*ISOLATION OF β-GLUCAN SYNTHETASE PARTICLES FROM PLANT CELLS AND IDENTIFICATION WITH GOLGI MEMBRANES**

By Peter M. Ray, Terry L. Shininger, and Margery M. Ray

DEPARTMENT OF BIOLOGICAL SCIENCES, STANFORD UNIVERSITY
STANFORD, CALIFORNIA

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Abstract.—A variety of particle-bound synthetases that use sugar nucleotides as glycosyl donors for the formation of polysaccharides similar to those of the cell wall have been demonstrated in mung beans and other plant tissues,[1] but the particles in question have not been previously identified.[2, 3] The polysaccharide synthetase particles from peas that form mainly β-1,4-glucan from UDPG and GDPG have now been separated from other cell particles by combinations of velocity and isopycnic density gradient centrifugation. The particles have an effective density of about 1.15 gm cm^{-3}, exhibit latent nucleoside diphosphatase activity upon IDP, UDP, GDP, and to a lesser extent upon ADP, and also possess acid phosphatase and weak ATPase activity. The isolated synthetase particles consist of somewhat condensed Golgi dictyosomes and free dictyosomal membranes bearing vesicles. It is concluded that the synthetase particles are Golgi membranes. The nucleoside diphosphatase activity of these particles may represent inactivated polysaccharide synthetase.

Methods.—Segments 8 mm long were cut from the third internode of etiolated pea seedlings (*Pisum sativum* L., cv. Alaska) 7-days old. Tissue (100–200 segments, 2–4 gm) was chilled on ice, and either ground in an ice-cold mortar for 2 min at 0°C in 4 ml of 40 mM Tris buffer, pH 8.0 containing 0.4 M sucrose, 10 mM KCl, 1 mM EDTA, 0.1 mM $MgCl_2$, 1 mM dithiothreitol, and 0.1% bovine serum albumin, or else chopped thoroughly with a stainless steel razor blade in 3 ml of this medium contained in a Pyrex dish on ice. The homogenate was squeezed through cheesecloth and centrifuged 2 min at 1000 × *g*. The precipitate, which contained negligible synthetase activity, was discarded and the supernatant was used for isolation of synthetase particles.

Density gradients were linear in sucrose concentration and otherwise had the same composition as the homogenization medium except for omission of KCl and $MgCl_2$; gradients were centrifuged in the Spinco SW 25.1 rotor. Isopycnic separations were obtained after 2 hr at 25,000 rpm.

Polysaccharide synthetase was assayed as 70% ethanol-insoluble radioactivity formed from 0.025 μc of ^{14}C-sugar nucleotide (normally UDPG) by 100 μl of enzyme preparation in 10 min at 25°C.

Acid phosphatase was assayed with 2.2 mM p-nitrophenylphosphate in 80 mM cacodylate buffer, pH 5.5, containing 2.2 mM $MgCl_2$, at 37°C. Results are given as ΔE_{420} in 60 min.

ATPase was assayed with 1.1 mM ATP and 1.1 mM $MgCl_2$ in 61 mM Tris buffer, pH 7.5, at 37°C. IDPase was assayed similarly, with 2.4 mM IDP, using enzyme preparation held at 0°C for 2 to 4 days to allow activation. Results are given as E_{710} from determination[4] of P_i formed in 60 min; E_{710} of 1.0 is equivalent to 0.43 μ moles P_i.

Cytochrome oxidase was assayed by spectrophotometrically following the oxidation of reduced cytochrome *c*. Malic dehydrogenase was assayed by following reduction of 2,6-dichlorophenolindophenol in presence of malate and NAD. Protein was determined with

Folin reagent[5] upon particles that had been recovered from the preparations by centrifugation.

Particles shown in Fig. 5 were fixed with 2.5% glutaraldehyde in 0.4 M sucrose (pH 7.4 with phosphate) followed by 0.9% OsO_4 (pH 7.4 with veronal). Epon sections were stained with lead citrate.

Results and Discussion.—Most of the UDPG-dependent glucan synthetase activity in pea homogenates could be sedimented by centrifugation at 40,000 × *g* for 30 minutes and accordingly in initial experiments the behavior of resuspended particles from pellets so obtained was examined by isopycnic density gradient centrifugation. As illustrated in Figure 1*A*, glucan synthetase activity banded sharply at a density of about 1.15 gm cm^{-3}, indicating that the responsible particles are fairly homogeneous in density and, therefore, presumably homogeneous in composition. This procedure afforded a nearly complete separation of synthetase particles from mitochondria, which were responsible for the major turbidity band at a density of 1.18-1.19 gm cm^{-3}, as is typical of mitochondria from many sources. A similar isopycnic density gradient distribution was obtained with mung bean particles prepared as described for peas.

Products formed by synthetase particles.—The synthetase particles that band at density 1.15 gm cm^{-3} produce both alkali-soluble and alkali-insoluble products from UDPG (Fig. 1*A*), and they also form an insoluble product from GDPG (Fig. 1*B*). A similar peak of activity was obtained with UDP-galactose as substrate.

Streptomyces QM B 814 cellulase[8] hydrolyzed both alkali-soluble and alkali-insoluble products derived from both UDPG and GDPG largely to cellobiose; a typical chromatogram scan is shown in Figure 2. No laminaribiose or laminaritriose was formed even though the enzyme could degrade laminarin to these oligosaccharides. Treatment of the radioactive products from UDPG or GDPG with concentrated HCl at 25°C released glucose and cellobiose in about equal amounts. These data indicate that the glucan formed by these synthetase particles was mainly β-1,4-linked.

Enzyme profiles.—Figure 3*A* shows the distribution of several enzymes on isopycnic gradients prepared from particles sedimented by 40,000 × *g* for 30

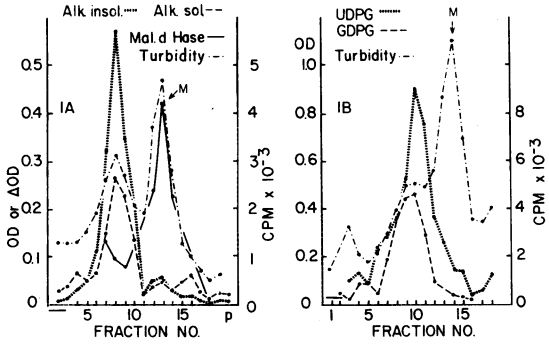

FIG. 1.—Distribution on isopycnic linear 20–50% sucrose density gradients of turbidity and of polysaccharide synthetase activities of particles pelleted from pea homogenates by centrifugation at 40,000 × *g* for 30 min. Fig. 1*A*, alkali-soluble and alkali-insoluble products from UDP-^{14}C-glucose (malic dehydrogenase distribution also shown). Fig. 1*B*, hot ethanol-insoluble products from UDPG and GDPG. In this and subsequent figures, M indicates position of mitochondrial band; P is fraction containing pelleted material at bottom of tube; the bar that appears along the abscissa at left denotes the fractions that correspond to the sample that was applied originally to the gradient.

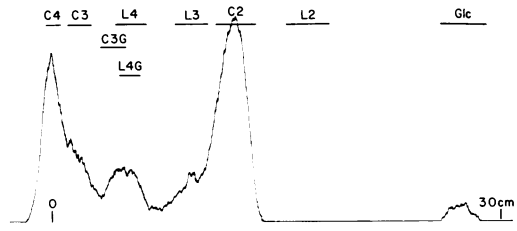

FIG. 2.—Scan of paper chromatogram of cellulase hydrolysate of alkali-insoluble product formed from UDPG by pea particles; solvent was *n*-propanol, ethyl acetate, water (7:1:2 by vol.). Positions of comparison compounds shown above: L2, L3, L4, laminaribiose, -triose, -tetraose; C2, C3, C4, cellobiose, -triose, -tetraose; L4G, laminaribiosyl-1,4-glucose; C3G, cellobiosyl-1,3-glucose. The radioactive peak in position of C2 rechromatographed like C2 in ethyl acetate, pyridine, water (10:4:3 by vol.). The minor product in position of L4, L4G, and C3G ran slower than these compounds in this latter solvent and proved to be a mixture of at least two compounds which have not yet been identified. Chromatograms of cellulase hydrolysates of alkali-soluble product from UDPG and of alkali-insoluble product from GDPG were similar to the scan illustrated.

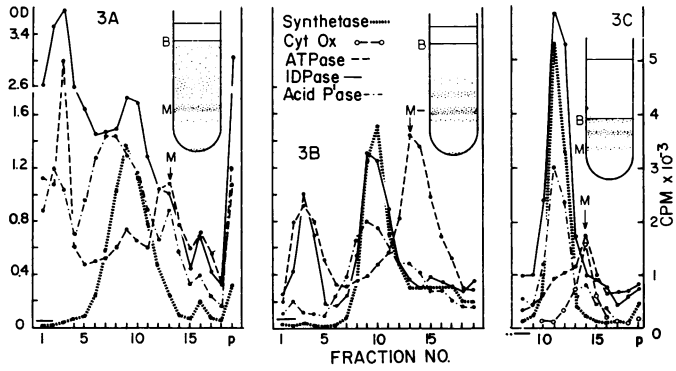

FIG. 3.—Distributions, on isopycnic gradients, of glucan synthetase (UDPG as donor, *right-hand ordinates*), ATPase, IDPase and acid phosphatase (*left-hand ordinates*), of particles from pea tissue: Fig. 3*A*, pellet from centrifugation of mortar-ground homogenate at 40,000 × *g* for 30 min; Fig. 3*B*, pellet from centrifugation of a similar homogenate through 10 ml of 0.5 *M* sucrose medium 30 min at 10,000 × *g*; Fig. 3*C*, particles from synthetase peak region of velocity gradient (zone marked with bracket in inset diagram in Fig. 4*B*), prepared from tissue homogenized by chopping with a razor blade (cytochrome oxidase distribution also shown). 3*A* and 3*B* were 20–50% gradients (25 ml), 3*C* was 25–45% gradient (15 ml). B shows boundary between the applied sample and top of gradient.

minutes. Peaks of ATPase and IDPase activity coincide with the synthetase peak, while a broad zone of acid phosphatase activity overlaps but peaks at a slightly lower density than the synthetase activity, indicating the presence of a separate class of particles, which have been termed "heavy lysosomes."[9] It was found that this phosphatase peak and the broad zone of turbidity associated with it in the upper part of the gradient could be eliminated by using the particles that sediment at 10,000 × *g* for 10 minutes. If the particles were initially pelleted by centrifuging through a layer of 0.5 M sucrose for 30 minutes at 10,000 × *g*, the ATPase peak previously associated with the synthetase band would be reduced to a minor shoulder compared with the mitochondrial ATPase peak (Fig. 3*B*). This indicates that the ATPase peak at fraction 9 in Figure 3*A* was associated with a different class of particle than that which bears the synthetase

activity. A very prominent peak of IDPase, however, accompanied the synthetase peak (Fig. 3*B*) as did some phosphatase activity. The IDPase activity is latent, developing to a maximum over about 4 days at 0°C.

Velocity sedimentation behavior.—If the particles from a synthetase peak similar to that in Figure 3*B* were centrifuged through a sucrose gradient of lower density for velocity separation (Fig. 4*A*), the peaks of ATPase and phosphatase activity moved more slowly than the peak of synthetase activity, whereas IDPase activity coincided with synthetase activity. This indicated that some of the phosphatase and ATPase activity found at the synthetase peak in Figure 3*B* was due to contaminating particle(s), whereas IDPase appears to be borne by the synthetase particles.

Velocity sedimentation profiles obtained for synthetase particles by layering unpelleted homogenates directly upon 15 to 35 per cent sucrose gradients and centrifuging are shown in Figure 4*B*. The peak of synthetase activity traveled about half as rapidly as the mitochondrial peak. Comparison of the profiles in Figure 4*A* and 4*B* shows that a marked decline in sedimentation coefficient, and hence presumably in size of the synthetase particles, was taking place during the isolation procedure. Other velocity sedimentation experiments showed that most of this degradation resulted from pelleting and resuspension of the particles.

As shown in Figure 4*B*, the velocity sedimentation profile for synthetase particles that had been extracted from the tissue by chopping it in the homogenizing medium with a razor blade exhibited relatively little slow-traveling synthetase activity between the synthetase peak and the starting point, as compared to that

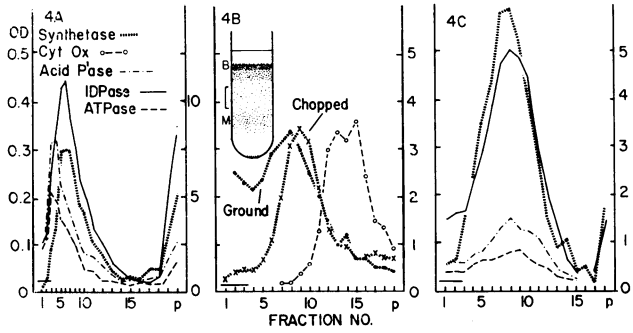

FIG. 4.—Velocity sedimentation of synthetase particles and other particulate enzyme activities in 15–35% linear sucrose gradients. Fig. 4*A*, particles from synthetase peak region of a gradient like that of Fig. 3*B;* 25 min at 13,750 rpm (ca. 20,000 × *g*); Fig. 4*B*, unpelleted homogenate, prepared by grinding in a mortar or by chopping with a razor blade, and layered onto gradients; 25 min at 10,000 rpm (ca. 10,000 × *g*); Fig. 4*C*, particles from synthetase peak region of an isopycnic gradient similar to Fig. 3*C*, after dilution with buffer; 36 min at 10,000 rpm. Right-hand ordinates are synthetase assay (UDPG) in cpm × 10^{-2} (10^{-3} in case of 4*B*). Cytochrome oxidase distribution shown in 4*B* is for chopped homogenate; that of ground homogenate was closely similar. Fractions were 1.5 ml except in 4*A* where fractions 1–10 were 1 ml and the rest were 2 ml; distance on abscissa is proportional to distance through the gradient, the entire abscissa representing 65 mm. The inset diagram in Fig. 4*B* pertains to the chopped homogenate, but ground homogenate gives a gradient of similar appearance; the bracket to the left of this diagram shows the portion of the gradient that is used to prepare the type of isopycnic gradient shown in Fig. 3*C*.

of homogenates prepared by grinding in a mortar. This indicates that grinding degrades the size of many of the synthetase particles, and that these particles, when extracted without grinding, comprise a population relatively homogeneous in size and possess a sedimentation coefficient about one half that of mitochondria.

Isolation and characterization.—To isolate synthetase particles in the least degraded state we remove, as shown in the inset in Figure 4*B*, a zone (the "velocity cut") containing the synthetase peak, from three similar velocity gradients, and layer this upon a gradient that is then centrifuged isopycnically. Such gradients (Fig. 3*C*) show one sharp major peak of turbidity, in which synthetase activity is localized, plus fainter bands due to those mitochondria that fall within the velocity cut, and to particles of density lower than the synthetase particles. Sharp peaks of IDPase and phosphatase activity accompany the synthetase peak, as well as a low level of ATPase activity. The purification of synthetase activity that is achieved by this procedure is shown in Table 1. That the specific activity of synthetase is essentially the same in all three fractions from the synthetase zone of the isopycnic gradient suggests that synthetase particles comprise virtually all the material in this region.

When synthetase particles, isolated from chopped tissue by the velocity cut/isopycnic density gradient procedure, were recentrifuged through a velocity gradient, the velocity profile obtained (Fig. 4*C*) was broader and the peak was somewhat slower traveling than that of the synthetase activity of the original homogenate (Fig. 4*C*). However, very small synthetase particles were not present (contrast Fig. 4*A*), indicating that fragmentation in size during this procedure is much less extensive than when grinding or pelleting are involved. The distribution of IDPase on this velocity gradient closely matched that of synthetase activity (Fig. 4*C*), confirming that these activities are borne by the same particles. Moreover, the phosphatase activity and the feeble ATPase activity that was present conformed with the distribution of the preceding activities,

TABLE 1. *Purification of glucan synthetase particles by combined velocity and isopycnic density gradient centrifugation*

	Glucan Synthetase*			Nucleoside Diphosphatase*		
Fraction†	Units / 100 μl	Units / mg protein	Total activity (units)	Total activity (units)	Activation factor	IDPase / ATPase
Total particles	1.25	11.8	199	317	1.1	1.3
Velocity gradient cut	0.74	69	108	102	2.7	3.8
Isopycnic gradient fractions 10	0.86	95	15	15	4.4	9.6
Isopycnic gradient fractions 11	1.64	93	28	31	3.9	10.7
Isopycnic gradient fractions 12	1.37	97	24	26		7.4
Fractions 10 + 11 + 12			67	72		

* Glucan synthetase activity is given in units of percentage of incorporation (from UDPG) into polysaccharide per min; nucleoside diphosphatase activity in units of μmoles P_i from IDP per hr, using fully activated enzyme. IDPase/ATPase activity ratio was calculated from activities expressed in the same units; activation factor denotes maximum IDPase (after activation of latent enzyme) divided by initial IDPase.

† Particles were obtained by chopping 12.8 gm of pea tissue with razor blades; after low-speed centrifugation an aliquot of the supernatant was centrifuged at 40,000 × *g* for 30 min to obtain "Total particles," while the rest was carried through velocity gradients followed by an isopycnic gradient, as described in text, affording the yields indicated.

indicating that these activities are found on the synthetase organelle and that the preparation is now substantially free of the contaminating ATPase-bearing particle mentioned previously.

The synthetase activity using UDPG, GDPG, and UDPGal as donors was in the ratio 10:4:2 with either the purified particles or the total particles obtainable by pelleting the crude homogenate. Therefore, these activities seem to be borne by the same (or very similar) particles.

The relative activity of the IDPase (nucleoside diphosphatase) of the purified particles on UDP was 72 per cent, GDP 49 per cent, ADP 11 per cent, and TPP (thiamine pyrophosphate) $< 1\%$ of the activity on IDP.

Electron micrographs of isolated synthetase particles (Fig. 5*A*) revealed a uniform population of smooth double-membrane segments bearing vesicles at their edges, which appear to be Golgi cisternae, and aggregates of two or more such membranes, often with an apparent concentric shell structure that was also observed in Golgi dictyosomes found in crude particle preparations (Fig. 5*B*) and in other isolated Golgi material.[6, 7, 10]

The conclusion that the synthetase particles are Golgi membranes is reinforced

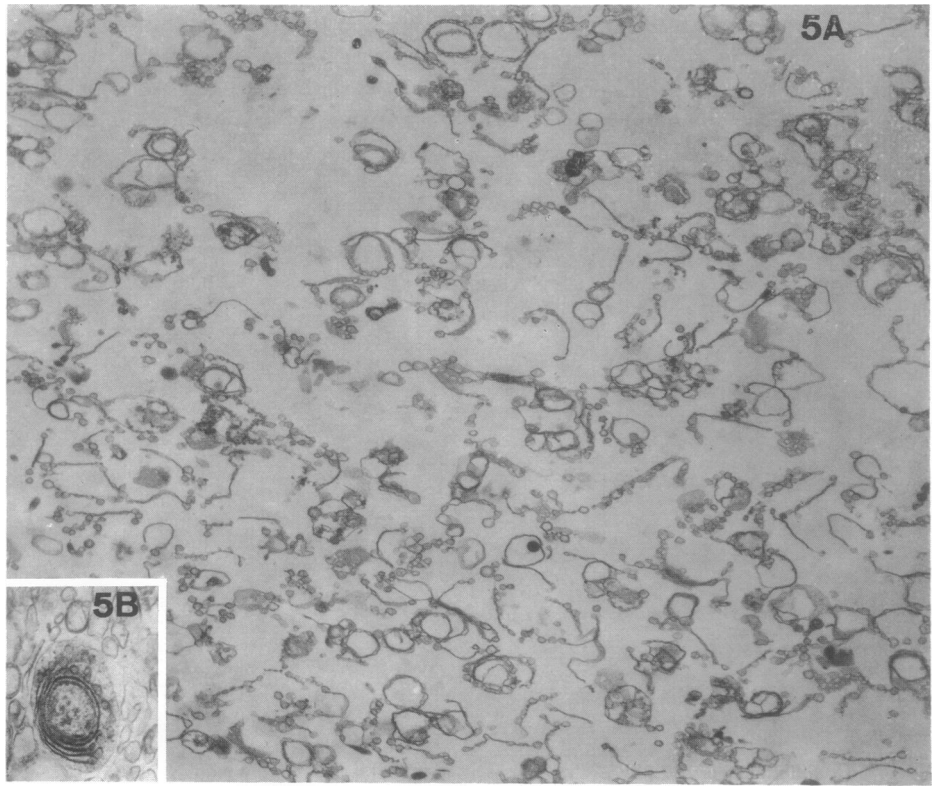

FIG. 5*A*.—Electron micrograph of particles in the peak synthetase fraction of a gradient similar to Fig. 3*C*. ×6,720. Fig. 5*B*, membrane-bound cytoplasmic vesicle containing a dictyosome among particles obtained from tissue that had been homogenized by chopping with a razor blade. ×10,752. Note resemblance to the membrane forms seen in 5*A*.

by the enzyme observations, since IDPase has been found by cytochemical methods to be associated universally with Golgi apparatus,[11] including dictyosomes of plant cells.[12, 13] The substrate range of the nucleoside diphosphatase activity also resembles what has been found for the Golgi enzyme.[11] Acid phosphatase has also been detected cytochemically in various Golgi systems of plant origin,[12, 14] as has weak ADPase and ATPase activity.[13] However, thiamine pyrophosphatase activity, which is characteristic of the Golgi apparatus of many cells,[11-13] is not found in the synthetase particles, but neither could we detect this enzyme in the total particles sedimented at 40,000 × g.

Significance of the nucleoside diphosphatase activity of Golgi structures.—IDPase activity of the purified synthetase particles is latent, as previously mentioned, whereas the ATPase and p-nitrophenyl-phosphatase activities are not. Activation of IDPase at 0°C follows a time course (over 4–5 days) similar to the decline of synthetase activity which, as is well known, is labile. Treatment with digitonin or Triton X-100, which inactivates the synthetase, activates the IDPase of these particles. IDP, UDP, and, to a lesser extent, GDP, which are substrates for the nucleoside diphosphatase activity, inhibit synthetase activity indicating that these nucleotides interact with the synthetase, whereas ADP, which is a poor substrate for nucleoside diphosphatase, does not inhibit the synthetase.

From these facts we infer that *the nucleoside diphosphatase activity of synthetase particles represents inactivated polysaccharide synthetase.* The close correspondence between synthetase and activated IDPase activities seen in the purified synthetase fractions 10–12 (Table 1) supports this view. It accounts for the correlation between apparent secretory activity of dictyosomes and presence of IDPase activity detected cytochemically.[12]

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