Preparation of Quality Inositol Pyrophosphates

Omar Loss¹, Cristina Azevedo¹, Zsolt Szijgyarto¹, Daniel Bosch¹, Adolfo Saiardi¹
¹Medical Research Council (MRC), Cell Biology Unit and Laboratory for Molecular Cell Biology, University College London

Correspondence to: Adolfo Saiardi at dmcbado@ucl.ac.uk

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

Keywords: Molecular Biology, Issue 55, Polyacryllyamide Gel Electrophoresis (PAGE), inositol hexakisphosphate (IP6), phytic acid, diphosphoinositol pentakisphosphate (IP7), bisdiphosphoinositol 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 derivatives. Of the latest, the two most abundant in eukaryotic cells are inositol pentakisphosphate (IP₅) and inositol hexakisphosphate (phytic acid or IP₆). IP₅ and IP₆ are the precursors of inositol pyrophosphate molecules that contain one or more pyrophosphate bonds⁷. Phosphorylation of IP₆ generates diphosphoinositolpentakisphosphate (IP₇ or PP-IP₆) and bisdiphosphoinositol tetrakisphosphate (IP₈ or (PP)₂IP₆). 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 evolution⁸-¹⁴.

The IP₆ kinases (IP₆Ks) posses an enormous catalytic flexibility, converting IP₆ to IP₅ and IP₆ to PP-IP₅ and IP₇ respectively and subsequently, by using these products as substrates, generate the more complex molecules⁴,⁵. Recently, a second class of pyrophosphate generating enzymes was identified in the form of the yeast protein VIP₁ (also referred as PP-IP₅K), which is able to convert IP₅ to IP₇ and IP₆ to IP₈⁷-⁸.

Inositol pyrophosphates regulate many disparate cellular processes such as insulin secretion⁸, telomere length¹⁰,¹¹, chemotaxis¹², vesicular trafficking¹³, phosphate homeostasis¹⁴ and HIV-1 gag release¹⁵. Two mechanisms of actions have been proposed for this class of molecules. They can affect cellular function by allosterically interacting with specific proteins like AKT¹⁶. Alternatively, the pyrophosphate group can donate a phosphate to pre-phosphorylated proteins¹⁷. 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 apparatus¹⁸,¹⁹. These procedures use acidic conditions that might lead to inositol pyrophosphate degradation²⁰ 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 IP₆-kinase and PP-IP₅K kinases reactions. This method was possible by the ability of polyacrylamide gel electrophoresis (PAGE) to resolve highly phosphorylated inositol polyphosphates²⁰. Following IP₅K1 and PP-IP₅K enzymatic reactions using IP₆ 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 IP₅K1 or VIP1 convert IP₅ to the pyrophosphorylated isoforms.
   2. We use His-IP₅K1 and GST-Vip1 enzymes purified from E. coli according to the protocol previously described¹⁷,¹⁸.
   3. Prepare 50 μL reactions containing 1X reaction buffer (30 mM Hepes pH 6.8, 50 mM NaCl, 6 mM MgSO₄, 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-IP₅K1 or GST-Vip1.
   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 IP₇ 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 IP₇ - 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 IP₂ standard and one sample lane, as shown in Figure 1. Stain the cut portion of the gel with Toluidine Blue (0.1% (w/v) toluidine blue, 20% (w/v) methanol, 2% (v/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 IP₂ band should be visible since it runs slightly slower than the IP₃ standard. ATP, which runs faster than IP₆, 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 (IP₆ and IP₇) 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 IP₂ band on the unstained portion of the gel using as reference the IP₃ migrating position determined with the stained gel (Figure 1).
4. Put the IP₂ band that was cut from the gel on a 15 mL tube and add 10 mL of MilliQ ddH₂O. 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 IP₂ and rotate at room temperature for 2 hours. Transfer the gel slice to a new 15 mL tube containing 5 mL of MilliQ ddH₂O and rotate at room temperature for 2 hours. Do not discard the methanol and MilliQ H₂O 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 IP₇, dry the 10 mL together (5 mL MilliQ ddH₂O 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 a 1.5 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 IP₇ 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 IP₇ concentration and purity.

1. Use 2-5 μL of the recovered IP₇ sample to run on a PAGE gel, similarly to Sections 2.2-2.5. Load several dilutions of IP₆ (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 IP₆ and IP₇, using imaging software such as Image-J, as shown in Figure 2B.

5. Representative Results:

The preparative enzymatic conversion of IP₆ to IP₇ using IP₇K1 and VIP1 enzymes can be easily resolved using PAGE analysis (Figure 1). The loading of IP₆ 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 IP₇. The analysis of the purified inositol pyrophosphate by PAGE revealed the purity of our IP₇ (Figure 2A). Interestingly, the 1/3PP/IP₇ isomer of IP₇ product of VIP1 migrates slightly slower than the SPP/IP₇ isomer of IP₇ that is generated by the IP₇K1. Use of IP₆ standards permit an easy quantification of the concentration of the purified IP₇ (Figure 2B). Before using IP₇ for further experiments, its biological activity can be assessed (Figure 3). SPP/IP₇ is incubated with VIP1 and with the IP₇ phosphatase DDP1 (diphosphoinositol polyphosphate phosphohydrolase). Routinely, the purified IP₇ is converted to IP₆ by VIP1 and to IP₆ 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_7) 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 IP₇, biological activity.** To determine the quality of the purified IP₇ we incubated 5PP-IP₅ (IP₇K₁ generated IP₇) with VIP1 or with the IP₇ phosphatase DDP1 and then resolved the reaction on PAG. The DAPI and Toluidine staining revealed the expected production of IP₇ by VIP1 and the conversion of IP₇ to IP₅ 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 pyrophosphate isoforms opening new avenues of research.

The described use of PAG technology to purify inositol pyrophosphate products of the enzymatic reaction carried out by either IP₇K₁ or VIP1 is a simple, economic and reliable method that allows for the production of large amounts of high quality IP₇. The method described above is not limited to the simple purification of IP₇ 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 IP₅ or different amounts of IP₇ as a substrate. These inositol pyrophosphates can be detected by increasing the length of the staining procedure and subsequently purified (section 3.2). Moreover, the use of IP₅ as substrate for the enzymatic reaction would allow the purification of PP-IP₅ 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).

### References

1. Bennett, M., Onnebo, S.M., Azevedo, C., & Saiardi, A. Inositol pyrophosphates: metabolism and signaling. *Cell Mol Life Sci.* 63 (5), 552-564 (2006).
2. Burton, A., Hu, X., & Saiardi, A. Are inositol pyrophosphates signalling molecules? *J Cell Physiol.* 220 (1), 8-15 (2009).
3. Barker, C.J., Illies, C., Gaboardi, G.C., & Berggren, P.O. Inositol pyrophosphates: structure, enzymology and function. *Cell Mol Life Sci.* 66 (24), 3851-3871 (2009).
4. Shears, S.B. Diphosphoinositol polyphosphates: metabolic messengers? *Mol Pharmacol.* 76 (2), 236-252 (2009).
5. Saiardi, A., Erdjument-Bromage, H., Snowman, A.M., Tempst, P., & Snyder, S.H. Synthesis of diphosphoinositol pentakisphosphate by a newly identified family of higher inositol polyphosphate kinases. *Curr Biol.* 9 (22), 1323-1326 (1999).
6. Draskovic, P., et al. Inositol hexakisphosphate kinase products contain diphosphate and triphosphate groups. *Chem Biol.* 15 (3), 274-286 (2008).
7. Mulugu, S., et al. A conserved family of enzymes that phosphorylate inositol hexakisphosphate. *Science.* 316 (5821), 106-109 (2007).
8. Fridy, P.C., Otto, J.C., Dollins, D.E., & York, J.D. Cloning and characterization of two human VIP1-like inositol hexakisphosphate and diphosphoinositol pentakisphosphate kinases. *J Biol Chem.* 282 (42), 30754-30762 (2007).
9. Illies, C. et al. Requirement of inositol pyrophosphates for full exocytotic capacity in pancreatic beta cells. *Science.* 318 (5854), 1299-1302 (2007).
10. Saiardi, A., Resnick, A.C., Snowman, A.M., Wendland, B., & Snyder, S.H. Inositol pyrophosphates regulate cell death and telomere length through phosphoinositide 3-kinase-related protein kinases. *Proc Natl Acad Sci U S A.* 102 (6), 1911-1914 (2005).
11. York, S.J., Armbruster, B.N., Greenwell, P., Petes, T.D., & York, J.D. Inositol diphosphate signaling regulates telomere length. *J Biol Chem.* 280 (5), 4264-4269 (2005).
12. Luo, H.R., et al. Inositol pyrophosphates mediate chemotaxis in Dictyostelium via pleckstrin homology domain-PtdIns(3,4,5)P₃ interactions. *Cell.* 114 (5), 559-572 (2003).
13. Saiardi, A., Sciambi, C., McCaffery, J.M., Wendland, B., & Snyder, S.H. Inositol pyrophosphates regulate endocytic trafficking. *Proc Natl Acad Sci U S A.* 99 (22), 14206-14211 (2002).
14. Auesukaree, C., Tochio, H., Shirakawa, M., Kaneko, Y., & Harashima, S. Pct1p, Arg82p, and Kcs1p, enzymes involved in inositol pyrophosphate synthesis, are essential for phosphate regulation and polyphosphate accumulation in Saccharomyces cerevisiae. *J Biol Chem.* 280 (26), 25127-25133 (2005).
15. Azevedo, C., Burton, A., Ruiz-Mateos, E., Marsh, M., & Saiardi, A. Inositol pyrophosphate mediated pyrophosphorylation of AP3B1 regulates HIV-1 Gag release. *Proc Natl Acad Sci U S A.* 106 (50), 21161-21166 (2009).
16. Chakraborty, A. et al. Inositol pyrophosphates inhibit Akt signaling, thereby regulating insulin sensitivity and weight gain. *Cell.* 143 (6), 897-910.
17. Bhandari, R. et al. Protein pyrophosphorylation by inositol pyrophosphates is a posttranslational event. *Proc Natl Acad Sci U S A.* 104 (39), 15305-15310 (2007).
18. Azevedo, C., Burton, A., Bennett, M., Onnebo, S.M., & Saiardi, A. Synthesis of InsP7 by the Inositol Hexakisphosphate Kinase 1 (IP$_6$K1). *Methods Mol Biol*. **645**, 73-85 (2010).

19. Otto, J.C., et al. Biochemical analysis of inositol phosphate kinases. *Methods Enzymol.* **434**, 171-185 (2007).

20. Losito, O., Szijgyarto, Z., Resnick, A.C., & Saiardi, A. Inositol pyrophosphates and their unique metabolic complexity: analysis by gel electrophoresis. *PLoS One*. **4**(5), e5580 (2009).