, Lee and Braun 1973, Burr and Nelson 1975, Singh and Sanwal 1976, Matheson and Richardson 1978). Recent studies on plant phosphorylase have been reviewed (Kumar 1989).

Recently purification and characterization of phosphorylase from two yam species have been reported (Oluoha 1990, Oluoha and Ugochukwu 1991). The authors showed that each form of the enzyme showed distinct kinetic properties. Pyridoxal-5' -phosphate (P5'P) has been found to be a cofactor of α-glucan phosphorylase from animals and its role has been fairly well established (Graves *et al.* 1975). Phosphorylases from plants and microorganisms do not show allosteric transition or covalent modification control mechanism like the animal enzyme, nevertheless, it has been suggested that all phosphorylases irrespective of the source contain P5'P-phosphate (Shimomura *et al.* 1980). Although the presence of P5'P has been reported in pea seed (Matheson and Richardson 1978) and potato phosphorylase (Shimomura *et al.* 1980), the question whether or not all plant phosphorylases contain P5'P and require it for catalytic activity remains unresolved (Shimomura *et al.* 1980, Kumar 1989). This is because the requirement of P5'P has been demonstrated only for phosphorylases originating from animals and no evidence has been obtained for phosphorylase originating from plants and microorganisms (Shimomura *et al.* 1980, Kumar 1989).

There have been very few studies on kinetic reaction mechanism of plant phosphorylase except those on potato enzyme (Gold *et al.* 1971) and pea seed phosphorylase (Matheson and Richardson 1978). The authors demonstrated that phosphorylase from these sources showed sequential reaction mechanism. Therefore it is essential to study the type of reaction mechanism indicated by yam tuber phosphorylase.

The aim of this investigation was to isolate, purify and characterize phosphorylase forms from *Dioscorea dumentorum* tuber. The presence and role of P-5'-P in the yam tuber enzymes was examined and the kinetic mechanism of yam phosphorylase was studied.

Materials and methods

Materials: All the reagents and chemicals were analytical grade and were obtained from *Sigma* except Sephadex series which were the products of *Pharmacia Fine Chemicals*. *Dioscorea dumentorum* tubers used in this study were supplied from Experimental farm X21 in Benin City, Nigeria and were planted, harvested and stored as previously described (Oluoha and Ugochukwu 1991).

Extraction and purification: The enzyme was extracted as previously described (Oluoha and Ugochukwu 1991) except the inclusion of 0.7 % sodium dithionite and 20 mM NaF in the extraction mixture. The chilled supernatant was brought to 20 % saturation with solid $(NH_4)_2SO_4$, centrifuged at 10 000 *g* for 10 min and the precipitate discarded. The supernatant was saturated to 30 % with $(NH_4)_2SO_4$,

centrifuged and the precipitate dissolved in 30 cm^3 of the buffer and labelled Dd_1. The resulting supernatant was saturated to 40 % with $(NH_4)_2SO_4$ and centrifuged. The precipitate was discarded and the supernatant brought to 50 % saturation and the mixture centrifuged. The precipitated protein was dissolved in 30 cm^3 of 0.1 M citrate buffer pH 6.5 and labelled Dd_2. The enzyme fractions were separately purified, using $(NH_4)_2SO_4$ gradient solubilization (Oluoha and Ugochukwu 1991), starch adsorption (Burr and Nelson 1975) and ion exchange chromatography (Oluoha 1990). The fractions from DEAE-Sephadex showing phosphorylase activity were pooled, centrifuged and protein precipitated with 30 % $(NH_4)_2SO_4$ saturation for Dd_1 and 50 % saturation for Dd_2. The precipitated proteins were dissolved in 40.0 cm^3 of 0.1 M citrate buffer pH 6.5 for Dd_1 and 6.2 for Dd_2 and used as purified enzymes.

Analytical methods: Protein was assayed using protein-dye binding method previously described (Oluoha 1990). Bovine serum albumin was used as the standard protein. Sodium was estimated using flame photometer, while ammonium ions were determined employing phenol-hypochlorite/nitroprusside reaction (Varley 1969).

Assay of phosphorylase activity: Phosphorylase activity was assayed in the direction of starch synthesis by P_i release (Oluoha 1990) and P_i released was assayed using malachite green dye-binding method (Hohenwalline and Winner 1973). Activity of phosphorylase was also assayed using production of iodine-staining glucan (Oluoha and Ugochukwu 1991) except that 5 mg of amylopectin was used instead of soluble starch. Purified enzymes and G-1-P were further treated with glucoamylase and norite/celite (Oluoha 1990) to remove contaminating primer and activity determined. Phosphatase activity was assayed using p-nitrophenyl-phosphate (Oluoha 1990). In direction of starch degradation, phosphorylase activity was assayed by following the rate of disappearance of starch-iodine complex (Singh and Sanwal 1976). Amylase activity was similarly assayed except the omission of phosphate buffer.

pH optimum: pH optimum of each enzyme form was determined in 0.1 M citrate buffer in pH range of 5.0 to 7.5 using the assay methods. Enzyme forms were also incubated for 10 min at each pH and the activity assayed at pH optimum found for each enzyme fraction. To determine the amino acid residue at the active site, the enzyme forms were incubated at each pH at saturating substrate levels and the activities assayed.

Starch extraction: 200 g of peeled yam tubers were homogenized in 1 dm^3 of distilled water and starch extracted by squeezing the homogenate through three layers of muslin. The starch in the supernatant was allowed to settle and the liquid decanted. The starch was washed with more distilled water and centrifuged. The precipitated starch was dried at 70 °C until a constant mass was obtained. The dried starch was ground to fine powder and stored in vacuum dessicator. Amylopectin was prepared as described by Matheson and Richardson (1978).

Identification of pyridoxal-5'-phosphate: Solutions of phosphorylase forms at pH 7.0 and pH 13.0 containing 5.0 mg protein cm^{-3} were scanned between 300 nm and

500 nm using *Pye Unican SP 1800* spectrophotometer. The removal of P-5'-P and reconstitution of deformed enzymes were carried out according to the method of Shimomura *et al.* (1980) using semicarbazide as an aldehyde reagent.

Gel electrophoresis: Polyacrylamide disc gel electrophoresis of purified yam phosphorylase was performed at various pH values and gel concentrations containing 2 % amylopectin using the method of Hedrick and Smith (1968) at constant current of 5 mA per tube. The activity of separated enzymes was detected by incubating the gels in 50 mM G-1-P for 1 h, rinsing in buffer and staining with dilute iodine. The enzyme proteins were also stained with *Coomassie Blue R 250*. Molecular mass of each phosphorylase form was determined employing disc gel electrophoresis, SDS gel electrophoresis (Weber and Osborne 1969) and gel filtration (Oluoha 1990) using the following standard proteins: Ovalbumin (45 000), bovine serum albumin (68 000), hexokinase (96 000) and catalase (240 000).

Table 1. Summary of purification steps

	Total protein [mg]	Total activity [nkat]	Specific activity [nkat mg^{-1} (protein)]	Purification (fold)	Yield [%]
Crude extract	551.70	2000.0	3.63	1.00	100.0
0 - 20 % $(NH_4)_2SO_4$ precipitation (supernatant)	200.00	1500.0	7.50	2.06	75.0
$(NH_4)_2SO_4$ gradient solubilization (Dd_1)	5.00	750.0	150.00	41.40	37.5
Starch adsorption and DEAE-Sephadex chromatography (Dd_1)	2.80	700.0	250.00	68.90	35.0
$(NH_4)_2SO_4$ gradient solubilization (Dd_2)	8.00	1000.0	125.00	34.50	50.0
Starch adsorption and DEAE-Sephadex chromatography (Dd_2)	2.76	800.0	290.00	80.00	40.0

Results

α-Glucan phosphorylase extracted from *Dioscorea dumentorum* tuber was fractionated using $(NH_4)_2SO_4$ precipitation and further purified (Table 1). Two forms of the enzyme were obtained and designated as Dd_1 and Dd_2. Dd_1 was purified 68.9 fold with the yield of 35 % and specific activity of 250 nkat mg^{-1} protein, while 80 fold purification of Dd_2 gave specific activity of 290 nkat mg^{-1} protein and a recovery of 40 %. The enzyme fractions were stable at room temperature but lost about 80 % of their activity after two days. In frozen state, the enzyme forms remain viable for over 5 months. In order to prevent proteolytic degradation and denaturation, 0.2 % thymol was added to the enzyme solutions and they were kept at 0 °C. In ammonium sulphate gradient solubilization method, Dd_1 eluted between 24 % and 28 % $(NH_4)_2SO_4$ saturation while Dd_2 was solubilized between 44 % and

48 % $(NH_4)_2SO_4$. Dd_1 eluted between O.11 M and 0.16 M NaCl from ion exchange column, while Dd_2 eluted between 0.12 M and 0.15 M NaCl. The purified enzymes separated in polyacrylamine gels and stained in dilute iodine solution showed single bands of phosphorylase activity that corresponded to the protein bands stained with Coomassie blue.

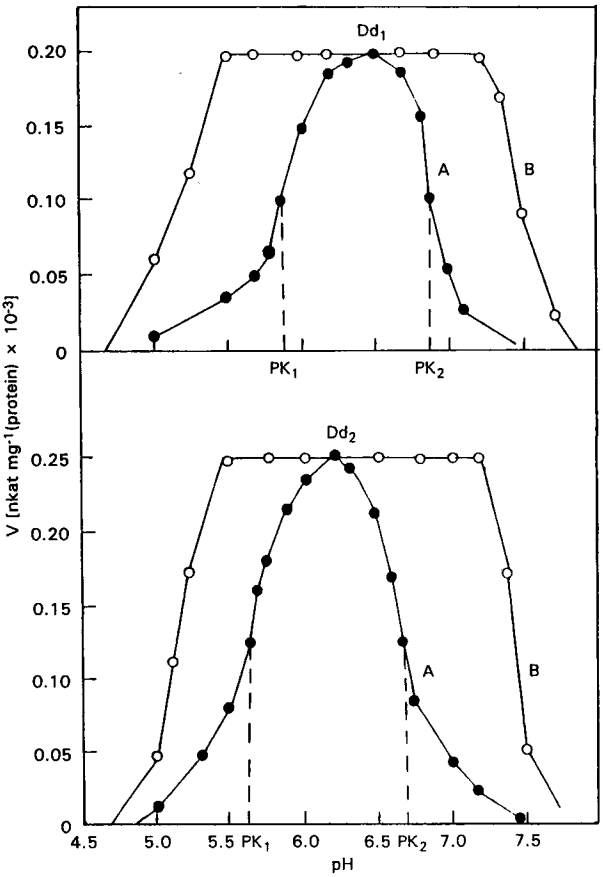

Fig. 1. Photophosphorylase activity in direction of starch synthesis assayed by P_i release using malachite green binding method as affected by pH.
Curve A: Optimum pH determination. Enzyme activity was assayed at various pH values as indicated.
Curve B: pH stability curve. Enzyme forms were incubated for 10 min at various pH and activity assayed at optimum pH found for each enzyme fraction. Each point represents the mean of 4 determinations. pK_a values extrapolated as indicated were the mean of 4 determinations.

pH optima obtained for Dd_1 and Dd_2 were 6.5 and 6.2, respectively (Fig. 1). When the enzyme fractions were incubated at different pH values between 5.5 and 7.2 and the activities assayed at pH optimum found for each enzyme form, full activities were recovered (Fig. 1). However, below pH 5.5 and above pH 7.2, full activities of the enzymes were not recovered, which shows irreversible inactivation. The pK_a values

extrapolated from Fig. 1 were 5.9 ± 0.1 and 6.95 ± 0.17 for Dd_1 and 5.65 ± 0.11 and 6.7 ± 0.2 for Dd_2. Temperature optima found for Dd_1 and Dd_2 were 35 °C and 40 °C, respectively, with activation energies of 30.9 kJ mol^{-1} K^{-1} for Dd_1 and 28.13 kJ mol^{-1} K^{-1} for Dd_2. Both enzyme forms were irreversibly denatured above 45 °C. In order to

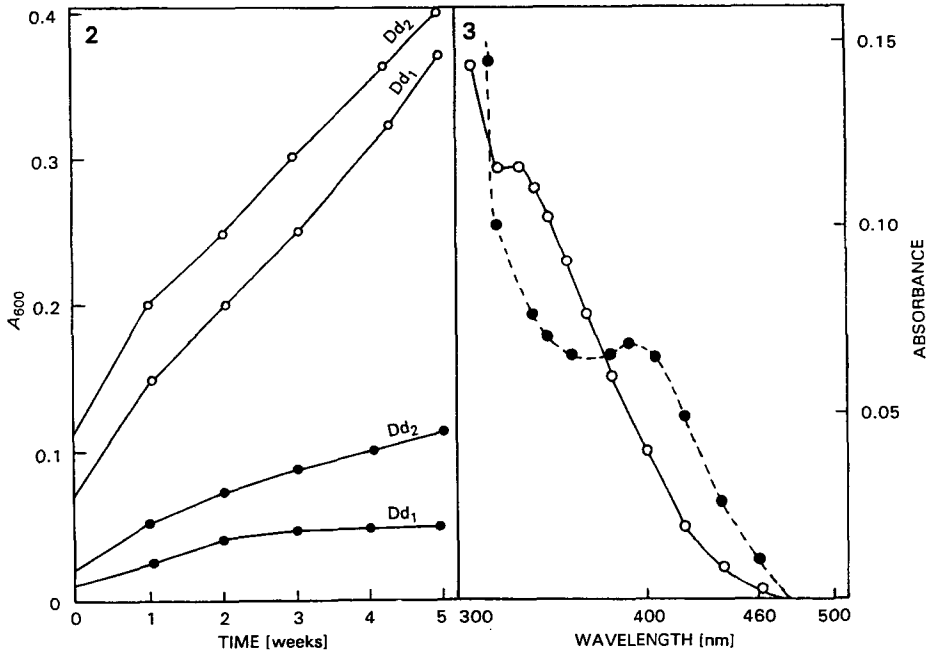

Fig. 2. Activity of phosphorylase during tuber formation (*closed circles*) and sprouting (*open circles*). The tubers were removed at one week intervals, the enzyme forms separated and activities determined using iodine staining glucan method. Each point represents the mean activity of 4 determinations. The significance of activity differences was evaluated ($P < 0.01$).

Fig. 3. Spectral identification of pyridoxal phosphate in *D. dumentorum* phosphorylase.
Solutions of the purified enzyme forms containing 5.0 mg protein per cm^3 at pH 7.0 (*open circles*) and pH 13.0 (*closed circles*) were scanned between 300 nm and 500 nm.

ascertain if yam phosphorylase can synthesize starch in the absence of added primer, all traces of contaminating starch were removed from the enzymes and G-1-P. When the enzymes were assayed in the presence of purified G-1-P as the only substrate, the enzymes were active. This indicates that the phosphorylase forms could also synthesize polyglucan without added primer. The activity of Dd_1 was constant during tuber formation, while that of Dd_2 increased significantly during the same period ($P < 0.01$). However, the activities of both enzymes increased sharply during sprouting (Fig. 2). This suggests that Dd_2 may be involved in starch synthesis.

Solutions of purified enzymes at pH 7.0 and protein concentration of 5 mg cm^{-3} showed adsorption peaks at 330 nm. This disappeared at pH 13.0 when a new peak became evident at 388 nm (Fig. 3). These results indicate that the two enzyme forms

contain P-5'-P as their prosthetic group. Addition of further quantities of the coenzyme had no effect on the activities of the yam enzymes. Removal of P-5'-P with semicarbazide inactivated the phosphorylase fractions. However, the activity of the enzyme was recovered on incubation with P-5'-P with semicarbazide inactivated phosphorylase fractions. This indicates that P-5'-P is essential for the activity of yam phosphorylase.

The results of disc gel electrophoresis at various gel concentrations indicate that the enzyme forms exist as size and charge isomers. The M_r mass found for the yam phosphorylase forms using various methods are shown below:

Method	Dd_1	Dd_2
Gel electrophoresis	105 000	198 000
SDS gel electrophoresis	98 000	96 000
Gel filtration	104 000	194 000
Average molecular mass	102 000 ± 5 000	195 000 ± 7 000

These data are the means of 4 determinations ± the standard errors of mean.

The K_m obtained for Dd_1 and Dd_2 in synthetic direction are 2.0 mM and 1.5 mM respectively (Table 2), while the V_{max} are 200.0 nkat mg^{-1}(protein) and 259.7 nkat mg^{-1}(protein), respectively. This shows that Dd_2 is relatively more active in this direction and shows more affinity for G-1-P than Dd_1. The presence of ATP, ADP, Na_2SO_4 and IAA increased the K_m while the V_{max} decreased, indicating mixed-type inhibition for both enzymes. The K_i for both phosphorylase forms are shown in Table 2. ADP and IAA showed the same inhibition effect on Dd_1. In the presence of ADP-glucose, the K_m for Dd_1 increased while V_{max} decreased, showing mixed-type inhibition. However, ADP-glucose non-competitively inhibited Dd_2 as indicated by unaltered K_m and decreased V_{max}. When the activities were assayed in the presence of Mg^{2+}, AMP and Ca^{2+}, the K_m of both enzymes decreased, while the V_{max} increased. This is consistent with enzyme activation. The two cations exerted the same activation effect on Dd_2. The K_m in direction of starch degradation obtained for Dd_1 and Dd_2 are 1.26 mM and 0.9 mM P_i, respectively with V_{max} of 2857.0 nkat mg^{-1}(protein) for Dd_1 and 2531.6 nkat mg^{-1}(protein) for Dd_2 (Table 3). These show that Dd_1 is more active in this direction. Inhibition of the enzyme forms by ADP-glucose and Na_2SO_4 increased their K_m while V_{max} decreased. This indicates mixed-type inhibition for the enzymes. Assaying the activity of Dd_1 in the presence of ATP and ADP gave K_m of 2.17 mM and 1.0 mM P_i respectively, while the V_{max} obtained were 2000.0 nkat mg^{-1}(protein) and 2083.3 nkat mg^{-1}(protein) respectively. These results indicate that ATP is a mixed-type inhibitor for Dd_1 while ADP uncompetitively inhibited it. Activity of Dd_2 in the presence of ATP increased the K_m but the V_{max} remained unchaged. However, the presence of ADP increased the K_m with decreased V_{max}. These results are consistent with ATP being competitive inhibitor for Dd_2 while ADP a mixed-type inhibitor. Both enzymes were activated by AMP, Ca^{2+}, and IAA.

Table 2. Kinetic constants of *D. dumentorum* phosphorylase in direction of synthesis. Phosphorylase activity was assayed in direction of glucan synthesis. P_i released was determined using malachite green method as described under materials and methods. Each value is the mean of 4 determinations ± standard error of mean and Dd_2 and Dd_1 activity differences were computed ($P < 0.05$).

Addition to assay	K_m [mM]	V_{max} [nkat mg^{-1} prot.]	K_i [mM]
	Dd_1		
None	2.00 ± 0.02	200.00 ± 5.0	-
ATP (2.0 mM)	2.94 ± 0.09	161.30 ± 1.7	4.25 ± 0.11
ADP (2.0 mM)	2.70 ± 0.10	168.06 ± 5.0	5.71 ± 0.20
IAA (2.0 mM)	2.70 ± 0.08	168.06 ± 5.0	5.71 ± 0.25
ADP-glucose (2.0 mM)	2.22 ± 0.04	134.00 ± 2.0	4.06 ± 0.20
Na_2SO_4 (5.0 mM)	4.40 ± 0.10	90.00 ± 3.0	3.75 ± 0.15
Mg^{2+} (3.0 mM)	2.40 ± 0.07	246.90 ± 10.0	-
Ca^{2+} (2.0 mM)	2.10 ± 0.10	253.16 ± 13.0	-
AMP (0.2 mM)	1.29 ± 0.03	384.61 ± 20.0	-
	Dd_2		
None	1.50 ± 0.05	259.70 ± 3.9	-
ATP (2.0 mM)	2.00 ± 0.01	181.80 ± 4.0	3.53 ± 0.10
ADP (2.0 mM)	1.86 ± 0.09	222.20 ± 5.1	8.33 ± 0.20
IAA (2.0 mM)	1.92 ± 0.10	196.10 ± 4.5	6.20 ± 0.12
ADP-glucose (2.0 mM)	1.50 ± 0.06	152.40 ± 3.0	1.68 ± 0.03
Na_2SO_4 (5.0 mM)	3.90 ± 0.20	100.00 ± 5.0	3.10 ± 0.08
Mg^{2+} (3.0 mM)	1.47 ± 0.08	294.10 ± 6.0	-
Ca^{2+} (2.0 mM)	1.40 ± 0.07	294.10 ± 7.3	-
AMP (0.2 mM)	1.47 ± 0.09	454.50 ± 12	-

Bisubstrate kinetics: The K_m intercepts and slopes for Dd_1 were affected by amylopectin concentration (Fig. 4A). The K_m for Dd_2 was independent of variation in amylopectin (Fig. 4B). Symmetrical results were obtained when amylopectin concentration was varied at fixed levels of G-1-P. The results suggest sequential reaction mechanism for both enzyme fractions. Fig. 5 shows the effect of various fixed starch concentrations when P_i was varied in direction of phorphorolysis for the phosphorylase forms. The lines of reciprocal plots were parallel with constant slopes. 1/V intercept replots were linear. Symmetrical results were obtained when starch was varied at fixed P_i.

Discussion

Two forms of phosphorylase were isolated from *D. dumentorum* tuber and this contrasts with one from *D. rotundata* tuber (Oluoha 1990), four from maize (Tsai and Nelson 1969), nine from potato tubers (Gerbrandy and Doorgeest 1972) and five from banana fruits (Singh and Sanwal 1976). They showed hyperbolic saturation curves in the presence and absence of the modifiers.

Table 3. Kinetic constant of *D. dumentorum* phosphorylase in direction of phosphorolysis. Phosphorylase activity was assayed in direction of starch degradation as detailed under materials and methods section. Each value represents the mean of the values of 4 determinations ± standard error of mean. The significance of enzyme activity differences were evaluated ($P < 0.01$).

Addition to assay	K_m [mM]	V_{max} (nkat mg^{-1} protein)	K_i [mM]
	Dd_1		
None	1.26 ± 0.04	2857.00 ± 80	-
ATP (2.0 mM)	2.17 ± 0.10	2000.00 ± 70	2.76 ± 0.10
ADP (2.0 mM)	1.00 ± 0.03	2083.00 ± 100	5.40 ± 0.15
ADP-glucose (2.0 mM)	2.40 ± 0.12	1980.10 ± 75	1.06 ± 0.03
Na_2SO_4 (5.0 mM)	1.75 ± 0.04	1785.70 ± 60	8.33 ± 0.25
Mg^{2+} (3.0 mM)	0.95 ± 0.03	3030.30 ± 90	-
Ca^{2+} (2.0 mM)	1.10 ± 0.06	3030.30 ± 100	-
IAA (2.0 mM)	0.78 ± 0.02	3225.80 ± 100	-
AMP (0.2 mM)	0.80 ± 0.03	3125.00 ± 78	-
	Dd_2		
None	0.90 ± 0.03	2531.60 ± 60	-
ATP (2.0 mM)	3.38 ± 0.01	2531.60 ± 80	0.75 ± 0.02
ADP (2.0 mM)	1.25 ± 0.03	2247.20 ± 100	5.14 ± 0.11
ADP-glucose (2.0 mM)	1.58 ± 0.06	2127.60 ± 95	2.64 ± 0.08
Na_2SO_4 (5.0 mM)	3.20 ± 0.10	2298.85 ± 92	1.96 ± 0.05
Mg^{2+} (3.0 mM)	0.83 ± 0.02	3174.60 ± 110	-
Ca^{2+} (2.0 mM)	0.87 ± 0.04	3030.30 ± 120	-
AMP (0.2 mM)	0.72 ± 0.025	2941.17 ± 85	-
IAA (2.0 mM)	0.94 ± 0.03	2857.14 ± 130	-

Although Mg^{2+} has been reported to activate maize enzyme (Tsai and Nelson 1969), Burr and Nelson (1975) found this salt inhibitory to purified major sweet maize isoenzyme. Activation of phosphorylase from *D. cayenensis* by Mg^{2+} and Ca^{2+} has been reported (Oluoha and Ugochukwu 1991), while *D. rotundata* enzyme was inhibited (Oluoha 1990). In this study, the two cations activated the phosphorylase forms. Na_2SO_4 has been reported to activate maize phosphorylase (Burr and Nelson 1975) while that of *D. cayenensis* was inhibited (Oluoha and Ugochukwu 1991). However, it has been reported that concentration lower than 12.5 mM had no effect on *D. rotundata* phosphorylase but the enzyme was inhibited at higher concentration (Oluoha 1990). In this study, both enzymes were inhibited at all concentrations. Dd_2 was more sensitive to this inhibition.

Nucleotides have been reported to have variable effects on phosphorylase from plants. For example, ATP and AMP were found to exert no effect on isoenzyme from banana fruits (Singh and Sanwal 1976) and sweet maize (Lee and Braun 1973), to activate mistletoe leaf phosphorylase (Khanna *et al.* 1971), to inhibit maize endosperm enzyme (Tsai and Nelson 1969) and barley grain phosphorylase (Baxter and Duffus 1973). c-AMP has been found to inhibit barley grain enzyme (Baxter and

Duffus 1973). In this study, ATP, ADP and ADP-glucose inhibited the enzymes, while AMP activated them. c-AMP had no effect on the enzyme forms.

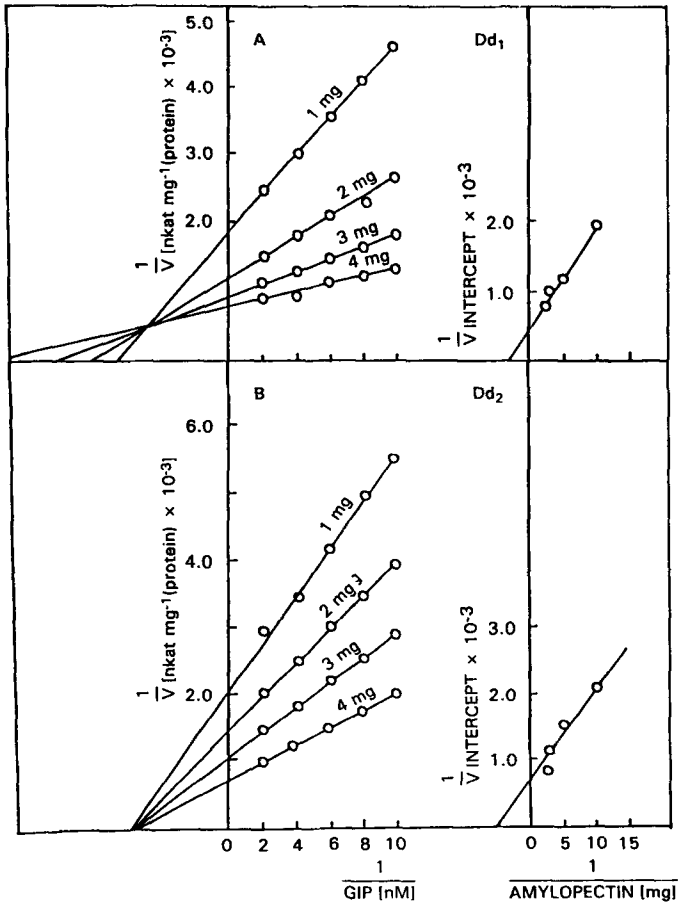

Fig. 4. Effect of fixed levels of amylopectin on *D. dumentorum* phosphorylase forms (Dd_1 - *A*, Dd_2 - *B*) in direction of glucan synthesis. Enzyme activity was assayed by varying concentrations of G-1-P as indicated with fixed levels of amylopectin. Each point represents the mean of the results from 4 samples. The standard error of the mean was less than 2 %.

ADP-glucose has been reported to inhibit phosphorylase from some plant tissues (Matheson and Richardson 1978). Although Dd_2 showed about 1.4 times more affinity for P_i than Dd_1, the activity of Dd_1 is higher than that of Dd_2. This indicates that the degree of affinity for P_i does not dictate the rate of glucan degradation. It is also found that Dd_1 is more sensitive to inhibition while Dd_2 is more sensitive to activation (Table 3). The reverse is the case in synthetic direction. The K_m values reported here (Tables 2 and 3) are considerably lower than those reported for phosphorylase from maize (Burr and Nelson 1975), pea seeds (Matheson and

Richardson 1978), *D. rotundata* tubers (Oluoha 1990), and *D. cayenensis* (Oluoha and Ugochukwu 1991).

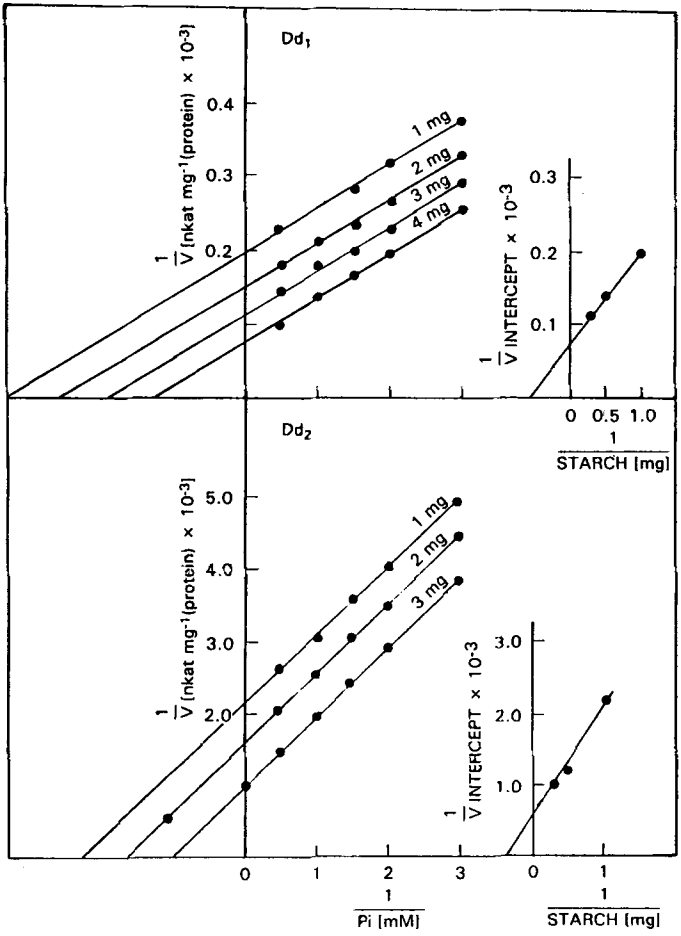

Fig. 5. Primary plots of 1/V versus $1/P_i$ at fixed levels of starch for *D. dumentorum* phosphorylase forms in direction of starch degradation. Enzyme activity was assayed by varying the concentrations of inorganic phosphate at fixed levels of starch. Each point represents the mean value of the results of 4 samples. The standard error of the mean was less than 1.2 %.

Bisubstrate studies in direction of phosphorolysis indicate double displacement mechanism for *D. dumentorum* enzymes (Fig. 5), in which the leading substrate binds to the enzyme with a release of the product before the second substrate binds with the release of the final product. In direction of synthesis, the enzyme forms indicated sequential reaction mechanism in which the substrates must bound to the enzyme to form a ternary complex before any products are released. Similar sequential mechanism has been reported for potato tuber phosphorylase (Gold *et al.*

1971) and pea seed enzymes (Matheson and Richardson 1978) when assayed in both synthetic and degradation directions.

The two enzyme forms differ with respect to pH optima. The pH 6.2 found for Dd_2 is lower than the values reported for *D. rotundata* phosphorylase (Oluoha 1990) and *D. cayenensis* enzymes (Oluoha and Ugochukwu 1991) but the values for both enzymes are higher than that found for pea seed phosphorylase forms (Matheson and Richardson 1978). The phosphorylase forms also differ in their optimum temperatures. These optimum temperatures are lower than those reported for banana fruit phosphorylase (Singh and Sanwal 1976) and mistletoe leaf enzymes (Khanna *et al.* 1971). pH stability studies indicate that the decline in activity of the enzyme forms between pH 5.5 and pH 7.5 was due to unsuitable ionic forms of the enzyme or substrate or both and not due to denaturation but below pH 5.5 and above pH 7.5 the enzymes were denatured. Instabilities of pea seed enzymes at pH 5.0 (Matheson and Richardson 1978) and potato phosphorylase at pH 5.5 have been reported (Shimomura *et al.* 1980).

P-5′-P has been identified as the prosthetic group of yam phosphorylase forms using spectral analysis (Lee and Braun 1973, Matheson and Richardson 1978) and this is essential for enzyme activity. Excess amount of this coenzyme has been reported to inhibit pea seed phosphorylase (Matheson and Richardson 1978) and some other enzymes that do not contain it as well as some in which it is a cofactor (Avramic-Zikic and Madsen 1972, Cozzani *et al.* 1974).

Activity of Dd_2 increased during yam tuber formation and this strongly suggests that Dd_2 may play a synthetic role. Similar result has been reported for phosphorylase from developing wheat grains and it was suggested that the enzyme was important in grain starch synthesis in wheat during the early stages of grain filling (Konrad and Judel 1981). The finding that Dd_2 is more active in direction of starch synthesis supports the possible involvement of this fraction in starch synthesis.

The subunit M_r determined for Dd_2 and Dd_1 using SDS gel electrophoresis are 96 000 and 98 000 respectively, while the intact M_r found for Dd_2 is 195 000 and for Dd_1 102 000. These results suggest a dimer for Dd_2 and a monomer for Dd_1. The pK_a values obtained from pH/activity studies (Fig. 1) vary from 5.65 to 6.85 and this is within the pK_a values of 5.6 to 7.0 of histidine residue in protein. Histidine may therefore be the amino acid around the active site which might be involved in catalytic activity.

In conclusion, two forms of phosphorylase have been isolated from *D. dumentorum* tuber and purified to apparent homogeneity. Dd_1 has been suggested to have degradative function while Dd_2 appears to play a synthetic role. P-5′-P has been identified as prosthetic group of yam phosphorylase and this is essential for enzyme activity. Histidine is indicated as the amino acid that might be involved in catalysis. Bisubstrate studies indicate ping pong mechanism in direction of phosphorolysis while in synthetic direction sequential mechanism operates.

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