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The phytase RipBL1 enables the assignment of a specific inositol phosphate isomer as a structural component of human kidney stones†

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Inositol phosphates (InsPs) are ubiquitous in all eukaryotes. However, since there are 63 possible different phosphate ester isomers, the analysis of InsPs is challenging. In particular, $InsP_1$, $InsP_2$, and $InsP_3$ already amass 41 different isomers, of which some occur as enantiomers. Profiling of these "lower" inositol phosphates in mammalian tissues requires powerful analytical methods and reference compounds. Here, we report an analysis of $InsP_2$ and $InsP_3$ with capillary electrophoresis coupled to electrospray ionization mass spectrometry (CE-ESI-MS). Using this method, the bacterial effector RipBL1 was analyzed and found to degrade $InsP_6$ to Ins(1,2,3)P_3, an understudied $InsP_3$ isomer. This new reference molecule then aided us in the assignment of the isomeric identity of an $InsP_3$ while profiling human samples: in urine and kidney stones, we describe for the first time the presence of defined and abundant $InsP_3$ isomers, namely Ins(1,2,3)P_3, Ins(1,2,6)P_3 and/or Ins(2,3,4)P_3.

Introduction

myo-Inositol phosphates (InsPs) are molecules with various numbers of phosphate groups modified on one to six of the OH groups of *myo*-inositol (hereafter Ins), which are present in all eukaryotes. By sequential phosphorylation, 63 different InsPs can in principle be generated, of which over one third are thought to be relevant to mammalian metabolism as signalling molecules.[1,2]

Analysis of these InsPs is important to better characterize InsP identity and abundance in mammalian metabolism, which might finally lead to a deciphering of the alleged "inositol phosphate code".[1,3,4] A variety of analytical methods have been reported, including, for example, strong anion exchange high performance liquid chromatography (SAX-HPLC), high performance liquid chromatography coupled to mass spectrometry (HPLC-MS), and capillary electrophoresis coupled to electrospray ionization mass spectrometry (CE-ESI-MS).[5–13] Most of these methods are sensitive and efficient for the analysis of highly phosphorylated InsPs (*e.g.* $InsP_4$, $InsP_5$, $InsP_6$).[5–11] Moreover, CE-MS methods are powerful tools for profiling of inositol pyrophosphates (PP-InsPs), such as $InsP_7$ and $InsP_8$ that carry one or several diphosphate groups.[6,14–16]

Analyzing identity and abundance of $InsP_1$, $InsP_2$ and $InsP_3$ isomers in biological samples remains a significant challenge for several reasons. First, due to the non-specificity of the current extraction and separation methods, it is difficult to identify $InsP_1$ and $InsP_2$ isomers from the isobaric and more abundant sugar mono- and diphosphates. Second, the number of possible $InsP_1$ to $InsP_3$ isomers amasses to 41 and out of these alone 20 isomers of $InsP_3$ exist, including 8 enantiomeric pairs. Enantiomer separation on a chiral stationary phase remains an unsolved issue for these molecules. Additionally, it is generally assumed that the *myo*-configuration is the relevant one, but also other inositol configurations occur in biology.[17] Moreover, the high negative charge density and the absence of a chromophore generate significant issues regarding sensitive detection. The most commonly used analytical methods for $InsP_2$ and $InsP_3$ separation are SAX-HPLC and

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HPLC-MS.[9,18,19] The latter can profit from a heavy isotope labelling methylation strategy to enable ESI^+ measurements.[9] Ins(1,4,5)P_3, the Ca^{2+} release factor,[20] is the most characterized $InsP_3$ and represents the textbook example of a second messenger. Other $InsP_3$ isomers have been described in biology, such as Ins(1,3,4)P_3 and Ins(1,2,3)P_3.[18] $InsP_{(1-5)}$ with a phosphate group in the 2-position are usually not described in mammalian metabolite overviews,[2,21–24] although such esters of $InsP_2$ and $InsP_3$ have been found since 1995.[18,25,26] Recently, using ^{13}C enrichment and 2D-NMR analysis, it was discovered that Ins(2,3)P_2 and Ins(2)P are major metabolites in immortalized mammalian cell lines, calling into question the general notion that InsPs with a phosphate in the axial 2-position are biologically irrelevant.[27] A recent finding that 2-PP-InsP_5 carrying a pyrophosphate in the 2-position is a biologically relevant species, further underscores the need to reassess inositol phosphate structures.[16] Recently developed new analytical technologies are now able to reveal several 'discarded' isomers, whose biological importance must now be evaluated.

Our approach to analyze $InsP_{4-6}$ and PP-InsPs by CE-ESI-MS is extended herein to study $InsP_{2-3}$. We were interested to profile human samples and since the involvement of PP-InsPs in systemic human phosphate homeostasis has become clearer,[28–31] we decided to analyze urine samples where excess phosphate is excreted. Surprisingly, we detected several $InsP_3$ isomers in urine. This led us to postulate that these $InsP_3$ might be structural components of kidney stones. If this would be the case, we could potentially use urine InsP presence as a biomarker for impending stone formation. To achieve our objectives, we reveal that RipBL1, a bacterial phytase effector protein,[32] selectively dephosphorylates $InsP_6$ to Ins(1,2,3)P_3. Our CE-ESI-MS method combined with [$^{13}C_6$] $InsP_3$ produced from [$^{13}C_6$] $InsP_6$[33,34] by RipBL1 now enables us to assign $InsP_3$ in human kidney stone and urine and demonstrates in patient samples (healthy *vs.* kidney stone formers) that the most abundant isomers host a phosphate in the 2-position.

Results

$InsP_2$ and $InsP_3$ isomers are well separated by CE-ESI-MS

The CE-ESI-MS analytical method has been originally developed to investigate inositol pyrophosphate metabolism and is a particularly effective separation platform for isomers of $InsP_{4-6}$ and also for PP-InsPs.[6] CE-ESI-MS was not further developed to analyze $InsP_{1-3}$ in biological samples, despite the potential for the determination of spiked $InsP_3$ in plasma by capillary zone electrophoresis-mass spectrometry.[12] There are 15 isomers of $InsP_2$ that include 6 enantiomeric pairs, and 20 $InsP_3$ isomers that include 8 enantiomeric pairs. We disclose a CE-ESI-MS method for analyzing six commercially available isomers of $InsP_2$ and seven commercially available isomers of $InsP_3$ by baseline separation, other than enantiomers, which one cannot discriminate by using an achiral bare fused silica capillary.

The set of commercial $InsP_2$ and $InsP_3$ are shown in Fig. 1A: Ins(4,5)P_2, Ins(1,2)P_2, Ins(1,5)P_2, Ins(2,4)P_2, Ins(1,3)P_2, Ins(1,4)P_2, Ins(3,4,5)P_3, Ins(1,2,6)P_3, Ins(1,3,4)P_3, Ins(1,4,5)P_3, Ins(2,4,5)P_3, Ins(1,3,5)P_3, Ins(2,3,5)P_3. A bare fused silica capillary with a length of 100 cm was implemented for separations by applying 30 kV across the capillary (Agilent 7100 CE). Detection was achieved with an ESI-QQQ-MS (Agilent 6495C Triple Quadrupole with Agilent Jet Stream electrospray ionization source) in the negative ionization mode connected with an Agilent CE-ESI-MS interface. Initially, 35 mM ammonium acetate titrated with ammonium hydroxide to pH 9.75 was used as background electrolyte (BGE). With this BGE, separation of six $InsP_2$ isomers is achieved, but Ins(1,5)P_2, Ins(2,4)P_2, and Ins(1,3)P_2 were not baseline separated (Fig. 1B). Also, the separation of eight $InsP_3$ isomers was achieved with the exception of Ins(1,3,4)P_3, Ins(1,4,5)P_3, and Ins(1,4,6)P_3 (Fig. 1C). After pH optimization and BGE screening, a near baseline separation of Ins(1,5)P_2 and Ins(2,4)P_2 was achieved with 50 mM ethylamine titrated with formic acid to pH 10.0 (Fig. 1D). Separation of previously coeluting Ins(1,3,4)P_3 and InsP(1,4,5)P_3 was also resolved using this BGE (Fig. 1D) and a baseline separation of InsP(1,3,5)P_3 and Ins(2,3,5)P_3 was achieved as well (Fig. 1D). Ins(1,4,6)P_3 still coeluted with Ins(1,4,5)P_3.

Method validation was performed with InsP standards, including linearity, limit of detection (LOD) and limit of quantification (LOQ). The external calibration curves of $InsP_2$ and $InsP_3$ were constructed at eight concentration levels by regression of concentrations against the analyte peak area (Fig. S1, ESI†). The calibration curves were linear and had a coefficient of determination > 0.997 over the investigated range of 0.1–10 μg mL^{-1} for Ins(1,2)P_2 and 0.4–40 μg mL^{-1} InsP(1,2,6)P_3. With 20 nL sample injection, the LODs are 0.025 μg mL^{-1} for $InsP_2$ (*i.e.* 1.3 fmol) and 0.020 μg mL^{-1} for $InsP_3$ (*i.e.* 0.8 fmol), the LOQs are 0.05 μg mL^{-1} for $InsP_2$ (*i.e.* 2.6 fmol) and 0.10 μg mL^{-1} for $InsP_3$ (*i.e.* 4 fmol; Fig. S1, ESI†). The limit of detection of this method is significantly lower compared to other chromatography-related methods summarized in ref. 35.

RipBL1 degrades $InsP_6$ to Ins(1,2,3)P_3

RipBL1 is an effector protein of the phytopathogenic Gram-negative bacteria *Ralstonia solanacearum*, that shares homology with the bacterial effector XopH from *Xanthomonas campestris*. Previous work demonstrated that XopH displays an unusual phytase activity that dephosphorylates $InsP_6$ stereoselectively at the C1 position only, resulting in the generation of Ins(2,3,4,5,6)P_5.[32] We tested $InsP_6$ dephosphorylation by phytases, such as RipBL1 *in vitro*, to see if we could generate more high-quality non-commercial standards. Importantly, after 45 min 100% of $InsP_6$ was degraded by RipBL1, and one defined $InsP_3$ was detected as constituting about 95% of the digestion product. There was very little dephosphorylation to $InsP_4$ and $InsP_2$ (Fig. S2B, ESI†). CE-qTOF-MS analyses of the enzymatic reaction product confirmed a main molecular ion peak at m/z 418.9551 corresponding to $InsP_3$ (Fig. S2A, ESI†). To structurally assign the $InsP_3$ product, we generated a [$^{13}C_6$] $InsP_3$ by [$^{13}C_6$] $InsP_6$[33] digestion with RipBL1, and then analyzed its identity through spiking-in of all commercially available $InsP_3$ standards. As shown in Fig. 2A, the [$^{13}C_6$] $InsP_3$ was

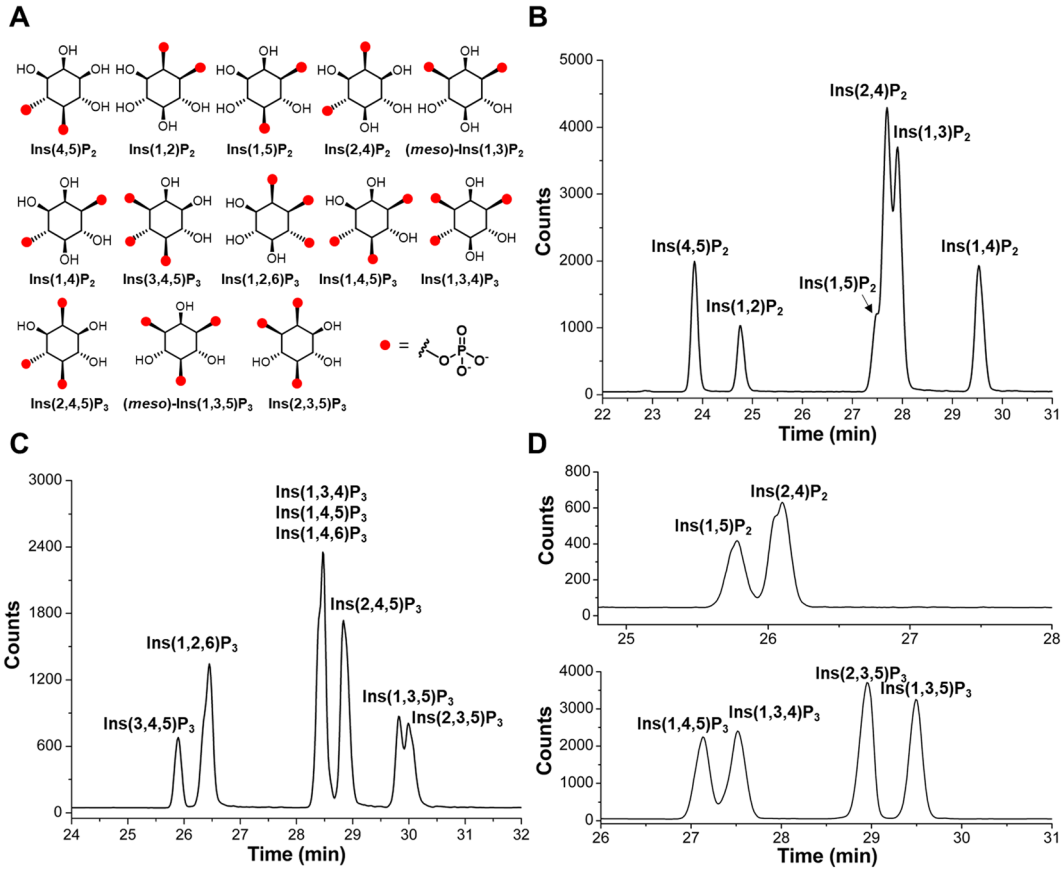

Fig. 1 Separation of InsPs by CE-ESI-MS. (A) Structures of commercial $InsP_2$ and $InsP_3$ isomers. Achiral isomers are labeled as "*meso*". (B and C) Separation of $InsP_2$ and $InsP_3$ standards by CE-ESI-MS, BGE: 35 mM ammonium acetate titrated with ammonium hydroxide to pH 9.75. (D) Separation of $InsP_2$ and $InsP_3$ standards by CE-ESI-MS, BGE: 50 mM ethylamine titrated with formic acid to pH 10.0.

baseline separated from all commercially available $InsP_3$ standards, with the exception of Ins(3,4,5)P_3 but also for this isomer, there was no perfect comigration. Previous work suggests that pyrohydrolysis can partially degrade "higher" InsPs and generate"lower" InsPs through dephosphorylation, and the pyrohydrolysis does not cause phosphate migration.[19,36] Analysis of pyrohydrolysis products of Ins(3,4,5)P_3 and the [$^{13}C_6$] $InsP_3$ solution after heating to 100 °C for 2.5 h confirmed that [$^{13}C_6$] $InsP_3$ is different from Ins(3,4,5)P_3, as the labeled *vs.* non-labeled $InsP_2$ products were different (Fig. 2B).

As Ins(4,5,6)P_3, Ins(2,3,6)P_3 (or its enantiomer Ins(1,2,4)P_3), as well as the *meso*-compounds Ins(1,2,3)P_3, and Ins(2,4,6)P_3 were not readily available, the further assignment was performed differently. We compared the migration time of [$^{13}C_6$] $InsP_3$ with the pyrohydrolysis products of Ins(2,3,5,6)P_4 and Ins(1,4,5,6)P_4 solution, respectively. The [$^{13}C_6$] $InsP_3$ product of RipBL1 does not comigrate with any $InsP_3$ isomers found as pyrohydrolysis products of Ins(2,3,5,6)P_4 or Ins(1,4,5,6)P_4 (Fig. S3, ESI†). These results provide evidence that the identity of the [$^{13}C_6$] $InsP_3$ product is either Ins(1,2,3)P_3 or Ins(2,4,6)P_3, both of which are symmetric *meso*-compounds.

Pyrohydrolysis products of Ins(1,2,3)P_3 and Ins(2,4,6)P_3 are expected to be different. For Ins(1,2,3)P_3, three $InsP_2$, namely Ins(1,2)P_2, Ins(2,3)P_2 and Ins(1,3)P_2, should be the pyrohydrolysis products. On the other hand, Ins(2,4)P_2, Ins(2,6)P_2, and Ins(4,6)P_2 would be the expected pyrohydrolysis products of Ins(2,4,6)P_3. By comparing the electrophoretic mobility with the commercially available $InsP_2$ standards, two $InsP_2$ peaks were identified as Ins(1,2)P_2 and its enantiomer Ins(2,3)P_2 (major product) as well as Ins(1,3)P_2 (minor product) in the pyrohydrolysis solution of [$^{13}C_6$] $InsP_3$ (Fig. 2C). Consequently, [$^{13}C_6$] $InsP_3$ generated by RipBL1 treatment of [$^{13}C_6$] $InsP_6$ represents Ins(1,2,3)P_3.

Dephosphorylation of $InsP_6$ by different types of phytases to produce Ins(1,2,3)P_3, either as the final product or as intermediates, has been investigated.[37–41] Ins(1,2,3)P_3 as an intermediate in barley aleurone tissue was described as well.[42] That $InsP_6$ metabolism by phytase in plants and fungi might pass through Ins(1,2,3)P_3 was discussed.[18] Ins(1,2,3)P_3 as the final product of $InsP_6$ hydrolysis, as shown for RipBL1, is however unusual, only alkaline phytase from Lily Pollen is a candidate enzyme but the Ins(1,2,3)P_3 purity was not described.[38] Thus, to the best of our knowledge, RipBL1 is the first bacterial effector phytase that with high selectivity and yield generates Ins(1,2,3)P_3.

Ins(1,2,3)P_3, Ins(1,2,6)P_3 and/or its enantiomer Ins(2,3,4)P_3 exist in human kidney stones and urine

Kidney stones are solid objects in the kidney or bladder that can cause different disease symptoms and consist of various low

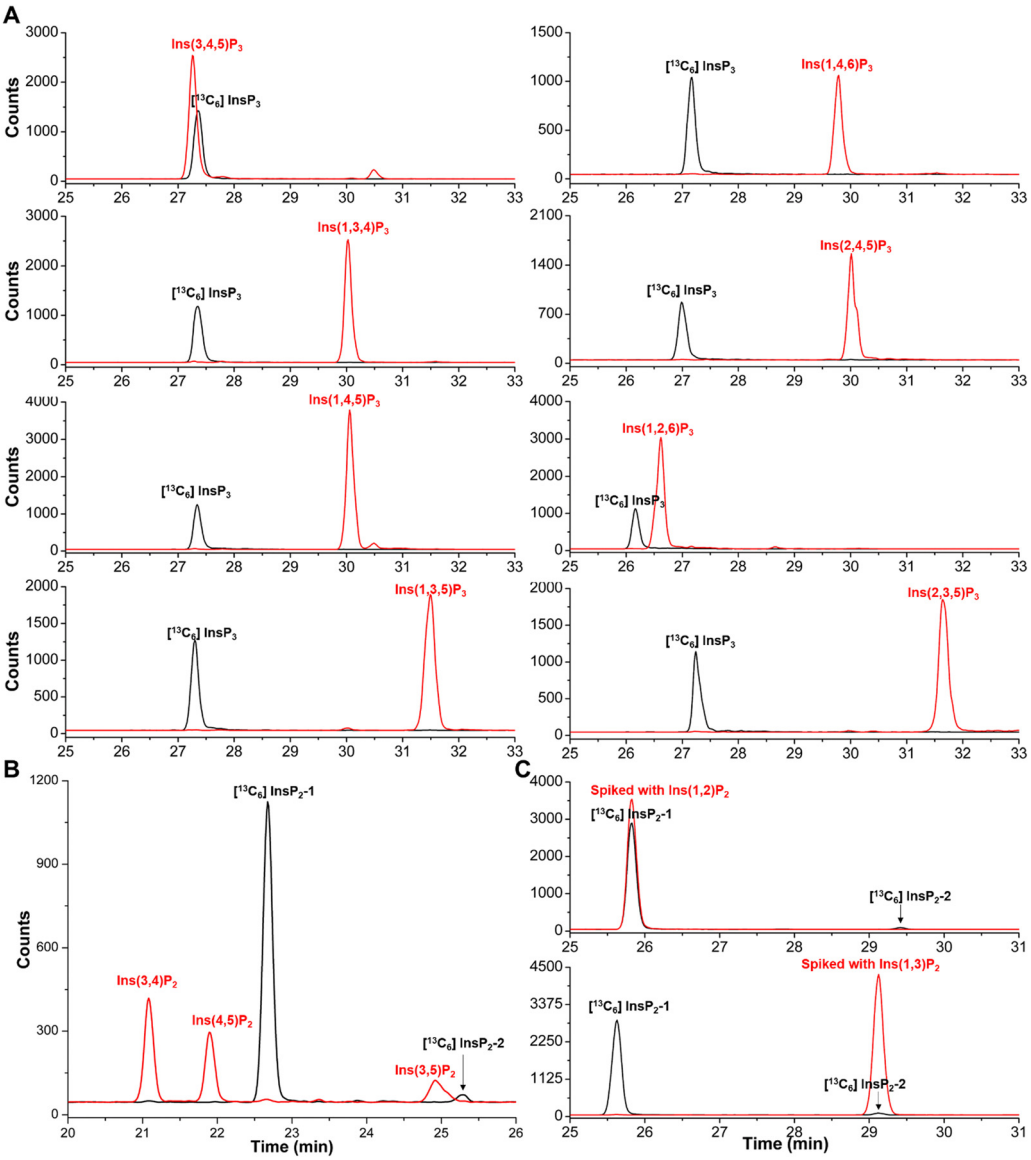

Fig. 2 Identification of the $InsP_3$ (black line) dephosphorylation product of [$^{13}C_6$] $InsP_6$ by the RipBL1 enzyme. (A) CE-ESI-MS analysis of $InsP_3$ individually spiked (red line) with Ins(3,4,5)P_3 standard, Ins(1,4,6)P_3 standard, Ins(1,3,4)P_3 standard, Ins(2,4,5)P_3 standard, Ins(1,4,5)P_3 standard, Ins(1,2,6)P_3 standard, Ins(1,3,5)P_3 standard or Ins(2,3,5)P_3 standard, as indicated. (B) Analysis of $InsP_2$ generated from Ins(3,4,5)P_3 and the [$^{13}C_6$] $InsP_3$ isomer by heating to 100 °C for 2.5 h. Extracted ion electropherograms of [$^{13}C_6$]-labelled $InsP_2$ (black lines) generated by [$^{13}C_6$] $InsP_3$ and $InsP_2$ (red trace) generated by Ins(3,4,5)P_3. (C) [$^{13}C_6$] $InsP_2$ (black line) generated from [$^{13}C_6$] $InsP_3$ after heating to 100 °C for 2.5 h spiked with Ins(1,2)P_2 standard or Ins(1,3)P_2 standard as indicated (red line). Note that the [$^{13}C_6$] $InsP_2$-1 isomer has the same migration time as the Ins(1,2)P_2 standard and that the [$^{13}C_6$] $InsP_2$-2 isomer has the same migration time as the Ins(1,3)P_2 standard.

molecular weight compounds as well as proteins.[43] Further, kidney stones can be categorized into calcium containing stones and non-calcium containing stones. Calcium containing stones are the most common forms of kidney stones, including calcium oxalate monohydrate (COM) or dihydrate (COD) as well as calcium phosphate and mixtures of these.[43] The formation of kidney stones is mainly driven by urinary supersaturation and crystallization. These processes are environment dependent, influenced by urine pH, concentration of specific substances and effective molecules (promoters, and inhibitors of kidney stone formation).[44] Studies have shown that for example urinary $InsP_6$ can inhibit crystallization during the process of kidney stone formation in a model system.[45–48] Recently, $InsP_6$ analogues with PEG modifications were reported to completely inhibit such crystallization processes in the nanomolar range.[49] Additionally, studies with SNF472 (a hexasodium salt of $InsP_6$) as an inhibitor of vascular calcification in a phase 2 clinical trial are ongoing,[50] highlighting a strong relationship between $InsP_6$ and calcification. Whether some other inositol phosphates might actually promote crystallization and whether these

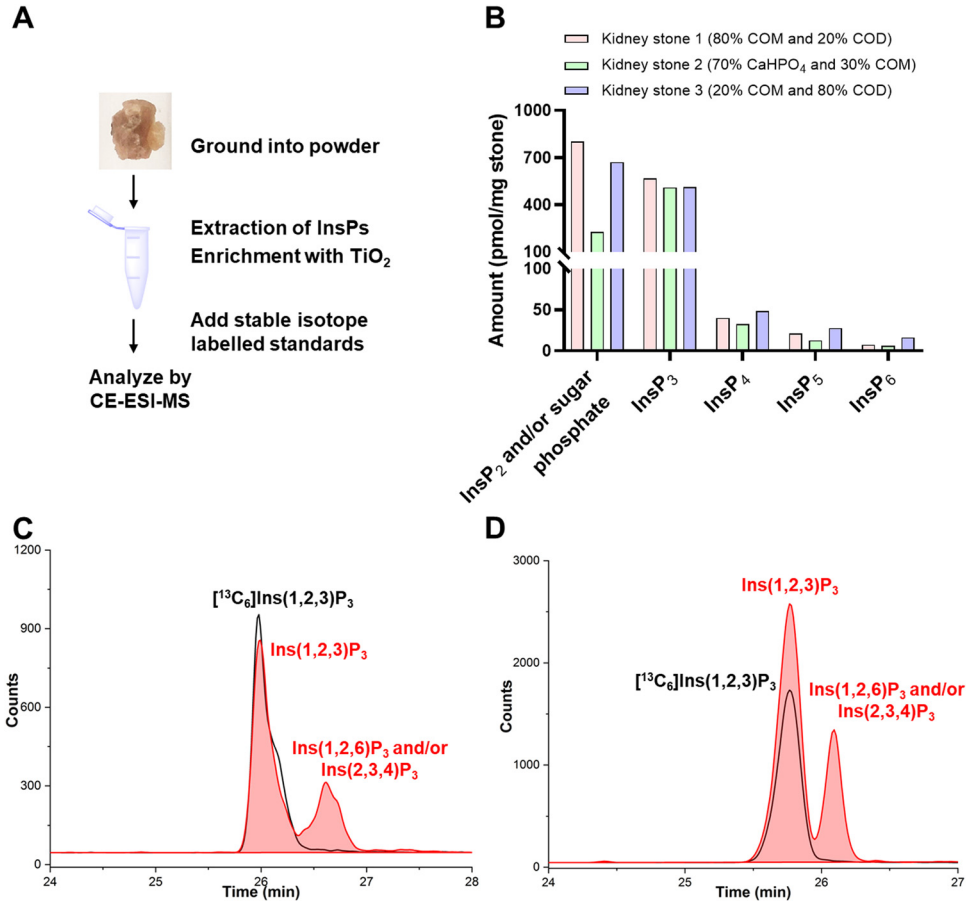

Fig. 3 Profiling of $InsP_5$ in human kidney stones. (A) Extraction and analysis workflow of kidney stones for CE-ESI-MS. (B) InsPs distribution in kidney stone 1 (contains 80% COM and 20% COD), kidney stone 2 (contains 70% $CaHPO_4$ and 30% COM) and kidney stone 3 (contains 20% COM and 80% COD) from three different patients. (C) Extracted ion electropherograms of [$^{13}C_6$] Ins(1,2,3)P_3 (black line) and $InsP_3$ in kidney stone 1 (red area). (D) Extracted ion electropherograms of [$^{13}C_6$] Ins(1,2,3)P_3 (black line) and $InsP_3$ in kidney stone 2 (red area).

substances are then incorporated into the kidney stone has not been studied. We therefore asked the question if $InsP_6$ or other InsPs could be structural components of kidney stones.

To investigate the presence and profiles of InsPs in kidney stones, different ground kidney stones of several compositions (calcium oxalate * xH_2O (x = 1 COM, x = 2 COD) and calcium hydrogen phosphate ($CaHPO_4$)), determined by IR spectroscopy, were extracted with perchloric acid according to the reported TiO_2 purification method[51] and then analyzed by CE-ESI-MS (Fig. 3A).[6] We identify several InsPs as part of kidney stones providing the first such profiles (for a representative example see Fig. S4, ESI†). The high-resolution masses of the InsPs were confirmed by a CE-qTOF analysis (Fig. S5, ESI†). Some of the InsP isomers were identified with internal [$^{13}C_6$] labelled reference compounds. Fig. 3B summarizes the observed levels of different InsPs in calcium oxalate stones (kidney stone 1 contains 80% COM and 20% COD, kidney stone 3 contains 20% COM and 80% COD) and a calcium phosphate stone (kidney stone 2 contains 70% $CaHPO_4$ and 30% COM), which roughly show the same trend in abundance of the analytes.

While inositol pyrophosphates were not detectable in these three types of calcium containing kidney stones, we noticed a decrease in InsP abundance proportional to the number of phosphate groups. $InsP_6$ was the least abundant InsP followed by $InsP_5$ and $InsP_4$ isomers. One of the $InsP_5$ isomers was assigned as 2-OH $InsP_5$ (Fig. S4, ESI†) by spiking with an internal [$^{13}C_6$] reference. The most abundant InsP detected in kidney stone were $InsP_{2-3}$ species and or isobaric sugar bisphosphates.

Full recovery for $InsP_{6-7}$ and good recovery for 2-OH $InsP_5$ from mammalian cell extracts were reported previously.[6] Here our analysis shows good recovery for $InsP_6$ (84%), 2-OH $InsP_5$ (70%) and $InsP_3$ (71%) from kidney stone extracts (kidney stone 2 is used as a representative example) by spiking with the [$^{13}C_6$] reference before extraction (pre-spiking) and after extraction but before measurement (post-spiking) (Fig. S6, ESI†). Since kidney stones are rich in Ca^{2+}, which could affect InsP recovery, we reassessed TiO_2 mediated InsP retrieval by adding 15 mM ethylenediaminetetraacetic acid (EDTA) during extraction. However, the recovery of $InsP_6$ and $InsP_3$ did not critically rely on presence or absence of additional EDTA.

Three peaks belonging to $InsP_2$ and/or isobaric sugar bisphosphates were separated in kidney stone (Fig. S7, ESI†). Spiking indicated that none of these three peaks represents glucose-1,6-bisphosphate. The most intense peak has an

identical migration time with [$^{13}C_6$] Ins(1/3,2)P_2 generated by pyrohydrolysis from [$^{13}C_6$] Ins(1,2,3)P_3 (Fig. S7, ESI†), indicating Ins(1,2)P_2 and/or Ins(2,3)P_2 are present in kidney stones. This is in line with recent findings that Ins(2,3)P_2 is present in the μM range in immortalized mammalian cells.[27]

InsP_3 was also comparably abundant in the calcium containing stones. Two peaks of InsP_3 were recorded and identified as Ins(1,2,3)P_3, representing the most intense peak, and Ins(1,2,6)P_3 and/or its enantiomer Ins(2,3,4)P_3. Identification was achieved by spiking with [$^{13}C_6$] Ins(1,2,3)P_3 (Fig. 3C and D) and Ins(1,2,6)P_3 (Fig. S8, ESI†). Ins(1,2,3)P_3 was reported in mammalian cell models in a concentration range of 1–10 μM.[18] A possible role for Ins(1,2,3)P_3 as an intracellular iron chelator in the process of iron transport has been considered.[52,53] Ins(1,2,6)P_3 and/or the enantiomer Ins(2,3,4)P_3 was proposed in mammalian B-cells in 1992,[54] however, as discussed above, since then very few studies have been conducted to characterize their identity and functional roles and these isomers are missing in discussions in recent literature reviews.[21,22]

The formation of kidney stone is a result of urinary supersaturation and crystallization. To study potential correlations of the profiles of InsPs in kidney stones and urine, we additionally profiled InsPs in 0.4 mL urine samples both from patients who have kidney stones (9 urine samples from different donors) and matched healthy people (10 urine samples from different donors, Table S1, ESI†). The accurate masses of InsPs identified in urine samples were confirmed by a CE-qTOF analysis (for representative example see Fig. S9 and S10, ESI†). Since we cannot yet distinguish sugar mono and bisphosphates from InsP_1 and InsP_2 because of limitations of our current method, we must assume that *m*/*z* 259.0229 and *m*/*z* 338.9889 correspond to InsP_1 and/or sugar phosphates and InsP_2 and/or sugar bisphosphates, respectively. CE-QQQ was then used to profile InsP levels also in urine samples. The CE-QQQ results indicated that InsP_2 and/or sugar bisphosphates are the most abundant species, followed by InsP_1 and/or sugar phosphates. Interestingly, InsP_3 was in the same concentration range as the sugar phosphates/InsP_1 group of analytes and InsP_4 was also present in the samples, but much less concentrated (*ca.* 7–8 fold) (Fig. 4A).

InsP_6 was detectable in five of the urine samples from ten healthy people and only one of the urine samples from nine kidney stone patients. In all cases, the concentration of InsP_6 was lower than the limit of quantification (LOQ). 2-OH InsP_5 was also in between the LOD and LOQ in seven of the urine samples from ten healthy people and only three of the urine sample from nine kidney stone patients. According to the signal-to-noise of spiked 1 μM [$^{13}C_6$] InsP_6 and [$^{13}C_6$] 2-OH InsP_5, we determined limits of detection (LOD) of InsP_6 and 2-OH InsP_5 in the urine samples analyzed were approx. 7.5 nM and 1.5 nM InsP_5, respectively. Limits of quantitation (LOQ) of InsP_6 and 2-OH InsP_5 were approx. 22.5 nM and 4.5 nM, respectively. Therefore, less than 7.5 nM of InsP_6 exist in most of these urine samples, which is in accordance with the reported detection using InsPs specific assays.[51,55,56] In half of these samples, approx. 1.5 nM to 4.5 nM 2-OH InsP_5 exist. InsP_4 were also present in urine samples. InsP_5 and InsP_4 received little attention so far in human urine, and our results provide an initial overview of these species. A study of InsPs in rat urine after giving InsP_6 dietary treatment indicated that InsP_6 as well InsP_2, InsP_3, InsP_4 and InsP_5 is excreted in the urine.[55] The source of those isomers remains obscure.

Similar to the results obtained for kidney stones, InsP_2 and/or sugar bisphosphates are most abundant. The most intense species comigrates with [$^{13}C_6$] Ins(1/3,2)P_2 generated by pyrohydrolysis of [$^{13}C_6$] Ins(1,2,3)P_3 (Fig. S9, ESI†). Also in agreement with the results found in kidney stones, two peaks corresponding to the mass of InsP_3 in urine samples can be identified as Ins(1,2,3)P_3 and Ins(1,2,6)P_3 and/or the enantiomer Ins(2,3,4)P_3 (Fig. 4B, C and Fig. S11, ESI†). According to our current data set, there is no significant difference of InsPs and/or sugar phosphates in urine from patients and healthy people regarding levels of InsP_1 to InsP_4. For InsP_{5-6} the picture is less

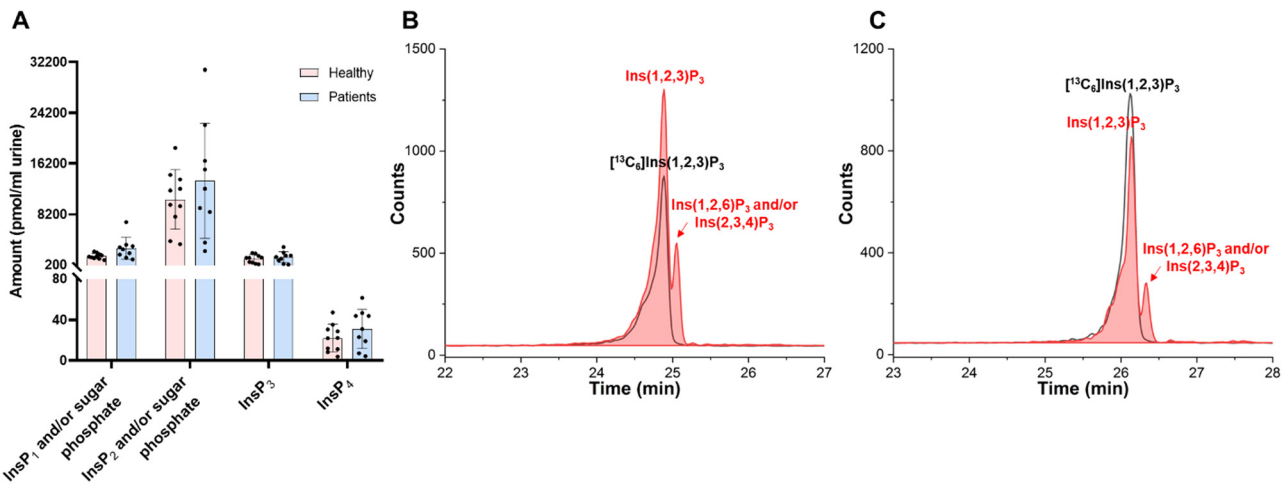

Fig. 4 InsPs in urine samples from patients with kidney stones *vs.* healthy people. (A) InsPs distribution in urine samples: ten samples from healthy individuals, and nine samples from patients with kidney stones. (B) Extracted ion electropherograms of [$^{13}C_6$] Ins(1,2,3)P_3 (black line) and InsP_3 in urine from patients (red trace). (C) Extracted ion electropherograms of [$^{13}C_6$] Ins(1,2,3)P_3 (black line) and InsP_3 in urine from healthy individuals (red trace).

clear as the measured concentrations were in between our current LOD and LOQ for most samples.

Conclusions

We have developed a CE-MS method to separate ''lower'' InsPs, particularly focusing on $InsP_2$ and $InsP_3$. Using this method combined with a pyrohydrolysis strategy, we were able to delineate the identity of a major human $InsP_3$, *i.e.* Ins(1,2,3)P_3 in urine and kidney stones. To achieve this goal, a phytase (RipBL1) was used that selectively degrades $InsP_6$ to Ins(1,2,3)P_3 and that also served for production of a ^{13}C labelled Ins(1,2,3)P_3 internal reference. Our profiling of InsPs in human kidney stone and urine also unveiled Ins(1,2,6)P_3 and/or the enantiomer Ins(2,3,4)P_3 besides the major Ins(1,2,3)P_3. Importantly, the assignments of $InsP_3$ are based on an accurate mass determination and comigration with standards. As a next step, further studies must delineate the stereoisomeric identity of the newly identified $InsP_3$, since chiral selectors have already been developed for certain InsP enantiomers for assignments by ^{31}P NMR spectroscopy.[32] Additionally, methods to distinguish $InsP_1$ and $InsP_2$ from sugar (bis)phosphates will have to be developed. Derivatization of sugar phosphates to separate them from InsPs could be considered.[57] Further improvements in LOQs will be helpful to establish, if potentially $InsP_5$ or $InsP_6$ concentrations in urine can be used as biomarkers for kidney stone formation.

Ins(1,2,3)P_3 was described in mammalian cells more than 25 years ago[18,26] and other inositol phosphates with a phosphate ester in the 2-position clearly exist. However, the interest in these isomers in human biology has faded over time, and recent literature is not reporting on them anymore so their roles remain unresolved. Our study in combination with work from the Fiedler group[27] demonstrates that Ins(2)Ps are abundant cellular, kidney stone and body fluid components. These discoveries warrant reassessment of the classically discussed InsP metabolism. New attention must be given to noncanonical InsP species to fully and properly appreciate the inositol phosphate code.

Author contributions

G. L., A. S., and H. J. J. designed research and wrote the paper. G. L., D. Q. and H. J. J. analyzed data, E. R., R. S., G. S., A. S., T. L. cloned and analyzed RipBL1, D. C., O. B., C. A. W., J. P. J., T. K. provided materials and analyzed data. All authors discussed the paper and provided input for the final version. Further Swiss Kidney Stone Cohort investigators are listed in the acknowledgments.

Conflicts of interest

The authors declare no conflicts of interest.

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