Inositol Metabolism in Plants. VI. Conversion of Myo-Inositol to Phytic Acid in Wolffiella floridana

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Abstract. When Wolffiella floridana, an aquatic angiosperm in the family, Lemnaceae, was grown in axenic culture under continuous light in E medium containing 1.0% sucrose and a micromolar amount of \(^{14}\text{C}\)-labeled myo-inositol (MI), MI was taken up by the growing plants and converted to phytic acid. After 13 weeks in labeled medium during which time there was a 1000-fold increase in fresh weight, 30% of the \(^{14}\text{C}\) was recovered in ethanol insoluble residue. Extraction of this residue with EDTA released 70% of the label into solution. Phytic acid, identified by paper electrophoresis, ion exchange chromatography, and hydrolysis with phytase, accounted for most of this radioactivity although some label was also found in pentaphosphate and lower phosphate esters of MI. Very little MI was converted to cell wall polysaccharides under the conditions used. Results of this study indicate that Wolffiella floridana is a convenient tissue for the study of phytic acid biosynthesis under laboratory conditions.

Lemna gibba G3, grown under short day conditions in medium of the same composition as that used for W. floridana, also formed labeled phytic acid as well as other labeled lower phosphate esters of MI.

Phytic acid, the hexaphosphoric acid ester of myo-inositol, is a plant constituent of long-standing familiarity yet its biosynthesis remains as an unsolved problem (8). As a complex salt, it is found in most plant tissues; root, stem, leaf, flower, and fruit, as well as seed. The latter usually contains most of the phytic acid present in the mature fruit. Since seed or grain accumulates phytic acid, there have been several attempts to demonstrate phytic acid biosynthesis by incorporation studies in which labeled myo-inositol (MI) or isotopic inorganic phosphate (P\(_\text{i}\)) was given to plants during the ripening process (1, 3, 4, 25). In addition, others have measured the accumulation process in terms of changes in P\(_i\), free MI, and phytic acid (16, 19, 24). One study extended experiments with labeled MI to include young plants not yet in the ripening stage (14). In that study, young plants given MI-2\(^{\text{3}}\text{H}\) did form labeled phytic acid but much of the tritium no longer was attached to C2 of the MI fragment in contrast to greater retention of tritium by C2 in older plants. Except for this result, more findings reported thus far have concluded that biosynthesis of phytic acid involves direct phosphorylation of MI, either by successive steps of phosphate esterification or by a more obscure process in which only the end product, phytic acid, is detected. Use of tritiated MI (3, 4, 14), particularly MI-2\(^{\text{3}}\text{H}\), may give results that can be misinterpreted in the absence of detailed information concerning the metabolic fate of tritium attached to C2. Moreover, experiments reported thus far have not achieved substantial incorporation of label into phytic acid.

In the course of an investigation of MI metabolism in Lemnaceae (18), we noted that Wolffiella floridana forms a substantial amount of phosphorylated MI during vegetative growth in axenic culture. Experiments reported here relate to the identification of this material as phytic acid.

Materials and Methods

Plant Cultures. Wolffiella floridana and Lemna gibba G3, grown from cultures generously provided by Dr. C. F. Cleland and Dr. W. R. Briggs, were cultured in liquid E medium containing sucrose (1% w/v) as recommended by Cleland and Briggs (7). Growth conditions were described in an earlier paper (18). Unlike L. gibba, W. floridana showed no tendency to flower, even under continuous light.

Recovery of Phytic Acid. Plants were harvested on a Buchner funnel, washed repeatedly with fresh portions of distilled water, and then freed of excess water by drawing air through the funnel for several min. Plant tissue was ground in ethanol (80% v/v) either in a glass homogenizer (Kontes No. 885454) or a Servall Omnimixer fitted with a 50 ml stainless steel container. Ground tissue was centrifuged, washed repeatedly with fresh portions of...
ethanol (80% v/v) until washes were free of chlorophyll and $^{14}$C, then washed successively with ethanol (100%), chloroform-methanol (1:2, v/v), ethyl ether, and dried. The final product (1.44 g), a white powder, referred to in this paper as ethanol insoluble residue, contained phytic acid as an insoluble salt. Extraction of the residue with disodium EDTA (0.2% w/v, 40°, 30 min) released phytic acid into solution along with other components. Separation of these components from phytic acid was accomplished by paper electrophoresis and ion exchange chromatography.

**Ion Exchange Chromatography.** The procedure devised by Wilson and Harris (28) was used to separate phosphate esters of MI. It was modified slightly to accommodate the smaller samples involved in this study. The column had a resin bed that was 5 by 0.9 cm and each chamber of the reservoir initially held 100 ml of eluant. Uniform flow of 100 ml per hr was maintained with a peristaltic pump. Fraction volume was 5 or 10 ml as circumstances demanded. With this system, we could separate a mixture of mono-, di-, tri-, tetra-, penta-, and hexaphosphates of MI in about 5 hr. The elution pattern of a partially hydrolyzed phytate (from a sample of sodium phytate kindly provided by Dr. M. E. Tate, Waite Institute, Adelaide, Australia) that had been treated with phytase (27) is given in figure 1.

**Paper Electrophoresis.** Separations were made on Whatman No. 1 or 3MM paper strips in 0.1 m ammonium formate buffer (pH 3.8, 20 volts per cm, 100 min) (10) or in 0.1 m sodium oxalate buffer pH 1.58, 16 volts per cm, 150 min) (23). Mobilities were compared to that of picric acid. Sodium oxalate buffer gave an excellent separation of a mixture of mono-, di-, tri-, tetra-, penta-, and hexaphosphates of MI. Recently Tate (26) used this buffer to resolve 4 isomeric pentaphosphates of MI with the aid of moving paper electrophoresis. Phosphates were detected by spraying dried papers with ammonium molybdate reagent (12) and exposing sprayed papers to UV light. Radioactivity was detected with a Packard Model 7200 windowless strip scanner.

**Other Analytical Procedures.** Gel filtration was run on Sephadex G-100 and G-200 columns prepared according to the manufacturer’s recommendations. Effluent fractions from these and other columns were assayed for phosphate (5), total carbohydrate (9), and radioactivity. Radioactive samples were counted in a liquid scintillation spectrometer according to methods used previously (18). The efficiency of the spectrometer count was 78% for $^{14}$C.

Monosaccharide components in hydrolyzates of cell wall polysaccharides were separated on thin layer plates of cellulose powder with the following solvent systems: ethyl acetate-pyridine-water-acetic acid (5:5:3:1, v/v) or methyl ethyl ketone-n butanol-acetic acid-water (3:2:2:2, v/v). The $R_f$ values were reported earlier (18).

**Results**

The sample of ethanol insoluble residue of *W. floridana* used to isolate and identify phytic acid was obtained from an axenic culture that had been grown in E-plus-sucrose medium (200 ml) to which had been added MI-2-$^{14}$C (7.8 $\mu$C; 11.5 mc/mmole). The inoculum was 4 frond clusters (approx 10 mg) and the final fresh weight (13 weeks) was 12.5 g, an increase of over 1000-fold. Plants were grown...
under continuous light. The distribution of $^{14}$C into various extracts and the ethanol insoluble residue is given in Table I.

**Acid Hydrolysis of Ethanol Insoluble Residue.** Treatment with 0.1 N hydrochloric acid (100°, 30 min) released 80% of the $^{14}$C present in the residue. Thin layer chromatography failed to mobilize any of the $^{14}$C present although considerable quantities of unlabeled apiose, xylose, arabinose, and glucose were separated on the plate and could be detected by conventional spray reagents for sugars. Even hydrolysis with N hydrochloric acid (121°, 30 min sealed tube) failed to produce a hydrolyzate in which $^{14}$C could be separated from the origin of the chromatogram with the 2 solvent systems used.

**Disodium EDTA Extraction of Ethanol Insoluble Residue.** This procedure released 75 to 80% of the $^{14}$C present in the residue. Paper electrophoresis of a portion of this extract in formate buffer resulted in the separation of about 65% of the radioactivity present in the extract as a single spot with

**Fig. 2.** Radiochromatogram scan of EDTA extracts of ethanol insoluble residues of *W. floridana* and *L. gibba* after paper electrophoresis in a) ammonium formate or b) sodium oxalate buffer as indicated in the figure. For comparative purposes, a partially hydrolyzed, phytase-treated sample of phytate was included in (b) to indicate the relative positions of MI phosphates. Partially shaded spots correspond to color produced by phosphorus reactive spots or picric acid. Numerals refer to the number of phosphorus ester groups assumed to be attached to MI.

**Table I. Recovery of Carbon-14 From Wolffiella floridana After Labeling With myo-Inositol-2-$^{14}$C**

<table>
<thead>
<tr>
<th>Carbon-14 fraction</th>
<th>Radioactivity</th>
<th>% Of total $^{14}$C supplied</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial amount in medium (200 ml)</td>
<td>14,000</td>
<td>100</td>
</tr>
<tr>
<td>Final amount in medium after 13 weeks growth</td>
<td>3151</td>
<td>22.5</td>
</tr>
<tr>
<td>Amount recovered in ethanol-soluble fraction</td>
<td>5450</td>
<td>39</td>
</tr>
<tr>
<td>Amount recovered in subsequent organic washes</td>
<td>256</td>
<td>2</td>
</tr>
<tr>
<td>Amount recovered in ethanol insoluble residue</td>
<td>3816</td>
<td>27</td>
</tr>
<tr>
<td>Total recovery</td>
<td>12,773</td>
<td>90.5</td>
</tr>
</tbody>
</table>

Fig. 3. Gel filtration on a column of Sephadex G-200 of an EDTA extract of ethanol insoluble residue from *W. floridana*. The radioactivity (---), total carbohydrate content (----), total phosphorus content (- - - - - - -), and phosphorus content (-----) per unit volume in the effluent are compared. Arrow refers to the final increment of the void volume.

$R_{ext} = 2.33$, identical with that of phytic acid (fig 2a). A portion of the $^{14}$C remained at the origin and a small peak of radioactivity was found in the region of MI monophosphates. Electrophoresis in oxalate buffer also gave a major radioactive spot corresponding to MI hexaphosphate as well as a small radioactive peak corresponding to MI pentaphosphate (fig 2b).

A portion of the EDTA extract was gel-filtered through a column of Sephadex G-200. The elution pattern is seen in figure 3. All the radioactivity present in this extract was recovered in 2 peaks. A high molecular weight fraction (55-75 ml of effluent volume) in the region of the excluded
Radioactivity bound to the Phytase.

Two intermediate phosphate peaks were devoid of carbohydrate or $^{14}$C. Paper electrophoresis in oxalate buffer of the material present in the low molecular weight fraction revealed that nearly all the radioactivity that was present in this fraction had the mobility of MI hexaphosphate (fig 2b). This corresponds to about 55% of all the $^{14}$C present in the ethanol insoluble residue.

To further characterize this EDTA-extracted radioactive material, a portion corresponding to 70 mg of ethanol insoluble residue was passed through a Dowex 1 formate resin column ($5 \times 0.9$ cm). The resin retained 82% of the $^{14}$C. Elution with a formic acid-ammonium formate gradient removed over 90% of the $^{14}$C that was bound to the column as 3 radioactive peaks (fig 4), 2 small ones in the region of MI mono- and pentaphosphates, and a third corresponding to MI hexaphosphate. The latter contained 75% of the $^{14}$C in the extract and was accompanied by 70 $\mu$g of phosphorus.

Hydrolysis of Ethanol Insoluble Residue With Phytase. Incubation at 37° of the ethanol insoluble residue (38 mg) in a phytase preparation (4 ml) prepared from endosperm tissue of 3 day old Zea mays seedlings (17) slowly released the MI portion of MI phosphate as free cyclitol. Within 45 min after the start of incubation, 70% of the residue-bound radioactivity was soluble but most of this soluble fraction still retained one or more phosphate groups attached to MI as indicated by its retention on Dowex 1 formate resin. Incubation for 16 hr did not increase the amount of soluble label appreciably but it did remove phosphate groups as indicated by the fact that now 75% of the label was no longer retained by the anionic resin. The neutral portion of the hydrolysate, freed of ionic components, was evaporated to a small volume, diluted with carrier MI (150 mg), and crystallized from water with ethanol. The specific activity of this MI, 11,900 cpm per mmole, decreased only slightly (to 11,700 cpm per mmole) after 2 recrystallizations. Conversion of this MI to its hexaacetate followed by 2 recrystallizations from ethanol with water reduced the specific activity slightly to 11,100 cpm per mmole. It was estimated that at least 92% of the $^{14}$C present in the neutrals from the 16 hr phytase hydrolysis was present as MI.

![Fig. 4. Ion exchange chromatography of an EDTA extract of ethanol insoluble residue of $W$. floridana. Radioactivity (- - - -) and phosphorus content ( - - - -) per unit volume are compared. See figure 1 for gradient as well as other details.](image-url)

Fig. 5. Radiochromatogram scan of phytase-hydrolyzed extracts of ethanol-insoluble residue from MI-labeled $W$. floridana following paper electrophoresis in sodium oxalate buffer. Hydrolyses were run on a) untreated residue, b) water-extracted residue, and c) water-extracted residue in presence of 0.05 M phosphate. Samples were removed for separation by paper-electrophoresis at intervals of 0, 0.5, 1, 2, 4, and 24 hr. Partially shaded spots correspond to color produced by phosphorus reactive spots of partially hydrolyzed phytate control. Numerals refer to the number of phosphate ester groups assumed to be attached to MI.

The unusually slow appearance of free MI from ethanol insoluble residue after treatment with phytase prompted further study of the time course of phytase digestion. Residue (25 mg) was incubated at 37° in the phytase preparation (1.5 ml) and at intervals (0, 0.5, 1, 2, 4, and 24 hr), aliquots were removed and applied to Whatman No. 1 paper (1.5 in streaks). Paper electrophoresis in oxalate buffer followed by scanning gave the radiochromatograms reproduced in figure 5a. Delay in applying and drying the zero-time control resulted in some hydrolysis but the first significant shift from MI hexaphosphate was evident at 0.5 hr. Within 2 hr most of the MI hexa- and pentaphosphate label had disappeared as the amount of label in lower phosphates increased. At 4 hr, di- and monophosphates accounted for most of the phosphate ester label while at 24 hr, only these 2 esters remained in addition to a very large free MI peak at the origin and a persistent radioactive component, unaffected by phytase, that had a very slight positive mobility. Nagai and Funahashi (15) have observed a sac-
cessive dephosphorylation of MI hexaphosphate by wheat bran phytase similar to that reported here.

The Zea mays phytase preparation used in this study was a very active one. The amount of enzyme used in the time course study was sufficient to hydrolyze completely to P1 and MI as much as 1 mg of pure phytic acid in 6 hr, an amount much greater than that present in 25 mg of ethanol insoluble residue. Results suggested that ethanol insoluble residue contained an inhibitor. To test this possibility, a small quantity of labeled phytic acid (37,000 cpm) that had been extracted with disodium EDTA from ethanol insoluble residue (30 mg) and then purified by electrophoresis in oxalate buffer, was incubated with Zea mays phytase, employing the same conditions used for hydrolysis of residue-bound material. After 15 min only mono- and diphosphates of MI were detected. Within 60 min the digestion was complete. all 14C was present as free MI.

Ethanol insoluble residue that had been extracted with boiling water prior to phytase treatment released labeled free MI much more rapidly during phytase hydrolysis than unextracted residue (fig 5b). Four hr after addition of phytase, nearly all the label was present as free MI or its monophosphate. Water extraction had removed about 35% of the label from the ethanol insoluble residue, only one-third of which could be identified as phosphate esters. This treatment also removed the labeled component, noted above, that had been characterized by its slight positive mobility in oxalate buffer. Very little MI hexaphosphate was removed by water extraction. When water-extracted ethanol insoluble residue was incubated with Zea mays phytase in the presence of 0.05 M potassium phosphate (pH 5.3), hydrolysis was strongly inhibited (fig 5c). Even after 24 hr of hydrolysis, tetra- and tri-orthophosphates as well as the two lower phosphates of MI were still present in the reaction mixture. Considerable P1 was present in ethanol insoluble residue as can be seen by the amount of P1 released by EDTA (fig 4). It appears very likely that this bound phosphate was responsible for much of the phytase inhibition observed in the time-course study shown in figure 5a. Others have reported that P1 inhibits the action of crude phytase (11, 22).

MI Phosphates of Ethanol Insoluble Residue From Lemma gibba. When MI-2-14C was given to growing cultures of L. gibba, part of the MI was utilized for biosynthesis of labeled D-apiosyl and D-xylosyl units of cell wall polysaccharides (18) but a considerable portion of the administered label was present in a soluble anionic fraction recovered from ethanol insoluble residue after hydrolysis with 0.1 N hydrochloric acid. At least a part of this material was thought to be labeled D-galacturonan since it was known from the work of Beck (6) that the apiose-containing polysaccharide of Lemma is a pectic substance rich in D-galacturonic acid. To examine this possibility, an EDTA extract of ethanol insoluble residue (experiment 1, reference 18) from MI-2-14C labeled L. gibba that had been labeled with MI-2-14C. See figure 1 for gradient as well as other details with regard to comparison with authentic phytate.

Fig. 6. Distribution of radioactivity after ionexchange chromatography of an EDTA extract of ethanol insoluble residue from L. gibba that had been labeled with MI-2-14C. See figure 1 for gradient as well as other details with regard to comparison with authentic phytate.

Discussion

In previous studies on the metabolism of MI in plant tissues, it was found that this cyclitol invariably served as a general precursor of all uronosyl and pentosyl units of cell wall polysaccharides. Presumably, this conversion was preceded by an oxidative cleavage of the cyclitol to yield D-glu-
curonic acid. Subsequent metabolism of d-glucuronic acid accounted for the observed pattern of metabolism. The broad applicability of these observations was confirmed in studies of apiose biosynthesis in L. gibba. Therefore, it was most surprising to discover that W. floridana, another member of Lemnaceae, directed MI utilization in the direction of phytic acid accumulation rather than cell wall biosynthesis. Although we still have no answer for this interesting contrast in modes of MI utilization, the fact that such an accumulation does occur, particularly in a vegetative tissue that is readily cultured under ordinary laboratory conditions, prompted this report.

From the amount of phosphorus found in the MI hexaphosphate peak of EDTA-extracted label (fig 4), it can be estimated that the 1.4 g of residue recovered from labeled W. floridana (table 1) contained at least 3 to 4 mg of extractable phytic acid. Exogenously supplied MI could only account for 90 µg of this; the rest must have been formed as a normal product of metabolism. Why it accumulates in this green tissue is not known. Its accumulation in seeds and other storage tissues is regarded as a functional device whereby phosphate is stored (8). More recently, we have proposed that the MI portion of phytic acid is also utilized for cell wall biosynthesis during germination (17). The possibility exists that phytic acid accumulation in W. floridana fronds is related to turion formation, the formation of resting-stage fronds (13).

As regards phytic acid biosynthesis, present results favor the view that this process involves phosphorylation of MI. The way in which this takes place is not clear. Preliminary experiments in this laboratory involving short term pulse labeling of W. floridana and L. gibba with MI-U-14C reveal labeling patterns that resemble, in part, the long term W. floridana experiment reported here. That is, MI mono- and hexaphosphates accumulate label during the pulse, but in the subsequent chase, there is no increase in hexaphosphate at the expense of a decrease in the mono-phosphates. In fact, neither mono- nor hexaphosphate show any change in isotope content. In this respect our results mirror those of Asada, Tamaka, and Kasai (4), of Saio (19), and of Sobolev (24), a distinctive accumulation of MI hexaphosphate accompanied by little or no evidence of lower phosphate esters when conditions favor phytic acid biosynthesis. Differences in phytic acid biosynthesis between young and mature plants as reported by Kindl and Hoffman-Ostenhof (14) are difficult to assess until more information is available concerning the path of MI biosynthesis in plants and the fate of C2 bound tritium during MI metabolism.

In seed endosperm, phytic acid accumulates as a complex salt in characteristic protein-rich bodies called aleurone grains (2). Sobolev and Rodionova (25) were able to demonstrate cell-free incorporation of labeled P1 into aleurone grains prepared from ripening sunflower seeds but only if they included a mitochondrial preparation from the same source. Incorporation of label into phytic acid was low but the care with which these workers identified their product left little doubt but that they had, indeed, obtained labeled phytic acid. Whether structures similar to aleurone grains are present in the vegetative tissue of W. floridana is not known. The phytic acid present in ethanol insoluble residue appears to be bound, at least in part, to protein through a complex salt linkage. Treatment with pronase releases considerable phytic acid into solution over and above that released by EDTA (unpublished observation) but is not yet established that this protein is structurally associated with phytic acid in intact tissue. Phytic acid readily complexes with protein and the solubility of this association is markedly influenced by phosphate and calcium over a wide range of pH (20, 21). Considerable research is still needed to clarify this complex relationship as it exists in the plant cell.

**Literature Cited**