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COMPARISON OF STRUCTURE AND KINETIC MECHANISM OF PHOSPHORYLASE FORMS ISOLATED FROM WATER YAM (*DIOSCOREA ALATA*) AND WHITE YAM (*DIOSCOREA ROTUNDATA*) TUBERS

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KeyWord Index—*Dioscorea alata*; *Dioscorea rotundata*; Dioscoreacae; phosphorylase; yams; kinetic mechanism; structure; pH function.

Abstract—Two forms of phosphorylase from *Dioscorea alata* and one from *D. rotundata* were purified using various techniques of chromatography and electrophoresis. The molecular masses obtained for fractions I and II of *D. alata* phosphorylase were 120 000 ± 3000 and 170 000 ± 6800, respectively, while the molecular mass obtained for the phosphorylase from *D. rotundata* was 188 000 ± 10 000. SDS gel electrophoresis indicated two subunit structures for fraction II and *D. rotundata* enzyme, while fraction I is a monomeric protein with a single polypeptide chain. Kinetic data are consistent with a degradative role of phosphorylase from white yam, while water yam tuber phosphorylase may play a synthetic role. Bisubstrate kinetics showed a double displacement mechanism in the direction of phosphorolysis for *D. alata* phosphorylase forms except fraction II, which indicated a single displacement mechanism only when P_i was varied at fixed starch concentrations. In the direction of glucan synthesis, a sequential mechanism was indicated for all the yam enzymes studied. However, *D. rotundata* phosphorylase differs from that of *D. alata* enzyme in that the latter showed a ping-pong mechanism only in the direction of starch degradation. Histidine has been identified as an amino acid residue that might be involved in catalysis.

INTRODUCTION

There have been a number of studies on the kinetic properties of starch phosphorylase (E.C. 2.4.1.1) from plants such as potato tubers [1–4], hybrid maize [5, 6], sweet corn [7], banana fruits [8, 9], pea seeds [10] and yam tubers [11–13], and the apparent K_m values reported were variable. However, very little work has been done on the kinetic mechanism of plant phosphorylase except for that emanating from studies on potato enzyme [3] and pea seed phosphorylase [10]. The results indicated a sequential mechanism when assayed in both synthetic and degradative directions. Subsequent studies showed that the reaction catalysed by potato phosphorylase is a random-order equilibrium bibi mechanism [3]. Recently, phosphorylase forms from *D. dumentorum* were characterized and the kinetic mechanism studied [13]. The results showed that the yam tuber enzymes had a different kinetic mechanism from that of other plant phosphorylases so far examined and that the yam tuber contains both monomeric and dimeric phosphorylase molecules. This investigation describes a study of the kinetic mechanism of *D. alata* phosphorylase and compares it with that exhibited by phosphorylase from white yam tuber.

RESULTS AND DISCUSSION

Discorea alata phosphorylase fractions I and II obtained from ammonium sulphate gradient solubilization were further purified using starch adsorption, gel filtration and DEAE-Sephadex chromatography [12]. The final specific activity obtained for fraction I (F_1) and fraction II (F_2) were 120.3 nkat mg^{-1} protein and 200 nkat mg^{-1} protein, respectively. *Discorea rotundata* phosphorylase was similarly purified [11] except for the omission of the ammonium sulphate gradient solubilization to give a preparation with a specific activity of 300 nkat mg^{-1} protein. Disc gel electrophoresis at various gel concentrations, pH values and using different enzyme concentrations showed single bands of phosphorylase activity when the gels were stained in dilute iodine solution, and these coincided with the protein bands obtained by staining some gels in Coomassie blue. Moreover, each enzyme eluted from the chromatographic columns with symmetrical peaks with constant specific activity. These preparations seem to be homogeneous.

Figure 1 shows the effect of fixed levels of amylopectin on *D. alata* phosphorylase forms when glucose-1-phosphate (G-1-P) was varied. The lines of reciprocal plots

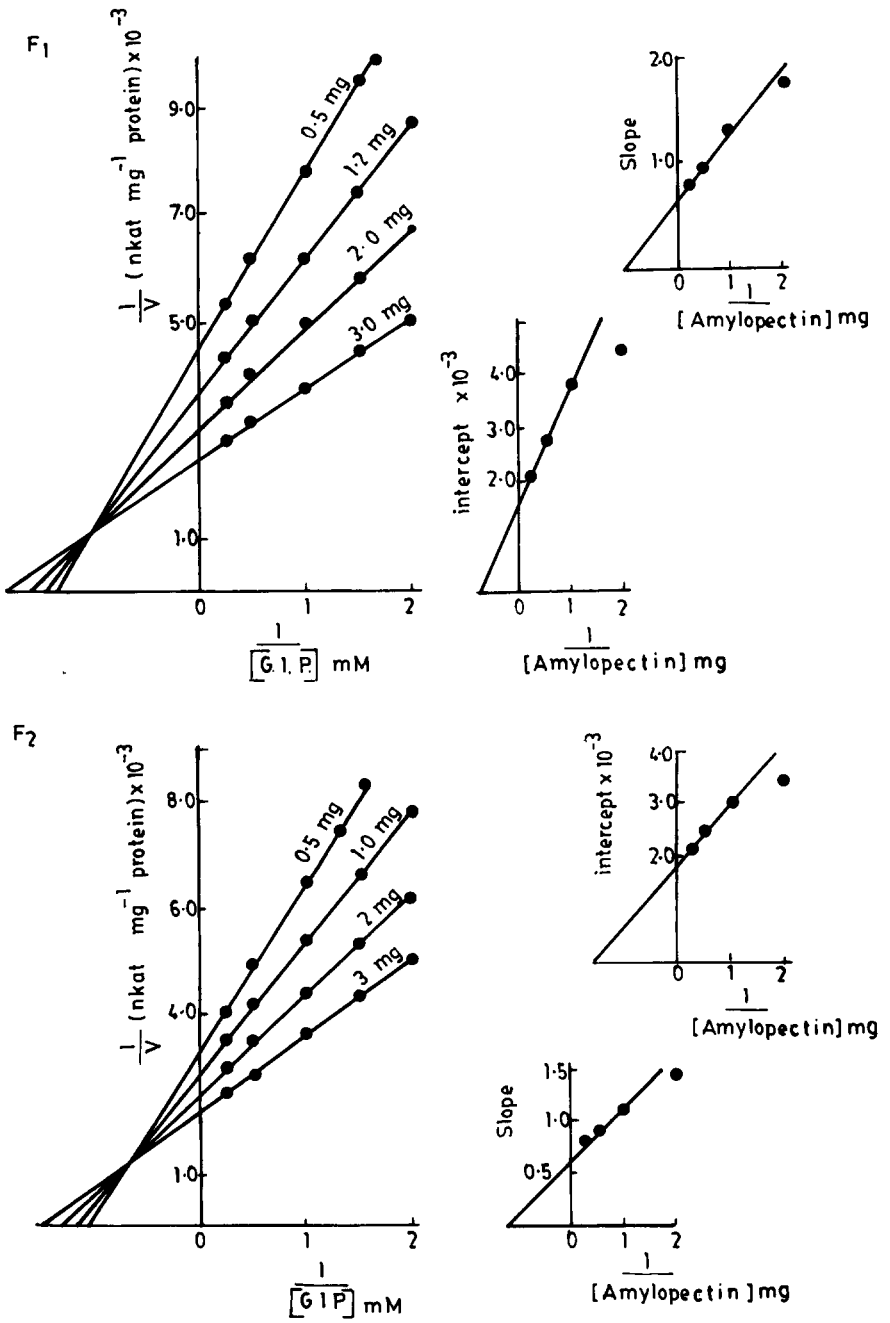

Fig. 1. The effect of fixed levels of amylopectin on *D. alata* F_1 and F_2 in the direction of glucan synthesis when G-1-P was varied.

intersected at a point on the left of the vertical axis and above the $\frac{1}{S}$ axis for both enzyme fractions. The intercepts and slopes were affected as the amylopectin concentrations were altered. Symmetrical results were obtained when the amylopectin concentration was varied at fixed levels of G-1-P. Figure 2 shows the effect of fixed levels of amylopectin when G-1-P was varied for *D. rotundata* phosphorylase in the direction of glucan synthesis. The lines of reciprocal plots intersected on the $\frac{1}{S}$ axis. The apparent K_m values were independent of amylopectin concentrations. Symmetrical results were obtained when amylopectin was varied at fixed levels of G-1-P. These results are characteristic of a sequential reaction mechanism for all the yam enzymes in which both substrates must be added to the enzymes before any products can be released. This obeys the general Alberty rate equation where

$$v = \frac{V_{max}\,(A)\,(B)}{K_{m_B}(A) + K_{m_A}(B) + K_{s_A} \times K_{m_B} + (A)\,(B)}$$

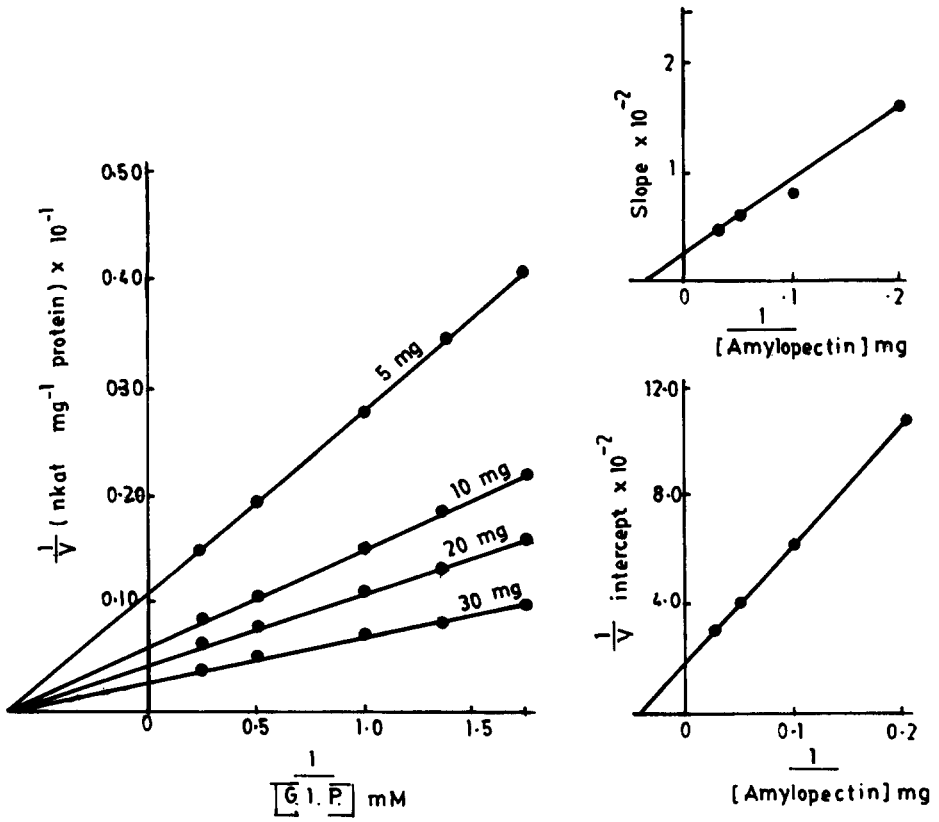

Fig. 2. The effect of fixed levels of amylopectin on *D. rotundata* phosphorylase when G-1-P was varied.

and refers to the synthetic reaction in which A = G-1-P, and B = polysaccharide. In the degradation reaction A = Pi. Here V_{max} takes its usual meaning. $K_{m_A} = K_m$ for A when B is saturating; $K_{m_B} = K_m$ for B when A is saturating. K_{s_A} = dissociation constant for $E + A \rightleftharpoons AE$.

A single displacement rapid equilibrium random bibi mechanism has been reported for potato phosphorylase [3]. Also reported is a sequential mechanism for pea seed phosphorylase forms [10], *E. coli* maltodextrin enzyme [14] and other glucose transferring phosphorylase with the exception of sucrose phosphorylase that showed a ping-pong mechanism [15].

The effect of fixed levels of starch on F_1 and F_2 in the direction of phosphorolysis when Pi was varied is shown in Fig. 3. The lines of the reciprocal plots intersected on the left of the vertical axis and slightly above the $\frac{1}{S}$ axis for F_2. The apparent K_m, intercepts and slopes varied as the fixed substrate concentrations, while intercept and slope replots were linear. However, the lines were parallel for F_1 with constant slopes and varied intercepts at fixed levels of starch. The $\frac{1}{V}$ intercept replot was linear. The effect of fixed levels of Pi on F_1 and F_2, when starch was the variable substrate, is shown in Fig. 4. The lines of reciprocal plots were parallel for all the enzyme fractions with constant slopes. The intercepts were affected by changes in fixed Pi concentrations. The $\frac{1}{V}$ intercept replots were linear. These results are consistent with a ping-pong reaction mechanism for the water yam enzymes. The rate equation for the initial reaction is

$$v = \frac{V_{max}\,(A)\,(B)}{K_{m_A}(B) + K_{m_B}(A) + (A)\,(B)}$$

Cleland [16] and Palmer [17] have pointed out that, in using initial velocity patterns alone in interpretation of a ping-pong mechanism, one must be cautious in interpreting an apparently parallel pattern as a double displacement mechanism in the absence of corroborative data. However, the constant slopes obtained from the parallel lines in this study coupled with zero slope replots are consistent with the ping-pong mechanism. It is interesting to note that in phosphorolysis, F_2 differs from F_1 in showing a sequential mechanism only when Pi was varied at fixed starch concentrations (Fig. 3).

Figure 5 shows the effect of fixed levels of starch when Pi was varied for *D. rotundata* enzyme in the degradation direction. The lines of primary plots intersected on the left of the $\frac{1}{V}$ axis and above the $\frac{1}{S}$ axis. The slopes and intercepts were affected by changes in fixed starch concentrations. Symmetrical results were obtained when Pi was the fixed substrate. These results differ from those obtained for *D. alata* phosphorylase forms in showing a sequential mechanism in this direction irrespective of which substrate was varied, but similar to other plant phosphorylases [3, 10]. Although sucrose phosphorylase from *Pseudomonas saccharophile* [15] has been reported

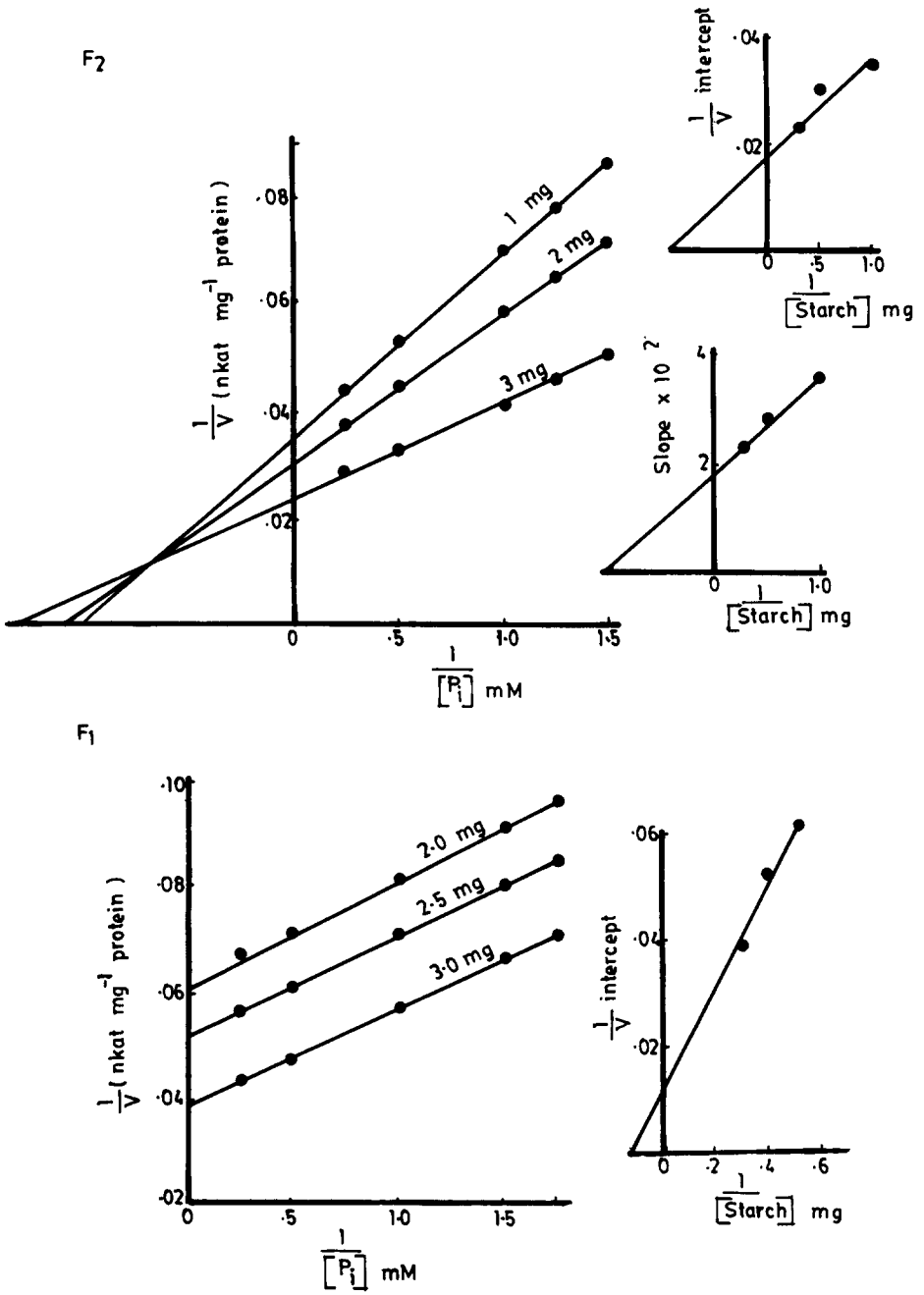

Fig. 3. The effect of starch concentration on double reciprocal plots of *D. alata* fractions I and II in the direction of phosphorolysis when *Pi* was the variable substrate.

to show a ping-pong mechansim, this differs from *D. alata* phosphorylase forms in that the latter showed a double displacement mechanism only in the direction of phosphorolysis, while in the synthetic direction a sequential mechanism was indicated. *Discorea dumentorum* phosphorylase forms showed a similar reaction mechanism to that of *D. alata* enzyme with the exception of F_2, which showed a sequential mechanism only when *Pi* was varied.

One of the most important numerical values used for the description of macromolecules is the molecular mass [18] and this indicates their complexity. In this study the average molecular mass found for F_1, F_2 and *D. rotundata* phosphorylase were 123 000 ± 3000, 170 000 ± 6800 and 188 000 ± 10 000, respectively (Table 1), while the SDS polyacrylamide gel electrophoresis indicated subunit molecular masses of 120 000 for F_1, 86 000 for F_2 and 94 000 for white yam enzyme (Table 1). These results showed a dimer for F_2 and *D. rotundata* phosphorylase with two identical polypeptide chains, while F_1 is a monomer with a single polypeptide chain. The molecular mass of 170 000 found for F_2 is lower than the molecular mass

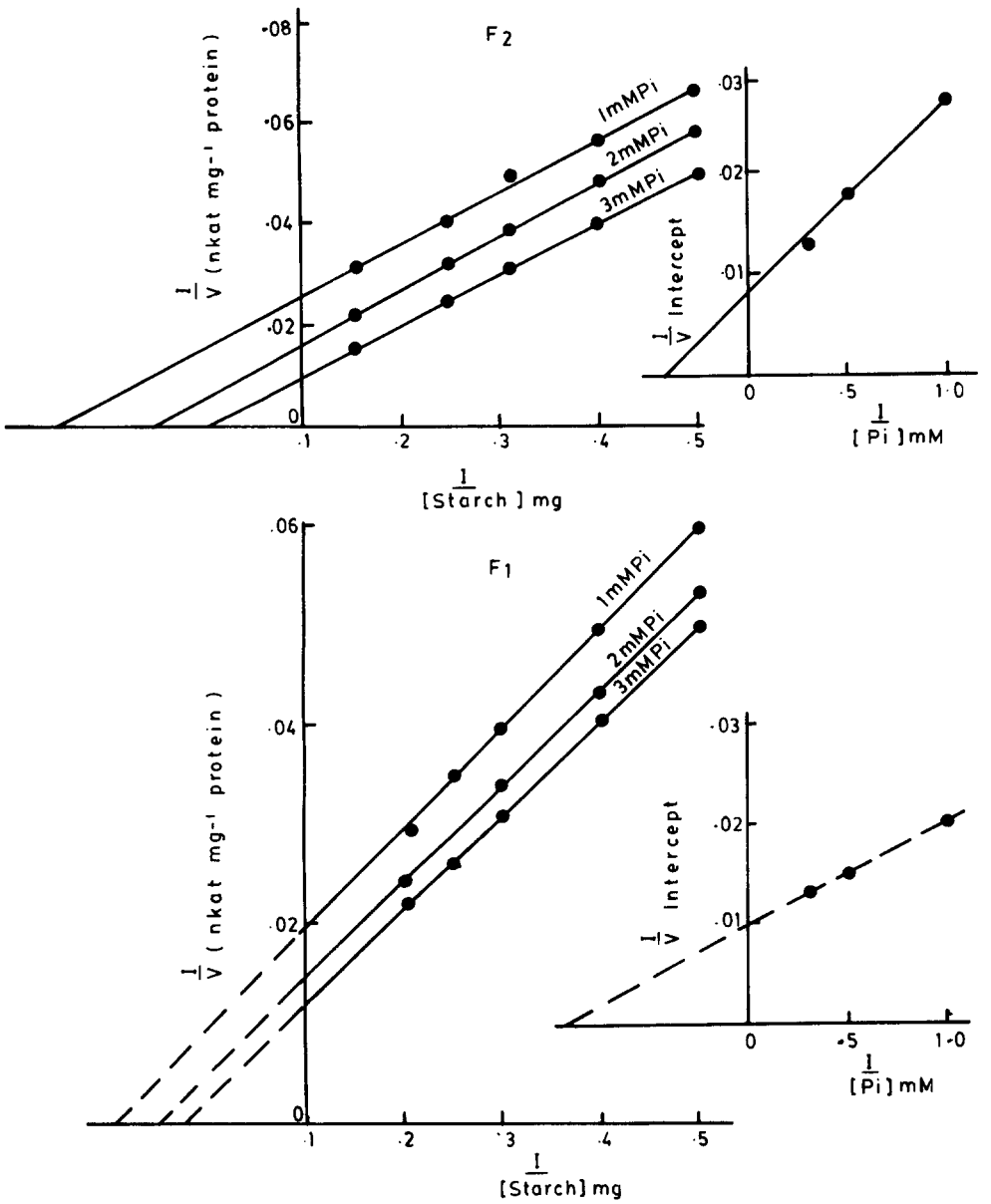

Fig. 4. The effect of fixed levels of *Pi* on double reciprocal plots of *D. alata* phosphorylase fractions I and II in the direction of glucan degradation when starch was varied.

(192 000) obtained for DC_1 of *D. cayenensis* [25] and the molecular mass (196 000) reported for Dd_2 of *D. dumentorum* [13]. A molecular mass of 180 000 to 600 000 has been reported for four of the nine potato tuber phosphorylase fractions [19] while Lee [1] reported a molecular mass of 207 000 for purified potato phosphorylase with two subunit structures. The molecular mass of 600 000 is too high and seems to be an aggregate of three dimer molecules. The discrepancy between the molecular mass reported by the two authors might be due to the nature of the sample and the methods used. Lee [1] used purified enzyme, while Gerbrandy and Doorgeest [19] used crude extract and gel filtration. From the results of structural studies of potato and rabbit phosphorylase, Nakano *et al.* [20] concluded that all phosphorylases so far isolated from various sources are either dimers or tetramers with a subunit molecular mass of 100 000. However, sucrose phosphorylase has been reported to have a molecular mass of 80 000 to 100 000 [15] while that of fraction I of pea seed α-glucan phosphorylase [21] was found to be 135 000, all indicating monomeric enzymes. Fraction II of pea seed phosphorylase is a tetramer with a molecular mass of 490 000 [21]. However, phosphorylase forms isolated from yam tuber studied are mixtures of monomeric and dimeric molecules.

The activity of an enzyme depends on the degree of ionization of the side chain of certain amino acid residues in the protein, and the pK_a values obtained from pH

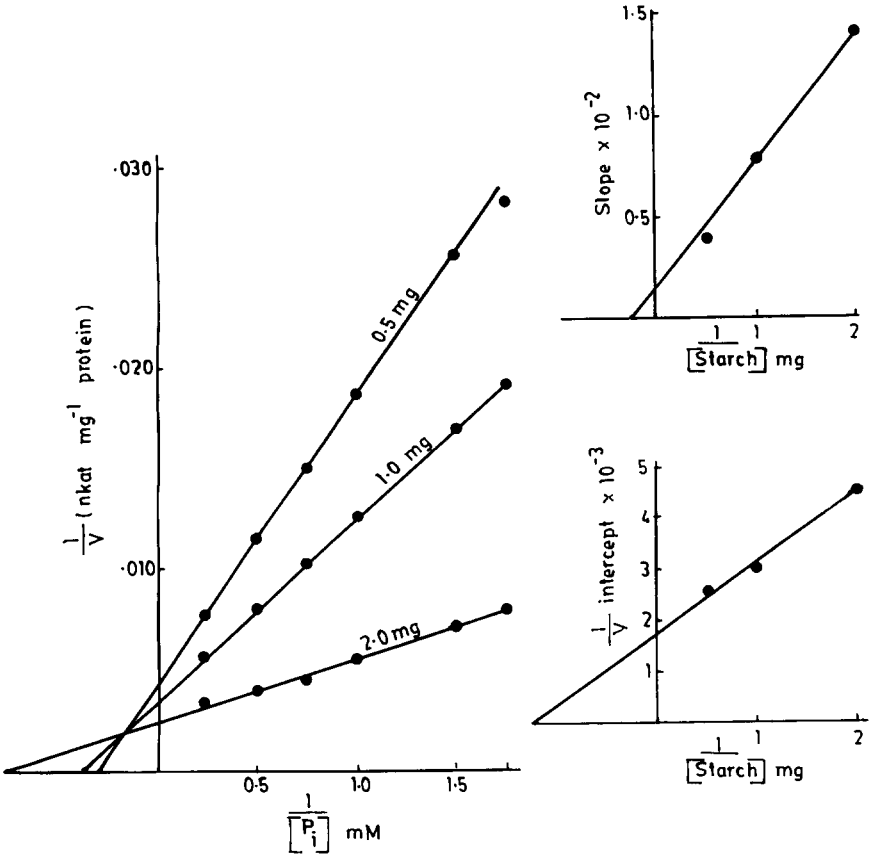

Fig. 5. The effect of fixed levels of starch on *D. rotundata* phosphorylase in the direction of glucan degradation when *Pi* was varied.

Table 1. The molecular mass of phosphorylase from water yam and white yam tubers, determined using various methods

Method used		Molecular mass
D. rotundata phosphorylase		
Gel filtration		189 000
Gel electrophoresis		187 000
SDS gel electrophoresis		94 000 × 2
Average		188 000 ± 10 000
D. alata phosphorylase forms		
Gel filtration	125 000	168 000
Gel electrophoresis	123 000	170 000
SDS gel electrophoresis	121 000	86 000 × 2
Average	123 000 ± 3000	170 000 ± 6800

activity plots of the enzyme when compared with characteristic pK_a values established for acidic and basic groups in protein [22, 23] (Table 2) may suggest the identity of those amino acid residues around the active site [24]. In this study the pK_a values obtained from pH/activity curves for F_1 were 5.8 and 7.2, while the values for F_2 were 5.95 and 7.25. The pK_a values extrapolated for *D. rotundata* enzyme were 5.7 and 7.0. As this is within the range of pK_a of imidazolium group [22, 23] it therefore suggests that histidine might be the amino acid residue around the active site that might be involved in catalytic activity. This supports the suggestion that histidine is involved in catalytic activity of yam phosphorylase [25].

The result of log R_m versus gel concentration plots (Fig. 6) obtained using disc gel electrophoresis in a non-denaturing system for *D. alata* phosphorylase showed

Table 2. Characteristic pK_a values for acidic and basic groups in protein [22]

Groups	pK_a at 25°
α-Carboxyl (terminal)	3.0–3.2
β-Carboxyl (aspartate)	3.0–4.7
γ-Carboxyl (glutamate)	ca 4.4
Imidazolium (histidine)	5.6–7.0
α-Amino (terminal)	7.6–8.4
ε-Amino (lysine)	9.4–10.6
Guanidinum (arginine)	11.6–12.6
Phenolic hydroxyl (tyrisine)	9.8–10.4
Sulphydryl (cysteine)	8.0–9.0

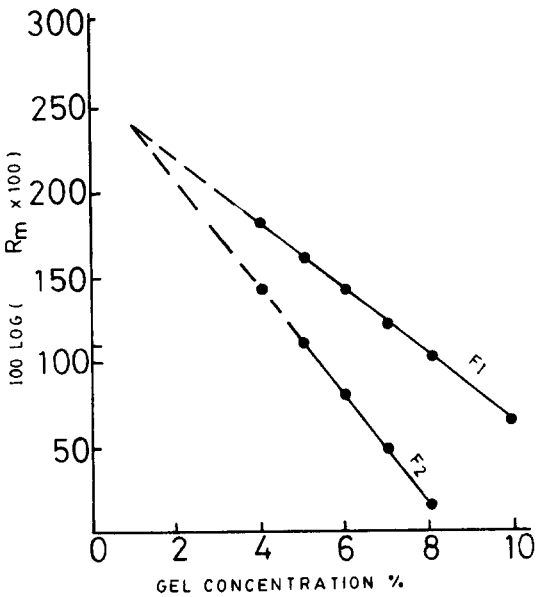

Fig. 6. Plots of log R_m vs gel concentration for *D. alata* phosphorylase forms separated using disc gel electrophoresis.

two non-parallel lines which, when extrapolated backwards, met at a common point at about 1% gel concentration. This idicates that the phosphorylase forms exist as size isomers, i.e. they differ in molecular size. This helps in the selection of appropriate purification methods to be employed. *Discorea dumentorum* phosphorylase forms have been reported to exist in size and charge isomers [13].

The kinetic constants obtained for yam phosphorylase in the direction of glucan synthesis are shown in Table 3, while Table 4 shows the kinetic constants obtained in the direction of phosphorolysis. These results indicate that the affinity of the enzymes for G-1-P in the direction of synthesis determines the rate of starch synthesis. Thus, the affinity of the *D. alata* phosphorylase forms is about 2.5 times higher than that of the *D. rotundata* enzyme and this is shown in their high rate of synthetic activities (10 to 13 times more active in this direction than white yam tuber phosphorylase). The K_m and K_B obtained with amylopectin for *D. rotundata* enzyme are very high (Table 3) and seem to be non-physiological. In the direction of starch degradation, the reverse is the case in that the affinity of *D. rotundata* phosphorylase for starch is about 2 to 11 times higher than that of F_1 and F_2, respectively, and this reflects its high rate of starch degradation [6 to 10 times more active in this direction than *D. alata* enzymes (Table 4)].

These results confirm the report that phosphorylase from *D. rotundata* functions mainly as a starch degrading enzyme [11], while *D. alata* phosphorylase forms may have a synthetic role [25].

EXPERIMENTAL

Materials. The yam tubers used in this study were obtained from one of the farms, University of Benin, They were planted and harvested as previously described [12]. Spectrophotometric measurements were made using a SP 1800 Pye Unicam double-beam spectrophotometer. All the reagents used were of analyt. grade.

Isolation and purification of enzymes. Sepn and purification of *D. alata* phosphorylase were carried out as previously described [12], while *D. rotundata* enzyme was purified as in ref. [11].

Enzyme assay. Phosphorylase activity was assayed by Pi release and the iodine-staining glucan method using 5 mg amylopectin [11]. The activity of the enzymes was also assayed in the direction of starch degradation by following the rate of dissappearance of blue colour of the starch–iodine complex [9]. Amylase activity was similarly assayed except for the omission of phosphate buffer.

Table 3. Kinetic constants of *D. alata* and *D. rotundata* phosphorylase forms in the direction of glucan synthesis. The values were extrapolated from intercept and slope replots of Figs 1 and 2

Enzyme form	$K^*_{m_A}$ (mM)	K_A (mM)	V_{max} (nkat mg^{-1} protein)	$K^*_{m_B}$ (mg)	K_B (mg)
F_1	0.57	0.45	666.70	1.43	1.0
F_2	0.69	1.0	540.50	6.30	0.83
D. rotundata phosphorylase	1.50	2.50	50.0	22.20	28.6

*A = G-1-P; B = amylopectin.

Table 4. Kinetic constants in the direction of phosphorolysis obtained from secondary replots in Figs 3, 4 and 5

Enzyme form	$K^*_{m_A}$ (mM)	K_A (mM)	V_{max} (nkat mg^{-1} protein)	$K^*_{m_B}$ (mg)	K_B (mg)
F_1	1.20	—	91.0	10.0	—
F_2	0.79	0.68	58.8	1.1	0.95
D. rotundata phosphorylase	1.33	7.90	558.2	0.84	5.0

*A = Pi; B = starch.

pH function. Phosphorylase activity was determined at various pH values ranging from 5.0 to 7.5 at saturating substrate concns using the assay methods. V_{max} was plotted against pH and the pK_a values were extrapolated.

Analytical method. Protein was estimated using protein–dye binding method [26]. Bovine serum albumin was used as the standard protein. Na was estimated using a flame photometer, while NN_4^+ was determined employing phenol–OCl^-/nitroprusside reaction [27].

Molecular mass determination. The molecular mass of each enzyme fraction was determined using disc gel electrophoresis [28], SDS gel electrophoresis [29] and gel filtration [11]. The following standard protein markers were used: ovalbumin (45 000), bovine serum albumin (68 000), hexokinase (96 000), β-amylase (215 000) and catalase (240 000).

Bisubstrate kinetics. Phosphorylase activity was assayed in the direction of glucan synthesis by varying G-1-P at fixed levels of amylopectin prepd as in ref. [25], while in the direction of phosphorolysis, enzyme activity was assayed at fixed levels of starch with Pi as the variable substrate. Phosphorylase activity was also assayed by varying starch concn at fixed levels of Pi.

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