The oxygen isotope composition of phosphate released from phytic acid by the activity of wheat and *Aspergillus niger* phytase

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**Abstract.** Phosphorus (P) is an essential nutrient for living organisms. Under P-limiting conditions plants and microorganisms can exude extracellular phosphatases that release inorganic phosphate (P$_i$) from organic phosphorus compounds (P$_{org}$). Phytic acid (*myo*-inositol hexakisphosphate, IP$_6$) is an important form of P$_{org}$ in many soils. The enzymatic hydrolysis of IP$_6$ by phytase yields available P$_i$ and less phosphorylated inositol derivates as products. The hydrolysis of organic P compounds by phosphatases leaves an isotopic imprint on the oxygen isotope composition ($\delta^{18}O$) of released P$_i$, which might be used to trace P in the environment. This study aims at determining the effect of phytase on the oxygen isotope composition of released P$_i$. For this purpose, enzymatic assays with histidine acid phytases from wheat and *Aspergillus niger* were prepared using IP$_6$, adenosine 5’-monophosphate (AMP) and glycerophosphate (GPO$_4$) as substrates. For a comparison to the $\delta^{18}O$ of P$_i$ released by other extracellular enzymes, enzymatic assays with acid phosphatases from potato and wheat germ with IP$_6$ as a substrate were prepared. During the hydrolysis of IP$_6$ by phytase, four of the six P$_i$ were released, and one oxygen atom from water was incorporated into each P$_i$. This incorporation of oxygen from water into P$_i$ was subject to an apparent inverse isotopic fractionation ($\varepsilon \sim 6$ to 10‰), which was similar to that imparted by acid phosphatase from potato during the hydrolysis of IP$_6$ ($\varepsilon \sim 7$‰), where less than three P$_i$ were released. The incorporation of oxygen from water into P$_i$ during the hydrolysis of AMP and GPO$_4$ by phytase yielded a normal isotopic fractionation ($\varepsilon \sim -12$‰), similar to values reported for acid phosphatases from potato and wheat germ. We attribute this similarity in $\varepsilon$ to the same amino acid sequence motif (RHGXRXP) at the active site of these enzymes, which leads to similar reaction mechanisms. We suggest that the striking substrate dependency of the isotopic fractionation could be attributed to a difference in the $\delta^{18}O$ values of the C–O–P bridging and non-bridging oxygen atoms in organic phosphate compounds.

## 1 Introduction

*myo*-Inositol hexakisphosphate (phytic acid, IP$_6$) is a very important storage molecule for P, Mg, K, Fe and Zn located in plant seeds (Cosgrove and Irving, 1980; Raboy, 1997; Shears and Turner, 2007). It is crucial for seedling growth. Lott et al. (2000) estimated the yearly global production of IP$_6$ in seeds and fruits to be close to $3.5 \times 10^7$ t, containing $9.9 \times 10^6$ t of P. Plant residues introduce IP$_6$ to soil. There it can be stabilized on particles by sorption mechanisms (Ogunalaga et al., 1994) and can comprise up to 50% of organic phosphorus (Dalal, 1977; Anderson, 1988), becoming in some instances the dominant form of organic phosphorus (Turner, 2007). In other cases, however, IP$_6$ can be rapidly mineralized after its introduction in a soil (Doolette et al., 2010).

Plants and soil microorganisms take up phosphorus (P) as inorganic phosphate (P$_i$) from the soil solution and low P$_i$ concentrations can limit biological growth and crop production in many ecosystems (Ehlers et al., 2010; Richardson et al., 2011). Under P-limiting conditions, some plants and mi-
croorganisms can exude phytases, which catalyze the hydrolysis of phosphomonoester bonds in IP₆, leading to the release of inorganic phosphate (Pᵢ) (Hayes et al., 1999; Richardson et al., 2000, 2001; Lung and Lim, 2006; Li et al., 1997a, b). The exudation of phytases might therefore be an important mechanism of plants and microorganisms to utilize a fraction of soil organic phosphorus. For example, Zimmermann et al. (2003) showed that a transgenic potato expressing a synthetic gene encoding for phytase was able to take up a significant amount of P from IP₆, whereas the potato wild type was not. However, the cycling and bioavailability of IP₆ and the role of phytase in terrestrial ecosystems are still poorly understood (Turner et al., 2000, 2002).

The oxygen isotopes associated with phosphorus might be used to trace these enzymatic processes and to shed new light on the cycling and bioavailability of IP₆ in soils (Frossard et al., 2011). In the terrestrial environment, the oxygen isotope composition (δ¹⁸O) of phosphate has been used as a tracer in the terrestrial environment to study the cycling of P in soils (Zohar et al., 2010a and b; Tamburini et al., 2010, 2012; Angert et al., 2011, 2012; Gross and Angert, 2015), in plants (Young et al., 2009; Pfahl et al., 2013) and in aerosols (Gross et al., 2013). Under ambient conditions and in the absence of biological activity, the δ¹⁸O of phosphate does not change (Kolodny et al., 1983; O’Neil et al., 2003). However, biological uptake of phosphate leads to a substantial alteration of δ¹⁸O values (Paytan et al., 2002; Blake et al., 2005; Stout et al., 2014). This alteration is due to the activity of intracellular pyrophosphatases, which catalyze a complete oxygen exchange between Pᵢ and water, leading to an equilibrium isotope fractionation (Cohn, 1958; Longinelli and Nuti, 1973; Blake et al., 2005; Chang and Blake, 2015). Furthermore, the hydrolysis of organic P compounds by extracellular phosphomonooesterases and phosphodiesterases leads to the incorporation of one or two oxygen atoms from water into released Pᵢ (Cohn, 1949; Liang and Blake, 2006, 2009; von Sperber et al., 2014). This incorporation of oxygen from water is subject to a kinetic isotope fractionation (ε), which has been determined for alkaline phosphatases (Liang and Blake, 2006), phosphodiesterases and nucleotidases (Liang and Blake, 2009), and acid phosphatases (von Sperber et al., 2014). To date, the effect of phytases on the δ¹⁸O of the released inorganic phosphate is not known.

In the soil–plant system it is important to distinguish between two types of phytases: 3-phytase and 6-phytase. The 3-phytases, EC 3.1.3.8, which are typical for microorganisms and most likely the prevalent phytase in the soil environment, first hydrolyze the ester bond at the 3-position of IP₆ (myo-inositol hexakisphosphate), which leads to the formation of IP₅ (myo-inositol 1,2,4,5,6-pentakisphosphate) and free inorganic phosphate. In contrast, 6-phytases first hydrolyze the 6-position, which leads to the formation of IP₅ (myo-inositol 1,2,3,4,5-pentakisphosphate) and free inorganic phosphate. The numbering of the carbon atoms corresponds to the numbering for the D configuration.

### 2 Material and methods

#### 2.1 Preparation of enzymatic assays

Enzymatic assays with phytases from two different organisms (phytase from wheat, Sigma Aldrich P1259, and phytase from Aspergillus niger, BASF, Natuphos®, Natuphos 5000) were prepared to determine their effect on the oxygen isotope composition of released Pᵢ. Assays consisted of 200 mM acetate buffer (pH 5.5), with either 2 mM phytic acid (Sigma Aldrich P8810), or 7 mM glycerophosphate (Sigma Aldrich G6501) or 7 mM adenosine 5′-monophosphate (Sigma Aldrich A1752) as a substrate and with 0.5 UN of phytase (1 UN is defined as the activity required to convert 1 µmol of substrate per minute).

Assays with acid phosphatases from potato and wheat germ consisted of 200 mM acetate buffer (pH 4.8), 3 mM phytic acid and 3 UN of enzyme (Sigma Aldrich p3752 and Sigma Aldrich p3627). Proteins were further purified by dialysis with a dilution factor of 10 000, using a SnakeSkin dialysis tubing 10K MWCO 16 mm (Thermo Scientific, SnakeSkin, PI88243). All assay reagents were prepared in ¹⁸O-labeled and non-labeled double deionized water (dd-H₂O) and filter-sterilized. Batch assays had a volume of 3 mL and were

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**Figure 1.** Phytic acid (IP₆) degradation to IP₅ by 3-phytases and 6-phytases (modified from Dvořáková, 1998). 3-Phytases first hydrolyze the ester bond at the 3-position of IP₆ (myo—inositol hexakisphosphate), which leads to the formation of IP₅ (myo—inositol 1,2,4,5,6-pentakisphosphate) and free inorganic phosphate. In contrast, 6-phytases first hydrolyze the 6-position, which leads to the formation of IP₅ (myo—inositol 1,2,3,4,5-pentakisphosphate) and free inorganic phosphate. The numbering of the carbon atoms corresponds to the numbering for the D configuration.
were prepared in 15 mL centrifuge tubes. Directly after the addition of the reagents, the tubes were closed and only opened for sampling. The concentration of released Pi in the assays was monitored using the colorimetric malachite green method (Ohno and Zibilske, 1991). After 48 h, Pi yield was usually close to 65 % and did not change any more, despite the enzyme being still active, which indicates that the original substrate IP6 molecule was degraded to myo-inositol biphosphate (IP2) and four Pi molecules (4 × Pi/6 × Pi = 66.6 %). Enzymatic reactions were terminated after 72 h by adding 2 mL of 7 M ammonia solution. Experiments were carried out in a temperature-controlled water bath at 37 °C. To test whether temperature had an effect on the isotopic fractionation, enzymatic assays were also prepared at 4 °C. The δ18O of water in the assays was measured at the beginning and at the end of each experiment and did not vary over the course of the experiment. Released Pi was purified according to the protocol of Tamburini et al. (2010). In brief, Pi is first precipitated as magnesium ammonium phosphate (MAP), which can be retrieved by filtration and subsequently re-dissolved, purified and precipitated as silver phosphate (Ag3PO4).

2.2 Ultraviolet radiation (UVR) digestion

The δ18O of IP6 and of the filtrate after the precipitation of MAP were analyzed after UVR digestion. IP6 and the filtrate were transferred in a solution with 20 mL of 18O-labeled and unlabeled dd-H2O and 3 mL of 28 % H2O2 and left overnight in a 25 mL quartz tube. The next day, the solutions were exposed to UVR (500 W mercury lamp) for 4 h at 27 °C. During the photodecomposition of organic P compounds, only C–O bonds are cleaved, whereas O–P bonds remain intact, leading to the release of the original PO4 moiety from the organic P compound without any incorporation of oxygen from water (Liang and Blake, 2006). UVR-released Pi was then processed following the protocol of Tamburini et al. (2010). The δ18O of phosphate from the organic P compound (δ18O) was calculated according to the modified equation from McLaughlin et al. (2006):

$$\delta^{18}O_{S} = \left( \delta^{18}O_{P-UVR} \times \delta^{18}O_{W}^{***} \right) \div \left( \delta^{18}O_{P-UVR} - \delta^{18}O_{W}^{***} \right),$$

with δ18O*** and δ18O being the δ18O of labeled and unlabeled water and δ18O being the δ18O of UVR-released phosphate in water which was labeled (***). The fraction of oxygen which exchanged with water during UVR digestion (Fexch) can be calculated according to

$$F_{exch} = \left( \delta^{18}O_{P-UVR} \times \delta^{18}O_{W}^{***} \right) \div \left( \delta^{18}O_{S} - \delta^{18}O_{W}^{***} \right).$$

2.3 Determination of δ18O values of phosphate and water

Oxygen isotope analysis of Ag3PO4 was carried out with a vario PYRO Cube (Elementar, Hanau, Germany) connected in continuous flow to an Isoprime 100 isotope ratio mass spectrometer (Isoprime, Manchester, UK). The pyrolysis of Ag3PO4 took place at 1450 °C in a carbon-based reactor. A temperature-controlled purge and trap chromatography system was used to separate CO from N2. Results were calibrated against an internal Ag3PO4 standard (Acros Organics, Geel, Belgium; δ18O = 14.2 ‰ Vienna Standard Mean Ocean Water (VSMOW)) and two benzoic acid standards distributed by the International Atomic Energy Agency (IAEA) (IAEA 601: δ18O = 23.1 ‰; IAEA 602: δ18O = 71.3 ‰). Analytical error calculated on replicate analysis of standards was better than ±0.4 ‰.

For oxygen isotopes analysis of water, a 0.3 % CO2 and He mixture was equilibrated for 18 h at 25 °C with the samples in airtight Exetainers. Aliquots of the CO2/He mixture from the headspace were sampled and transferred to a Delta V Plus mass spectrometer (Thermo Fisher Scientific Inc.) using a gas bench (GasBench II, Thermo Scientific Inc.). The oxygen isotope composition of water was derived from the isotope analysis of CO2. The system was calibrated with the international standards VSMOW, Standard Light Antarctic Precipitation (SLAP), and Greenland Ice Sheet Precipitation (GISP), distributed by the IAEA. Analytical error calculated on replicate analysis of standards was better than ±0.06 ‰.

Oxygen isotope compositions are reported in the conventional delta notation ($\delta(\%e) = (R_s / R_t - 1) \times 1000$, where $R_s$ denotes the ratio of the heavy to light isotope and $R_t$ are the ratios of the sample and standard, respectively) with respect to VSMOW.

2.4 Statistical analyses

Standard deviations (SD), linear regressions, ANOVA and Tukey’s HSD tests were calculated using the statistical software R. A one-way ANOVA was carried out for isotopic fractions caused by different phytases and substrates. After rejecting the null hypothesis of the ANOVA, isotopic fractions were compared with Tukey’s HSD tests.

3 Results

3.1 Incorporation of oxygen from water into Pi during hydrolysis of IP6 by phytases

Purified phytase from wheat and Aspergillus niger hydrolyzed approximately 65 % of the phosphate molecules bound to IP6. Control experiments with crude protein extract from wheat phytase without any substrate revealed a substantial contamination of approximately 20 μmol Pi UN−1 protein extract. In order to remove this contamination, crude pro-
3.2 Incorporation of oxygen from water into P\textsubscript{i} during UVR digestion

The $\delta^{18}O$ of P\textsubscript{i} produced during UVR digestion of IP\textsubscript{6} in water with a $\delta^{18}O$ of $-9.8\%$ was 21.0 and 24.4 $\%$ for water with a $\delta^{18}O$ of 51.2 $\%$, corresponding to an incorporation of 6 $\%$ of oxygen from water into released P\textsubscript{i} (Table 1). The filtrate retrieved after precipitation of MAP contains IP\textsubscript{2}, which was also analyzed for its $\delta^{18}O$. The $\delta^{18}O$ of P\textsubscript{i} produced during UVR digestion of IP\textsubscript{2} in water with a $\delta^{18}O$ of $-10.4\%$ was 21.7 and 22.4 $\%$ for water with a $\delta^{18}O$ of 73.3 $\%$, corresponding to an incorporation of 1 $\%$ of oxygen from water into the formed P\textsubscript{i} (Table 1). These findings confirm that the UVR-induced release of the original PO\textsubscript{i} moiety from the organic P compound proceeded with little incorporation of oxygen from water.

3.3 Oxygen isotope composition of P\textsubscript{i} released after hydrolysis of AMP and GPO\textsubscript{4} by phytase and after hydrolysis of IP\textsubscript{6} by acid phosphatase

Phytases can hydrolyze single phosphomonoester substrates, and some acid phosphatases can partly hydrolyze IP\textsubscript{6} (Gibson and Ullah, 1988; Oh et al., 2004; Annaheim et al., 2013). For this reason, the effect of wheat phytase on adenosine 5'-monophosphate (AMP) and on glycerophosphate (GPO\textsubscript{4}) was tested. Wheat phytase hydrolyzed approximately 72 $\%$ of AMP and approximately 80 $\%$ of GPO\textsubscript{4}. Experiments with AMP as a substrate ($\delta^{18}O_S = 15.8\%$), which were carried out in assays with a $\delta^{18}O_W$ of $-45.5\%$, resulted in a mean $\delta^{18}O_P$
of $-1.9\%e$. Experiments with GPO$_4$ as a substrate ($\delta^{18}O_S = 16.6\%e$), which were carried out in assays with a $\delta^{18}O_W$ of $-50.4\%e$, resulted in a mean $\delta^{18}O_P$ of $-2.4\%e$ (Table 3).

In addition, two acid phosphatases from potato and wheat germ with IP$_6$ as a substrate were tested. Acid phosphatase from wheat germ hydrolyzed approximately 10\% of IP$_6$ and acid phosphatase of potato hydrolyzed approximately 40\% of IP$_6$. Experiments with acid phosphatase from wheat germ were carried out in assays with a $\delta^{18}O_W$ of $-58.5\%e$ and resulted in a mean $\delta^{18}O_P$ of 3.0\%. Experiments with acid phosphatase from potato were carried out in assays with a $\delta^{18}O_W$ of $-9.8\%e$ and resulted in a mean $\delta^{18}O_P$ of 16.7\%e (Table 3).

4 Discussion

4.1 Implications of incorporation of oxygen from water into P$_i$ during hydrolysis of IP$_6$ by phytases

The slopes from assays with purified phytases are close to 0.25, similar to experiments conducted with phosphomonooesterases like alkaline and acid phosphatases (Liang and Blake, 2006; von Sperber et al., 2014). However, both slopes (0.23 and 0.24) are significantly different from 0.25 (ANOV A, $p < 0.05$). This indicates that the contamination with P$_i$ from the crude extract, where we observe a strong deviation in the slope, may not have been fully removed by our purification step.

The finding of a 0.25 slope implies that one oxygen atom from water is incorporated into each released P$_i$. From this observation follows that the enzymatic release of P$_i$ from IP$_6$ proceeds by cleaving the P–O bond of the oxygen connected to myo-inositol via the addition of oxygen from water, a process that is different from the abiotic photodecomposition, where C–O bonds are cleaved and P–O bonds remain intact.

4.2 Oxygen isotope fractionation during the incorporation of oxygen from water into P$_i$

Assuming that released P$_i$ consists of three oxygen atoms from the original substrate and one oxygen which has been incorporated from water, the following mass balance can be applied to determine the oxygen isotope fractionation ($\varepsilon$) caused by phytases (Liang and Blake, 2006):

$$\delta^{18}O_P = 0.75 \times \delta^{18}O_S + 0.25 \times (\delta^{18}O_W + \varepsilon), \quad (3)$$

where $\delta^{18}O_P$ is the $\delta$ value of released P$_i$, $\delta^{18}O_S$ is the $\delta$ value of the substrate (meaning the average value of the 4-phosphate released from IP$_6$), $\delta^{18}O_W$ is the $\delta$ value of the water and $\varepsilon$ is the isotopic fractionation.

The analysis of $\delta^{18}O_P$ and $\delta^{18}O_W$ is straightforward, but the determination of $\delta^{18}O_S$ is more complicated. Compared to single phosphomonooesters, such as glycerophosphate or adenosine 5’-monophosphate, phytic acid consists in total of six phosphate molecules, which all might have different $\delta^{18}O$ values. The direct determination of the $\delta^{18}O$ of each of the phosphate molecules attached to myo-inositol is not possible. However, the bulk isotope composition of the phosphate moieties from IP$_6$ and IP$_2$ can be determined, allowing for the calculation of $\delta^{18}O_S$. Our results indicate that the original substrate IP$_6$ molecule was degraded to IP$_2$ and 4 P$_i$ molecules ($IP_6 \rightarrow IP_3 + P_i \rightarrow IP_4 + 2P_i \rightarrow IP_3 + 3P_i \rightarrow IP_2 + 4P_i$). In this case, $\delta^{18}O_S$ corresponds to the $\delta^{18}O$ of the 65\% of phosphate molecules that were cleaved from IP$_6$. By using a simple mass balance, $\delta^{18}O_S$ can be derived indirectly from $\delta^{18}O$ of IP$_6$ ($\delta^{18}O_{IP_6}$) and IP$_2$ ($\delta^{18}O_{IP_2}$) as follows:

$$\delta^{18}O_{IP_6} = 2/3 \times \delta^{18}O_S + 1/3 \times \delta^{18}O_{IP_2}. \quad (4)$$

And solving for $\delta^{18}O_S$:

$$\delta^{18}O_S = 3/2 \times \delta^{18}O_{IP_6} - 1/2 \times \delta^{18}O_{IP_2}. \quad (5)$$

The $\delta^{18}O_{IP_2}$ value was determined by oxidation photodigestion of the filtrate, which consists of IP$_2$, after the MAP precipitation step. Digestion of the organic P compounds by UVR led to the release of P$_i$ with a $\delta^{18}O_{IP_2}$ value of 22.8\%e ($\pm 0.4\%e$) and a $\delta^{18}O_{IP_2}$ value of 22.0\%e ($\pm 0.4\%e$) (Table 1). Using these values in Eq. (5) we calculate a $\delta^{18}O_S$ value of 23.2\%e ($\pm 0.7\%e$). Solving Eq. (3) with the obtained $\delta^{18}O_S$
Table 3. δ18O values of water (δ18OW), released phosphate (δ18Op) and phosphate in organic P compound (δ18OS) as well as isotopic fractionation (ε), which was calculated according to Eqs. (6) and (7) with an assumed δ18OCON value of 15‰. Results are from experiments with IP6, AMP and GPO4 as substrates and with phytase from wheat and acid phosphatases from wheat germ and potato. * Values from von Sperber et al. (2014).

<table>
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<th>Enzyme</th>
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<th>δ18Op</th>
<th>δ18OS</th>
<th>ε</th>
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value results in an average ε of 6.4‰ (±2.9‰) in assays with wheat phytase and in an average ε of 6.7‰ (±3.4‰) in assays with Aspergillus niger phytase (Table 2). The isotopic fractionation is not significantly different between the two types of phytases (ANOVA; p value > 0.05).

We can refine our results by addressing the fact that the linear regression of δ18Op vs. δ18OW yields a slope of 0.23 in the case of wheat phytase and in a slope of 0.24 in the case of Aspergillus niger phytase (Fig. 2). These values are slightly below a slope of 0.25, indicating small contaminations with P1 that was not derived from IP6. These small contaminations are the reason for the linear relationship between δ18OW values and ε (Table 2). In the case of wheat phytase, only 23% of oxygen in free inorganic phosphate in solution is derived from water. This means that free inorganic phosphate in solution, which has been released from the organic P substrate by enzymatic activity, only accounts for 92% of total inorganic phosphate in solution (4 × 23%). Therefore, 8% of free inorganic phosphate in solution is due to contamination. To account for this contamination, another term has to be included into the mass balance and Eq. 3 needs to be rewritten for experiments with wheat phytase as

\[
\delta^{18}O_{\text{P}} = 0.92 \times \left(0.75 \times \delta^{18}O_{\text{S}} + 0.25 \times \delta^{18}O_{\text{W}} + 0.25 \times \varepsilon \right) \\
+ 0.08 \times \delta^{18}O_{\text{CON}},
\]

and for experiments with Aspergillus niger phytase as

\[
\delta^{18}O_{\text{P}} = 0.96 \times \left(0.75 \times \delta^{18}O_{\text{S}} + 0.25 \times \delta^{18}O_{\text{W}} + 0.25 \times \varepsilon \right) \\
+ 0.04 \times \delta^{18}O_{\text{CON}},
\]

with δ18OCON being the δ18O of the contaminant. Analysis of δ18OCON was not possible; however, δ18Op values under environmental conditions usually lie within the range of 15‰ (±5‰) (Tamburini et al., 2014). Assuming a δ18OCON value of 15‰ results in an average ε of 8.2‰ (±0.9‰) in assays with wheat phytase and in an average ε of 7.7‰ (±1.0‰) in assays with Aspergillus niger phytase. Taking into account a possible contamination, ε will change depending on the assumed δ18OCON value. An assumed δ18OCON value of 20‰ would result in an ε of 6.4‰ (±0.9‰) in the case of wheat phytase and in an ε of 6.9‰ (±1.0‰) in the case of Aspergillus niger phytase, while an assumed δ18OCON value of 10‰ would result in an ε of 9.9‰ (±0.9‰) in the case of wheat phytase and in an ε of 8.6‰ (±1.0‰) in the case of Aspergillus niger phytase.

These results provide an estimate of 6 to 10‰ for the oxygen isotopic fractionation during the release of P1 from IP6, i.e., the oxygen incorporated is enriched in 18O relative to the water it derived from. True inverse kinetic isotope fractionations are rare, and so far have not been observed for oxygen isotope effects in phosphorus cycling. It is unlikely that the apparent inverse isotope effect is caused by the contaminant, as δ18OCON would have to be +65‰ in experiments with wheat phytase and +117‰ in experiments with Aspergillus niger phytase to accommodate for a normal isotope effect (i.e., ε < 0‰). These high δ values are not realistic, and we therefore assume that there is another reason for the observed positive isotopic fractionation.

An inverse kinetic isotope effect can be caused by a hidden equilibrium isotope fractionation. Unlike kinetic isotope fractionation, equilibrium isotope fractionation is often strongly temperature dependent. The effect of temperature on the isotopic fractionation caused by phytases was tested at 4 and 37°C. In the case of wheat phytases, ε had a value of 4.9‰ (±1.0‰), and in the case of Aspergillus niger phytase, ε had a value of 8.0‰ (±0.9‰) at 4°C. The isotopic fractionation was not significantly different between the two temperatures (ANOVA; p value >0.05), mirroring the findings with phosphomonoesters (Liang and Blake, 2006, 2009; von Sperber et al., 2014). This indicates that a hidden
equilibrium isotope fractionation may not be the cause of the observed apparent inverse isotope fractionation.

4.3 Comparison of phytase to acid phosphatase oxygen isotope fractionation

Phytases can vary significantly in their catalytic properties and mechanisms. For example, depending on the optimal pH of catalysis, they can be either alkaline, neutral or acid phosphatases (Mullaney and Ullah, 2003). Most plant and fungal phytases belong to the histidine acid phosphatases, which share the same amino acid sequence motif (RHGXRXP) at their active sites as acid phosphatases and nucleotidases (van Etten et al., 1991; Oh et al. 2004; Kostrewa et al., 1997, 1999; Lim et al., 2000). The amino acid sequence motif at the active site of phosphatases drives the reaction mechanisms, which can lead to either the incorporation of an oxygen atom derived from a water molecule into the newly formed phosphate (Lindqvist et al., 1994; Knöfel and Strätter, 2001; Ortlund et al., 2004), e.g., acid phosphatases or nucleotidases, or to the incorporation of an oxygen atom derived from a hydroxide ion, e.g., alkaline phosphatases (Kim and Wickoff, 1991; Stec et al., 2000). It has been suggested that these two types of reaction mechanisms are the reason why different phosphomonoesterases cause different isotopic fractionations (von Sperber et al., 2014). Based on these findings it can be expected that the isotopic fractionation caused by phytases is similar to that of acid phosphatases and nucleotidases.

The action of wheat phytase led to an ε of −12.3‰ (±2.3‰) in the case of AMP and of −12.0‰ (±2.2‰) in the case of GPO4 (calculated according to Eqs. 6 and 7 with a δ18OCON value of 15‰; Table 3). These fractionations are similar to those reported for acid phosphatases from wheat germ and potato (approximately −10‰; von Sperber et al., 2014). Acid phosphatase from wheat germ hydrolyzed approximately 10% of IP6, while acid phosphatase of potato hydrolyzed approximately 40% of IP6. The δ18O of the myoinositol phosphate derivatives of these reactions were not analyzed. Using a value of 23.2‰ for δ18O of the myoinositol phosphate, the fractionation caused by abiotic photodecomposition would be lower than the actual δ18O of the three oxygen atoms cleaved from IP6 during enzymatic activity. This would result in an underestimate of δ18O which in turn would lead to a biased calculation of ε = i.e., the true value of ε could be smaller than 0‰ and thus be a normal isotope effect. We consider this issue to be a crucial aspect for the interpretation of the effect of phosphatases on the oxygen isotope composition of phosphate. Thereafter, if the C–O–P bridging oxygen atoms are depleted in 18O relative to the non-bridging oxygen atoms, the δ18O of P1 cleaved from IP6 and IP2 by abiotic photodecomposition would be lower than the actual δ18O of the three oxygen atoms cleaved from IP6 during enzymatic activity. It has been shown that some plants grown under P-limited conditions can exude phytases (Li et al., 1997a, b; Richardson et al., 2001; Lung and Lim, 2006). The measurements of these enzymatic activities in soils are usually conducted under pH-buffered and temperature-controlled conditions with artificial substrates, e.g., para-nitrophenyl phosphate – i.e., these measurements can only provide information on the potential enzymatic activity, and not on the actual activity. In the natural soil environment these conditions can vary substantially and rates of hydrolysis might be much lower. For example, in soils, phytic acid might undergo adsorp-
tion and/or precipitation reactions, prohibiting the diffusion of an IP₆ molecule into the active site of the enzyme (Anderson, 1980; McKercher and Anderson, 1989; Oginalaga et al., 1994). Similarly phytase can be rapidly sorbed onto soil particles (George et al., 2005). Moreover, the use of para-nitrophenyl phosphate as a substrate cannot distinguish between extracellular acid phosphatase activity and phytase activity. The isotopic imprint caused by phosphatases might be used to distinguish between the actual enzymatic processes occurring in situ. The effects of phosphomonoesterases and phosphodiesterases on the oxygen isotope composition of phosphate could be traced in alkaline Mediterranean soils (Gross and Angert, 2015). The enzymatic release of phosphate from added organic compounds led, on the one hand, to an increase in available Pᵢ concentration and, on the other hand, to a decrease in δ¹⁸O values of available Pᵢ (Gross and Angert, 2015). This decrease in δ¹⁸O values was attributed to the negative isotopic fractionation caused by alkaline phosphatases (Liang and Blake, 2006). Another recent study conducted on a 6500-year soil coastal dune chronosequence found that δ¹⁸O values of available Pᵢ were in isotopic equilibrium with soil water at younger sites and below isotopic equilibrium at older sites, with higher organic P contents. The low δ¹⁸O values at the older sites indicated higher mineralization rates of labile organic P compounds, in particular DNA, by extracellular phosphatases (Robert et al., 2015). The findings of our study are therefore of high value in future studies for the interpretation of δ¹⁸O values of available phosphate extracted from soils with high phytic acid contents.

5 Conclusions

The present study indicates that the isotopic fractionation caused by phytases from wheat and Aspergillus niger is similar compared to the fractionation reported for acid phosphatases from wheat germ and potato, and that there is no substantial difference between oxygen isotope fractionation by 6-phytases and 3-phytases. This observation is attributed to the similar reaction mechanisms of phytases and acid phosphatases. Temperature does not have an influence on the observed isotopic fractionations, which alleviates the interpretation of δ¹⁸O values of phosphate extracted from soils under natural conditions with large diurnal and seasonal temperature fluctuations. Furthermore, this study highlights the influence of the substrate on the calculated isotopic fractionation caused by phosphatases. Our results support the hypothesis that δ¹⁸O values of the bridging oxygen atom (C–O–P) and the non-bridging oxygen atoms (O–P) in phosphate molecules of organic P compounds are different. As the hydrolysis of different organic phosphorus substrates by different phosphatases can lead to very different isotopic signals, our findings highlight the potential of oxygen isotopes associated with phosphate as a tracer for enzymatic processes in the soil–plant system. Future research should focus on the substrate effect on δ¹⁸O values of phosphate during enzymatic hydrolysis. On the one hand, efforts should be directed to test whether the bridging oxygen atom (C–O–P) has a different δ¹⁸O values compared to the non-bridging oxygen atoms (O–P). On the other hand, it is important to test in the field whether the hydrolysis of different organic phosphate esters leads to different δ¹⁸O values of resin extractable Pᵢ.

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