Levels of Free Sugars, Intermediate Metabolites, and Enzymes of Sucrose–Starch Conversion in Developing Wheat Grains

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Activities of the enzymes presumably implicated in starch biosynthesis and the levels of free sugars and intermediate metabolites of proposed pathway were monitored throughout grain development in wheat (*Triticum aestivum* L.). Except invertase, which was absent after the 21-day stage, all other enzyme activities increased up to 28 and/or 35 days after anthesis (DAA), declining thereafter. Starch synthetase was slightly more active with ADPG than with UDPG. The highest levels of free sugars occurred at 14 DAA. The ratios of the levels of G-6-P:F-6-P:G-1-P and UDPG:ADPG were close to 25:5:1 and 3:1, respectively. High P_i:G-1-P ratios (100–870) make starch synthesis by phosphorylase highly unlikely. ADPG–starch synthetase appears to be the predominant route of starch biosynthesis. Desiccation toward grain maturation seems to be the major factor terminating starch accumulation.

Among various constituents of food, cereals, particularly wheat, contribute more than 60% of the food calories and are an important agricultural crop. Starch is the major constituent of wheat grains, comprising about 70% of the grain dry weight. The attempts to increase starch content in cereals, hence, have a direct bearing on the food industry. Starch content can be manipulated by chemical means provided we have adequate information on the pathway of starch biosynthesis and the control mechanisms associated with it. The enzymes of starch biosynthesis have been studied during grain development in maize (Tsai et al., 1970), barley (Batra and Mehta, 1981a,b), rice (Perez et al., 1975), and wheat (Kumar, 1982). However, in much of the work concerned with the biosynthesis of starch in developing cereal grains, no determination of in vivo concentrations of both substrates and products simultaneously with measurement of relevant enzyme activities has ever been made. Such studies might help in understanding the control mechanisms associated with the pathway of starch biosynthesis and thus provide chemical means to manipulate starch content vis-à-vis grain yield. The prevention of sucrose conversion to starch by pyrophosphate in sweet corn during the postharvest period is one such example (Amir and Cherry, 1971). With this in mind, an attempt was made to determine the activities of various enzymes and intermediate metabolites of sucrose–starch conversion at different stages of grain development in wheat.

MATERIALS AND METHODS

Plant Material. Wheat (*Triticum aestivum* L.) cv. C-591 was raised under field conditions in three replications and samples were harvested as described earlier (Kumar and Singh, 1980).

Enzyme Extraction. For all enzymes, except starch synthetase, extract was made in 0.1 M Tris–maleate buffer, pH 7.0 (Tsai et al., 1970; Tsay et al., 1983). Soluble and bound fractions of starch synthetase were obtained according to Baxter and Duffus (1971).

Enzyme Assays. Hexokinase (EC 2.7.1.1) was assayed according to Tsai et al. (1970). Phosphoglucoisomerase (EC 5.3.1.9) and phosphoglucomutase (EC 2.7.5.1) were assayed spectrophotometrically by coupling the product of the reaction to G-6-P dehydrogenase (Kumar and Singh, 1983). The assay mixtures for invertase (EC 3.2.1.26), sucrose–UDP glucosyltransferase (EC 2.4.1.13), and UDPG (ADPG) pyrophosphorylase (EC 2.7.7.9) were prepared according to Kumar and Singh (1980). Inorganic pyrophosphatase (EC 3.6.1.1) and starch synthetase (EC 2.4.1.21) were assayed according to Heppel (1950) and Leloir et al. (1961), respectively.

Carbohydrate Composition. The extraction and estimation of various carbohydrate components were achieved by the standard methods described earlier (Kumar and Singh, 1981).

Metabolites. From the frozen grains, metabolites were extracted according to Rasi-Caldogno and De-Michelis (1978). The metabolites were estimated at 30 °C in an enzymeter (Calbiometer 340, Calbiochem.). The assay mixture (1.5 mL) in the cuvette contained the following: buffered extract, 0.5 mL; Tris-HCl buffer (pH 7.8), 60 μmol; $MgSO_4$, 10 μmol; NADP, 1 μmol; sodium pyrophosphate, 3 μmol. The absorbance (E_1) at 340 nm was recorded. Two units (5 μL) of G-6-P dehydrogenase was added and increased absorbance (E_2) noted. The extinctions E_3, E_4, E_5, E_6, and E_7 were likewise recorded after successive additions of 2 units (5 μL) each of phosphoglucoisomerase, phosphoglucomutase, hexokinase, UDPG pyrophosphorylase, and 5 μL of ADPG pyrophosphorylase. From these values, concentrations were calculated as $\Delta OD(G\text{-}6\text{-}P) = E_2 - E_1$, $\Delta OD(F\text{-}6\text{-}P) = E_3 - E_2$, $\Delta OD(G\text{-}1\text{-}P) = E_4 - E_3$, $\Delta OD(ATP) = E_5 - E_4$, $\Delta OD(UDPG) = E_6 - E_5$, and $\Delta OD(ADPG) = E_7 - E_6$.

All biochemicals and auxillary enzymes were purchased from Sigma. Since ADPG pyrophosphorylase was not commercially available, it was purified from spinach leaves according to the procedure of Ghosh and Preiss (1966). This preparation was free of all the interfering enzyme activities. Recoveries of standard metabolites were in the range 84–93%.

Enzymes of Carbohydrate Metabolism. All the enzyme activities could be detected at the very first stage, viz., 7 DAA. Hereafter, the activity in each case increased progressively with grain development to attain a peak at 28 and/or 35 DAA (Table I). However, invertase could not be detected after the 21-day stage. A sharp increase in all the enzyme activities was observed after the 14-day stage. Out of the two pyrophosphorylases, activity with UDPG was considerably higher than with ADPG. Starch synthetase in both the fractions was slightly more active with ADPG as the glycosyl donor. During initial experimentation, sucrose–ADP glucosyltransferase was found to be very low as compared to sucrose–UDP glucosyltransferase.

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Table I. Enzymes of Starch Metabolism in Developing Wheat Grains[a]

enzyme[c]	days after anthesis					
	7	14	21	28	35	42
invertase, nmol	31.0 ± 0.62	28.1 ± 0.36	14.0 ± 0.19	ND[b]	ND	ND
sucrose–UDP glucosyl transferase, nmol	28.5 ± 1.52	34.5 ± 2.56	85.0 ± 4.03	149.0 ± 7.97	138.0 ± 5.12	83.0 ± 5.67
hexokinase, nmol	1.2 ± 0.04	10.3 ± 0.38	14.7 ± 0.27	17.3 ± 0.31	19.7 ± 0.37	17.0 ± 0.19
phosphoglucoisomerase, μmol	0.04 ± 0.00	0.9 ± 0.01	1.9 ± 0.06	1.7 ± 0.01	2.3 ± 0.05	1.2 ± 0.04
phosphoglucomutase, nmol	3.1 ± 0.02	201.0 ± 5.67	331.7 ± 8.84	453.3 ± 23.00	111.0 ± 5.02	114.7 ± 2.84
ADPG pyrophosphorylase, nmol	2.2 ± 0.00	102.0 ± 0.66	227.7 ± 4.17	235.3 ± 3.34	207.3 ± 2.50	19.3 ± 0.67
UDPG pyrophosphorylase, μmol	0.03 ± 0.00	0.8 ± 0.01	3.7 ± 0.09	6.8 ± 0.03	2.2 ± 0.02	0.6 ± 0.01
inorganic pyrophosphatase, nmol	15.8 ± 0.30	48.2 ± 0.73	84.5 ± 0.88	111.4 ± 3.51	64.6 ± 0.87	21.9 ± 2.34
starch synthetase, ΔA_{540}						
bound ADPG specific	0.4 ± 0.01	1.4 ± 0.04	1.4 ± 0.03	3.8 ± 0.11	1.8 ± 0.07	1.0 ± 0.05
soluble ADPG specific	0.5 ± 0.01	3.8 ± 0.09	25.0 ± 1.03	42.5 ± 1.70	37.6 ± 1.58	22.7 ± 1.06
bound UDPG specific	ND	1.4 ± 0.06	1.2 ± 0.06	3.5 ± 0.17	1.4 ± 0.11	0.7 ± 0.05
soluble UDPG specific	1.1 ± 0.04	2.5 ± 0.10	21.6 ± 0.50	38.2 ± 1.07	33.5 ± 1.04	20.6 ± 1.02

[a] Each value is the mean ± SE from six independent estimations. [b] Not detectable. [c] Units per minute per grain.

Table II. Carbohydrate Composition of Developing Wheat Grains[a]

sugar	mg/grain for days after anthesis of					
	7	14	21	28	35	42
starch	0.61 ± 0.02	2.71 ± 0.07	12.60 ± 0.30	25.60 ± 0.21	30.50 ± 0.43	35.40 ± 0.7
total soluble sugars	1.53 ± 0.05	2.49 ± 0.06	1.99 ± 0.10	1.73 ± 0.10	1.69 ± 0.08	1.51 ± 0.0
reducing sugars	0.26 ± 0.05	0.49 ± 0.02	0.42 ± 0.03	0.34 ± 0.01	0.31 ± 0.01	0.28 ± 0.0
nonreducing sugars	1.27 ± 0.06	2.00 ± 0.03	1.57 ± 0.05	1.39 ± 0.07	1.38 ± 0.06	1.23 ± 0.09
sucrose	0.76 ± 0.01	1.41 ± 0.03	1.05 ± 0.05	0.72 ± 0.02	0.42 ± 0.03	0.53 ± 0.04

[a] Each value is the mean ± SE from six independent estimations.

Table III. Metabolite Composition of Developing Wheat Grains[a]

metabolite[c]	days after anthesis					
	7	14	21	28	35	42
G-6-P, nmol	8.7 ± 0.28	41.0 ± 0.50	60.7 ± 3.60	53.7 ± 0.65	42.7 ± 1.35	ND[b]
F-6-P, nmol	1.3 ± 0.08	6.0 ± 0.20	10.7 ± 0.75	10.7 ± 0.34	7.0 ± 0.34	ND
G-1-P, nmol	0.3 ± 0.01	1.3 ± 0.05	3.3 ± 0.06	2.5 ± 0.10	2.0 ± 0.10	ND
ADPG, nmol	0.1 ± 0.00	1.6 ± 0.05	6.3 ± 0.29	7.7 ± 0.12	5.3 ± 0.05	0.3 ± 0.00
UDPG, nmol	0.9 ± 0.04	5.3 ± 0.60	18.0 ± 0.85	23.0 ± 0.12	17.0 ± 0.85	1.5 ± 0.10
P_i, μmol	0.28 ± 0.02	0.53 ± 0.04	0.76 ± 0.05	0.40 ± 0.03	0.41 ± 0.03	0.12 ± 0.01
ATP, nmol	1.3 ± 0.15	11.7 ± 0.62	22.0 ± 0.25	20.3 ± 0.25	17.0 ± 0.50	5.0 ± 0.13

[a] Each value is the mean ± SE from six independent estimations. [b] Not detectable. [c] Per grain.

Carbohydrate Compositions. As shown in Table II, small quantities of starch were present at 7 DAA. The quantity increased almost linearly from 0.6 mg at the 7-day stage to 39 mg at maturity. The highest levels of total, reducing, and nonreducing sugars and sucrose occurred at 14 DAA (Table II). Thereafter, a steady decline was observed until 28 days, and the contents remained fairly constant afterward. There was a slight increase in sucrose content toward maturity.

Metabolites. The amount of G-6-P was over 25 times higher than that of G-1-P and about 5 times higher than that of F-6-P throughout the period of grain development (Table III). The ratio of UDPG:ADPG at different stages was close to 3 and that of P_i:G-1-P in the range of 100–870. The level of all the metabolites, including ATP, increased during grain development, reaching maximum at 21 DAA and declining thereafter. Hexose phosphates were absent at maturity. However, both the nucleotide sugars were present in minute quantities at this stage. Levels of PP_i were also monitored by coupling with UDPG pyrophosphorylase, but it could not be detected at any of the stages.

DISCUSSION

Carbohydrates are present in wheat grain mainly as sucrose (Sakri and Shannon, 1975). Accumulation of sucrose until 14 DAA (Table II) may represent its rapid translocation from the photosynthesizing parts to the endosperm and less utilization, as active starch synthesis commenced from 14 days onward. A slight increase in the level of sucrose toward maturity may reflect the balance of sucrose left unutilized in starch biosynthesis.

Starch synthesis attained a maximum rate only after the 14-day stage. By this time, invertase activity was already declining and it was over by the 21-day stage. Hence, it cannot be implicated in the process of sucrose–starch conversion. The close coincidence of the patterns of sucrose–UDP glucosyltransferase with the rate of starch accumulation indicates that this enzyme plays a major role in the hydrolysis of sucrose. Very low levels of sucrose–ADP glucosyltransferase observed here coupled with the observations of Murata et al. (1964), who in rice grains, observed UDP levels to be substantially lower than ADP and UDPG levels to be higher than ADPG, make the participation of this enzyme unlikely in the process of sucrose hydrolysis. Greater concentrations of UDPG than ADPG observed here also (Table III) lend supporting evidence to the above suggestion. The role of invertase may be to provide substrates for energy liberating respiratory enzymes during the early periods of cell division phase, when high energy investment is required to sustain active cell division.

Fructose and UDPG formed as a result of sucrose–UDP glucosyltransferase activity can then be channeled toward starch biosynthesis through a well-known reaction sequence. Fructose can be converted to G-1-P via hexokinase, phosphoglucoisomerase, and phosphoglucomutase (deFekete and Cardini, 1964). ADPG pyrophosphorylase

may then bring about the conversion of G-1-P to ADPG. The nondetectability of PP_i throughout grain development suggests its rapid hydrolysis by inorganic pyrophosphatase, thus favoring ADPG formation. PP_i is a potent inhibitor of ADPG pyrophosphorylase (Amir and Cherry, 1971). UDPG may be utilized for starch synthesis either directly by UDPG–starch synthetase or indirectly through ADPG–starch synthetase after being converted to ADPG via G-1-P (Kumar and Singh, 1980).

The ratios of G-6-P:F-6-P:G-1-P were close to 25:5:1, whereas this ratio in spinach chloroplasts has been reported to be 19:8:1 (Latzko and Gibbs, 1969). Liu and Shannon (1981) found the G-6-P:G-1-P ratio in starch granule preparation from corn to be 40. G-1-P may be a substrate for phosphoglucomutase, phosphorylase, and pyrophosphorylases. Very low levels of G-1-P indicate its rapid utilization. This may mean that phosphoglucomutase may be regulating the rate of strarch synthesis by controlling the rate of G-1-P formation.

Hexose phosphates could not be detected at the 42-day stage. However, at the 35-day stage, these were present despite low sucrose content as compared to 42 DAA. UDPG and ADPG were present at the 42-day stage. It suggests that in vivo, activities of hexokinase, phosphoglucoisomerase, and phosphoglucomutase are lost somewhere between the 35- and 42-day stage, presumably due to desiccation. The in vitro detectability of these enzymes at the 42-day stage may be due to their rehydration following homogenization. Since both fine and coarse controls in the process of starch biosynthesis in reserve tissues have been ruled out (Jenner, 1982; R. Singh, Personal communication), desiccation toward grain maturation (Kumar and Singh, 1981) appears to be the major factor terminating starch accumulation. Translocation of sucrose from other parts to the grains that have already lost their capacity to metabolize it further can very well account for a slight increase in its content at 42 DAA. It conclusively proves that termination of starch accumulation as grain matures in not due to the nonavailability of sucrose but is attributable to the loss in synthetic capacity of endosperm.

Phosphorylase from plant sources has a very high K_m value for G-1-P [1–50 mM (Alexander, 1973; Chen and Whistler, 1976)]. In wheat grain, the concentration of G-1-P is very low and its maximum level (at the 21-day stage) approached 0.1 μM. Furthermore, the observed P_i:G-1-P ratio is very high (100–870). Since the phosphorylase reaction in vitro has an equilibrium constant of 2.4 (Cohn, 1961), the formation of α-1,4 linkages by phosphorylase in highly unlikely. Thus unless a very high concentration of G-1-P is assumed at the site of phosphorylase, it cannot be implicated in starch biosynthesis. The rate of glucose transfer from UDPG is usually $^1/_3$ to $^1/_{10}$ of that from ADPG, and the K_m for UDPG is about 15–30-fold higher than for ADPG (Cardini and Frydman, 1966). UDPG concentrations in the present case were just 3-fold higher than those of ADPG. Moreover, the presence of UDPG-linked mechanisms may be concerned with the synthesis of endosperm cell wall components (Nikaido and Hassid, 1971). Hence, it seems that the ADPG route is by far the preferred route for starch synthesis in developing wheat grains.

ATP, the substrate of ADPG pyrophosphorylase, was present thoughout grain development. High levels of P_i may be representative of rapid PP_i hydrolysis and considerable phosphatase activity in developing wheat grains.

Registry No. G-6-P, 56-73-5; G-1-P, 59-56-3; F-6-P, 643-13-0; UDPG, 133-89-1; ADPG, 2140-58-1; P_i, 14265-44-2; EC 2.7.1.1, 9001-51-8; EC 5.3.1.9, 9001-41-6; EC 2.7.5.1, 9001-81-4; EC 3.2.1.26, 9001-57-4; EC 2.4.1.13, 9030-05-1; EC 2.7.7.9, 9026-22-6; EC 3.6.1.1, 9024-82-2; EC 2.4.1.21, 9030-10-8; starch, 9005-25-8; sucrose, 57-50-1.

LITERATURE CITED

Alexander, A. G. *Ann. N.Y. Acad. Sci.* **1973**, *210*, 64.
Amir, J.; Cherry, J. H. *J. Agric. Food Chem.* **1971**, *19*, 954.
Batra, V. I. P.; Mehta, S. L. *Phytochemistry* **1981a**, *20*, 635.
Batra, V. I. P.; Mehta, S. L. *Phytochemistry* **1981b**, *20*, 1827.
Baxter, E. D.; Duffus, C. M. *Phytochemistry* **1971**, *10*, 2641.
Cardini, C. E.; Frydman, R. B. *Methods Enzymol.* **1966**, *8*, 387–394.
Chen, M.; Whistler, R. L. *Int. J. Biochem.* **1976**, *7*, 433.
Cohn, M. *Enzymes, 2nd Ed.* **1961**, *5*, 179–206.
deFekete, M. A. R.; Cardini, C. E. *Arch. Biochem. Biophys.* **1964**, *104*, 173.
Ghosh, H. P.; Preiss, J. *J. Biol. Chem.* **1966**, *241*, 4491.
Heppel, L. A. *Methods Enzymol.* **1950**, *2*, 570–576.
Jenner, C. F. In "Encyclopedia of Plant Physiology"; Loewus, F. A.; Tanner, W., Eds.; Springler-Verlag: Berlin, Heidelberg, and New York, 1982; Vol. 13, pp 700–737.
Kumar, R. Ph.D. Thesis, HAU Hissar, India, 1982.
Kumar, R.; Singh, R. *Phytochemistry* **1980**, *19*, 2299.
Kumar, R.; Singh, R. *J. Sci. Food Agric.* **1981**, *32*, 229.
Kumar, R.; Singh, R. *Trop. Plant Sci. Res.* **1983**, *1*, 109.
Latzko, E.; Gibbs, M. *Plant* Physiol. **1969**, *44*, 396.
Leloir, L. F.; deFekete, M. A. R.; Cardini, C. E. *J. Biol. Chem.* **1961**, *236*, 636.
Liu, T. Y.; Shannon, J. C. *Plant Physiol.* **1981**, *67*, 525.
Murata, T.; Sugiyama, T.; Akazawa, T. *Arch. Biochem. Biophys.* **1964**, *107*, 92.
Nikaido, H.; Hassid, W. Z. *Adv. Carbohydr. Chem. Biochem.* **1971**, *26*, 351.
Perez, C. M.; Perdon, A. A.; Rusurreccion, A. P.; Villareal, R. M.; Juliano, B. O. *Plant Physiol.* **1975**, *56*, 579.
Rasi-Caldogno, F.; De-Michelis, M. I. *Plant Physiol.* **1978**, *61*, 85.
Sakri, F. A. K.; Shannon, J. C. *Plant Physiol.* **1975**, *55*, 881.
Tsai, C. Y.; Salamini, F.; Nelson, O. E. *Plant Physiol.* **1970**, *46*, 299.
Tsay, J. S.; Kuo, W. L.; Kuo, C. G. *Phytochemistry* **1983**, *22*, 1573.

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