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3-Phosphoglycerate Phosphatase in Plants

III. ACTIVITY ASSOCIATED WITH STARCH PARTICLES[1]

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ABSTRACT

A particulate form of 3-phosphoglycerate phosphatase represents about 20% of this activity in spinach (*Spinacia oleracea* var. Longstanding Bloomsdale) leaves. By differential and isopycnic sucrose density gradient centrifugation, all the particulate activity was found in starch grains that pelleted through 2.5 M sucrose. This particulate phosphatase was extremely stable, had a pH optimum of 5.8, and an apparent Michaelis constant (3-phosphoglycerate) of 9×10^{-4} M. No cation requirement for activity could be demonstrated, and the enzyme was inhibited by 0.5 mM Zn^{2+} or Cu^{2+}. The enzyme was most active in catalyzing the hydrolysis of 3-phospho-D-glycerate, but it was not substrate specific. The phosphatase from the starch grains could not be removed by washing, dialysis, homogenization, or treatment with a French pressure cell, but it was solubilized by prolonged sonication or by addition of 0.25 M $MgCl_2$, when the particles were suspended in 0.8 M sucrose. The solubilized enzyme was partially purified. The properties of the enzyme on the particles or after solubilization were similar to those previously described for the cytosol form of this phosphatase. It is conjectured that the phosphatase of the starch grain regulates glucan synthesis by controlling a 3-phosphoglycerate pool which is an effector for ADP-glucose pyrophosphorylase.

In the two previous papers of this series the isolation and characterization of a soluble form of 3-P-glycerate phosphatase from sugarcane leaves were described (5), and the distribution and physiological properties of the enzyme were explored (6). The enzyme is very active in leaves of C_4-plants and is located mainly in the cytosol of the mesophyll cells. In leaves of C_3-plants much of it also appeared in the soluble fraction. However, a substantial portion (about 20%) of the 3-P-glycerate phosphatase activity of spinach leaves has been found firmly bound to starch-like particles. The properties of this enzyme in starch grains of spinach leaves are similar to 3-P-glycerate phosphatase in the cytosol of sugarcane leaves. Current investigations indicate that this particulate phosphatase of the starch grain is present in other plants besides spinach leaves (J. Hallett, D. D. Randall and N. E. Tolbert, unpublished). Conjecture about its function in the starch grain deals with regulation of starch biosynthesis through the control of ADP-glucose pyrophosphorylase activity by the effector, 3-P-glycerate (1).

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MATERIALS AND METHODS

Fresh field-grown spinach (*Spinacia oleracea* var. Longstanding Bloomsdale), leaves were used shortly after harvest or after storage for several days at 4 C. The procedure of Tolbert *et al.* (8) for peroxisome isolation from spinach leaves was followed. The leaves were ground in a Waring Blendor for 10 sec in medium containing 0.5 or 0.8 M sucrose and 20 mM glycylglycine at pH 7.5. Particles were removed by differential centrifugation at 0 to 6000*g* for 10 or 20 min and resuspended in the grinding medium. They were then separated by discontinuous isopycnic sucrose gradient centrifugation at 47,000 to 107,000*g* through 1.5 to 2.3 or 2.5 M sucrose. The pellet that sedimented through the densest sucrose was the source of the particulate 3-P-glycerate phosphatase.

The assay for 3-P-glycerate phosphatase and other methods were the same as used previously (4–6). Heavy metal salts of the substrates were converted by resins to K^+ or Na^+ salts before use. Determination of reducing sugar was by the method of Nelson (3). Particles were stained with I_2 by boiling for 5 min in 70% ethanol, washing with H_2O, and staining with 0.5% I_2 in 5% KI. Excess stain was removed by washing with H_2O and centrifuging.

RESULTS

Distribution of 3-P-Glycerate Phosphatase Among Particles. Spinach leaves were homogenized, and the suspension was separated into fractions by differential centrifugation (Table I). The 6000*g* pellet contained 38% of the 3-P-glycerate phosphatase activity as well as the peroxisomes, the bulk of the broken chloroplasts, and some mitochondria. The particles in the 6000*g* pellet were then separated by sucrose gradient centrifugation, and the small pellet at the bottom of the tube represented material of sufficient density to pass through 2.5 M sucrose during centrifugation. It contained about half of the 3-P-glycerate phosphatase activity, which had been placed on the gradient (Table I), or at least 19% of the total activity of the homogenate. Only traces of activity were found in all the other particulate fractions. Activity at the top of the gradient represented solubilized enzyme that had been in the 6000*g* pellet, and its original source is not clear. Upon resuspension of the sucrose gradient pellet

in 0.8 M sucrose and recentrifugation on a second gradient (data not shown), all of the phosphatase activity again pelleted through the densest sucrose at the bottom of the gradient, and no further phosphatase was obtained in the supernatant fraction.

Microscopic examination of the gradient pellet, which was gray to gray-violet in color, showed that the major constituent was small ellipsoidal starch-like grains. There were also a few whole cells, cell fragments, and broken chloroplasts, which were essentially all removed during the second gradient centrifugation. The pellet stained blue-purple with I_2 and KI. These observations are consistent with the speculation that a particle with a specific density greater than 2.5 M sucrose would be a polysaccharide such as starch. No nucleotidase or diesterase activities were found in this starch pellet, suggesting the absence of nuclei.

Stability. The stability and storage characteristics of the 3-P-glycerate phosphatase activity in the starch pellet were excellent. Pellets from preparations have been stored frozen for as long as 3 months before resuspending without loss of 3-P-glycerate phosphate activity. The resuspended particulate enzyme was stable at 4 C at pH 5.8 to 7.0 until microbial growth started (several weeks). Frozen suspensions were stable indefinitely. Preparations of the solubilized phosphatase or partially purified enzyme from the starch particles were stable and could be stored frozen at −18 C.

pH Optimum. 3-P-Glycerate phosphatase in the starch particles from spinach had a sharp optimum of activity around pH 5.8 (Fig. 1). The addition of Mg^{2+} did not affect this optimum or the amount of activity. The pH optimum is very similar to that of the soluble sugarcane 3-P-glycerate phosphatase (pH 5.9–6.5) (5).

Kinetics. 3-P-Glycerate phosphatase activity in the starch grains before or after dialysis and in solubilized enzyme preparations from the particles all showed a linear function of activity with enzyme concentration (data presented in Ref. 4). Normal enzyme saturation kinetics were obtained with the particulate enzyme (Fig. 2) and the apparent K*m* for 3-P-glycerate was 9×10^{-4} M.

Effect of Divalent Cations. The 3-P-glycerate phosphatase in the starch particle had no requirement for a divalent cation (Table II). The addition of 1 mM EDTA or dialysis for 24 hr against 20 volumes of 1 mM EDTA did not inhibit the enzyme activity, but rather increased activity about 10%. Zn^{2+} and Cu^{2+} were inhibitory and caused greater than 50% inhibition at 0.5 mM. These results are all similar to those with the soluble enzyme from sugarcane leaves (5).

Table I. *Separation of a Particulate Form of 3-P-Glycerate Phosphatase*

Longstanding Bloomsdale spinach (300 g) was ground in a Waring Blendor and particles were separated by differential and sucrose density gradient centrifugation by procedures developed by Tolbert *et al.* (8). The pellet, obtained by centrifugation through a gradient up to 2.5 M sucrose, was resuspended in 0.8 M sucrose containing 20 mM sodium cacodylate buffer, pH 6.3, and 1 mM EDTA.

Fraction	3-P-Glycerate Phosphatase		
	Units[1]	% of total	Specific activity
Original	7,280	100	0.03
6000*g* pellet fraction from differential centrifugation	2,850	38	0.27
All sucrose gradient fractions	Trace	...	...
Sucrose gradient supernatant	1,200	16.5	...
Sucrose gradient pellet	1,370	18.8	1.28

[1] Micromoles P_i released min^{-1} mg $protein^{-1}$.

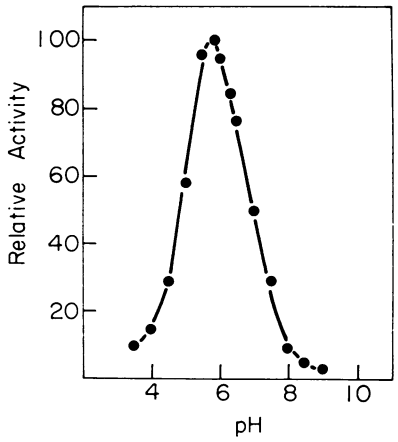

FIG. 1. Effect of pH upon activity of 3-P-glycerate phosphatase in starch particles. The enzyme was assayed in the particulate form and values are the average of assays in three buffer systems: 0.1 M sodium cacodylate + 0.1 M sodium acetate; 0.2 M sodium cacodylate; 0.1 M sodium cacodylate + 0.1 M glycylglycine.

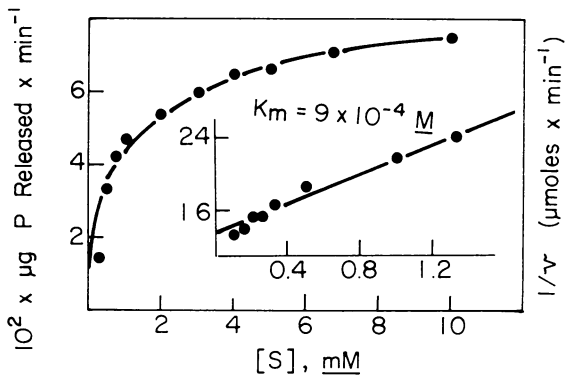

FIG. 2. Michaelis-Menten and Lineweaver-Burk plots for 3-P-glycerate phosphatase in starch particles from spinach leaves. The initial velocity as a function of substrate concentration was measured in 0.3 ml reaction mixtures containing 0.1 substrate of indicated concentration, 0.15 ml 0.2 M sodium cacodylate at pH 5.9 and 0.05 ml of suspended enzyme. The reaction was terminated after 1 min with 0.3 ml of 10% trichloroacetic acid. In the insert K*m* is calculated from a plot of 1/V (µmoles min^{-1}) *versus* 1/S (mM^{-1}).

Substrate Specificity. The relative specificity of the starch particle 3-P-glycerate phosphatase (Table III) was similar to that for the soluble phosphatase from sugarcane leaves. The particulate enzyme was most active but not specific for 3-P-glycerate. The substrates were assayed in the presence of 1 mM $MgSO_4$, which had no effect on the hydrolysis of 3-P-glycerate and had little effect on the hydrolysis of the other substrates. Thus other phosphatases requiring Mg^{2+} probably were absent.

Solubilizing the Particulate 3-P-Glycerate Phosphatase. Resuspension of the starch particles was facilitated by the use of approximately 0.8 M sucrose in 20 mM cacodylate buffer at pH 6.3. Extensive sonication had to be used to solubilize the enzyme. When the particles were suspended in buffer alone and sonicated for as long as 20 min, only 50% of the 3-P-glycerate phosphatase was solubilized to the extent that it was not pelleted by centrifugation at 14,000*g* for 20 min. However, if the

Table II. *Effect of Divalent Cations on the 3-P-Glycerate Phosphatase in Starch Particles*

Aliquots of the particles which had been dialyzed against 20 mM cacodylate buffer at pH 5.9 and 1 mM EDTA were equilibrated at 30 C for 15 min in 0.5 ml of a solution containing 100 μmoles of sodium cacodylate buffer, pH 5.9, and the designated cation. The reaction was initiated by the addition of substrate.

Addition	Relative Activity	
	5 mM Cation	0.5 mM Cation
Control	100	100
Control + EDTA	102	100
$MgSO_4$	104	101
$MnCl_2$	102	101
$ZnSO_4$	5	15
$CoSO_4$	78	85
$CuSO_4$	14	45
$CaCl_2$	98	101
$Pb(acetate)_2$	104	99
$NiSO_4$	80	101

Table III. *Substrate Specificity of 3-P-Glycerate Phosphatase in the Starch Particles*

The enzyme was used in the resuspended particulate form. The Mg^{2+} was equilibrated 15 min with the enzyme before the reaction was initiated with 10 μmoles (13.3 mM) of each substrate.

Substrate	Relative Activity	
	$-Mg^{2+}$	$+Mg^{2+}$
3-P-Glycerate (control)	100	100
P-Glycolate	4	5
2-P-Glycerate	14	15
Glucose-6-P	47	40
Fructose-1,6-diP	67	66
β-Glycerol-P	50	56
p-Nitrophenylphosphate	96	112
bis-*p*-Nitrophenylphosphate	9	8
Adenosine triphosphate	70	52
Adenosine diphosphate	67	62
5′-Adenylic acid	60	59
5′-Deoxyadenylic acid	4	4
3′-Adenylic acid	30	32
3′-Deoxyadenylic acid	3	8

starch pellet was suspended in 0.8 to 1.3 M sucrose, 90 to 94% of the phosphatase was solubilized when sonicated. Homogenization in a Potter-Elvejhem homogenizer did not release the enzyme from the particles, nor did repeated passage through a French pressure cell. Repeated freezing and thawing did not solubilize the enzyme. Dialysis of the suspended particles against 20 volumes of 1 mM EDTA in 20 mM sodium cacodylate buffer at pH 6.3 did not release the enzyme but did result in about a 10% increase in the activity. These procedures broke all traces of whole cells and chloroplasts in the suspension. Limited solubilization of the phosphatase from the starch grains seemed to be favored at pH values about 7. After suspension of the particles at pH 7.0 to 7.5 a maximum of 36% of the enzyme was released from them. Above pH 7.5 the stability of the particulate phosphatase began to decrease with a parallel decrease in the activity that was solubilized.

Incubation of the starch particle suspension with β-amylase at 4 C resulted in solubilization of the 3-P-glycerate phosphatase and the concomitant release of increasing amounts of reducing sugar (Fig. 3). These results support the idea that the particles contain some form of starch or similar polysaccharide. The reason for the loss in total activity for the suspended particles when incubated with amylase for 48 hr is not known, but in the absence of amylase the phosphatase activity was stable for weeks.

Incubation of the starch particles in 0.25 M $MgCl_2$ solubilized 100% of the phosphatase (Fig. 4). NaCl was not as effective as $MgCl_2$. At 0.30 M NaCl, 55% of the phosphatase had been released from the particle, but only 10% more of the phosphatase was solubilized even up to 0.8 M NaCl. The ionic strength of either salt for 50% solubilization of the phosphatase was the same, but solubilization above that point did not coincide. Phosphatase release from the particle seemed to be more dependent upon the Mg^{2+} cation. As the $MgCl_2$ concentration was increased to 0.35 M or greater, the total soluble activity, when measured in the supernatant after removal of the particles, increased 15 to 20% over the total activity of the suspension in $MgCl_2$ before centrifugation. Mg^{2+} did not stimulate the enzyme activity with the particles present. The significance of the in-

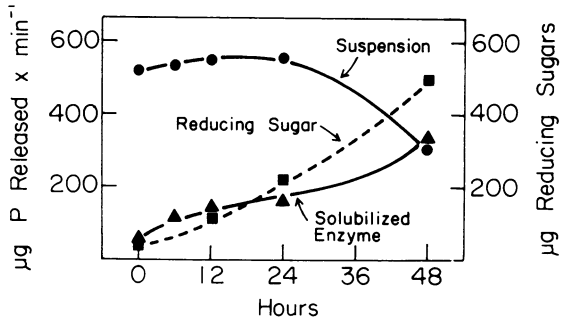

FIG. 3. Solubilization of 3-P-glycerate phosphatase in suspended starch particles by β-amylase. Samples (0.5 ml) of the starch grains containing 3-P-glycerate phosphatase were incubated at 4 C for 48 hr in 50 mM acetate buffer at pH 5.5 with 700 units of β-amylase (Worthington). Reducing sugar (■) and phosphatase activity of the suspension (●) and the supernatant (▲) after centrifugation at 10,000*g* for 20 min were followed.

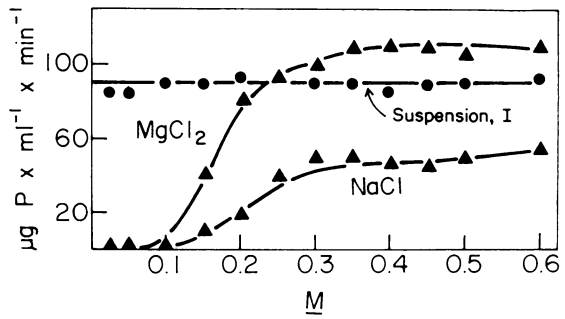

FIG. 4. Solubilization of 3-P-glycerate phosphatase from starch particles as a function of salt concentration. To samples (0.10 ml) of the starch particles in 0.8 M sucrose, 1 mM EDTA, and 20 mM cacodylate buffer at pH 6.3 was added 0.10 ml $MgCl_2$ or NaCl to give the final salt concentration. The samples were incubated for 30 min at 4 C. Total enzyme activity is shown for the untreated suspension (●) and for the supernatant (▲) after treating the suspension with $MgCl_2$ or with NaCl and removing the particles by centrifuging at 10,000*g* for 20 min.

crease in activity at high $MgCl_2$ concentrations, after the enzyme was solubilized, is not understood.

Purification of 3-P-Glycerate Phosphatase from Starch Particles. In the isolation of the starch pellet by sucrose density gradients the 3-P-glycerate phosphatase associated with the particles was enriched 46-fold over the homogenate. The 3-P-glycerate phosphatase from the particle was solubilized and further purified by procedures used to purify the soluble form of 3-P-glycerate phosphatase from sugarcane leaves (Table IV) (5). The enzyme was solubilized by sonification for 20 min at 4 C in 0.8 M sucrose with 20 mM cacodylate buffer at pH 6.3 and 1 mM EDTA, and residual particles were removed by centrifugation. To the enzyme solution at 4 C was added dropwise 0.4 of a volume of reagent grade acetone at −5 C. The system stood for 20 min, then was centrifuged for 10 min at 14,000*g* and the precipitate discarded. More cold acetone, equal to 20% of the starting volume, was added, and the system was equilibrated and centrifuged. The phosphatase activity was in the precipitate, which was redissolved in 20 mM cacodylate buffer at pH 6.3 and 1 mM EDTA. The recovery of the enzyme was 68% with about a 7.5-fold enrichment. Addition of 40 g of $(NH_4)_2SO_4$ per 100 ml precipitated about 25% of the enzyme with no purification. Addition of 20 gm more of $(NH_4)_2SO_4$ per 100 ml precipitated 34% of the enzyme with a small enrichment. After this partial purification, as shown in Table IV, the phosphatase from starch grains of spinach leaves had a specific activity of about 11 and was enriched 384-fold. Our best preparations of the soluble phosphatase from sugarcane leaves had a specific activity of 740 and were enriched 2530-fold (5).

Table IV. *Purification of the Particulate 3-P-Glycerate Phosphatase from 30 g of Spinach Leaves*

Fraction	Units[1]	Specific Activity	Yield	Enrichment	Total Protein
	μmoles/min		%		*mg*
Original extract	74.8	0.03	100	1	2490
6000*g* Pellet	29.0	0.27	38	9	110
Sucrose density gradient pellet	14.0	1.3	19	46	11
Solubilization (sonicated)	13.8	1.3	18	46	11
Acetone fractionation	8.4	9.9	11	352	0.9
$(NH_4)_2SO_4$ fractionation	2.9	10.8	3	384	0.3

[1] Micromoles P_i released min^{-1} mg $protein^{-1}$.

DISCUSSION

The identification of the particles which contained the 3-P-glycerate phosphatase was tentatively designated as starch grains for several reasons. The density of the particle was sufficient to sediment during centrifugation through 2.5 M sucrose. The size and appearance of the particles microscopically resembled starch grains. The particles stained with I_2-KI. Amylase released the phosphatase activity from the particle simultaneously with formation of reducing sugar. The extreme stability of the particles to dialysis and homogenization was atypical of other subcellular organelles.

Since the properties of 3-P-glycerate phosphatase in the cytosol (5) or from the starch grains appear similar, it is probable that the two phosphatases are closely related. However, release of the phosphatase from the starch grain during grinding of the spinach leaf does not seem likely. The procedure was a brief homogenization in buffered sucrose in order to preserve peroxisomes and chloroplasts (8). In contrast, the phosphatase with the starch particles could not be removed by exhaustive washing, dialysis, homogenization, or passage through a French pressure cell. For these reasons it seems that multiple pools of this phosphatase must exist in the leaf cell.

Although 3-P-glycerate phosphatase was so tightly bound to the starch grain that it could hardly be removed by physical means, incubation with 0.25 M $MgCl_2$ in 0.8 M sucrose solubilized it. NaCl was less effective. The function of Mg^{2+} is not known, particularly when Mg^{2+} did not stimulate the starch-bound phosphatase activity. Mg^{2+} in this case seems to be controlling the particle-protein binding site rather than the active site of hydrolysis.

Since the starch grains are in the chloroplast, in a sense the 3-P-glycerate phosphatase of the starch grain is a chloroplastic enzyme. If 3-P-glycerate phosphatase of the starch grain was partially solubilized *in vivo*, then one would expect to find some of this activity in the isolated plastids. For comparison, an analogous phosphatase for P-glycolate hydrolysis (7) does remain with the chloroplasts (6). No 3-P-glycerate phosphatase was found in the chloroplast isolated on sucrose gradients, buffered with 20 mM glycylglycine at pH 7.5. Phosphatase activity on the top of the gradient (Table I) suggests that the enzyme may have been loosely associated with the chloroplasts during differential centrifugation but dissociated during the gradient centrifugation. Apparently the dense starch grains tear out of the choroplast and pellet as a separate band.

During destarching of leaves by storage in the dark, the starch pellet largely disappears. Preliminary experiments indicate that a particulate form of 3-P-glycerate phosphatase remains that passes through 2.5 M sucrose during high speed centrifugation. This might be a limited form of the starch grain with enzymes and primer polysaccharide that is resistant to further breakdown. On the other hand, some preparations from spinach leaves were observed which contained only a very small amount of particulate 3-P-glycerate phosphatase, but no physiological correlation with the age and storage condition of the leaves has hitherto been possible.

Many factors appear to regulate carbohydrate metabolism and gluconeogenesis. In the case of starch formation, P-glycerate phosphatase of the starch grain may be a part of this regulatory mechanism by controlling the P-glycerate level at a specific site. 3-P-Glycerate in turn is an effector for ADP-glucose pyrophosphorylase (1), which forms the precursor for starch synthesis. This type of regulation may be prevalent in plants in contrast to the use of secondary effectors such as cyclic-AMP. Spinach chloroplasts also contain a glycerate kinase (2). Thus enzymes for increasing and decreasing the 3-P-glycerate level are present. A diurnal variation in activity of 3-P-glycerate phosphatase in sugarcane leaves has been noted, which if present in other plants (6), would regulate the pool size of 3-P-glycerate, activity of ADP-glucose pyrophosphorylase and starch synthesis. The location of the phosphatase in the starch grain is puzzling, because 3-P-glycerate and ADP-glucose pyrophosphorylase are in the chloroplast stroma. However, if large amounts of the phosphatase were in the stroma of the chloroplast, too much of the 3-P-glycerate, which is an essential part of the photosynthetic carbon cycle, might be removed. Complex regulation of starch synthesis by components of the starch grains and the chloroplasts are probably involved, and the exact function of 3-P-glycerate phosphatase in the starch grain is not known.

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