Zinc and iron speciation in the cereal grain

by:
Jan K. Schjoerring
Daniel P. Persson
Thomas H. Hansen
Kristian H. Laursen
and Søren Husted

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Objectives

• To quantify how Zn and Fe are distributed in the cereal grain
• To identify the dominating ligands in cereal tissue fractions
Background

- Zn and Fe speciation is largely unknown.
- The dominating ligand is considered to be the P-rich compound IP6 (the phytate dogma).
- Proteins/peptides may also be important, e.g. ferritin, metallothioneins or nicotianamine with higher bioavailability.
- P and S are indicators of the ligand type.

PO$_2^-$  CN$^-$  C$_2^-$

Myo-inositol (1,2,3,4,5,6 hexakis-phosphate Phytic acid, IP$_6$)

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Quantification of Zn, Fe, P and S distribution in rice grain

Hansen et al. 2009 Plant Methods 5, 12

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Quantification of Zn, Fe, P and S distribution in rice grain

Zn distribution

- Bran: 34%
- Endosperm: 57%
- Embryo: 9%

Fe distribution

- Bran: 55%
- Endosperm: 32%
- Embryo: 13%

Dry matter distribution

- Endosperm: 86%
- Bran layers, including the aleurone: 12%
- Embryo: 2%

>40% of both Zn and Fe is localized to the embryo and the bran layers and therefore lost in polishing

Hansen et al. 2009 Plant Methods 5, 12
Nutrient concentration in rice grain as a function of pre-ceeding polish time

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Multi-elemental speciation analysis by LC-ICP-MS

- HPLC pump
- Scavenger column
- Superdex 75 10/300 SEC column
- Superdex Peptide 10/300 HR-SEC column
- UV-DAD
- Agilent 7500cx ICP-MS
- Automatic fraction collection (Agilent 1100 SFC)
- P
- IP-ICP-MS
- Demetalization Ultra-Filtration
- S
- MALDI-TOF-MS
- RPC-ESI-MS

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Analytical challenges: The S sensitivity in ICP-MS is very low

- The major isotope ($^{32}$S) is a prohibited mass due to e.g. the double oxygen interference.

- S is usually measured in standard mode as the low abundant $^{34}$S isotope.

- S has a high ionization potential (10.36 eV).

### S-isotopes

<table>
<thead>
<tr>
<th>Mass</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>32</td>
<td>94.9%</td>
</tr>
<tr>
<td>33</td>
<td>0.8%</td>
</tr>
<tr>
<td>34</td>
<td>4.3%</td>
</tr>
<tr>
<td>35</td>
<td>0.02%</td>
</tr>
<tr>
<td>36</td>
<td></td>
</tr>
</tbody>
</table>

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Increasing the sensitivity of S by \( \text{O}_2/\text{He} \) addition to the octopole

- The reaction is thermodynamically favourable:
  \[
  \Delta H_f = -6.2 \text{ kJ mol}^{-1}
  \]
  (Bandura et al. 2002)

- Can Fe, P and Zn also be analysed as oxides?
Signal-to-noise ratio and LOD was significantly improved

- S/N markedly improved for $^{48}\text{SO}^+$ compared to $^{34}\text{S}$ in standard mode (LOD~3 ppb)

- LOD for $^{72}\text{FeO}^+$ was improved >20 times compared to $^{57}\text{Fe}$ in standard mode (~0.5 ppb)

Persson et al. 2009 Metallomics 1, 418-426
The method was validated against three metalloproteins with known S:metal ratio

<table>
<thead>
<tr>
<th>Enzyme/Protein</th>
<th>Zn-protein I</th>
<th>Zn-protein II</th>
<th>Fe protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Carbonic anhydrase (bovine erythrocytes)</td>
<td>Metallothionein 2A (rabbit liver)</td>
<td>Myoglobin (horse heart)</td>
</tr>
<tr>
<td>Molecular weight (Da)</td>
<td>28 983</td>
<td>6581</td>
<td>16 954</td>
</tr>
<tr>
<td>Histidines</td>
<td>11</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>Methionines</td>
<td>4</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Cysteines</td>
<td>0</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Theoretical S:metal ratio</td>
<td>4</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Analysed S:metal ratio</td>
<td>$4.08 \pm 0.02$</td>
<td>$3.06 \pm 0.06$</td>
<td>$1.91 \pm 0.05$</td>
</tr>
</tbody>
</table>

Persson et al. 2009
Speciation pattern of barley embryo sample in oxygen mode

- The speciation of Fe and Zn is clearly de-coupled

- Zn elutes as only single peak (3.0 kDa) overlapping with the LMW S-peak (ratio of S:Zn peak 43±6.2 S atoms per Zn)

- The major Fe peak overlaps with P as a major (12.3 kDa) and a minor (0.5 kDa) complex

- Which P-peak contains phytic acid?

Persson et al. 2009 Metallomics 1, 418-426
Dephosphorylation of fractionated P species by phytase incubation - phytate identification by IP-ICP-MS

Fe$_4$(IP$_6$)$_{18}$

Retention time

Persson et al. 2009 Metallomics 1, 418-426

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Mass balance - Enzymatic extractions

Extraction with phytase doubled Fe extractability, but did not increase that of Zn.

Extraction with protease XIV increased Zn extractability 5 times, but did not increase that of Fe.

P extractability increased with phytase (86%), but not with protease.

S extractability increased with protease (96%), but not with phytase.

Persson et al. 2009 Metallomics 1, 418-426
Size exclusion chromatography of rice endosperm

Retention time, s

Ion intensity, counts s⁻¹

Fe

P

Retention time, s

Ion intensity, counts s⁻¹

S

Zn

$^{66}\text{Zn}$

$^{48}\text{SO}^+$

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Fe associated with phytate but not Zn
Extraction of unprepared rice versus cooked rice (superdex 200: 20-500 kDa)

[Graphs showing ion intensity and retention time for TSN_TRIS/NaCl and TSN_TRIS/NaCl_Boiled]
Endosperm Fe speciation in rice overexpressing nicotiamine synthetase

- Activation-tagging mutant lines in which expression of a rice NAS gene, OsNAS3, was increased by introducing 35S enhancer elements - Prof. Gynheung An, Pohang Univ., South Korea
- WT and OsNAS3-D1 had an equal amount of Fe bound in a medium molecular weight complex co-eluting with P in a Fe: IP6 oligomer complex
- OsNAS3D-1 had significantly more (7.0±0.3 times) Fe bound in a low molecular weight complex
- The low molecular weight Fe peak did not co-elute with neither P nor S. Fe was thus not bound to IP6, or to cysteine/methionine containing ligands (peptides or proteins)
- Mass calibration indicated an apparent molecular mass of approximately 1300±400 Da for the Fe complex, suggesting a cluster with Fe bound to the –NH+ and/or –COO- functional groups of several NA ligands

Lee et al. 2009 PNAS (in press)
Conclusions & perspectives

• The speciation of Fe and Zn in rice and barley grain were clearly de-coupled
  – Fe is predominantly speciated with IP$_6$ polymers
  – Zn with LMW peptides
• The phytate dogma is challenged: Neither the speciation analysis nor the enzymatic extraction indicated Zn:IP$_6$ binding
• Ligand exchange is always an issue to consider when analysing coordination complexes…..
• LC-ICP-MS is a powerful tool to study the elemental speciation of grain tissues
• Ideal tool for testing e.g. the various transgenic approaches used to increase bioavailability of Fe and Zn in the cereal grains
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