Video Article

Preparation of Quality Inositol Pyrophosphates

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URL: http://www.jove.com/video/3027
DOI: doi:10.3791/3027

Keywords: Molecular Biology, Issue 55, Polyacrilamyde Gel Electrophoresis (PAGE), inositol hexakisphosphate (IP6), phytic acid, diphosphoinositol pentakisphosphate (IP7), bisdiphoshoinositol tetrakisphosphate (IP8), IP6-kinase (IP6K), PP-IP5K, VIP1

Date Published: 9/3/2011

Citation: Loss, O., Azevedo, C., Szijgyarto, Z., Bosch, D., Saiardi, A. Preparation of Quality Inositol Pyrophosphates. *J. Vis. Exp.* (55), e3027, doi:10.3791/3027 (2011).

Abstract

Myo-inositol is present in nature either unmodified or in more complex phosphorylated derivates. Of the latest, the two most abundant in eukaryotic cells are inositol pentakisphosphate (IP_5) and inositol hexakisphosphate (phytic acid or IP_6). IP_5 and IP_6 are the precursors of inositol pyrophosphate molecules that contain one or more pyrophosphate bonds[1]. Phosphorylation of IP_6 generates diphoshoinositolpentakisphosphate (IP_7 or PP-IP_5) and bisdiphoshoinositoltetrakisphosphate (IP_8 or $(PP)_2$-IP_4). Inositol pyrophosphates have been isolated from all eukaryotic organisms so far studied. In addition, the two distinct classes of enzymes responsible for inositol pyrophosphate synthesis are highly conserved throughout evolution[2-4].

The IP_6 kinases (IP_6Ks) posses an enormous catalytic flexibility, converting IP_5 and IP_6 to PP-IP_4 and IP_7 respectively and subsequently, by using these products as substrates, promote the generation of more complex molecules[5,6]. Recently, a second class of pyrophosphate generating enzymes was identified in the form of the yeast protein VIP_1 (also referred as PP-IP_5K), which is able to convert IP_6 to IP_7 and IP_8[7,8].

Inositol pyrophosphates regulate many disparate cellular processes such as insulin secretion[9], telomere length[10,11], chemotaxis[12], vesicular trafficking[13], phosphate homeostasis[14] and HIV-1 gag release[15]. Two mechanisms of actions have been proposed for this class of molecules. They can affect cellular function by allosterically interacting with specific proteins like AKT[16]. Alternatively, the pyrophosphate group can donate a phosphate to pre-phosphorylated proteins[17]. The enormous potential of this research field is hampered by the absence of a commercial source of inositol pyrophosphates, which is preventing many scientists from studying these molecules and this new post-translational modification. The methods currently available to isolate inositol pyrophosphates require sophisticated chromatographic apparatus[18,19]. These procedures use acidic conditions that might lead to inositol pyrophosphate degradation[20] and thus to poor recovery. Furthermore, the cumbersome post-column desalting procedures restrict their use to specialized laboratories.

In this study we describe an undemanding method for the generation, isolation and purification of the products of the IP_6-kinase and PP-IP_5-kinases reactions. This method was possible by the ability of polyacrylamide gel electrophoresis (PAGE) to resolve highly phosphorylated inositol polyphosphates[20]. Following IP_6K1 and PP-IP_5K enzymatic reactions using IP_6 as the substrate, PAGE was used to separate the generated inositol pyrophosphates that were subsequently eluted in water.

Video Link

The video component of this article can be found at http://www.jove.com/video/3027/

Protocol

1. Enzymatic Reaction - day 1 (1 hour in the afternoon)

1. The first step is to prepare 10-20 independent enzymatic reactions in which IP_6K1 or VIP1 convert IP_6 to the pyrophosphorylated isoforms.
2. We use His-IP_6K1 and GST-Vip1 enzymes purified from *E. coli* according to the protocol previously described[17,18].
3. Prepare 50 µL reactions containing 1X reaction buffer (30 mM Hepes pH 6.8, 50 mM NaCl, 6 mM $MgSO_4$, 1 mM DTT), 6 mM PhosphoCreatine (PCr), 25 U/mL CreatinePhosphoKinase (CPK), 5 mM ATP (Mg salt), 0.3 mM IP6, 0.05-0.1 µg His-IP_6K1 or GST-Vip1. Adjust volume with MiliQ ddH_2O.
4. Briefly spin the reaction and incubate at 37°C overnight with rotation.

2. Polyacrylamide gel casting and loading - day 2 (4 hours in the afternoon)

1. The polyacrylamide gel is prepared using 24 cm long,18 cm wide glass plates and 1.5 mm wide spacers. Usually a 16 lane or a preparative single lane comb is used.

2. Prepare a mix (50 mL/gel) containing the following: 35.5% (w/v) Acrylamide:Bis-Acrylamide 19:1, 1X Tris/Borate/EDTA (TBE), 0.05% (w/v) ammonium persulfate (APS), 0.05% (w/v) Temed. Pour the mix between the pre-casted glass plates, insert the comb and let polymerize for 30-60 minutes at RT.
3. Once the gel has polymerized, transfer the apparatus to the cold room and pre-run in 1X TBE for about 30-60 minutes at 200-300 Volts.
4. Add 1X of OrangeG dye (10 mM Tris-HCl pH7.0, 1 mM EDTA, 30% glycerol, 0.1% OrangeG) to each reaction. Prepare a sample containing 2 nmol of IP_6 to load as a standard control.
5. Wash each well thoroughly with running buffer using a syringe and a 21G needle to remove any precipitate, then load the gel. Avoid loading on the side wells.
6. Run the gel overnight at 450-550 Volts (7 mAmp/gel), until the OrangeG dye band is within the last 10 cm from the bottom of the gel.

3. Isolation of IP_7 - day 3 (4 hours) and day 4 (6-7 hour SpeedVac drying process)

1. Disassemble the gel apparatus and carefully remove one glass plate leaving the gel on the other one. Cut a small portion of the gel from just above the OrangeG dye band to the bottom containing the IP_6 standard and one sample lane, as shown in **Figure 1**.
2. Stain the cut portion of the gel with Toluidine Blue (0.1% (w/v) toluidine blue, 20% (w/v) methanol, 2% (w/v) glycerol) for a few minutes (1-3 min) or until the inositol pyrophosphate band appears. Put the glass plate previously removed back on top of the gel to prevent the unstained gel from drying.

The IP_7 band should be visible since it runs slightly slower than the IP_6 standard. ATP, which runs faster than IP_6, should also be visible (**Figure 1**). Transfer the stained portion of the gel in a de-staining solution (20% (w/v) methanol) for a few minutes, wash away any excess of Toluidine Blue and reposition the gel with the unstained gel.

If visualization of higher pyrophosphorylated inositol isoforms (IP_8 and IP_9) is required, stain the gel with Toluidine Blue staining solution for 20 minutes at room temperature. Subsequently, wash away the Toluidine Blue with the de-staining solution for about 15 minutes.

3. With a razor blade cut the IP_7 band on the unstained portion of the gel using as reference the IP_7 migrating position determined with the stained gel (**Figure 1**).
4. Put the IP_7 band that was cut from the gel on a 15 mL tube and add 10 mL of MilliQ ddH_2O. Put tubes in rotation for 10 minutes at room temperature. Discard the liquid to remove excess of TBE and microscopic acrylamide particles.
5. Subsequently, perform two dehydration-hydration cycles. Add 5 mL of 50% (w/v) methanol to the tube with the gel containing IP_7 and rotate at room temperature for 2 hours. Transfer the gel slice to a new 15 mL tube containing 5 mL of MilliQ ddH_2O and rotate at room temperature for 2 hours. Do not discard the methanol and MilliQ H_2O from the tubes. Repeat the dehydration-hydration cycle once more by re-transferring the polyacrylamide gel in the 15 mL tubes previously used. One of the washes can be performed over night.
6. To concentrate the eluted IP_7, dry the 10 mL together (5 mL MilliQddH_2O and 5 mL 50% (w/v) methanol) using a SpeedVac heated at 60°C.
7. Once the samples are nearly dry, transfer the remaining liquid (300-600 µl) to a1.5 mL centrifuge tube and spin for 2 minutes at 5000 rpm.
8. Collect supernatant and transfer into a fresh 1.5 mL centrifuge tube; leave the bottom 20-30 µL since it may contain acrylamide particles.
9. If necessary continue the drying process using an unheated SpeedVac. The recovery of IP_7 is dramatically reduced if the samples dry completely, therefore terminate the drying process when the samples reach the volume of 100-300 µL.

4. Determination of IP_7 concentration and purity.

1. Use 2-5 µL of the recovered IP_7 sample to run on a PAGE gel, similarly to Sections 2.2-2.5. Load several dilutions of IP_6 (i.e. 0.5, 1, 2, 4 nmol) as concentration standard and 4 nmol of Poly-P marker. After running the gel, visualize the inositol pyrophosphate isoforms by staining and de-staining the entire gel with Toluidine Blue solution, following the procedure described in Section 3.2 (**Figure 2A**).
2. After Toluidine staining, the concentrations can be determined by scanning the gel and comparing the differences in intensity between IP_6 and IP_7, using imaging software such as Image-J, as shown in **Figure 2B**.

5. Representative Results:

The preparative enzymatic conversion of IP_6 to IP_7 using IP_6K1 and VIP1 enzymes can be easily resolved using PAGE analysis (**Figure 1**). The loading of IP_6 as a size control together with Toluidine Blue gel staining allows the identification of the pyrophosphorylated derivates, since they run slower depending on the number of phosphate groups present on the inositol ring. The procedure described above allows the easy purification of IP_7. The analysis of the purified inositol pyrophosphate by PAGE revealed the purity of our IP_7 (**Figure 2A**). Interestingly, the 1/3PP-IP_5 isomer of IP_7 product of VIP1 migrates slightly slower than the 5PP-IP_5 isomer of IP_7 that is generated by the IP_6K1. Use of IP_6 standards permit an easy quantification of the concentration of the purified IP_7 (**Figure 2B**). Before using IP_7 for further experiments, its biological activity can be assessed
(**Figure 3**). 5PP-IP_5 is incubated with VIP1 and with the IP_7 phosphatase DDP1 (diphosphoinositol polyphosphate phosphohydrolase). Routinely, the purified IP_7 is converted to IP_8 by VIP1 and to IP_6 by DDP1 (**Figure 3**).

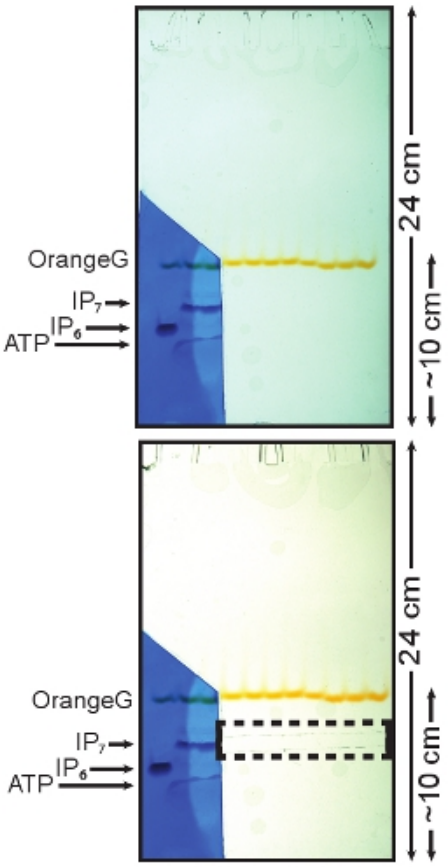

Figure 1: Toluidine staining of PAGE and isolation of the IP_7 band. The portion of the gel containing the standard (IP_6) was cut and stained using a Toluidine Blue solution. The three bands represent (top to bottom) IP_7, IP_6 and ATP. The stained portion of the gel was then aligned with the remaining of the gel. This allows the localization of the portion of the gel containing IP_7, which can then be cut and purified (dashed box).

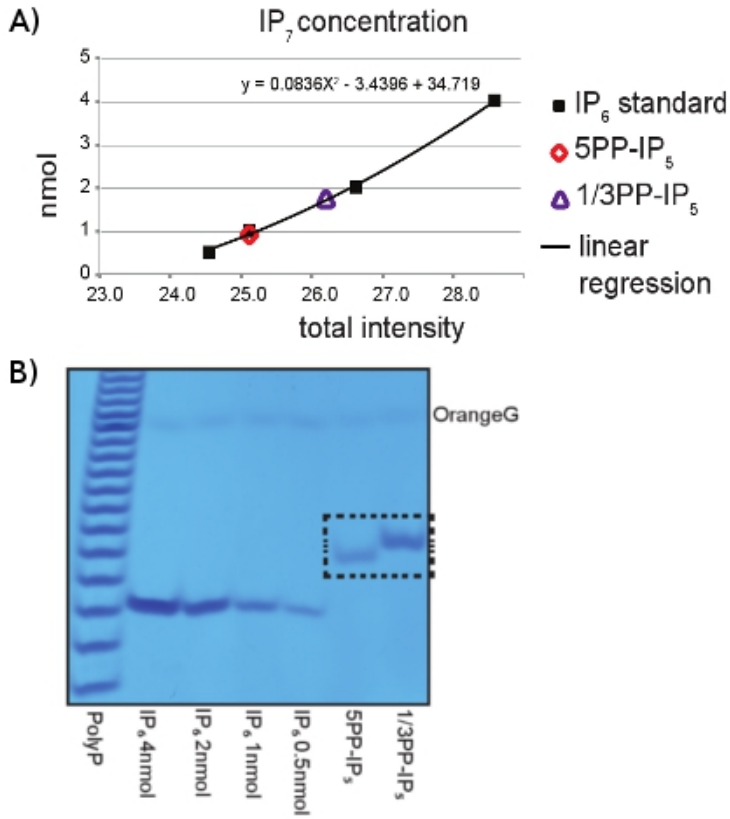

Figure 2: PAGE analysis of IP$_6$K1 and VIP1 reaction products. A) Analysis of IP$_6$ (4, 2, 1, 0.5 nmol) by Toluidine Blue staining was used in order to determine the IP$_7$ concentration purified from both IP$_6$K1 (5PP-IP$_5$) and VIP1 (1/3PP-IP$_5$) reactions. **B)** Scatter plot analysis to determine the concentration of the purified IP$_7$. Concentrations were determined according to band intensity, calculated using imageJ software, compared to pre-determined amounts of IP$_6$. The X-axis represents intensities; the Y-axis represents concentrations expressed in nmol.

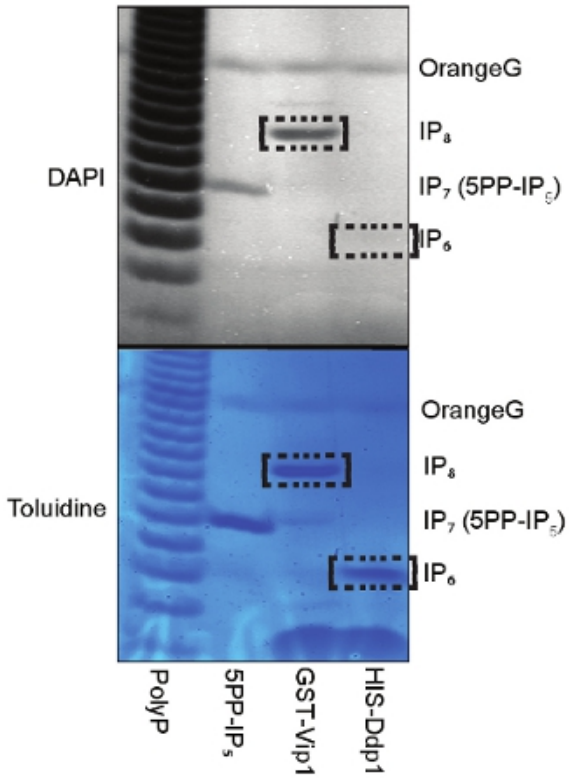

Figure 3: Analysis of IP_7 biological activity. To determine the quality of the purified IP_7 we incubated 5PP-IP_5 (IP_6K1 generated IP_7) with VIP1 or with the IP_7 phosphatase DDP1 and then resolved the reaction on PAGE. The DAPI and Toluidine staining revealed the expected production of IP_8 by VIP1 and the conversion of IP_7 to IP_6 by DDP1.

Discussion

The use of inositol pyrophosphate in biochemistry is severely limited by the commercial unavailability of such compounds and the poor sensitivity of the existing detection methods. The combination of PAGE, which enables the separation of molecules possessing different number of phosphate groups, and Toluidine Blue (**Figure 1**), a metachromatic dye which binds to phosphate groups, enables the easy detection of inositol pyrophoshate isoforms opening new avenues of research[20].

The described use of PAGE technology to purify inositol pyrophosphate products of the enzymatic reaction carried outby either IP_6K1 or VIP1 is a simple, economic and reliable method that allows for the production of large amounts of high quality IP_7. The method described above is not limited to the simple purification of IP_7 but minor modifications of the described protocol may allow the purification of a different range of inositol pyrophosphates. Higher phosphorylated inositol pyrophosphate isoforms, containing more than eight phosphate groups can be detected using IP_7 or different amounts of IP_6 as a substrate[20,6]. These inositol pyrophosphates can be detected by increasing the length of the staining procedure and subsequently purified (section 3.2). Moreover, the use of IP_5 as substrate for the enzymatic reaction would allow the purification of PP-IP_5 and other inositol pyrophosphates containing a hydroxyl group on the inositol ring.

In conclusion, this undemanding method allows for the reliable purification of milligram quantities of inositol pyrophosphates with widely available instruments, thus opening new avenues for this exciting research field.

Disclosures

No conflicts of interest declared.

Acknowledgements

We thank A. Riccio for helpful comments and to read the manuscript. This work was supported by the Medical Research Council (MRC) funding to the Cell Biology Unit and by a Human Frontier Science Program Grant (RGP0048/2009-C).

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