Inositol 1,3,4,5,6-pentakisphosphate 2-kinase was purified from immature soybean seeds harvested approximately 5 weeks post-anthesis. A crude extract was clarified using polyethyleneimine and purified by chromatography on DEAE-cellulose, Cibacron Blue 3GA-agarose, Toyopearl DEAE 650M, and Toyopearl phenyl 650M columns. The enzyme had a relative molecular mass, \( M_r \), of 52,000 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and retained 50% of its activity after 6 weeks at 0 °C. The \( K_m \) values for inositol 1,3,4,5,6-pentakisphosphate and MgATP, respectively, were 2.3 \( \mu \)M and 8.4 \( \mu \)M, and the \( V_{\text{max}} \) was 243 nmol/min/mg. The pH and temperature optima, respectively, were 6.8 and 42 °C. Maximum activity was obtained when the magnesium ion concentration was 4 mM. The kinase specifically phosphorylated the 2-position on the inositol ring and could also utilize D-inositol 1,4,5,6-tetrakisphosphate as a substrate. The \( K_m \) for the reaction was 14, indicating that the enzyme may be involved in both inositol hexakisphosphate formation in maturing seeds and ATP resynthesis in germinating seeds. Substrate concentrations in mature seeds were favorable for ATP formation, whereas additional factors appeared to drive the accumulation of inositol hexakisphosphate in maturing seeds.

**Experimental Procedures**

**Materials**

\( \gamma^3\text{P} \text{ATP} \) and \(^{3} \text{H}\text{InsP}_6 \) were obtained from DuPont NEN. Inositol phosphates were prepared from \( \text{InsP}_6 \) by chemical hydrolysis (13, 14), except for D-Ins(1,4,5)P_3 and D-Ins(3,4,5,6)P_4, which were obtained from Sigma, and Ins(1,3,4,6)P_4, which was from Calbiochem. DEAE-cellulose, Cibacron Blue 3GA-agarose, and polyethyleneimine were purchased from Sigma. Toyopearl DEAE 650M, Toyopearl phenyl 650M, and the Progel-TSK HA-1000 column were from Tosoh. The SynChropak GPC100 column was a product of SynChrom Inc. AG 1-X8 200–400-mesh resin and Bio-Gel P-6DG were from Bio-Rad. AS3 and AS7 columns were from Bio-Rad. Absorption (Glycine max L. Merr. var. 2973) seeds generously provided by Pioneer Hi-Bred International were planted outdoors and harvested approximately 5 weeks post-anthesis, when the developing green seeds were reaching maximum size but had not started to turn yellow.

**Assay of Ins(1,3,4,5,6)P_6 2-Kinase Activity**

Enzyme activity was determined by incubation of 5 \( \mu \)l of enzyme in a total volume of 200 \( \mu \)l of 20 mM HEPES buffer (pH 7.0) containing 5 mM EGTA, 50 mM KCl, 5% glycerol, 5 mM \( \beta \)-mercaptoethanol, 0.1 mM PMSF, 5 mM MgCl_2, 50 mM Ins(1,3,4,6,5)P_6, 200 \( \mu \)M ATP, 50,000–100,000 cpm \( \gamma^3\text{P} \text{ATP} \) (3000 C/\( \mu \)mol), and either 0.0025% bovine serum albumin or 0.01% Tween 20 at 30 °C for 30 min. The reaction was stopped by the addition of 200 \( \mu \)l of 0.75 \% HCl. The mixture was diluted with 400 \( \mu \)l of \( \text{H}_2\text{O} \) and loaded onto a 200-\( \mu \)l column of AG 1-X8 200–400-mesh resin in the chloride form. ATP was eluted with two 5-ml aliquots of 0.3 \% \( \text{HCl} \). \( \text{InsP}_6 \) was eluted with 5 ml of 1 \% \( \text{HCl} \) into a 7-m1 polyethylene scintillation vial for Cerenkov counting. Under these conditions, less than 0.5% of the ATP coeluted with \( \text{InsP}_6 \).

The abbreviations used are: InsX\( \text{P}_n \), mgcv-inositol phosphates with \( X \) = positions of phosphate groups, and \( Y \) = number of phosphate groups when more than 1 (the \( y \) definition is assumed when none is indicated. In the \( l \) configuration, the positions are numbered in the opposite direction. For example, \( l \)-Ins(3,4,5,6)P_4 is the same as \( l \)-Ins(1,4,5,6)P_4. PMSF, phenethylmethylsulfonyl fluoride; MES, 2(N-morpholino)ethanesulfonic acid.
Inositol Pentakisphosphate 2-Kinase from Soybean Seeds

Purification of Ins(1,3,4,5,6)P_6 2-Kinase

Preparation of Cell Extract—Two hundred g of immature soybean seeds was homogenized 60 s in a Waring blender with 800 ml of 20 mM HEPES buffer (pH 7.8) containing 2 mM EDTA, 10 mM β-mercaptoethanol, 0.1 mM PMSF, 5% glycerol, and 1 μg/ml leupeptin. The crude mixture was filtered through several layers of cheesecloth and centrifuged for 30 min at 10,000 × g at room temperature. The supernatant was again filtered and applied to a 1-ml Bio-Gel P-6DG desalting column and eluted with 60 ml of 20 mM HEPES buffer (pH 7.8) containing 5 mM EGTA, 10 mM β-mercaptoethanol, 0.1 mM PMSF, 5% glycerol, and 1 μg/ml leupeptin (buffer A).

Anion Exchange Chromatography I—The clarified extract was applied from an ice bath at 3 ml/min to a 2.5 × 40-cm DEAE-cellulose column pre-equilibrated with buffer A at room temperature. Twenty 15-ml fractions were eluted at 3 ml/min with a gradient of 0–0.5 M KCl in buffer A.

Dye Affinity Chromatography—Sixty ml containing the 2-kinase peak from the DEAE-cellulose column was dialyzed overnight at 4 °C against 1 liter of buffer A and applied from an ice bath at 1.5 ml/min to a 2.5 × 14-cm Cibacron Blue 3GA-agarose column at room temperature. Twenty 10-ml fractions were eluted at 1.5 ml/min with a gradient of 0–1.5 M KCl in buffer A.

Anion Exchange Chromatography II—Fifty ml containing the Cibacron Blue 3GA-agarose column was dialyzed twice at 4 °C against 500 ml of buffer A and applied from an ice bath at 1.0 ml/min to a 1.5 × 3.0-cm Toyopearl DEAE 650M column at room temperature. Twenty 5-ml fractions were eluted at 1.0 ml/min with a gradient of 0–0.1 M KCl in buffer A.

Hydrophobic Interaction Chromatography—Twenty-five ml containing the 2-kinase peak from the Toyopearl DEAE 650M column was combined with 1.5 volumes of 3% (NH_4)_2SO_4 added dropwise with stirring at room temperature and separated on a 1.5 × 3.0-cm column of Toyopearl phenyl 650M with a 100-ml gradient of 0–4 × (NH_4)_2SO_4 in buffer A at 1 ml/min. Before assay, protein and activity, 200-μl aliquots were desalted by mixing with 200 μl buffer A and centrifuging for 30 min at 12,000 × g in Microcon 10 microcentrators. The retentates were reconstituted with 200 μl of buffer A.

Size Exclusion Chromatography—Four ml containing the 2-kinase peak from the Toyopearl phenyl 650M column was dialyzed against 500 ml of buffer A at 4 °C and diluted with 10 ml of 10 mM potassium phosphate buffer (pH 7.0) containing 10 mM β-mercaptoethanol, 5% glycerol, 0.1 mM PMSF, and 1 μl/ml leupeptin (buffer B). The enzyme was applied at 1.0 ml/min to a 7.5 × 75-mm Progel-TSK HA-1000 column pre-equilibrated with buffer B at room temperature, and 30 1-ml fractions were eluted at 1.0 ml/min with buffer B containing a phosphate gradient of 10–50 mM for 5 min, 50–100 mM for 20 min, and 100–200 mM for 5 min.

Gradient Ion Chromatography—Fifty-μl samples were separated on AG3 and AS4 columns as described previously (14) except the final isocratic step was extended an additional 5 min. Five hundred-μl fractions were collected at 30-s intervals. 32P was detected by Cerenkov counting, and H fractions were counted with 5 ml of scintillation fluid.

Electrophoresis and Protein Determination

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed with 10% gels according to Laemmli (15). Gels were silver-stained by the procedure of Blum et al. (16). Protein was determined according to Bradford (17) using ovalbumin as the standard.

ATP Determination

ATP was determined by the luciferase reaction using an ATP Bioluminescence CLS kit. Sample aliquots were diluted to 500 μl with 10 mM HEPES buffer (pH 7.8) in 7-ml polyethylene vials, mixed with 500 μl of reagent solution, and counted for 30 s in a Beckman LS18000 scintillation counter. A standard curve was made with 1–7 × 10^{-18} mol of ATP.

Substrate Determinations in Soybean Seeds

Inositol Phosphates—To conserve the life of the HPLC columns, InsP_6 was determined separately from the other inositol phosphates. Ten g of immature seeds was homogenized 30 s in a Waring blender with 100 ml of 0.57 M NaCl, or 5 g of mature seeds was ground 50 g in a 10 ml KSM2 coffee mill and stirred for 30 min with 100 ml of 0.37 M HCl. Portions of each mixture were centrifuged for 30 min at 100,000 × g, and aliquots of the supernatant were diluted appropriately and analyzed for InsP_6 by ion chromatography on a Dionex AS3 column (18). The other inositol phosphate substrates, which were much less abundant than InsP_6, had to be concentrated prior to analysis. Twenty g of immature seeds was homogenized 30 s with 100 ml of 0.75 M HCl, or 5 g of mature seeds was processed as above, and the mixtures were centrifuged for 30 min at 20,000 × g and 4 °C. The supernatant was washed with volumes of deionized water and applied to a 5 × 0.9-cm column of AG 1-X8 at 2 ml/min. Inositol phosphates were eluted with 500 ml of 0–1 M HCl and 25–200 ml fractions were collected. The column was calibrated with 50 mg each of n-Ins(1,2,5,6)P_4 and Ins(1,3,4,5,6)P_5. One hundred-μl aliquots from each fraction were combined with 2 ml of H_2O and 1 ml of 0.1% Fe(NO_3)_3 in 2% HClO_4, and the absorbance was measured at 290 nm. Aliquots of the above reagent, it was possible to pool fractions containing InsP_4 and InsP_5 based on the calibration while avoiding InsP_6, which gave the only observable response. n-Ins(1,4,5,6)P_5 was the last InsP_6 to elute prior to the InsP_4, and all of the other InsP_6 eluted prior to Ins(1,3,4,5,6)P_5 on ion exchange columns eluted with mineral acids (14, 19). The pooled fractions were lyophilized, and the residue was dissolved in 1 or 2 ml of H_2O. Inositol phosphates were quantified by gradient ion chromatography on an AS3 column (14). When necessary the detection level was increased approximately 10-fold using isocratic ion chromatography on an AS7 column. Inositol phosphates were measured with 0.13 or 0.155 M HNO_3, respectively. To ensure that no loss of the inositol phosphates was occurring during analysis, 5 g of mature soybeans was spiked with 5 mg of n-Ins(1,2,5,6)P_4 upon extraction with 0.37 M HCl. After correcting for the amount present in an unspiked sample, the recovery was 115%.

Adenosine Phosphates—ADP and ATP were determined similarly to the procedure of Ching et al. (20). Immature seeds (1 g) were homogenized for 20 s with 35 ml of 0.25 M NaClO_4 at 4 °C, or 500 mg of ground mature soybeans was stirred for 5 min with 25 ml of the same. The mixtures were centrifuged for 10 min at 10,000 × g, and the supernatants were neutralized to pH 6 with K_2CO_3. After centrifugation, the ADP and ATP were determined as described above. ADP was calculated as the difference between the value for both nucleotides combined and the value for ATP alone. Recoveries were performed by spiking the initial extractions with 2.5 nmol of ADP or ATP. A total of 9% of the added ADP was detected as ATP, and the individual recoveries were 78% and 99% for ATP and ADP, respectively. The soybean values were corrected accordingly.

RESULTS

Purification of Ins(1,3,4,5,6)P_5 2-Kinase—Initially, mature dehydrated seeds were used as a source of Ins(1,3,4,5,6)P_5 2-kinase. When a 20–50% (NH_4)_2SO_4 precipitate was assayed with 50 μM of each of the four InsP_6 separable by ion exchange chromatography, the most InsP_6 was formed from Ins(1,3,4,5,6)P_5. Ins(1,2,3,4,5,6)P_6 gave slightly less product, while n-Ins(1,2,3,4,5,6)P_5 and n-Ins(1,2,4,5,6,5,6)P_5 yielded negligible activity. Protocols for purifying the 2-kinase were modified repeatedly and scaled up, but it was not possible to identify a protein band by electrophoresis that was unequivocally aso-
associated with the activity. Immature seeds were substituted because the specific activity was approximately double that of dry seeds on a protein basis. With immature seeds the ammonium sulfate precipitation became inefficient, possibly because of the lower lipid content, and was accordingly abandoned.

The results of a typical purification are given in Table I. The relative efficiencies of the individual columns varied according to the composition of the fractions pooled from the previous step. On average each column gave more than a 2-fold purification, and the combination represented in Table I gave the best overall results. The DEAE-cellulose column had to be replaced after each use, while the Blue agarose column could be used twice before it became noticeably fouled. The Toyopearl columns were small enough to be replaced each time out of convenience. The HA-1000 column was used in a polishing step to prepare enzyme for the characterization experiments.

Identification of Product of 2-Kinase Reaction—Gradient ion chromatography was used to identify the product formed from Ins(1,3,4,5,6)P₅ by two methods. First, the product formed from Ins(1,3,4,5,6)P₅ eluted at 28 min (Fig. 1A), which is the exact retention time of authentic Ins₅P (Fig. 1C and Ref. 14). Second, when the product was hydrolyzed by wheat phytase, the breakdown appeared to be identical to that of [1H]Ins₅P (Fig. 1B and C, and Ref. 6). The product formed from d-Ins(1,4,5,6)P₅ eluted at 23 min (Fig. 1D), which is consistent with b- and/or l-Ins(1,2,4,5,6)P₅. Under these chromatographic conditions Ins(1,2,3,4,5)P₅ elutes 1 min before b- and l-Ins(1,2,3,4,5)P₅, while Ins(1,3,4,5,6)P₅ elutes within 1 min after each of b- and l-Ins(1,2,4,5,6)P₅ (14). Further characterization of the apparent Ins(1,2,4,5,6)P₅ was beyond the scope of this study.

Purity, Size, and Stability of Ins(1,3,4,5,6)P₅ 2-Kinase—The purification process was monitored with sodium dodecyl sulfate-polyacrylamide gel electrophoresis of fractions collected at each step. Kinase activity from the Toyopearl phenyl 650M column was clearly associated with a band with a relative molecular mass, Mᵣ, of 52,000 (Fig. 2). The distribution of this band also corresponded to the activity of fractions from HA-1000 and GPC100 columns. The Mᵣ observed upon GPC100 chromatography was similar to that determined by electrophoresis. Ins(1,3,4,5,6)P₅ 2-kinase from the HA-1000 column retained 50% of its initial activity after 6 weeks at 0°C.

Effect of pH and Temperature—A pH profile of Ins(1,3,4,5,6)P₅ 2-kinase was constructed from pH 5.5 to 8.0 (Fig. 3). The optimum was pH 6.8, while approximately 50% of the maximum activity was obtained at each of the extremes. A temperature curve is shown in Fig. 4. The optimum was 42°C, and an Arrhenius plot of the data from 0–97°C was linear (Fig. 4, inset). The activation energy, Eₐ, was calculated to be 11.7 kcal/mol.

Effect of Salts and Detergents—As seen in Fig. 5, M₂⁺ was essential for activity with 4 mM yielding the highest activity. The effects of other inorganic salts and detergents is shown in Table II. KCl was strongly inhibitory at 200 mM, although preliminary experiments had shown that 50 mM gave optimal activity. Twenty mM Ca²⁺ almost completely prevented the reaction, while 20 mM (NH₄)₂SO₄ or phosphate was only slightly inhibitory. Sodium deoxycholate and sodium dodecyl sulfate reduced the reaction rate at very low concentrations. In other experiments 0.005% bovine serum albumin was found to increase the activity of the purified 2-kinase by 40%, and 0.01% Tween 20 or Triton X-100 could replace bovine serum albumin and raise the activity an additional 10%.

Specificity of Ins(1,3,4,5,6)P₅ 2-Kinase—The activity of the 2-kinase was tested on various inositol phosphates at concentrations of 20 μM. No Ins₅P was formed from Ins(1,2,3,4,6)P₅ or Ins(1,2,4,5,6)P₅, but d-Ins(1,2,4,5,6)P₅ yielded 22% of the Ins₅P formed from Ins(1,5,4,6)P₃. d-Ins(1,2,4,5,6)P₅ could be converted to Ins(1,3,4,5,6)P₅ by cis-phosphate migration, the activity could possibly be attributed to the latter. With a Kₘ of 2.3 μM, Ins(1,3,4,5,6)P₅ could give this result if it were a contaminant. Subsequent analysis of the d-Ins(1,2,4,5,6)P₅ by gradient ion chromatography did in fact reveal approximately 5% Ins(1,3,4,5,6)P₅. Thus it appears that the kinase can utilize only the Ins₅P that lacks a phosphate on the 2-position. When Ins(1,3,4,6)P₅ and Ins(1,2,4,5,6)P₅ were tested as substrates for Ins(1,3,4,5,6)P₅ 2-kinase, no activity was detected with the former, but d-Ins(1,4,5,6)P₅ gave activity comparable to that of Ins(1,3,4,5,6)P₅. Twenty-five and 100 μM d-Ins(3,4,5,6)P₅ had 11 and 19%, respectively, as much activity as 50 μM d-Ins(1,4,5,6)P₅, thus indicating that d-Ins(1,4,5,6)P₅ was preferred. The sole product of the reaction was identified by gradient ion chromatography as d- and/or l-Ins(1,2,4,5,6)P₅ (Fig. 1D). The Ins₅P peak increased over the course of 30 min, but no Ins₅P formation was detected. No products were detected by gradient ion chromatography when d-Ins(1,4,5,6)P₅, Ins(4,5,6)P₃, or d-Ins(1,5,6)P₅ were assayed at concentrations of 20 μM.

Kinetic Data—Reciprocal plots of velocity versus substrate concentration were constructed from data obtained by simultaneously varying Ins(1,3,4,5,6)P₅ and MgATP concentrations. Typical Michaelis-Menten kinetics for a sequential rather than a ping-pong reaction mechanism were displayed by both substrates (Figs. 6 and 7). Secondary intercept plots were drawn to determine the kinetic constants (Figs. 6 and 7, inset). Ins(1,3,4,5,6)P₅ and MgATP were found to have Kₘ values of 2.3 and 8.4 μM, respectively, and the Vₘ₉ₙₙ for the purified enzyme was 243 nmoI/min/mg. The equilibrium of the reaction was determined with 10 μM each of ATP and Ins(1,3,4,5,6)P₅ and 10–68 μM each of ADP and Ins₅P, giving [ADP][Ins₅P]/[ATP][Ins₅P] quotients ranging from 1 to 46. When the substrate quotients were plotted against the change in ATP, the X intercept corresponded to a K of 14 (Fig. 8). AG for the synthesis of Ins₅P under these conditions was calculated to be -1.6 kcal/mol. Experiments to determine the K of the reaction with ATP, d-Ins(1,4,5,6)P₅, ADP, and d-Ins(1,2,4,5,6)P₅ gave results similar to those in Fig. 8. Time-course assays of ATP formation using 50 μM Ins₅P or d-Ins(1,2,4,5,6)P₅ produced comparable data (Fig. 9), again indicating negligible difference in the kinetics of these two reactions.
Inositol Pentakisphosphate 2-Kinase from Soybean Seeds

![Graphs and results](image)

**FIG. 1. Identification of Ins(1,3,4,5,6)P6 2-kinase activity products.** A and B. 200 μl of 20 mM HEPES buffer (pH 7.0) containing 700,000 cpm [γ-32P]ATP (3000 Ci/mmol), 50 μM Ins(1,3,4,5,6)P6, 5 mM EGTA, 50 mM KC1, 5% glycerol, 5 mM β-mercaptoethanol, 0.1 mM PMSF, and 5 mM MgCl2 was incubated with 4.5 μg of 2-kinase from the Toyopearl DEAE 650M column for 30 min at 30 °C. The reaction was stopped by the addition of 400 μl of 5 mM sodium phytate-HCl (pH 5.0). One mg of wheat phytase in 400 μl of H2O was added. After 0 min (A) or 60 min (B) at room temperature, a 200-μl aliquot was combined with 200 μl of 0.37 M HCl and analyzed by gradient ion chromatography as described under “Experimental Procedures.” C. 200 μl of 20 mM HEPES buffer (pH 7.0) containing 200,000 cpm [3H]InsP6, 5 mM EGTA, 50 mM KC1, 5% glycerol, 5 mM β-mercaptoethanol, 0.1 mM PMSF, and 5 mM MgCl2 was incubated with 400 μl of 5 mM sodium phytate-HCl (pH 5.0) and 1 mg of wheat phytase in 400 μl of H2O. After 60 min at room temperature, a 200-μl aliquot was combined with 200 μl of 0.37 M HCl. D. 200 μl of 20 mM HEPES buffer (pH 7.0) containing 350,000 cpm [γ-32P]ATP, 20 μM dl-Ins(1,4,5,6)P4, 5 mM EGTA, 50 mM KC1, 5% glycerol, 5 mM β-mercaptoethanol, 0.1 mM PMSF, and 5 mM MgCl2 was incubated with 4.5 μg of 2-kinase from the Toyopearl DEAE 650M column for 30 min at 30 °C. The reaction was stopped by the addition of 200 μl of 0.08% phytic acid random hydrolysate as a carrier.

**FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of Ins(1,3,4,5,6)P6 2-kinase.** Standards in lane 1 were phosphorylase b (95,000), bovine serum albumin (68,000), ovalbumin (43,000), lactate dehydrogenase (36,000), and carbonic anhydrase (29,000). Lane 2 contained 2.6 μg of Ins(1,3,4,5,6)P6 2-kinase after Toyopearl phenyl 650M chromatography.

**FIG. 3. pH profile of Ins(1,3,4,5,6)P6 2-kinase.** Enzyme activity was determined as described under “Experimental Procedures” except 20 mM MES was included in the assay buffers.

**DISCUSSION**

The possible biochemical pathways leading to phytic acid biosynthesis are numerous, and a good deal of effort will be required to determine which operate *in vivo* and their relative contributions. Not only could dozens of different InsP isomers be involved, but in seeds the puzzle is compounded by the uncertainty of whether an inositol monophosphate or an inositol lipid is the ultimate precursor. When a soybean seed was incubated with [3H]inositol for 48 h, 4% of the label was incorporated into InsP6, but no intermediate InsP5 were detected.2 Similar results have been reported by Graf (21) for wheat kernels and Asada et al. (22) for rice. Since the intermediates in InsP6 biosynthesis do not accumulate, they must be processed.

2 B. Q. Phillippy, unpublished observation.

**In Vivo Substrate Concentrations—**Levels of the substrates for the 2-kinase were determined in immature as well as mature soybean seeds (Table III). In every instance, the more phosphorylated of the inositol phosphate reaction pair was in excess of its precursor. In contrast, there was more ATP than ADP in immature seeds, while the converse was observed for mature seeds.
Inositol Pentakisphosphate 2-Kinase from Soybean Seeds

Table II

<table>
<thead>
<tr>
<th>Component</th>
<th>Activity</th>
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<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>200 mM KCl</td>
<td>14</td>
</tr>
<tr>
<td>20 mM CaCl₂</td>
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</tr>
<tr>
<td>20 mM (NH₄)₂SO₄</td>
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<td>7</td>
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<tr>
<td>1 mM CHAPS</td>
<td>107</td>
</tr>
<tr>
<td>0.1% Tween 20</td>
<td>117</td>
</tr>
</tbody>
</table>

*Components were assayed as described under “Experimental Procedures” with 0.0025% bovine serum albumin included in all incubations.

Fig. 4. Effect of temperature on Ins(1,3,4,5,6)P₅ 2-kinase. The inset is an Arrhenius plot of log activity versus the reciprocals of the temperatures. Enzyme activity was determined as described under “Experimental Procedures.”

Fig. 5. Effect of Mg²⁺ concentration on Ins(1,3,4,5,6)P₅ 2-kinase activity. Enzyme activity was determined as described under “Experimental Procedures.”

Fig. 6. Dependence of Ins(1,3,4,5,6)P₅ 2-kinase activity on the concentration of Ins(1,3,4,5,6)P₅ at fixed concentrations of MgATP. The concentrations of MgATP were 2 (○), 4 (△), 8 (▽), and 16 (□) μM. The inset is a plot of the 1/V intercepts versus the reciprocals of the MgATP concentrations.

InsP₆ showed much less activity. Ins(1,3,4,5,6)P₅, which is usually the predominant InsP₆ and presumed precursor of InsP₆ in animal cells (10, 14, 23), was therefore chosen as the first substrate for purification of kinase activity. Ins(1,3,4,5,6)P₅ 2-kinase has also been detected in mung bean seeds (9, 10, Xenopus oocytes (24), Dictyostelium (10, 12), and rat brain (10). Although mature seeds were initially used as a convenient source of the enzyme, green seeds were ultimately required to unequivocally associate enzyme activity with a specific electrophoretic band to be sequenced for cloning. This is consistent with the fact that InsP₆ synthesis is one of the major functions of maturing soybean seeds (25).

Ins(1,3,4,5,6)P₅ 2-kinase was the first inositol polyphosphate kinase to be purified to homogeneity from plants. The Kₘ values for InsP₆ and MgATP were 2.3 and 8.4 μM, respectively, and the Vₘₐₓ for the reaction was 243 nmol/min/mg at 30 °C. The same enzyme partially purified from mung bean seeds to an activity of 4.5 nmol/min/mg at 37 °C had Kₘ values for 41 and 88 μM for InsP₆ and ADP, respectively (9).

The K of Ins(1,3,4,5,6)P₅ 2-kinase was 14, indicating that the reaction can easily proceed in either direction depending on the substrate concentrations. This agrees with data on the same
Inositol Pentakisphosphate 2-Kinase from Soybean Seeds

FIG. 7. Dependence of Ins(1,3,4,5,6)P₅ 2-kinase activity on the concentration of MgATP at fixed concentrations of Ins(1,3,4,5,6)P₅. The concentrations of Ins(1,3,4,5,6)P₅ were 1 (○), 2 (●), 4 (▲), and 8 (◀) μM. The inset is a plot of the 1/Y intercepts versus the reciprocals of the Ins(1,3,4,5,6)P₅ concentrations.

FIG. 8. Equilibrium constant determination. The equilibrium of the reaction was determined with 10 μM each of ATP and Ins(1,3,4,5,6)P₅ and 10–68 μM each of ADP and InsP₆, giving [ADP][InsP₆]/[ATP][Ins(1,3,4,5,6)P₅] quotients ranging from 1 to 46. Two hundred-pl samples containing 0.9 μg of Ins(1,3,4,5,6)P₅ 2-kinase, 20 mM HEPES, 5 mM EGTA, 50 mM KCl, 5% glycerol, 5 mM β-mercaptoethanol, 0.1 mM PMSE, 5 mM MgCl₂, 0.01% Tween 20, 10 μl of purified Ins(1,3,4,5,6)P₅ 2-kinase, 50 μM ADP, and either 50 μM InsP₆ (●) or 50 μM DL-Ins(1,2,4,5,6)P₅ (▲) were incubated at 30 °C. The reaction was stopped by 250-fold dilution with 10 mM HEPES (pH 7.8), and ATP was determined immediately as described under "Experimental Procedures."

enzyme from ungerminated mung bean seeds described by Biswas et al. (9). In immature seeds the 2-kinase reaction probably dominates, while the reverse reaction may be significant in germinating seeds. Although the ATP/ADP ratio favors InsP₆ synthesis in immature seeds, the higher concentration of InsP₆ compared to the other reactants would normally tend to reverse the reaction. However, InsP₆ is sequestered as insoluble globoid crystals within protein bodies (26), thus permitting its extensive synthesis. In mature seeds the concentrations of all sub-

strates are favorable for the reverse reaction. However, there are other reactions such as oxidative phosphorylation (27) and the malate dehydrogenase pathway (28) that can also generate ATP during germination. The K values of the other soybean inositol polyphosphate kinases await their purification and will be critical to understanding the regulation of InsP₀ synthesis. Unfortunately none of the reports involving animal cells mentioned Kᵣ; it is possible that some of the activities attributed to separate phosphatases have been due in part to the reverse reactions of kinases.

The ability of Ins(1,3,4,5,6)P₅ 2-kinase to buffer ATP levels would seem to have broad implications for the role of InsP₀ in the energetics of intracellular metabolism. In mammalian cells, where Ins(1,3,4,5,6)P₅ and InsP₀ are the predominant two soluble inositol polyphosphates (29), a similar function has been suggested for the newly discovered inositol polyphosphate pyrophosphatases (29, 30). Beside serving as a physiological ligand for iron (31), the proposed function for InsP₀ as a cellular energy store could be relevant in all types of cells.

Although activities capable of phosphorylating each of the InsP₀ fractions separated by ion exchange chromatography appeared to be present in soybean seeds, purified Ins(1,3,4,5,6)P₅ 2-kinase did not utilize ε-Ins(1,2,3,4,5,6)P₆, Ins(1,2,3,4,6)P₅, or DL-Ins(1,2,4,5,6)P₅. The other major InsP₀ kinase in addition to the 2-kinase was a 5-kinase that phosphorylated Ins(1,2,3,4,6)P₅. The levels of the kinases at various stages of
seed development may clarify their roles in InsP₆ biosynthesis. Ins₃,4,5,6P₄ 2-kinase was able to phosphorylate the 2-position of n-Ins(1,4,5,6)P₄, n- and/or t-Ins(1,2,4,5,6)P₅ is the predominant InsP₅ in mature soybeans, which also contain a lesser amount of one or both isomers of Ins(1,4,5,6)P₄ (14). Therefore, this reaction may be responsible for the greater accumulation of n- and/or t-Ins(1,2,4,5,6)P₅ relative to Ins(1,3,4,5,6)P₅, which is rapidly converted to InsP₆ by the same enzyme.

In tracing the pathway of InsP₆ synthesis in soybean seeds, the next step will be to determine the precursors of Ins(1,3,4,5,6)P₅ and Ins(1,2,3,4,6)P₅. In various animal cells kinase activities that produce Ins(1,3,4,5,6)P₅ from Ins(3,4,5,6)P₄ (32-34), Ins(1,3,4,5,6)P₅ (33-38), Ins(1,3,4,5,6)P₄ (39), and Ins(1,4,5,6)P₄ (34, 40) have been observed. Shears (38) has also identified a kinase in rat liver that phosphorylates the 2-position of Ins(1,3,4,6)P₄. In the green alga Chlamydomonas, Irvine et al. (39) have demonstrated a pathway whereby Ins(1,4,5)P₃ is sequentially converted to Ins(1,3,4,5)P₄ and InsP₆. In the green alga Chlamydomonas, Irvine et al. (39) have demonstrated a pathway whereby Ins(1,4,5)P₃ is sequentially converted to Ins(1,3,4,5)P₄ and InsP₆. In various animal cells kinase activities that produce Ins(1,3,4,5,6)P₅ from Ins(3,4,5,6)P₄ (32-34), Ins(1,3,4,5,6)P₅ (33-38), Ins(1,3,4,5,6)P₄ (39), and Ins(1,4,5,6)P₄ (34, 40) have been observed. Shears (38) has also identified a kinase in rat liver that phosphorylates the 2-position of Ins(1,3,4,6)P₄. In the green alga Chlamydomonas, Irvine et al. (39) have demonstrated a pathway whereby Ins(1,4,5)P₃ is sequentially converted to Ins(1,3,4,5)P₄ and InsP₆.

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