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ENZYMES OF STARCH METABOLISM IN DEVELOPING GRAINS OF HIGH LYSINE BARLEY MUTANT

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Key Word Index—*Hordeum vulgare*; Gramineae; barley; Notch-2; NP 113; ADPG(UDPG)-pyrophosphorylase; starch phosphorylase; ADPP(UDPG)-starch synthetase; NDP kinase; inorganic pyrophosphatase; grain development.

Abstract—The absolute activities of ADPG(UDPG)-pyrophosphorylase, starch phosphorylase, ADPG(UDPG)-starch synthetase, NDP-kinase and inorganic pyrophosphatase have been studied in high lysine mutant barley Notch-2 and its parent NP 113 grains during development. In general, mutant Notch-2 grains had higher average activities of UDPG-pyrophosphorylase and starch phosphorylase and lower activity of ADPG(UDPG)-starch synthetase per grain than the parent NP 113 during grain development. Activities of NDP-kinase, ADPG-pyrophosphorylase and inorganic pyrophosphatase differed only to a small extent between the mutant Notch-2 and NP 113. It is suggested that the lower activity of ADPG(UDPG)-starch synthetase might be responsible for the reduced accumulation of starch in the mutant Notch-2 grain as compared with parent NP 113 during development.

INTRODUCTION

Sucrose–starch transformation in developing cereal grains involves several enzymes. The more important of the enzymes of starch metabolism are sucrose-synthetase, ADPG(UDPG)-pyrophosphorylase, starch phosphorylase, ADPG(UDPG)-starch synthetase and branching enzyme [1–4]. NDP-kinase and inorganic pyrophosphatase also play an indirect but important role in starch biosynthesis by forming substrates or depleting products of several enzymes catalysing multistep conversion of sucrose to starch.

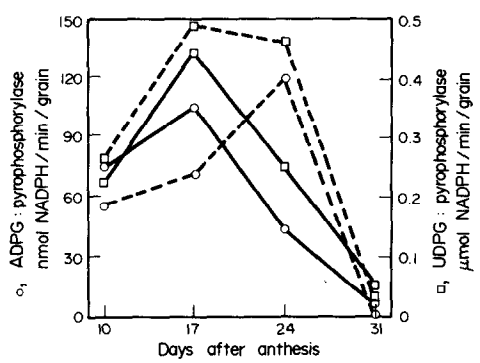

Fig. 1. ADPG-pyrophosphorylase and UDPG-pyrophosphorylase activity in developing grains of NP 113 (——) and Notch-2 (– – –) barley.

The EMS-induced barley mutant Notch-2 [5] has a higher protein percentage, increased lysine and improved nutritional quality than its parent NP 113 [6]. However, like other high lysine mutants of maize and sorghum, Notch-2 has lower grain yield than its parent NP 113 mainly as a result of the lower accumulation of dry matter during development [7]. It has been suggested that in high lysine Opaque-2 maize, reduced accumulation of starch is mainly due to lower level of enzymes of starch biosynthesis during the later stages of endosperm development [8,9]. Although the mutant Notch-2, as compared with NP 113 has higher amounts of soluble sugars including non-reducing sugars [6] and higher activity of ADP(UDP)-sucrose synthetase [10], the starch synthesis is lower. Therefore, in the present study the activities of ADPG(UDPG)-pyrophosphorylase, starch phosphorylase, starch synthetase, NDP-kinase and inorganic pyrophosphatase have been studied in developing grains of NP 113 and Notch-2 to understand the regulation of starch synthesis.

RESULTS

ADPG(UDPG)-pyrophosphorylase

ADPG-pyrophosphorylase activity during grain development in high lysine barley mutant Notch-2 and its parent NP 113 grain is shown in Fig. 1. Activity increased until day 17 in NP 113 and day 24 in Notch-2. Thereafter, it decreased sharply at day 31 in both. No activity could be detected in Notch-2 grains at day 31. Activity of ADPG-pyrophosphorylase was higher in NP 113 grain at days 10 and 17 while at day 24 it was nearly three-fold higher in Notch-2 than NP 113 grain.

The enzyme activity on a dry wt basis as well as the specific activity between NP 113 and Notch-2 (Table 1) followed a pattern similar to that obtained on a per-grain

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Table 1. ADPG-pyrophosphorylase and UDPG-pyrophosphorylase activity in developing grains of NP 113 and Notch-2 barley

Days after anthesis	Activity (μmol NADPH/min)			
	per g dry wt		sp. act. (per mg protein × 10)	
	NP 113	Notch-2	NP 113	Notch-2
ADPG-pyrophosphorylase				
10	7.50(0.92)	6.45(0.58)	0.85(0.09)	0.69(0.06)
17	4.83(0.39)	4.24(0.30)	0.72(0.07)	0.43(0.02)
24	1.33(0.04)	5.20(0.52)	0.26(0.01)	0.67(0.09)
31	0.17(0.02)	0.00(0.00)	0.04(0.00)	0.00(0.00)
UDPG-pyrophosphorylase				
10	22.42(2.00)	31.14(2.20)	2.61(0.16)	3.44(0.14)
17	20.42(0.65)	28.80(2.30)	3.04(0.11)	2.96(0.26)
24	7.53(0.30)	20.01(0.50)	1.45(0.02)	2.55(0.13)
31	1.43(0.10)	1.66(0.10)	0.33(0.02)	0.22(0.02)

Values in parentheses in this table and subsequent tables are standard errors.

basis. However, during development the activity/g dry wt in NP 113 decreased progressively during development to a very low level at day 31, whereas in mutant Notch-2 it decreased at day 17, increased at day 24 and then declined sharply and no activity could be detected at day 31.

UDPG-pyrophosphorylase activity per grain increased both in NP 113 and Notch-2 up to day 17 and showed a decline thereafter until day 31 (Fig. 1). The decrease in activity in Notch-2 at day 24 was much smaller than in NP 113 grain. This resulted in substantially higher activity in Notch-2 than NP 113 grain at this stage. However, at day 31, Notch-2 grains had lower activity than NP 113.

The UDPG-pyrophosphorylase activity/g dry wt decreased progressively in Notch-2 and NP 113 during development (Table 1). Compared with NP 113, the activity in Notch-2 on a dry wt basis was *ca* 1.4-fold until day 17, 2.66-fold on day 24 and 1.16-fold on day 31. The specific activity of UDPG-pyrophosphorylase in NP 113 increased at day 17 and decreased thereafter, whereas in Notch-2 grain it showed a decrease after day 10 (Table 1). The specific activity in Notch-2 was higher at days 10 and 24 and lower at day 31 as compared with NP 113. UDPG-pyrophosphorylase activity was substantially higher than ADPG-pyrophosphorylase both in NP 113 and Notch-2.

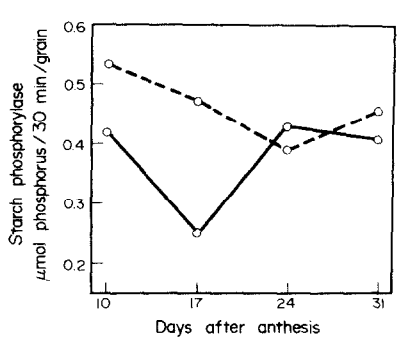

Fig. 2. Starch phosphorylase activity in developing grains of NP 113 (———) and Notch-2 (– – –) barley.

Starch phosphorylase

Starch phosphorylase activity per grain (Fig. 2) was higher in Notch-2 than NP 113 during early development while at later stages, the differences were much less. The activity was similar on days 10, 24 and 31 in NP 113 while at day 17, it was much less. In Notch-2 the activity decreased until day 24 and increased again on day 31.

Starch phosphorylase activity/g dry wt (Table 2) decreased throughout development in Notch-2 grain while in NP 113 barley, it decreased substantially at day 17 and did not change much thereafter. The activity/g dry wt in Notch-2 was substantially higher than in NP 113 grain during development. The specific activity of starch phosphorylase in Notch-2 was higher than in NP 113 at all stages except on day 24 (Table 2). During development, the specific activity decreased in NP 113 on day 17 and remained the same thereafter, while in Notch-2 a decrease was observed until day 24 and a small increase at day 31.

ADPG-starch synthetase

The soluble, bound and total ADPG-starch synthetase activity per grain (Fig. 3) increased until day 24 and decreased thereafter at day 31 in NP 113 and Notch-2

Table 2. Starch phosphorylase activity in developing grains of NP 113 and Notch-2 barley

Days after anthesis	Activity (μmol Pi released per 30 min)			
	per g dry wt		sp. act.	
	NP 113	Notch-2	NP 113	Notch-2
10	41.1(2.40)	61.6(1.01)	0.78(0.07)	1.02(0.04)
17	11.5(0.41)	27.5(1.20)	0.29(0.02)	0.56(0.02)
24	12.8(0.15)	17.1(0.20)	0.28(0.01)	0.25(0.01)
31	11.0(0.20)	16.6(0.71)	0.30(0.01)	0.37(0.01)

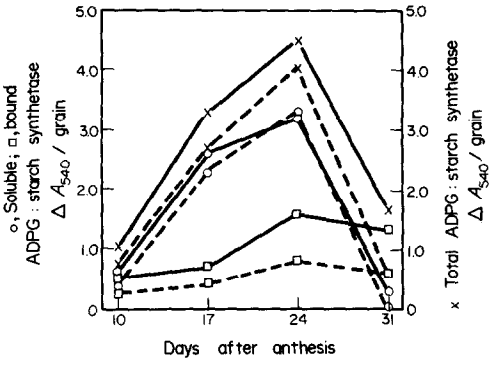

Fig. 3. Soluble, bound and total ADPG-starch synthetase activity in developing grains of NP 113 (———) and Notch-2 (– – –) barley.

grain. The decrease in soluble enzyme was much greater than in bound enzyme. The activity of the soluble enzyme in Notch-2 on day 31 was very small. Soluble enzyme activity per grain in NP 113 was higher than Notch-2 during development except on day 24, when it was nearly the same in both the varieties.

Bound and total ADPG-starch synthetase activity per grain was substantially lower in Notch-2 as compared with NP 113 during development. On day 31, the activity in Notch-2 grain was only 33% of that of the NP 113 grain. Of the total starch synthetase activity, a higher proportion was accounted for by soluble enzyme until day 24, when the rate of starch deposition was higher. However, on day 31, the situation was reversed and the bound enzyme contributed the most.

On the basis of dry wt, the soluble and bound ADPG-starch synthetase followed a different pattern. The soluble enzyme activity in Notch-2 was higher at days 17 and 24 and lower on days 10 and 31 as compared with NP 113 (Table 3). On the other hand, bound ADPG-starch synthetase/g dry wt was higher in NP 113 than Notch-2 at all stages. The specific activity of soluble ADPG followed a pattern similar to that obtained on a per-grain basis (Table 3). The specific activity of bound enzyme from NP 113 was higher than Notch-2 at all the stages (Table 3).

Total ADPG-starch synthetase/g dry wt in Notch-2 was 74% of that of the activity in NP 113 on day 10, whereas on days 17 and 24 it was higher in Notch-2 as compared with NP 113. The higher proportion of enzyme in Notch-2 on a dry wt basis is due to the smaller dry matter accumulation in Notch-2 than NP 113 rather than an actual increase in the enzyme.

UDPG-starch synthetase

The soluble, bound and total UDPG-starch synthetase activities per grain (Fig. 4) increased until day 24 in NP 113 and up to day 17 in Notch-2 grain, and decreased thereafter. The decrease in soluble enzyme was much greater than in bound enzyme. Notch-2 grain had relatively lower activities of soluble and bound UDPG-starch synthetase than NP 113 grain at all the stages except day 17 when the activities in Notch-2 were relatively higher. At day 31 soluble ADPG-starch synthetase in mutant Notch-2 was only 3% of the level in NP 113 grains.

The bound UDPG-starch synthetase activity in contrast to ADPG-starch synthetase was proportionally higher than the soluble enzyme in both NP 113 and Notch-2 grains.

On a dry wt basis, soluble UDPG-starch synthetase was higher in NP 113 at days 10, 24 and 31 than Notch-2 while on day 17, it was higher in Notch-2 (Table 4). Bound enzyme activity/g dry wt was higher at days 10 and 31 and lower at days 17 and 24 in NP 113 than in Notch-2 grain.

As a result the total UDPG-starch synthetase activity in NP 113 was higher on days 10, 24 and 31 and lower on day 17 as compared to mutant Notch-2 grain.

The pattern of changes in specific activity of the soluble UDPG-starch synthetase (Table 4) and the differences in specific activity between Notch-2 and NP 113 were similar

Table 3. ADPG-starch synthetase activity in developing grains of NP 113 and Notch-2 barley

Days after anthesis	Activity (A_{540} per 4 hr)			
	per g dry wt		sp. act.	
	NP 113	Notch-2	NP 113	Notch-2
Soluble enzyme				
10	56.8(1.1)	43.6(1.4)	0.72(0.01)	0.54(0.07)
17	121.7(3.0)	136.7(4.2)	2.44(0.02)	2.06(0.01)
24	92.2(0.6)	142.6(3.9)	2.25(0.01)	2.59(0.02)
31	8.8(0.1)	0.3(0.1)	0.34(0.01)	0.01(0.01)
Bound enzyme				
10	48.3(2.4)	34.1(2.0)	0.45(0.02)	0.27(0.04)
17	31.1(2.5)	23.7(1.7)	0.30(0.02)	0.20(0.01)
24	46.4(0.4)	33.2(2.0)	0.54(0.01)	0.31(0.04)
31	35.8(1.9)	19.4(0.6)	0.57(0.03)	0.29(0.01)
Total enzyme				
10	105.0	77.7	0.59	0.40
17	152.7	160.4	1.37	1.13
24	142.6	175.8	1.39	1.45
31	44.6	19.7	0.55	0.15

Table 4. UDPG-starch synthetase activity in developing grains of NP 113 and Notch-2 barley

Days after anthesis	Activity (A_{540} per 4 hr)			
	per g dry wt		sp. act.	
	NP 113	Notch-2	NP 113	Notch-2
Soluble enzyme				
10	31.9(0.02)	22.8(0.02)	0.41(0.01)	0.28(0.01)
17	23.3(0.02)	35.9(0.01)	0.47(0.01)	0.54(0.01)
24	23.6(1.10)	23.8(0.65)	0.55(0.02)	0.25(0.02)
31	8.8(0.01)	0.3(0.11)	0.34(0.01)	0.01(0.00)
Bound enzyme				
10	53.2(0.50)	47.0(1.15)	0.50(0.02)	0.38(0.01)
17	32.1(2.01)	53.1(0.69)	0.31(0.02)	0.45(0.03)
24	36.5(1.07)	41.5(0.39)	0.42(0.01)	0.39(0.01)
31	19.2(0.55)	14.1(0.25)	0.30(0.01)	0.21(0.01)
Total enzyme				
10	85.1	69.8	0.45	0.33
17	55.4	89.0	0.39	0.49
24	60.1	55.3	0.49	0.32
31	28.0	14.4	0.32	0.11

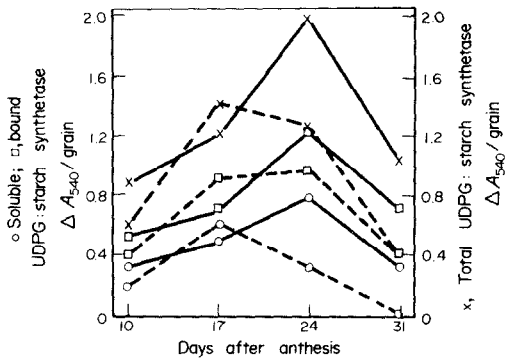

Fig. 4. Soluble, bound and total UDPG-starch synthetase activity in developing grains of NP 113 (———) and Notch 2 (– – –) barley.

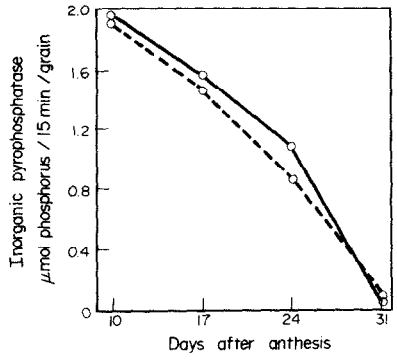

Fig. 6. Inorganic pyrophosphatase activity in developing grains of NP 113 (———) and Notch-2 (– – –) barley.

to the pattern obtained on a per-grain basis. The difference in the specific activity of bound enzyme between NP 113 and Notch-2 mutant was much less.

NDP-kinase

The NDP-kinase activity per grain increased between the 10- and 17-day stage in Notch-2, while it was practically unchanged in NP 113 (Fig. 5). The activity increased in both varieties at day 24 followed by a decline on day 31. The Notch-2 grain, compared with NP 113, had much lower activity on day 10 and substantially higher activity on day 24. No substantial differences could be observed on days 17 and 31.

The specific activity as well as the activity/g dry wt of NDP-kinase in NP 113 followed an erratic pattern during development (Table 5). In Notch-2 barley the activity remained at practically the same level until day 17, increased sharply on day 24 and decreased equally rapidly on day 31. On day 10, the Notch-2 barley contained relatively lower activity/g dry wt compared with NP 113 but it was relatively higher at subsequent stages.

Inorganic pyrophosphatase

The inorganic pyrophosphatase activity per grain decreased throughout grain development, to become almost negligible at day 31 post-anthesis (Fig. 6). The activity in Notch-2 grain did not differ much from that of NP 113 grain during development. However, on a dry wt basis the activity in Notch-2 was higher than NP 113 during development (Table 6). During development the activity/g dry wt also decreased. Compared with NP 113 the specific activity of enzyme in Notch-2 was higher on days 10 and 31 while at other stages the difference was not great (Table 6).

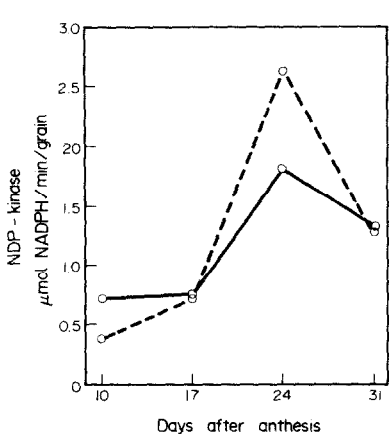

Fig. 5. NDP-kinase activity in developing grains of NP 113 (———) and Notch-2 (– – –) barley.

DISCUSSION

Induced mutant Notch-2 derived from NP 113 barley has substantially improved nutritional quality [6] due to the increased lysine content. However, the yield is low mainly due to decreased accumulation of starch. The reduced level of starch accumulation in Notch-2 could either be due to a lower level of enzymes involved in starch biosynthesis or to some kind of regulation in the activity of these enzymes.

During development, high lysine mutant Notch-2 had higher average activities per grain of ADPG-pyrophosphorylase, UDPG-pyrophosphorylase, and starch phosphorylase and lower activities of ADPG-starch synthetase and UDPG-starch synthetase, as compared to parent NP 113 grain. The quantitative differences for most enzymes between Notch-2 and NP 113 were more at day 24. This stage of grain development (day 24) was also characterized by the maximum difference in the rates of starch synthesis between two varieties [10] being lower in Notch-2 than NP 113.

Table 5. NDP-kinase activity in developing grains of NP 113 and Notch-2 barley

Days after anthesis	Activity (μmol NADPH per min)			
	per g dry wt		sp. act.	
	NP 113	Notch-2	NP 113	Notch-2
10	72.8(4.30)	43.3(3.30)	0.72(0.01)	0.42(0.02)
17	34.3(1.15)	42.9(0.45)	0.33(0.03)	0.38(0.01)
24	54.4(0.25)	115.2(1.10)	1.66(0.02)	2.41(0.05)
31	34.3(0.35)	38.2(0.75)	1.23(0.01)	1.17(0.09)

Table 6. Inorganic pyrophosphatase activity in developing grains of NP 113 and Notch-2 barley

Days after anthesis	Activity (μmol Pi released per 15 min)			
	per g dry wt		sp. act. (per mg protein)	
	NP 113	Notch-2	NP 113	Notch-2
10	197(11)	221(7)	3.51(0.15)	4.16(0.03)
17	72(1)	86(4)	1.59(0.02)	1.42(0.05)
24	32(1)	37(1)	0.96(0.08)	1.01(0.01)
31	1.3(0.1)	2.0(0.6)	0.05(0.01)	0.11(0.04)

The glucose moiety from ADPG or UDPG is transferred to the primer molecule in the presence of ADPG(UDPG)-starch synthetase, leading to increased primer chain length [11] and the polysaccharide molecule is further branched through α-1,6-glucosidic linkages by the action of a branching enzyme [12]. Relatively lower activities of ADPG(UDPG)- starch synthetase in Notch-2 grain appear to be mainly responsible for the decreased rate of starch synthesis in Notch-2 grain as compared to NP 113.

The ADPG(UDPG)-pyrophosphorylase reaction will be favoured in the direction of synthesis of ADPG(UDPG), particularly in the presence of high substrate (glucose-1-phosphate) concentration, provided that the inorganic pyrophosphate released during the reaction is removed from the site of action, and a sustained supply of high-energy phosphates (ATP and UTP) is maintained. Thus, associated with the pyrophosphorylase reaction are the enzymes inorganic pyrophosphatase and NDP-kinase. The Notch-2 mutant, on average, had similar inorganic pyrophosphatase activity and only slightly higher activity of NDP-kinase than NP 113 grain, thus making it unlikely for the pyrophosphorylase reaction in Notch-2 mutant to lead to increased ADPG(UDPG) synthesis. On the other hand, higher levels of soluble sugars and sucrose synthetase activity [10] in Notch-2 mutant will tend to form more nucleotide sugars (ADPG and UDPG), and reduced consumption of these nucleotide sugars through the ADPG(UDPG)-starch synthetase reaction will lead to their increased utilization through ADPG(UDPG)-pyrophosphorylase reaction. It thus appears more likely that excess of ADPG(UDPG) synthesized by sucrose synthetase, will be transformed into glucose-1-phosphate which will further accumulate in the mutant grain. The Notch-2 grain contains a substantially higher amount of glucose than the NP 113 grain [10].

Sucrose synthetase, which prefers UDP as a glucose acceptor, will produce more UDPG, which is the preferred substrate for pyrophosphorylase. The small amount of ADPG produced by ADP-sucrose synthetase might be utilized directly by starch synthetase which prefers ADPG to UDPG as the substrate. However, the rapidity of the pyrophosphorylase reaction towards glucose-1-phosphate will depend upon the availability of nucleoside diphosphates (UDP and ADP) and inorganic pyrophosphate. While UDP and ADP may become available through proposed futile cycling of fructose

$$(\text{fructose} \xrightarrow[\text{ADP}]{\text{ATP}} \text{fructose-6-PO}_4 \xrightarrow[\text{Pi}]{} \text{fructose}),$$

the source of pyrophosphate is difficult to visualize.

EXPERIMENTAL

Plant material. Seeds of barley (*Hordeum vulgare* L.) var. NP 113 and its high lysine mutant Notch-2 were grown under normal fertility in pots. The ears were harvested at 10, 17, 24 and 31 days after anthesis. The grains were dehusked and stored in liquid N_2 for further use.

Enzyme extracts. These were prepared by hand-grinding of seeds in liquid N_2 and extraction in a suitable medium (1:5 w/v). The extraction medium used for different enzymes was 0.1 M Tris–Cl buffer (pH 7.9) containing 5 mM glutathione and 1 mM EDTA for pyrophosphorylase; 0.1 M Tris–maleate buffer (pH 6.3) containing 1% PVP for phosphorylase; 50 mM HEPES buffer (pH 7.0) containing 10 mM EDTA, 5 mM DTT and 1% PVP for starch synthetase; 50 mM Tris–Cl buffer (pH 7.6) for inorganic pyrophosphatase and 20 mM HEPES (pH 7.0) containing 0.3 M sucrose and 1.0 mM DTT for NDP-kinase. The supernatant obtained after centrifugation of the homogenate at 20 000 g for 20 min was collected and used for enzyme assay.

In the case of starch-synthetase, the pellet obtained after the first extraction, was re-extracted in 4 vol. of extraction medium. The supernatants from both extractions were pooled to obtain the soluble enzyme fraction. The pellet left after the second extraction was resuspended in 10 vol. of extraction medium and used as the bound enzyme fraction. All operations, unless otherwise stated, were carried out at 4°.

Enzyme assay. ADPG- and UDPG-pyrophosphorylases were determined in the soluble fraction by coupling the product (glucose-1-phosphate) with phosphoglucomutase and glucose-6-phosphate dehydrogenase reactions and following the reduction of NADP spectrophotometrically against a control in which ADPG(UDPG) was omitted [13]. The reaction mixture contained Tris–Cl buffer (pH 7.9), 160 μmol; $MgSO_4$, 12 μmol; ADPG (or UDPG), 0.8 μmol; sodium pyrophosphate, 4 μmol; enzyme prep., 100 μl; glucose-6-phosphate dehydrogenase, 4 units; phosphoglucomutase, 2 units; NADP, 0.8 μmol; and H_2O to a final vol. of 2.85 ml. Cysteine, 9.6 μmol; BSA, 0.48 mg; and phosphoglyceric acid, 6 μmol were also included in the reaction mixture for ADPG-pyrophosphorylase.

The starch phosphorylase was assayed by measuring the release of Pi [14] from glucose-1-phosphate added as a substrate, using amylopectin as a primer. The reaction mixture (1.2 ml) containing Tris–maleate buffer (pH 6.3), 20 μmol; amylopectin 10 mg; enzyme prep. 100 μl; and glucose-1-phosphate, 10 μmol was incubated at 25° for 2 hr [15]. In the control set, glucose-1-phosphate was added only after the incubation and termination of the reaction by 1 ml of 5% TCA.

ADPG- and UDPG-starch synthetase were assayed in the soluble as well as insoluble fractions by the colorimetric method of ref. [16]. Some (0.2 ml) of the reaction mixture containing glycine buffer (pH 8.3), 4 μmol; EDTA, 0.1 μmol; amylopectin, 2.5 mg; glutathione, 1 mg; and ADPG (or UDPG), 0.3 μmol; was

incubated with 0.1 ml of the enzyme prep. at 37° for 4 hr. The ADP or UDP formed during the incubation was measured by the pyruvate kinase procedure [17]. In the control, the enzyme prep. was heat-denatured before addition to the reaction mixture.

NDP-kinase activity was assayed by coupling the product (ATP) with hexokinase and glucose-6-phosphate dehydrogenase reactions [18], 2.52 ml of the reaction mixture containing Tris–Cl buffer (pH 7.8), 200 μmol; $MgCl_2$, 10 μmol; glucose, 10 μmol; GTP, 1.2 μmol; ADP, 0.6 μmol; enzyme prep., 20 μl; hexokinase, 3 units, glucose-6-phosphate dehydrogenase 0.75 units; and NADP, 6 μmol. In the control, ADP was omitted from the reaction mixture.

Inorganic pyrophosphatase activity was assayed by estimating Pi [14] released after hydrolysis of pyrophosphate by the enzyme [19]. In the control, 0.5 ml of 5% TCA was added before addition of enzyme to the reaction mixture.

For all the enzymes, two independent extractions were done for each sample and then analysed in duplicate. The values reported in this study are an average of the values for two independent extractions which agreed closely. Sp. act. is expressed as the activity/mg protein.

Soluble protein. This was estimated as given in ref. [20].

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