Title
Zinc and iron speciation in the cereal grain

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Introduction

Iron (Fe) and zinc (Zn) deficiencies are very widespread nutritional disorder in the world, being most prevalent in developing countries where cereals constitute the major part of the diet (Welch and Graham, 1999). One reason is that the concentration of Fe and Zn are relatively low in the parts of the grain which is eaten, i.e. the endosperm, while more nutrient dense external layers are polished away. In addition, the bioavailability of Fe and Zn in cereals grains is generally low and believed to be controlled by phytic acid (myo-inositol-1,2,3,4,5,6-hexakisphosphate; IP_6), which is the main storage form of phosphorus (P) in cereals (Raboy, 2003). IP_6 as well as some of the less phosphorylated forms (IP_{1-5}) have a high affinity for various cations including Fe, Zn, Mn, Ni, Cd, Cu, Mg, Ca, Na and K (Torres et al., 2005), forming both water soluble and insoluble chemical species. Imaging techniques such as Secondary Ion Mass Spectrometry (SIMS) and Energy Dispersive X-ray analysis (EDX) have in combination with Electron Microscopy been used to confirm that electron-dense IP_6 molecules co-localize with a range of cations and proteins in globoids located in the protein storage vacuoles of the aleurone layer and the embryo (Mills et al., 2005). The co-localization of Fe, Zn and IP_6 in the embryo and aleurone tissues has been used to postulate that these cations are speciated with IP_6, but there is no evidence available to demonstrate that such species actually exists *in planta*. Therefore, we have analysed the molecular speciation of Fe and Zn in the cereal grain, using e.g. SEC-ICP-MS. Especially, we have focused on the interactions between Fe and Zn and their speciation with P and S, the latter two elements being major constituents of IP_6 and proteins, respectively, potentially involved in Fe and Zn binding.

Elemental concentrations in tissue fractions of barley seeds

The embryo was the most elemental dense tissue fraction in barley grains and especially for Zn and P the concentrations were 4-to-5-fold higher than those in the whole grain. The embryo was the only compartment where the Zn concentration exceeded that of Fe (Table 1). The dry weight of the embryo was only 4% of the total grain weight, but contributed to approximately 23% of the total Zn content. On the contrary, the endosperm contributed only with approximately 33% of the total Zn content even though it accounted for 68% of the total dry weight.

Table 1. Weight and elemental concentrations of whole barley grain and its main tissue fractions (values are means ± std, n=7). Each tissue type was weighed and analysed separately on ICP-OES. The recovery shows how much of the total (weight or amount of element) that was accounted for.

Tissue	Dry weight, mg	Zn, $\mu g\ g^{-1}$	Fe, $\mu g\ g^{-1}$	P, $mg\ g^{-1}$	S, $mg\ g^{-1}$
Whole grain	43 ± 3	30 ± 1	50 ± 3	3.74 ± 0.01	1.47 ± 0.02
Awns	2.4 ± 0.1	15 ± 0.6	67 ± 0.3	1.33 ± 0.07	0.49 ± 0.03
Bran layers	9.5 ± 0.3	47 ± 1	128 ± 6	6.71 ± 0.09	1.74 ± 0.01
Embryo	1.8 ± 0.1	164 ± 4	80 ± 3	13.6 ± 0.17	3.99 ± 0.12
Endosperm	29 ± 0.5	14 ± 1	25 ± 1	1.64 ± 0.08	1.16 ± 0.06
Recovery (%)	98	91	105	87	93

SEC-ICP-MS speciation analyses of barley embryos

Simultaneous analysis of Fe, Zn, P and S with a quadrupole-based ICP-MS system is an analytical challenge when analysing specific tissue fractions of which a limited quantity (low mg level) is available. In addition, $^{40}Ar^{16}O^{+}$ and $^{16}O_2^{+}$ polyatomic interferences on the most abundant ^{56}Fe and ^{32}S isotopes, respectively, imply that standard mode ICP-MS analysis is dependent on the detection of the much less abundant ^{57}Fe and ^{34}S isotopes, which severely reduces the sensitivity. The negative impacts of Ar-based interferences are typically reduced using an ICP-MS equipped with a reaction cell system. Pressurizing the ICP-MS reaction cell system with H_2 leads to a specific and efficient removal of $^{40}Ar^{16}O$ interferences on ^{56}Fe, enabling analysis of ^{56}Fe in the low ng L^{-1} concentration range. However, H_2 addition is detrimental to the ion transmissions of S, P and Zn. We developed a new approach involving addition of O_2 to the octopole reaction system. This enabled satisfactory signal intensities of S when measured as $^{48}SO^{+}$, Fe as $^{72}FeO^{+}$, P as $^{47}PO^{+}$ and Zn as $^{66}Zn^{+}$. The signal-to-noise ratios and LODs were markedly improved for Fe and S. The ion transmission of $^{66}Zn^{+}$ in O_2 mode decreased with 15%, but the background signal also decreased, which resulted in a slightly improved signal-to-noise ratio.

Using the oxygen mode, P, S, Fe and Zn in TRIS buffer extracts of barley embryos were analysed in one single chromatographic run (Fig. 1). Fe overlapped with P as a major (12.3 kDa) and minor peak (0.5 kDa), whereas Zn eluted in one single peak (3.0 kDa) between both the Fe and P signals. Sulphur appeared as two distinct peaks of which the first marginally co-eluted with the first eluting Fe/P signal. The second S peak perfectly co-eluted with the Zn signal at 864 s, indicating the presence of a peptide ligand. Thus, the chemical speciation of Zn and Fe differed. Fe mainly existed as an 12.3 kDa Fe:IP6 oligomer, whereas Zn was mainly speciated with proteins or peptides in a species with the apparent mass of 3.0 kDa.

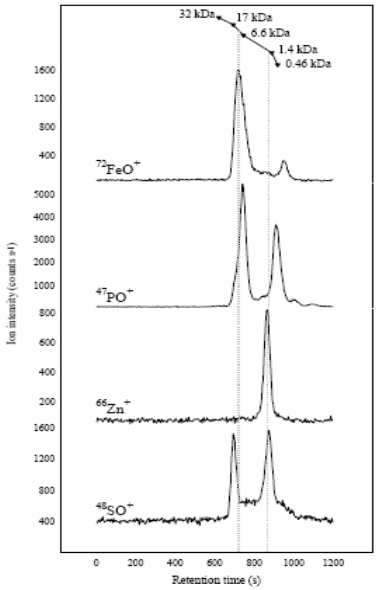

Fig. 1 SEC-ICP-MS speciation of Fe, P, Zn and S in an embryo sample, run in O_2 mode. The column was mass-calibrated with reference compounds as indicated at the top of figure.

Incubation of the embryo extractions incubated with phytase, the enzyme responsible for successive dephosphorylation of IP_6 in a stepwise and ordered manner (Lei and Porres, 2003), was injected on the SEC-column at times 0, 2, 4 and 8 h. The 12.3 kDa P-peak was gradually decreasing in size during incubation with the phytase enzyme (Fig. 2). At the same time, the 0.5 kDa P peak increased equivalently. The shift from the 12.3 kDa to the 0.5 kDa peak clearly showed that the apparent IP_6 oligomer was successively degraded into lower IPs (IP_{1-5}). Also the co-eluting Fe peak of the 12.3 kDa oligomer eluted later in the chromatogram (data not shown), confirming that Fe was indeed speciated with IP_6 or with a mixture of different IPs.

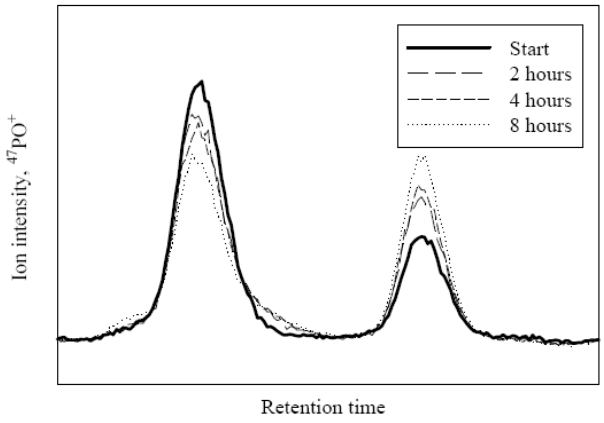

Fig. 2 Incubation of the extracted embryo sample using the IP_6 degrading enzyme phytase. The $^{47}PO^+$ signal at time zero is identical to the $^{47}PO^+$ signal in Fig. 3.

In order to identify the particular IPs involved in forming an oligomer with Fe, ion pairing chromatography was implemented in hyphenation to O_2 mode ICP-MS. The Fe/P peaks from the SEC chromatography (Fig. 1) were collected and re-injected on this system, where a standard containing IP_1-IP_6 had been identified. The largest Fe/P peak from the SEC chromatogram (peak 1) consisted mainly of IP_6 molecules (data not shown), and the smallest Fe/P peak (peak 2) was dominated by IP_1 and orthophosphate PO_4^{-3}. In the largest Fe/P peak there were also small amounts of IP_5, IP_4 and IP_1 present.

Since the molecular weight of IP_6 is only 660 Da, the 12.3 kDa peak may represent an oligomer of many IP_6 molecules, stabilized by hydrogen bonding and ionic bonding with cations, including Fe. This hypothesis was tested by analysing the sample under the same conditions but with pH lowered to 2, which destabilizes the strong tendency of IP_6 monomers to form hydrogen and ionic bonds in an IP_6 oligomer. Indeed, the low pH delayed the elution of the P peak with approximately 120 s, now eluting as a 0.7 ± 0.3 Da compound (data not shown), which fits well with the molecular size of one single IP_6 molecule.

The ratio between P and Fe in the 12.3 kDa Fe:IP_6 oligomer was quantified using external calibration of $^{47}PO^+$ and $^{72}FeO^+$. The ratio was found to be 27.7 ± 0.4 (n=3) P atoms per Fe atom. Since each IP_6 molecule contains 6 P atoms, the IP_6:Fe ratio would be 4.6. With the apparent mass of 12.3 kDa, these data indicate the following stoichiometry:

$Fe_4(IP_6)_{18}$ (molecular mass: 12.1 kDa). However, it should be noted that traces of several cations were found in the P-containing fractions and that the existence of pure Fe-P species *in planta* is rather unlikely.

Compared to the TRIS extraction, the extraction efficiency in the presence of phytase was doubled for Fe but was unchanged for Zn (data not shown). Also P was more than doubled whereas S was not affected. Conversely, incubation of embryo extracts with protease XIV caused the extraction efficiency of Zn to increase 5-fold compared to TRIS buffer extraction whereas the extraction efficiency of Fe was largely unaffected. At the same time, the amount of extracted S almost doubled compared to that in pure TRIS buffer and increased to 96% of the total S, whereas the extraction of P did not increase at all. The fact that phytase increased the extraction efficiency of Fe and P, but not that of Zn and S, and that protease XIV increased the extraction efficiency of Zn and S but not that of Fe and P, strongly support the results from the speciation analysis, showing that Fe was speciated with IP_6 in contrast to Zn, which was speciated with peptides

SEC-ICP-MS speciation analyses of rice endosperm

We are currently investigating the Fe and Zn speciation in the endosperm of rice seeds. This grain fraction is the most important grain fraction with respect to human nutrition. Preliminary data show similar Fe and Zn speciation as in the barley embryo, i.e. Zn appearing mainly bound to peptides, while Fe is mainly associated with phytic acid.

Conclusions

Simultaneous SEC-ICP-MS speciation analysis of S, P, Fe and Zn in cereal grain tissues was possible with a method based on the analysis of $^{66}Zn^+$ and the oxide product ions $^{48}SO^+$, $^{47}PO^+$ and $^{72}FeO^+$. Fe mainly co-eluted with P as a 12.3 kDa Fe:IP_6 oligomer, while Zn co-eluted only with S, indicating a peptide ligand. Incubation with phytase strongly increased the extraction efficiency of Fe and P from the embryo, but not that of Zn. In contrast, Zn and S extractability were strongly increased following incubation with protease. These data suggest that the well-established dogma stating that Zn and Fe share the same speciation and that their binding and bio-availability in the cereal grain are controlled by IP_6 is questionable.

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