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High-performance thin-layer chromatography method for inositol phosphate analysis

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Abstract

A simple and inexpensive high-performance thin-layer chromatography (HPTLC) method for the analysis of inositol mono- to hexakisphosphates on cellulose precoated plates is described. Plates were developed in 1-propanol–25% ammonia solution–water (5:4:1) and substance quantities as low as 100–200 pmol were detected by molybdate staining. Chromatographic mobilities of nucleotides and phosphorylated carbohydrates were also characterized. Charcoal treatment was employed to separate nucleotides from inositol phosphates with similar R_F values prior to HPTLC analysis. Practical application of the HPTLC system is demonstrated by analysis of grain extracts from wild type and low-phytate mutant barley as well as phytate degradation products resulting from barley phytase activity.

Keywords: Inositol phosphates; Nucleotides

1. Introduction

Analytical techniques for the separation of inositol phosphates (InsPs) have evolved from paper chromatography [1–3] and high-voltage paper electrophoresis (HVPE) [4] to liquid chromatography [5–7] and subsequently to high-performance liquid chromatography (HPLC) [8–15]. Although the most recent techniques undoubtedly allow both excellent separation and highly sensitive detection of inositol phosphates, there still exists a need for simple, robust and inexpensive methods. This need becomes especially obvious when the number of samples is so large that the application of ''high-tech'' equipment would be too time consuming and/or too costly. In our laboratory, a convenient method was sought to assay phytate ($InsP_6$) and P_i levels in several hundred grains from putative low-phytate barley mutants. Among the methods considered for this purpose were descending paper chromatography, HVPE and thin-layer chromatography (TLC). Since descending paper chromatography requires specially designed glass chambers, and HVPE can not be performed safely without a very efficient cooling unit, a simple TLC method seemed appropriate. Surprisingly, there are few reports [16,17] in which TLC is specifically used for inositol phosphate analysis, and since none of these methods seemed suitable for the processing of large numbers of samples, we have developed a TLC method by which up to 80 samples can be

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analyzed on conventional cellulose plates per day [18].

In this paper, development, methodology and practical applications of a simple and inexpensive high-performance thin-layer chromatography (HPTLC) system for inositol phosphate analysis are described. Our method offers reduced analysis time and a more sensitive detection than previously published TLC methods [16–18]. Apart from inositol phosphates, HPTLC characteristics of nucleotides and phosphosugars, which are often co-isolated with inositol phosphates [4,9] are also described.

2. Experimental

2.1. Chemicals

All inositol phosphates were obtained from Sigma (St. Louis, MO, USA); ultrapure ribonucleotide 5′-triphosphates, Fru(6)P, Fru(1,6)P_2 and Glc(1)P were from Boehringer (Mannheim, Germany). Ammonium heptamolybdate tetrahydrate, hydrochloric acid, perchloric acid, diethyl ether, 1-propanol, 25% ammonia solution and trichloroacetic acid (TCA) were from Merck (Darmstadt, Germany). Activated charcoal (Norit A) was from Serva (Heidelberg, Germany). Water, purified on a Milli-Q reagent grade water system (Millipore, Molsheim, France), was used in all experiments.

2.2. Plant material

Resting grains used for TCA extraction and phytase activity analysis were from barley (*Hordeum vulgare* L., cv. Alexis) and from the low-phytate grain mutant ALP4A, a mutant of cv. Alexis identified by screening techniques described previously [18].

2.3. TCA extraction of barley grains

A modified version of the extraction procedure of Pittet et al. [10] was used for TCA extraction of phospoinositols. Single barley grains were crushed with a pair of pliers, transferred to Eppendorf tubes and homogenized in 10× (v/w) 10% (w/v) TCA, 5 m*M* NaF and 5 m*M* EDTA using a small pestle. Homogenates were vortexed for 1.5 h and centrifuged at 5000 *g* for 5 min. TCA was removed from the supernatants by a three-fold extraction with two volumes of water-saturated diethyl ether. A 10-μl volume of each supernatant was lyophylized and resuspended in 3 μl water prior to HPTLC analysis.

2.4. Isolation of barley phytase products

Barley grains (cv. Alexis) were ground using a mortar and pestle. Two 40-mg portions of flour were suspended in 200 μl 50 m*M* sodium acetate, pH 4.6. Both samples were incubated at 35°C and vortexed briefly in intervals of 3 min. Enzyme activity was stopped by snap-freezing in liquid nitrogen after 30 min (sample 1) and 60 min (sample 2). Degradation products from phytase activity were then isolated by adding 200 μl 20% (w/v) TCA, 5 m*M* NaF and 5 m*M* EDTA to each sample, followed by vortexing, diethyl ether extraction and lyophylization as described above.

2.5. Charcoal treatment of an inositol phosphate-nucleotide mixture

Activated charcoal (Norit A) was pretreated with HCl as described by Mayr [12] and resuspended in 100 m*M* NaCl to obtain a 20% (w/v) suspension. An aqueous mixture of 100 μl, containing 15 nmol Ins(1)P, 15 nmol Ins(5,6)P_2, 25 nmol Ins(3,4,5,6)P_4, 25 nmol InsP_6 and 20 nmol ATP, CTP, GTP and UTP was divided into four 25-μl samples. Three of the four samples were treated 1–3 times with activated charcoal to remove nucleotides. This was carried out by adding 3 μl charcoal suspension and vortexing for 15 min at 4°C. After a centrifugation at 5000 *g* for 5 min, supernatants were removed and lyophylized or subjected to another round of charcoal treatment. Lyophylized samples were resuspended in 5 μl water and aliquots of 0.5 μl were analyzed by HPTLC.

2.6. HPTLC plates and development chamber

HPTLC cellulose precoated glass plates (without fluorescent indicator) were obtained from Merck (Darmstadt, Germany). Plate dimensions were 10× 10 cm and the coating thickness was 100 μm. Plates

were developed in a double-through chamber (internal dimensions: 12×12×4.2 cm) from Camag (Muttenz, Switzerland).

2.7. Sample application and plate development

Samples with a volume of 1–2 μl were applied in 0.2-μl aliquots at a distance of 7 mm from the plate border using a 0.1–2.0 μl precision pipette from Labsystems (Helsinki, Finland). A 10-ml volume of mobile phase consisting of 1-propanol–25% ammonia solution–water (5:4:1) [3] was used per experiment and care was taken to keep the distance between the upper level of the mobile phase and the lower borders of the application zones as short as possible. Plates were developed at room temperature until the solvent front was ca. 2 mm away from the upper plate border. Run time was approximately 5 h.

2.8. Detection of phosphorylated compounds

Detection of phosphocompounds with acidic molybdate solution followed by heating and UV-light exposure was first described by Bandursky and Axelrod [2]. A modified version of that method was carried out as follows: readily developed HPTLC plates were air dried and then sprayed with a molybdate reagent containing 8 m*M* ammonium heptamolybdate tetrahydrate, 0.1 *M* HCl and 0.5 *M* $HClO_4$ (modified after Hanes and Isherwood [1]). Plates were subsequently incubated at 85°C for 6.5 min and exposed to UV-light (254 nm) at a distance of 10 cm for another 6.5 min. Faint blue spots were immediately visible after UV exposure and maximal color intensity of spots was reached after 2 h. To avoid background formation and fading of spots, processed plates were kept out of bright light.

3. Results

3.1. HPTLC analysis of inositol phosphates, nucleotides and phosphorylated sugars

In order to compare chromatographic mobilities of various phosphocompounds, six different inositol phosphates, four nucleotides, three phosphorylated carbohydrates and orthophosphate were analyzed on a HPTLC cellulose plate (Fig. 1). From the very slowly migrating $InsP_6$ (lane 1) to $Ins(1,4,5)P_3$ (lane

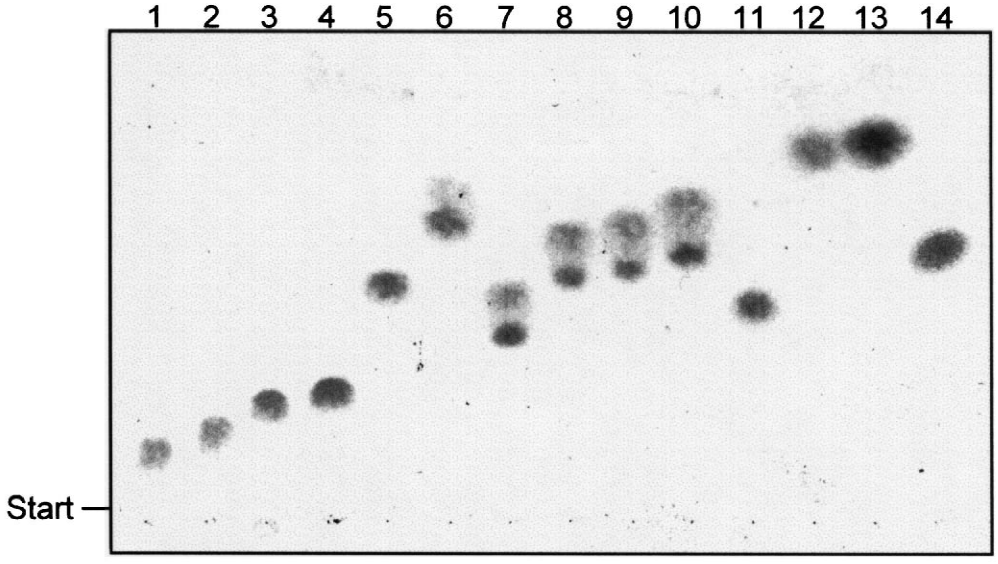

Fig. 1. HPTLC analysis of phosphorylated compounds. Samples were loaded in the following order: 1, $InsP_6$ (2.1 nmol); 2, $Ins(1,3,4,5,6)P_5$ (1.9 nmol); 3, $Ins(3,4,5,6)P_4$ (2.3 nmol); 4, $Ins(1,4,5)P_3$ (2.4 nmol); 5, $Ins(5,6)P_2$ (2.2 nmol); 6, Ins(1)P (2.3 nmol); 7, GTP (0.9 nmol); 8, UTP (0.9 nmol); 9, CTP (0.9 nmol); 10, ATP (0.9 nmol); 11, Fru(1,6) P_2 (1.9 nmol); 12, Fru(6)P (3.1 nmol); 13, Glc(1)P (2.7 nmol); 14, P_i (2.4 nmol). Plate development and molybdate staining were carried out as described in Experimental.

4) a steady increase in mobility could be observed, whereas Ins(5,6)P_2 (lane 5) and Ins(1)P (lane 6) migrated much faster. A faint colored spot, located just above the Ins(1)P spot (lane 6), represents a trace of the 2-monophosphate isomer (manufacturer's information).

Among the nucleotides, GTP migrated the slowest (lane 7), while UTP, CTP and ATP had similar mobilities (lanes 8–10). Above each nucleotide 5′-triphosphate a less intensely stained spot was observed, which most likely represents the respective nucleotide 5′-di- and/or monophosphate. These compounds were probably formed by slow hydrolysis of NTPs during the 5-h development period of the HPTLC plate.

Analysis of phosphorylated sugars showed a striking difference in mobility between Fru(1,6)P_2 (lane 11) and Fru(6)P (lane 12), whereas Fru(6)P and Glc(1)P (lane 13) had similar mobilities. Orthophosphate (lane 14) had a slightly higher mobility than ATP (lane 10).

R_F values of all substances analyzed in this experiment are listed in Table 1 alongside literature R_F values of inositol phosphates determined by descending paper chromatography [3].

Table 1
R_F values of inositol phosphates and other phosphocompounds

HPTLC		Paper chromatography	
Compound	R_F [a]	Compound	R_F [b]
$InsP_6$	0.08	$InsP_6$	0.09
Ins(1,3,4,5,6)P_5	0.11	$InsP_5$	0.10
Ins(3,4,5,6)P_4	0.15	$InsP_4$	0.17
Ins(1,4,5)P_3	0.16	$InsP_3$	0.22
Ins(5,6)P_2	0.27	$InsP_2$	0.27
Ins(1)P	0.34	InsP	0.38
GTP	0.20	GTP	ND[c]
UTP	0.27	UTP	ND
CTP	0.28	CTP	ND
ATP	0.29	ATP	ND
Fru(1,6)P_2	0.24	Fru(1,6)P_2	ND
Fru(6)P	0.41	Fru(6)P	ND
Glc(1)P	0.42	Glc(1)P	ND
P_i	0.32	P_i	0.34

[a] R_F values determined after HPTLC separation (Fig. 1).

[b] R_F values from a paper chromatography analysis performed by Desjobert and Petek [3]. The same mobile phase as for HPTLC was used but InsP standards were obtained by degradation of phytate through wheat bran phytase activity. The precise structural identities of the individual compounds were not given.

[c] ND: Not determined.

3.2. Estimation of detection sensitivity

The visual detection limit of the molybdate staining method described was estimated by employing a dilution series of several phosphocompounds, chromatographed on HPTLC plates. In the three examples shown in Fig. 2 the smallest visible quantities were 62.5 ng Ins(1)P, 125 ng Ins(1,4,5)P_3 and 125 ng $InsP_6$. Expressed in molar amounts these masses correspond to 114, 192 and 135 pmol, respectively. With respect to the inaccurate nature of such a visual estimation, these amounts are not to be considered as exact, quantitative data, but rather as an indication of detection limits in the 100–200 pmol range for all three compounds. Similar detection limits in the picomole range were found for other inositol phosphates as well as NTPs and phosphorylated sugars (data not shown).

3.3. Removal of nucleotides by treatment with activated charcoal

Initial HPTLC characterization of phosphocompounds (Fig. 1) showed that lower inositol phosphates ($InsP_x$, $x=1–3$) and nucleotides have similar R_F values (Table 1). Hence, distinguishing between these two compound classes would be difficult when tissue extracts with unknown composition were to be analyzed. In order to solve this problem, charcoal treatment was tested for its ability to remove interfering nucleotides prior to HPTLC (Fig. 3). Originally, charcoal treatment was employed as a clean-up procedure for samples in HPLC analysis of inositol phosphates [9–13].

HPTLC analysis of a defined mixture of InsP and NTP standards (Fig. 3, lane 1) revealed that ATP, CTP, UTP, Ins(1)P and Ins(5,6)P_2 were not separated sufficiently. A single round of charcoal treatment removed most of the GTP and ATP (lane 2); after two rounds only a small amount of GTP remained (lane 3) and three consecutive treatments were sufficient to remove the last trace of detectable nucleotide (lane 4). Staining intensities of inositol phosphate spots did not decrease from lane 1 to lane

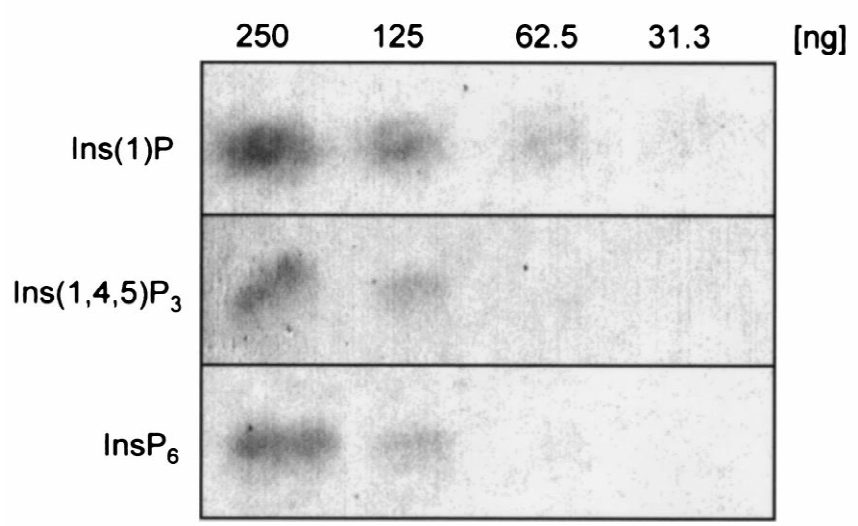

Fig. 2. Detection limits of inositol phosphates. Two-fold dilution series from 250 to 31.3 ng (top) of individual inositol phosphate standards (as indicated on the left-hand side) were separated by HPTLC and detected by spraying with Hanes–Isherwood molybdate reagent [1].

4; thus repeated charcoal treatment did not result in a loss of inositol phosphates.

3.4. Practical applications of the HPTLC system

To demonstrate routine applications of the HPTLC system in our work, we analyzed inositol phosphates from wild type vs. low-phytate barley grain mutants as well as phytate degradation products from endogenous barley phytase activity (Fig. 4).

In the grain extract of the low-phytate mutant ALP4A (lane 1) the amount of soluble, inorganic phosphate (R_F=0.31) was much higher in comparison to the wild type (cv. Alexis) extract (lane 2), whereas the content of phytate (R_F=0.08) was slightly reduced. Above the phytate spot in lane 1 a faint spot with a R_F value of 0.15 was observed, which indicated an $InsP_4$ isomer (Table 1).

Intensely stained application zones and ''tailing'' of $InsP_6$ spots in lanes 1 and 2 indicates that considerable amounts of phytate did not migrate at all or were insufficiently mobilized by the solvent mixture. A likely reason for this retarded migration is that phytate was present in partially unsoluble complexes together with minerals, proteins and/or starch [19].

Products of phytase activity (Fig. 4, lanes 3 and 4) were obtained by grinding wild type (cv. Alexis) barley grains and suspending two 40-mg portions of flour in sodium acetate buffer. After incubation periods of 30 min (sample 1) and 60 min (sample 2), both samples were extracted with TCA and diethyl ether, treated with charcoal and analyzed by HPTLC. Incubation for 30 min (lane 3) was sufficient to convert most of the phytate to P_i and three major degradation products with R_F values of 0.28, 0.22 and 0.15, corresponding to $InsP_2$, $InsP_3$ and $InsP_4$ isomers, respectively (see Table 1 and Section 4.2). After an incubation of 60 min (lane 4), only trace amounts of these products could be detected, reflecting a high level of enzymatic activity. In contrast to lanes 1 and 2, no phosphomolybdate staining was observed in the application zones of lanes 3 and 4, suggesting effective degradation of mineral-, protein- and/or starch-associated phytate.

4. Discussion

4.1. Development of a HPTLC system for inositol phosphate analysis

The HPTLC system described represents a sensitive and simple method for analysis of inositol

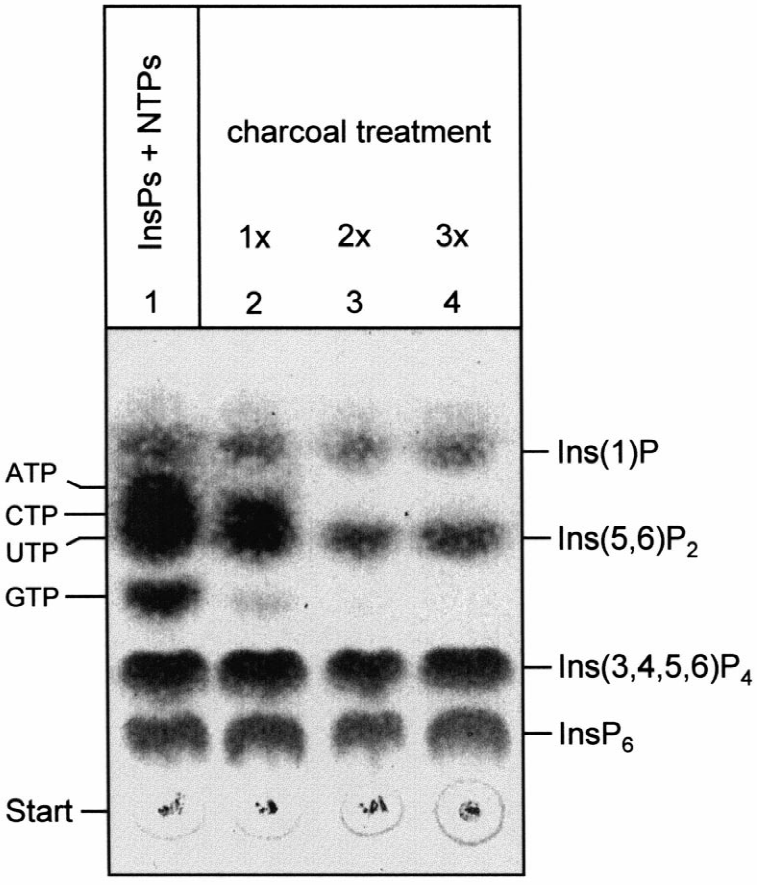

Fig. 3. Charcoal treatment of a mixture containing inositol phosphates and nucleotides. Twenty nmol of ATP, CTP, GTP and UTP was added to an aqueous solution of 15 nmol Ins(1)P, 15 nmol Ins(5,6)P_2, 25 nmol Ins(3,4,5,6)P_4 and 25 nmol InsP_6. The resulting mixture (total volume 100 μl) was divided into four 25-μl aliquots, which were treated up to three times with activated charcoal, lanes 2–4, as indicated. Lane 1, untreated mixture. Aliquots corresponding to 1/10 vol were analyzed by HPTLC. Nucleotides and inositol phosphates were assigned according to their R_F values using corresponding R_F values in Table 1 as a reference.

phosphates. Development of this system was largely inspired by paper chromatography methods [1–3]; cellulose precoated plates were chosen since cellulose coating and paper have a similar matrix chemistry. To find a suitable mobile phase for HPTLC, many solvent mixtures from paper chromatography protocols were tested until optimal separation of inositol mono- to hexakisphosphates was achieved with 1-propanol–25% ammonia solution–water (5:4:1). This solvent mixture was originally used for InsP chromatography on Whatman No.1 paper [3]. Our attempts to improve separation on HPTLC plates using minor modifications of this mixture were unsuccessful.

4.2. The HPTLC system compared with paper chromatography methods

Paper chromatography has been widely used to analyze phosphocompounds such as inositol phosphates [1–3], and with the introduction of HVPE an excellent degree of separation could be achieved [4]. Paper chromatography methods have a number of practical drawbacks, however, such as long development time (12–24 h) and the need for a very effective cooling system in the case of HVPE. Furthermore, the use of paper frequently limits the sensitivity of detection, which is due to spot diffusion and background problems, since paper gradually

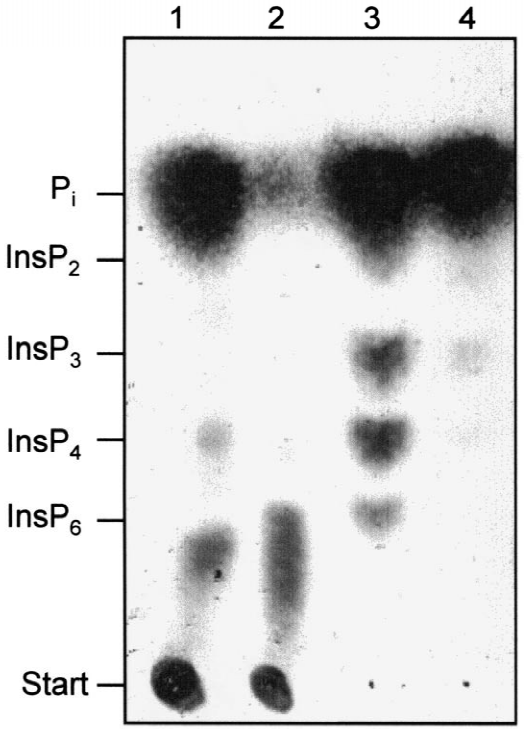

Fig. 4. Barley grain extracts and phytase degradation products analyzed by HPTLC. Single-grain extracts were prepared from the low-phytate mutant ALP4A and the wild type cv. Alexis. Following charcoal treatment samples of 0.5 μl (corresponding to 1/240 total extract volume) were spotted onto the plate (lanes 1 and 2). Degradation products from endogenous phytase activity in barley grain flour after incubation for 30 and 60 min are shown in lanes 3 and 4. Phosphoinositols were assigned by determining R_F values of spots (see text) and comparing those with reference values from Table 1.

turns blue after spraying with acidic molybdate reagents [20].

Nowadays, with the availability of HPTLC plates, chromatography of phosphocompounds can be performed on a refined cellulose matrix, a substantial improvement on cellulose in the form of paper. HPTLC cellulose coating represents a very homogenous layer with a thickness of only 100 μm, consisting of microcrystalline particles with a mean size of 4–8 μm (product information from Merck). These structural features make it possible to resolve highly polar phosphocompounds in a relatively short time period (ca. 5 h) and to detect minute quantities of substance in the 100–200 pmol range (Fig. 2). Compared to molybdate staining of paper chromatograms [1,2], this represents a 15- to 30-fold increase in sensitivity.

Where separation capacity is concerned, HPTLC does not offer a clear advantage over paper chromatography; R_F values of inositol phosphates and P_i determined from our analysis (Fig. 1 and Table 1) are quite similar to those published by Desjobert and Petek [3]. A noteworthy exception is Ins(1,4,5)P_3, which migrated considerably slower (R_F=0.16) than the $InsP_3$ standard with paper chromatography (R_F= 0.22). This difference in mobility is most likely due to a structural difference between Ins(1,4,5)P_3 and the $InsP_3$ compound used by Desjobert and Petek. All InsP standards employed in [3] were generated by digesting phytate with wheat bran phytase, and the precise isomeric identities of the degradation products were unknown at that time (1956). In later studies it was established that the major $InsP_3$ species produced by both wheat bran and barley phytase activity is Ins(1,2,3)P_3 [14,21]. It is important to note that the present HPTLC analysis of barley phytase degradation products (Section 3.4 and Fig. 4) also revealed a product with a R_F value of 0.22 which could very well be Ins(1,2,3)P_3 or an $InsP_3$ isomer with similar positional isomerism.

4.3. Inositol phosphate analysis by TLC and HPTLC

Examples of TLC-based inositol phosphate analysis are scarce in the literature and compared with the HPTLC method described here, these techniques are either laborious or designed for a limited spectrum of phosphoinositols, such as lower ($InsP_x$, x=1–3) or higher ($InsP_x$, x=4–6) phosphoinositols.

Angyal and Russel [16] developed a TLC method in which higher inositol phosphates were first suspended in anhydrous methanol and then methylated by diazomethane prior to separation on silica gel plates. Although several inositol pentakisphosphate isomers could be separated from each other, this technique seems impractical when a wider spectrum of inositol phosphates (i.e., isomers of $InsP_x$, x=1–6) or large numbers of samples are to be analyzed.

Another TLC protocol was employed by Emilsson and Sundler [17], who analyzed deacylation products obtained from macrophage lipids. Efficient sepa-

ration of InsP and $InsP_2$ molecules on polyethyleneimine cellulose plates was achieved, but since $InsP_3$ migrated very slowly, it is uncertain whether this system would be suitable for the separation of higher InsP compounds.

Our previously published TLC system [18], in which inositol phosphates were separated on conventional cellulose plates, was developed for the analysis of large numbers of samples. Using this method it was not only possible to assay $InsP_6$ and P_i in hundreds of barley grain extracts, but also to detect traces of lower inositol phosphates present in certain mutant grains. However, compared with the HPTLC system described in this paper, InsP analysis on conventional TLC plates requires six-fold larger quantities of sample (10–12 μl) in order to detect compound traces and rather long development periods (10–12 h) for optimal separation.

4.4. Analysis of phosphoinositols using extensive instrumentation

During the last decade several methods for high resolution analysis of inositol phosphates have been developed. Most of these methods are HPLC-based and employ radioactive as well as non-radioactive detection techniques (see Xu et al. [22] and Sandberg [23] for recent reviews). Typically, these HPLC systems use strong anion-exchange columns and acidic (HNO_3 [8] or HCl [9–13,15]), basic (NaOH [15]) or salt (tetramethylammonium chloride [12]) gradients for isomer-specific separation of inositol phosphates. The high separation capacity of these systems allows up to 25 different InsPs to be separated in a single run and UV–Vis detection is achieved by post-column derivatization with either Fe^{3+} alone [8,15] or with Y^{3+} in the presence of the dye 4-(2-pyridylazo)resorcinol [9–13]. Derivatization techniques of this kind have detection limits of 50–100 pmol and even higher sensitivity can be achieved with miniaturized micro-bore HPLC [13]. Alternative methods for InsP detection are conductivity measurement [15] or on-line scintillation counting, which is used in the case of radiolabeled phosphoinositols [14].

Finally, at the high end of technically advanced methods stands the two-dimensional total correlation (2D-TOCSY) nuclear magnetic resonance (NMR) spectroscopy of inositol phosphate mixtures. Using a 500-MHz spectrometer, Johnson et al. [24] succeeded in determining five different inositol phosphates in a mixture without any prior separation.

4.5. Advantages and limitations of the HPTLC system

Compared with the highly instrumentalized techniques discussed above, ''low-tech'' analysis of inositol phosphates by HPTLC offers a number of practical advantages: relatively low equipment costs, easy maintenance and the possibility to analyze a multitude of samples simultaneously. Moreover, detection by molybdate spraying is almost as sensitive as modern post-column derivatization techniques used in HPLC [8–13,15].

This paper demonstrates that the HPTLC system can be applied to single-grain analysis of barley mutants and that small-scale phytase reactions can be assayed (Fig. 4). Sample volumes used for this type of analysis corresponded to extract volumes obtained from just 200 μg grain material, a small fraction of a typical barley grain (weighing 40–50 mg). Thus, the high detection sensitivity makes the HPTLC system suitable for micro-analysis, and monitoring InsP-kinase or phosphatase activities seems feasible without having to use radiolabeled substrates.

An important limitation of the HPTLC system is its separation capacity, which is inferior to those of HVPE [4] or HPLC [8–15]. Therefore, care has to be taken when inositol phosphates from complex tissue extracts are to be analyzed; charcoal treatment is required to remove interfering nucleotides and co-isolated phosphosugars have to be identified by specific carbohydrate detection sprays in separate experiments [25–27]. Moreover, for final structural confirmation of HPTLC identified InsP molecules, isomer-specific HPLC [8–15] and/or NMR spectroscopy [11,24] still remain mandatory.

Taken together, the advantages of the HPTLC system are most ideally exploited in situations where technical convenience and sensitivity are more important than separation capacity.

5. Abbreviations

InsP, $InsP_2$, $InsP_3$, $InsP_4$, $InsP_5$ and $InsP_6$	D-*myo*-inositol mono-, di-, tri-, tetrakis-, pentakis- and hexakisphosphate with positional isomerism of phosphoesters indicated in parentheses
ATP, CTP, GTP and UTP	adenosine, cytidine, guanosine and uridine 5′-triphosphate
Fru(6)P	D-fructose-6-phosphate
Fru(1,6)P_2	D-fructose-1,6-diphosphate
Glc(1)P	D-glucose-1-phosphate
TCA	trichloroacetic acid
P_i	orthophosphate
cv.	cultivar

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