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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 (IP6) is an important form of P_{org} in many soils. The enzymatic hydrolysis of IP6 by phytase yields plant available inorganic phosphate (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 IP6, adenosine 5′monophosphate (AMP) and glycerophosphate (GPO4) 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 IP6 as substrate were prepared.

During the hydrolysis of IP6 by phytase, four P_i are released, and one oxygen atom from water is incorporated into each P_i. This incorporation of oxygen from water into P_i is subject to an apparent inverse isotopic fractionation ($\varepsilon \sim 6$ to 10 ‰), which is similar to that imparted by acid phosphatase from potato during the hydrolysis of IP6 ($\varepsilon \sim 7$ ‰) where less than three P_i are released. The incorporation of oxygen from water into P_i during the hydrolysis of AMP and GPO4 by phytase yielded a normal isotopic fractionation ($\varepsilon \sim -12$ ‰), again similar to values reported for acid phosphatases from potato and wheat germ. We attribute this similarity in ε 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 (IP6) is synthesized by plants to store phosphorus (P), inositol and nutritionally important minerals (Cosgrove and Irving, 1980). In soils, IP6 can accumulate to become the dominant form of organic phosphorus. Under P-limiting conditions, some plants and microorganisms exude phytases, which catalyze the hydrolysis of phosphomonoester bonds in IP6 leading to the release of available inorganic phosphate (P_i) (Hayes et al., 1999; Richardson et al., 2000, 2001; Lung and Lim, 2006). However, in terrestrial ecosystems the cycling and bioavailability of phytic acid are still poorly understood (Turner et al., 2002).

Recent studies have used the oxygen isotope composition ($\delta^{18}O$) of inorganic phosphate in terrestrial environments, e.g. in soils (Tamburini et al., 2010, 2012; Angert et al., 2011, 2012), in plants (Young et al., 2009; Pfahler et al., 2013) and in aerosols (Gross et al., 2013) as a tracer for its cycling. Under ambient conditions and in absence of biological activity, the $\delta^{18}O$ of phosphate does not change (O'Neil et al., 2003). However, the activity of intracellular and extracellular enzymes can lead to a substantial alteration of the $\delta^{18}O$ of enzymatically released P_i. Pyrophosphatases catalyze a complete oxygen exchange between P_i and water leading to an equilibrium isotope fractionation (Cohn, 1958; Longinelli and Nuti, 1973; Blake et al., 2005). Phosphomonoesterases and phosphodiesterases hydrolyze organic P-compounds leading to the incorporation of one or two oxygen atoms from water into released P_i (Cohn, 1949; Liang and Blake, 2006, 2009). 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, von Sperber et al., 2014), phosphodiesterases and nucleotidases (Liang and Blake, 2009), and acid phosphatases (von Sperber et al., 2014). To date, the effect of phytases on the $\delta^{18}O$ of 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 hydrolyzes 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, EC 3.1.3.26, which are typical for plants, first hydrolyze the 6-position of IP6 forming IP5 (*myo*-inositol 1,2,3,4,5-pentakisphosphate) and free inorganic phosphate (Wodzinski and Ullah, 1996; Dvorakova et al., 1998) (Fig. 1). The aim of this study was to investigate the effect of a phytase from wheat, which belongs to the 6-phytases, and a phytase from *Aspergillus niger*, which belongs to the 3-phytases, on the $\delta^{18}O$ values of released P_i.

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 P_i. Assays consisted of 200 mM acetate buffer (pH 5.5), with either 2 mM of phytic acid (Sigma Aldrich P8810), or 7 mM of glycerophosphate (Sigma Aldrich G6501) or 7 mM adenosine 5′monophosphate (Sigma Aldrich A1752) as substrate and with 0.5 UN of phytase (1 UN is defined as activity required to convert 1 µ mole of substrate per minute).

Assays with acid phosphatases from potato and wheat germ consisted of 200 mM acetate buffer (pH 4.8), 3 mM of 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 10000, using a SnakeSkin dialysis tubing 10 K MWCO 16 mm (Thermo Scientific, SnakeSkin, PI88243). All assay reagents were prepared in ^{18}O-labeled and non-labeled double deionized water (dd-H_2O) and filter-sterilized. Batch assays had a volume of 3 mL and were prepared in 15 mL centrifuge tubes. Directly after the addition of the reagents, the tubes were closed and only opened for sampling. The concentra-

tion of released P_i in the assays was monitored using the colorimetric malachite green method (Ohno and Zibilske, 1991). After 48 h P_i yield was usually close to 65 % and did not change any more, despite the enzyme being still active. 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 $\delta^{18}O$ 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 P_i was purified according to the protocol of Tamburini et al. (2010). In brief, P_i is first precipitated as struvite (NH_4MgPO_4), which can be retrieved by filtration and subsequently re-dissolved, purified and precipitated as silver phosphate (Ag_3PO_4).

2.2 Ultra violet radiation (UVR) digestion

The $\delta^{18}O$ of IP6 and of the filtrate after the precipitation of struvite were analyzed using UVR digestion. IP6 and the filtrate were transferred in a solution with 20 mL of ^{18}O labeled and unlabeled dd-H_2O and 3 mL 28 % H_2O_2 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 PO_4 moiety from the organic P-compound without any incorporation of oxygen from water (Liang and Blake, 2006). UVR-released P_i was then processed following the protocol of Tamburini et al. (2010). The $\delta^{18}O$ of phosphate from the organic P-compound ($\delta^{18}O_S$) was calculated according to the modified equation from McLaughlin et al. (2006b):

$$\delta^{18}O_S = \frac{\left(\delta^{18}O^{***}_{P\text{-}UVR} \times \delta^{18}O_W\right) - \left(\delta^{18}O_{P\text{-}UVR} \times \delta^{18}O^{***}_W\right)}{\left(\delta^{18}O^{***}_{P\text{-}UVR} - \delta^{18}O_{P\text{-}UVR}\right) - \left(\delta^{18}O^{***}_W - \delta^{18}O_W\right)} \quad (1)$$

with $\delta^{18}O^{***}_W$ and $\delta^{18}O_W$ being the $\delta^{18}O$ of labeled and unlabeled water and $\delta^{18}O_{P\text{-}UVR}$ being the $\delta^{18}O$ of UVR-released phosphate in water which was labeled (***) or nonlabeled. The fraction of oxygen which exchanged with water during UVR digestion (F_{exch}) can be calculated according to:

$$F_{exch} = \frac{\delta^{18}O^{***}_{P\text{-}UVR} - \delta^{18}O^{***}_W}{\delta^{18}O_S - \delta^{18}O^{***}_W} \quad (2)$$

2.3 Determination of $\delta^{18}O$ values of phosphate and water

Oxygen isotope analysis of Ag_3PO_4 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 Ag_3PO_4 took place at 1450 °C in a carbon-based reactor. A temperature controlled purge and trap chromatography system was used to separate CO from N_2. Results were calibrated against an internal Ag_3PO_4 standard (Acros Organics, Geel, Belgium; $\delta^{18}O$ = 14.2 ‰ Vienna Standard Mean Ocean Water (VSMOW)) and two benzoic acid standards distributed by the International Atomic Energy Agency (IAEA) (IAEA 601: $\delta^{18}O$ = 23.1 ‰ and IAEA 602: $\delta^{18}O$ = 71.3 ‰ VSMOW). Analytical error calculated on replicate analysis of standards was better than ±0.4 ‰.

For oxygen isotopes analysis of water, a 0.3 % CO_2 and He mixture was equilibrated for 18 h at 25 °C with the samples in airtight exetainers. Aliquots of the CO_2/He mixture from the headspace were sampled and transferred to a Delta V Plus mass spectrometer (Thermo Fisher Scientific Inc.) using a gas bench (Gas Bench II, Thermo Scientific Inc.). The oxygen isotope composition of water was derived from the isotope analysis of CO_2. The system was calibrated with the international standards VSMOW, Standard Light Antarctic Precipitation (SLAP), and Greenland Ice Sheet Precipitation (GISP), distributed by the IAEA. Reproducibility of the measurements was better than ±0.06 ‰. Oxygen isotope compositions are reported in the conventional delta notation (δ(‰) = $(R_x/R_s - 1)\cdot 1000$; where R denotes the ratio of the heavy to light isotope and R_x and R_s 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 fractionations caused by different phytases and substrates. After rejecting the null hypothesis of the ANOVA, isotopic fractionations were compared with Tukey's HSD tests.

3 Results

3.1 Incorporation of oxygen from water into P_i during UVR digestion

The $\delta^{18}O$ of P_i produced during UVR digestion of IP6 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_i (Table 1). The $\delta^{18}O$ of P_i produced during UVR digestion of IP2 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_i (Table 1). These findings confirm that the UVR-induced release of the original PO_4 moiety from the organic P-compound proceeded with little incorporation of oxygen from water.

3.2 Incorporation of oxygen from water into P_i during hydrolysis of IP6 by phytases

Purified phytase from wheat and *Aspergillus niger* hydrolyzed approximately 65 % of the phosphate molecules bound to IP6. This indicates that the original substrate IP6 molecule was degraded to inositol-biphosphate (IP2) and 4 P_i molecules ($4 \cdot P_i / 6 \cdot P_i$ = 66.6 %).

Control experiments with crude protein extract from wheat phytase without any substrate revealed a substantial contamination of approximately 20 µmol of P_i/UN protein extract. In order to remove this contamination, crude protein extracts were dialysed.

Mean $\delta^{18}O$ values of released P_i ($\delta^{18}O_P$) from assays with both, non-purified and purified protein, at different $\delta^{18}O$ values of water ($\delta^{18}O_W$) are shown in Fig. 2 and Table 2. Mean $\delta^{18}O_P$ values from assays with non-purified wheat phytase ranged from 6.3 to 33.9 ‰ and linear regression of mean $\delta^{18}O_P$ values against mean $\delta^{18}O_W$ values resulted in a slope of 0.17. Mean $\delta^{18}O_P$ values from assays with purified wheat phytase ranged from 6.5 to 31.0 ‰. Mean $\delta^{18}O_P$ values from assays with purified *Aspergillus niger* phytase ranged from 1.4 to 37.7 ‰. Linear regression of mean $\delta^{18}O_P$ values against mean $\delta^{18}O_W$ values from the assays with purified phytases resulted in 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.

3.3 Oxygen isotope composition of P_i released after hydrolysis of AMP and GPO4 by phytase and after hydrolysis of IP6 by acid phosphatase

Phytases can hydrolyze single phosphomonoester substrates and some acid phosphatases can partly hydrolyze IP6 (Anaheim et al., 2013). For this reason, the effect of wheat phytase on adenosine 5′-monophosphate (AMP) and on glycerophosphate (GPO4), which have been used in a previous study (von Sperber et al., 2014) was tested. Wheat phytase hydrolyzed approximately 72 % AMP and approximately 80 % of GPO4. Experiments with AMP as substrate ($\delta^{18}O_S$ = 15.8 ‰) which were carried out in assays with a $\delta^{18}O_W$ of −45.5 ‰ resulted in mean a $\delta^{18}O_P$ of −1.9 ‰. Experiments with GPO4 as substrate ($\delta^{18}O_S$ = 16.6 ‰) which were carried out in assays with a $\delta^{18}O_W$ of −50.4 ‰ resulted in mean a $\delta^{18}O_P$ of −2.4 ‰ (Table 3).

In addition, two acid phosphatases from potato and wheat germ with IP6 as substrate were tested. Acid phosphatase from wheat germ hydrolyzed approximately 10 % of IP6 and acid phosphatase of potato hydrolyzed approximately 40 % of IP6. Experiments with acid phosphatase from wheat germ were carried out in assays with a $\delta^{18}O_W$ of −58.5 ‰ and resulted in mean a $\delta^{18}O_P$ of 3.0 ‰. Experiments with acid phosphatase

from potato were carried out in assays with a $\delta^{18}O_W$ of −9.8 ‰ and resulted in mean a $\delta^{18}O_P$ of 16.7 ‰ (Table 3).

4 Discussion

4.1 Implications of incorporation of oxygen from water into P_i during hydrolysis of IP6 by phytases

The slopes from assays with purified phytases are close to 0.25, similar to experiments conducted with phosphomonoesterases like alkaline and acid phosphatases (Liang and Blake, 2006; von Sperber et al., 2014). The slight deviation from a slope of 0.25 for the purified phytases 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 and that the back-reaction of the enzymatic hydrolysis (the reformation of more phosphorylated inositols, e.g. IP3 or IP4) is too slow to have an effect on the oxygen isotope composition of released P_i during 48 h of reaction time. Otherwise, a higher slope would be observed, because reformed phosphoesterbonds would be, at least partly, hydrolyzed again leading to the incorporation of another oxygen from water into released P_i. From this observation follows that the enzymatic release of P_i from IP6 proceeds by cleaving the P-O bond of the oxygen connected to 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 (ε) caused by phytases (Liang and Blake, 2006):

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

where $\delta^{18}O_P$ is the δ-value of released P_i, $\delta^{18}O_S$ is the δ-value of the substrate (meaning the average value of the 4 phosphate released from IP6), $\delta^{18}O_W$ is the δ-value of the water and ε 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 phosphomonoesters, such as glycerophosphate or adenosine 5′-monophosphate, phytic acid consists in total of six phosphate molecules, of 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 inositol is not possible. However, the bulk isotope composition of the phosphate moieties from IP6 and IP2 can be determined, allowing for the calculation of $\delta^{18}O_S$. Our results indicate that the original substrate IP6 molecule was degraded to IP2 and 4 P_i molecules with $\delta^{18}O_S$ corresponding to the $\delta^{18}O$ of the 65 % of phosphate molecules that were cleaved from IP6. By using a simple mass balance, $\delta^{18}O_S$ and can be derived indirectly by from $\delta^{18}O$ of IP6 ($\delta^{18}O_{IP6}$) and IP2 ($\delta^{18}O_{IP2}$) as follows:

$$\delta^{18}O_{IP6} = 2/3 \times \delta^{18}O_S + 1/3 \times \delta^{18}O_{IP2} \quad (4)$$

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

$$\delta^{18}O_S = 3/2 \times \delta^{18}O_{IP6} - 1/2 \times \delta^{18}O_{IP2} \quad (5)$$

The $\delta^{18}O_{IP2}$ value was determined by oxidation of the filtrate, which consists of IP2, with UV radiation after the struvite precipitation step. Digestion of the organic P-compounds by UVR led to the release of P_i with a $\delta^{18}O_{IP6}$ value of 22.8‰ (±0.4‰) and a $\delta^{18}O_{IP2}$ value of 22.0‰ (±0.4‰) (Table 1). Using these values in Eq. (5) we calculate a $\delta^{18}O_S$ value of 23.2‰ (±0.7‰). Solving Eq. (3) with the obtained $\delta^{18}O_S$ 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 $\delta^{18}O_P$ vs. $\delta^{18}O_W$ 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 P_i that was not derived from IP6. These small contaminations are the reason for the linear relationship between $\delta^{18}O_W$ values and ε (Table 2). In 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 are 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 follows:

$$\delta^{18}O_P = 0.92 \times (0.75 \times \delta^{18}O_S + 0.25 \times \delta^{18}O_W + 0.25 \times \varepsilon) + 0.08 \times \delta^{18}O_{CON} \quad (6)$$

and for experiments with *Aspergillus niger* phytase as follows:

$$\delta^{18}O_P = 0.96 \times (0.75 \times \delta^{18}O_S + 0.25 \times \delta^{18}O_W + 0.25 \times \varepsilon) + 0.04 \times \delta^{18}O_{CON} \quad (7)$$

with $\delta^{18}O_{CON}$ being the $\delta^{18}O$ of the contaminant. Analysis of $\delta^{18}O_{CON}$ was not possible, however, $\delta^{18}O_P$ values under environmental conditions usually lie within the range

of 15‰ (± 5‰) (Tamburini et al., 2014). Assuming a $\delta^{18}O_{CON}$ 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 $\delta^{18}O_{CON}$ value. An assumed $\delta^{18}O_{CON}$ value of 20‰ would result in an ε of 6.4‰ (±0.9‰) in case of wheat phytase and in an ε of 6.9‰ (±1.0‰) in case of *Aspergillus niger* phytase, while an assumed $\delta^{18}O_{CON}$ value of 10‰ would results in an ε of 9.9‰ (±0.9‰) in case of wheat phytase and in an ε of 8.6‰ (±1.0‰) in case of *Aspergillus niger* phytase.

These results provide an estimate of 6 to 10‰ for the oxygen isotopic fractionation during the release of P_i from IP6, i.e. the oxygen incorporated is enriched in ^{18}O relative to the water it derived from. True inverse kinetic isotope fractionation are rather rare, and so far have not been observed for oxygen isotope effects in phosphorous cycling. It is unlikely that the apparent inverse isotope effect is caused by the contaminant, as $\delta^{18}O_{CON}$ 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. $\varepsilon < 0$‰). These high δ values are not realistic and we therefore assume that there is another reason for the observed positive the isotopic fractionation.

Apparently inverse kinetic isotope effects can be caused by 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 phosphomonoesterases (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 optimum pH of catalysis, they can either be alkaline, neutral or acid phosphatases (Mullaney and Ullah, 2003). Phytases from plants and fungi, as used in the experiments, usually belong to the histidine acid phosphatases (van Etten et al., 1991; Oh et al., 2004). The reported reaction mechanism of these phytases is very similar compared to the reported reaction mechanism of acid phosphatases. All of these enzymes share the same amino acid sequence motif (RHGXRXP) at their active sites (Kostrewa et al., 1997, 1999; Lim et al., 2000). It can therefore be expected that the isotopic fractionation caused by these phytases is similar to that of acid phosphatases.

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 ‰) (calculated according to Eqs. (6) and (7) with a $\delta^{18}O_{CON}$ 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 $\delta^{18}O$ of the inositol phosphate derivate of these reactions were not analyzed. Using a value of 23.2 ‰ for $\delta^{18}O_S$, obtained from the phytase experiment, resulted in an ε of −0.9 ‰ (±0.6) in the case of acid phosphatase from wheat germ and an ε of 7.2 ‰ (±2.9) in the case of acid phosphatase from potato (Table 3). The isotopic fractionation caused by potato acid phosphatase is similar to those caused by the two phytases used in this study. The isotopic fractionation caused by wheat germ acid phosphatase differs by approximately 8 ‰ compared to fractionation caused by phytases. The activity of wheat germ acid phosphatase with IP6 as substrate was very low, indicating that this enzyme was only able to cleave one phosphate moiety from IP6. One possibility to explain this observation is that the $\delta^{18}O_S$ value of this single moiety of IP6 is lower than 23.2 ‰. The determination of the $\delta^{18}O_S$ values of the single phosphate molecules is a challenge

beyond the scope of this study and which need to be addressed in future. The observation of generally strong similarities in the oxygen isotope fractionation between phytases and acid phosphatases supports the hypothesis that the reaction mechanisms catalyzed by these enzymes are similar.

4.4 Apparent substrate dependency of oxygen isotope fractionation

The isotopic fractionation caused by phytases and acid phosphatases differ systematically with varying substrates, and encompass a range from inverse (relative enrichment in ^{18}O, positive ε) to normal (relative depletion in ^{18}O, negative ε) isotope effects. At first sight, this striking substrate-dependency of the isotopic fractionation implies a substrate-dependent mode of function of hydrolysis that may involve a multi-step process, with competing inverse and normal isotope effects. While such a scenario is not impossible, there may be a more straightforward explanation for this phenomenon. We hypothesize that there may be a difference in the $\delta^{18}O$ of the bridging oxygen atom (C-O-P) and the three non-bridging oxygen atoms (O-P) in organic phosphate compounds. If the C-O-P bridging oxygen atoms are depleted in ^{18}O relative to the non-bridging oxygen atoms, the $\delta^{18}O$ of P_i cleaved from IP6 and IP2 by abiotic photodecomposition would be lower than the actual $\delta^{18}O$ of the three oxygen atoms cleaved from IP6 during enzymatic activity. This would result in an underestimate of $\delta^{18}O_S$ 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. We have not yet found a way to verify this hypothesis, which would be a highly interesting task for future research.

5 Conclusions

The present study indicates that the isotopic fractionation caused by phytases from wheat and from *Aspergillus niger* is the same 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. This study highlights the influence of the substrate on the calculated isotopic fractionation caused by phosphatases. Our results support the hypothesis that $\delta^{18}O$ 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. Verification of this hypothesis is a challenge which needs to be addressed in future studies.

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Table 1. Results from UVR digestion of organic P-compounds. The table shows measured $\delta^{18}O$-values of ^{18}O labeled water ($\delta^{18}O_W^{***}$) and non-labelled ($\delta^{18}O_W$) water, as well as $\delta^{18}O$-values of UVR-released phosphate in assays with ^{18}O labeled water ($\delta^{18}O_{IPx}^{***}$) and non-labelled water ($\delta^{18}O_{IPx}$). The $\delta^{18}O_S$ value was calculated according to Eq. (1). Exchanged F_{exch} is the fraction of oxygen atoms which exchanged with water calculated with Eq. (2).

Substrate	$\delta^{18}O_W$	$\delta^{18}O_W^{***}$	$\delta^{18}O_{P\text{-}UVR}$	$\delta^{18}O_{P\text{-}UVR}^{***}$	exch. F	$\delta^{18}O_{IPx}$	SD
IP6	−9.8	51.2	21.0	24.4	0.06	22.8	0.4
IP2 (filtrate)	−10.4	73.3	21.7	22.4	0.01	22.0	0.4

Table 2. $\delta^{18}O$-values (‰) of water ($\delta^{18}O_W$), released phosphate ($\delta^{18}O_P$) and phosphate in organic P-compound ($\delta^{18}O_S$) as well as isotopic fractionation (ε), which was calculated according to Eqs. (6) and (7) with an assumed $\delta^{18}O_{CON}$ value of 15 ‰. Results are from experiments with IP6 as substrate and with phytases from wheat and *Aspergillus niger*.

Enzyme	Substrate	°C	n	$\delta^{18}O_W$	$\delta^{18}O_P$	$\delta^{18}O_S$	ε	mean ε	SD
Phytase wheat (crude)	IP6	37	6	−67	6.3	23.2	NA	NA	NA
	IP6	37	3	−10	18	23.2	NA		
	IP6	37	6	42.3	24.1	23.2	NA		
	IP6	37	6	94.2	33.9	23.2	NA		
Phytase wheat (dialysed)	IP6	37	6	−53.7	6.5	23.2	7.0	8.2	0.9
	IP6	37	5	−10.4	16.7	23.2	8.0		
	IP6	37	6	29.0	25.9	23.2	8.7		
	IP6	37	6	51.0	31.0	23.2	9.0		
	IP6	4	6	−10.6	16.3	23.2	6.5	6.5	1.0
Phytase *A. niger* (dialysed)	IP6	37	2	−75.4	1.4	23.2	9.2	7.7	1.0
	IP6	37	3	−10.4	16.5	23.2	7.1		
	IP6	37	3	48.4	30.6	23.2	6.9		
	IP6	37	3	77.2	37.7	23.2	7.8		
	IP6	4	3	−10.6	17.1	23.2	9.5	9.5	0.9

Table 3. $\delta^{18}O$-values of water ($\delta^{18}O_W$), released phosphate ($\delta^{18}O_P$) and phosphate in organic P-compound ($\delta^{18}O_S$) as well as isotopic fractionation (ε), which was calculated according to Eqs. (6) and (7) with an assumed $\delta^{18}O_{CON}$ 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.

Enzyme	Substrate	°C	n	$\delta^{18}O_W$	$\delta^{18}O_P$	$\delta^{18}O_S$	ε	SD
Phytase wheat	IP6	37	5	−10.4	16.7	23.2	7.7	1.2
	AMP	37	3	−45.5	−1.9	15.8	−12.3	2.3
	GPO4	37	3	−50.4	−2.4	16.6	−12.0	2.2
Apase wheat germ	IP6	37	3	−58.5	3.0	23.2	−0.9	0.6
	AMP*	37	9	−10.0	7.1	15.8	−8.9	1.7
	GPO4*	37	3	−10.0	7.2	16.6	−11.0	1.3
Apase potato	IP6	37	2	−9.8	16.7	23.2	7.2	2.9
	AMP*	37	10	−10.0	7.3	15.8	−8.1	1.0
	GPO4*	37	6	−10.0	7.0	16.6	−11.8	1.3

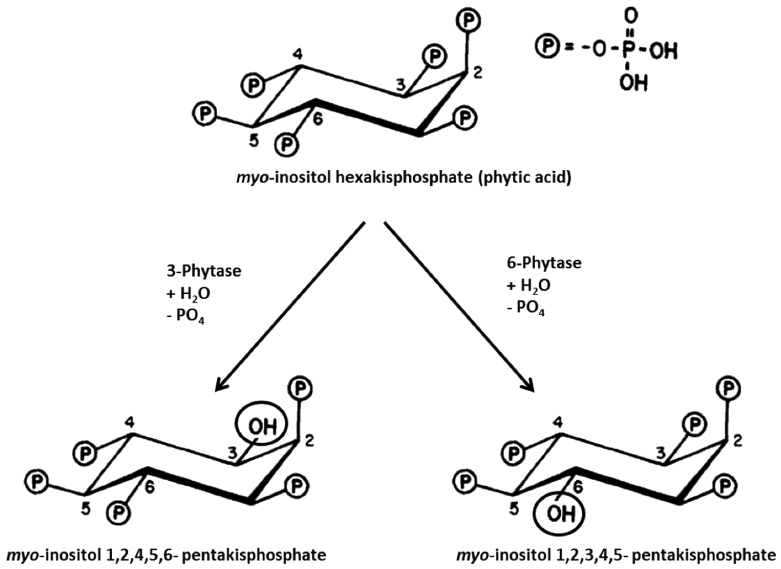

Figure 1. Phytic acid (IP6) degradation to IP5 by 3-phytases and 6-phytases (modified from Dvřákov, 1998). 3-phytases first hydrolyze the ester bond at the 3-position of IP6 (*myo*-inositol hexakisphosphate), which leads to the formation of IP5 (*myo*-inositol 1,2,4,5,6-pentakisphosphate) and free inorganic 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.

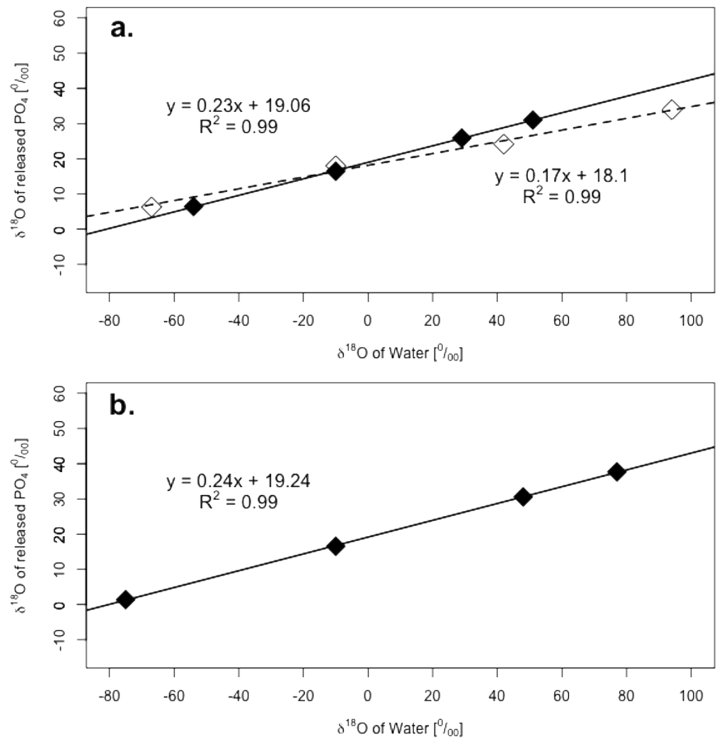

Figure 2. Mean $\delta^{18}O$ values of released P_i ($\delta^{18}O_P$) at different $\delta^{18}O$ values of water ($\delta^{18}O_W$) from **(a)** assays with non-purified wheat phytase (dashed line) and purified wheat phytase (solid line), **(b)** an assay with purified *Aspergillus niger* phytase.