Evidence of active NADP\(^+\) phosphatase in dormant seeds of Avena sativa L.

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Abstract

Freshly-harvested seeds of Avena sativa L. do not germinate when imbibed at temperatures higher than 25 °C. This high temperature dormancy is due to the impermeability of seed coats, and to the low activities of glycolysis and the oxidative pentose phosphate pathway (OPP) in the embryo. The analysis by exclusion chromatography of soluble NADP\(^+\) phosphatase activities of embryos revealed two isoforms: a 37 kDa isoform present in both dormant and after-ripened caryopses, and a second isoform, with an apparent molecular weight of 160 kDa, five times more active in embryos of dormant seeds than in the after-ripened ones, after 6 h of imbibition at 30 °C. Moreover, the activity of this 160 kDa isoform was three times less in embryos from dormant caryopses when they were grown at 10 °C, a permissive temperature for radicle protrusion. These results suggest a correlation between the activity of the 160 kDa NADP\(^+\) phosphatase and the dormancy state of the caryopsis. The two isoforms differed in the pH required for optimal activity: pH 5.7 and 6.5 for the 37 kDa and the 160 kDa phosphatases, respectively. Furthermore, the 160 kDa NADP\(^+\) phosphatase displayed a strong specificity for NADP\(^+\), whereas the 37 kDa isoform was able to hydrolyse numerous other phosphorylated compounds.

Key words: Avena sativa, dormancy, germination, NADP\(^+\) phosphatase.

Introduction

Freshly harvested seeds of Avena species do not germinate when placed at temperatures higher than 25 °C. This high temperature dormancy is partly due to the impermeability of seed coats and glumellae to oxygen (Côme et al., 1988), and to reduced rates of catabolism in embryos (Gallais et al., 1998). Numerous studies have reported the role of abscisic acid and gibberellins in the control of germination, but the action of these hormones on metabolism is unclear, indirect, and occurs after the protrusion of the radicle (Jacobsen and Chandler, 1987; Karssen, 1995; Fennimore and Foley, 1997). The earliest demonstrated metabolic event that distinguishes an imbibed seed able to germinate, is a rise in fructose 2,6-bisphosphate (Larondelle et al., 1987) which is supposed to activate glycolysis and, therefore, increase the NADH content. Such an increase in NADH has been observed before the protrusion of the radicle in Avena sativa L. caryopses (Gallais et al., 1998). In dormant (D) caryopses, many authors have reported low activities of the enzymes of the oxidative pentose phosphate pathway (OPP) (Adkins and Ross, 1981), and high redox charges of the NADP(H) (Fontaine et al., 1994). Nevertheless, in vivo analyses of the OPP enzymes have failed to indicate any difference between D and after-ripened (AR) seeds (Mayer and Poljakoff-Mayber, 1989; Lecat et al., 1992). Moreover, during the sensu stricto germination, comparable increases in pyridine nucleotide concentrations have been observed in both D and AR caryopses. If all pyridine nucleotides were strongly synthesized in embryos of AR caryopses after germination, their levels remained con-
stant in dormant material (Gallais et al., 1998). Therefore, dormancy cannot be explained only by insufficient glycolysis and/or OPP, and the phenomenon remains far more complex (Li and Foley, 1994).

In this study, an NADP⁺ phosphatase activity correlated with the dormancy state of the caryopses is evident in Avena sativa L. embryos, and some properties of the partially purified enzyme are presented. The high level of this enzymatic activity, only found in D caryopses, might explain the steady NADP⁺ and NADPH contents observed during seed imbibition (Gallais et al., 1998), in spite of the demonstrated NAD⁺ kinase activities (Gallais and Laval-Martin, 1999).

Materials and methods

Plant material

Oat seeds (Avena sativa L. cv. Noire de Moyencourt) were harvested in France in August 1993. Half of the seeds were kept dry at room temperature up to after-ripen; the other half was stored dry at −20°C to maintain dormancy. Caryopses were considered dormant when they could not germinate at 30°C, but germinated at 10°C (Fig. 1).

Germination controls were carried out using 100 naked caryopses. They were germinated at 30°C in absolute darkness, in Petri dishes (10 cm diameter) containing a cotton layer imbibed with deionized water (25 seeds per dish). A caryopsis was considered germinated when the radicle pierced the seed coat, approximately 10 h after the start of imbibition. All experiments were performed after 6 h of imbibition at 30°C, during the germination sensu stricto, i.e. in the time between the end of imbibition and the protrusion of the radicle.

Treatment of the seeds

Immediately before assays, the embryos were excised with a scalpel, leaving as little endosperm as possible. Endosperm refers to the rest of the seed, i.e. the seed coat, pericarp, aleurome layer, and starchy endosperm.

Extraction and assay of protein

Fifty to 250 excised embryos were ground in a mortar in the presence of liquid nitrogen. All subsequent steps were performed at 2–4°C. NADP⁺ phosphatase was extracted in 7 ml g⁻¹ fresh weight of buffer A (100 mM TRIS-HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 2 mM PMSF, 1 mM DTT, 1 μg ml⁻¹ leupeptin, and 1 μg ml⁻¹ pepstatin A). The homogenate was stirred for 10 min, then centrifuged at 105,000 g for 60 min (Rotor Beckman SW41). Aliquots of the supernatant were frozen in liquid nitrogen and stored at −20°C. Protein contents were determined according to Bradford using a Bio-Rad protein assay kit and bovine serum albumin as standard (Bradford, 1976).

Cell fractionation

Cells of embryo tissues were fractionated by differential centrifugations. All steps were performed at 0–4°C. Freshly harvested embryos (375) were gently ground with a pestle in a mortar for 5 min in 12 ml g⁻¹ fresh weight of buffer B (100 mM TRIS-HCl pH 7.5, 2 mM EDTA, 0.2% bovine serum albumin, 1 mM PMSF, 1 mM DTT, 0.5 M sucrose, 1 μg ml⁻¹ leupeptin, and 1 μg ml⁻¹ pepstatin A). The crude extract was centrifuged at 500 g for 10 min. The pellet containing nuclei, cell walls and whole cells, was discarded (Rickwood et al., 1994) and the supernatant (S₁₀₀) centrifuged for another 30 min at 20,000 g (Rotor Sorvall SS34) to gather the mitochondria (in P₁₀₀). The resulting supernatant, S₅₀, was submitted to a 105,000 g centrifugation to separate the pelletable microsomes (P₁₀₅) from the cytoplasm and vacuolar content (S₁₀₅). P₂₀ and the microsomal pellet were resuspended in a glass Potter homogenizer in buffer B (minus sucrose) to disrupt the organelle membranes. The membranes were isolated from the matrix of all organelles by a last centrifugation at 105,000 g for 60 min (Rotor Beckman SW41). Since only etiolated tissues were used, marker enzymes to estimate the subcellular distribution were: glucose-6-phosphate dehydrogenase (G6PDH) for cytosol (the studied embryos containing only non-photosynthetic pro-plastids) (according to Côme et al., 1988); and cytochrome c oxidase for mitochondrial membranes (according to Tolbert, 1974).

Partial purification of the NADP⁺ phosphatase

The S₁₀₅ (1 ml corresponding to 0.145 mg fresh weight extract) was directly applied to a Sephadex G200 column (5 × 55 cm, Pharmacia LKB) and eluted at a flow rate of 0.26 ml min⁻¹ with buffer C (100 mM TRIS-HCl pH 7.5, 50 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 μg ml⁻¹ leupeptin, and 1 mM DTT). The column calibration was performed with: dextran blue (2000 kDa), β-galactosidase (595 kDa), catalase (232 kDa), alcohol dehydrogenase (150 kDa), BSA (66 kDa), and cytochrome c (12.4 kDa).

Assay for NADP⁺ phosphatase activity

The NADP⁺ phosphatase activity of each fraction was assayed for 10 min, at 40°C, in 250 μl of a medium containing 25 μl of...
sample, 2.0 mM NADP\(^+\) and 50 mM Bicine-HCl pH 7.5. The reaction (linear for at least 30 min) was stopped by heating for 2 min in a boiling water bath.

The NAD\(^+\) formed was measured by a recycling procedure using an auxiliary enzyme, alcohol dehydrogenase (ADH, EC 1.1.1.1; from baker’s yeast, Sigma A3263), which, in the presence of excess ethanol, initiates a redox cascade starting with the reduction of NAD\(^+\) (Bernofsky and Swan, 1973). The reaction medium was analysed in a microplate (25 \(\mu\)l per well) with a final mixture containing: 8% (v/v) ethanol, 1% (v/v) PVP, 0.5 mM EGTA, 50 mM TRIS-HCl pH 8.5, 1 mM phenazine ethosulphate (PES), 0.44 mM 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT), and 2 units of ADH per 250 \(\mu\)l. The reduction rate of MTT, proportional to the amount of NAD\(^+\), was followed at 570 nm and computed NADP\(^+\) phosphatase activity at 10 °C and 30 °C: To confirm the correlation between the activity of NADP\(^+\) phosphatase and dormancy, at 6 h and 30 °C, the enzymatic activities were analysed in extracts from D caryopses able to germinate at 10 °C for 26 h. At 10 °C, the mean time for 50% germination of the D seeds was about 43 h, against 10 h for the AR caryopses at 30 °C (Fig. 1). The proteins of the D embryos were extracted after 26 h of culture at 30 °C seeds were in comparable stages of germination (arrows on Fig. 1). In addition, NADP\(^+\) phosphatase activities in AR and D embryos at 6 h and 10 °C and in AR and D embryos at 26 h and 30 °C were determined (Fig. 3).

**Results**

**Germination at 30 °C and 10 °C**

Germination of D seeds was highly inhibited at 30 °C whereas AR caryopses germinated within 10 h (Fig. 1). In contrast, the low temperature of 10 °C allowed D seeds to germinate after about 43 h and delayed the germination of AR seeds from 10 h to 31 h.

**Subcellular distribution of the NADP\(^+\) phosphatase activity**

Embryos of dormant caryopses (D embryos) imbibed for 30 h at 30 °C were fractionated and the NADP\(^+\) phosphatase activity was assayed in each of the subcellular fractions (Table 1). It has been verified that no activity was lost during fractionation (data not shown). The S\(_{105}\) fraction, containing cytoplasm and vacuolar content, was markedly enriched in NADP\(^+\) phosphatase activity. The low percentages of enzyme activity bound to mitochondrial membranes (2.6%) or detected in the microsomes matrix (1.7%) could correspond to liposomes formed during fractionation steps. These results suggested that the NADP\(^+\) phosphatase activity was mostly soluble and could be directly analysed by chromatography.

**Table 1. Subcellular distribution of the NADP\(^+\) phosphatase activity in embryos of dormant seeds of Avena sativa after 26 h of culture at 30 °C (means of two experiments)**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Total (nmol h(^{-1}))</th>
<th>Specific activity (nmol h(^{-1}) mg(^{-1}))</th>
<th>G6PDH (%)</th>
<th>Cyt c oxidase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoplasm and vacuoles</td>
<td>30.6</td>
<td>1071 (95.6)*</td>
<td>42.4</td>
<td>100%</td>
</tr>
<tr>
<td>Mitochondria</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Membranes</td>
<td>4.7</td>
<td>30 (2.6)</td>
<td>4.1</td>
<td>0%</td>
</tr>
<tr>
<td>Matrix</td>
<td>10.5</td>
<td>0 (0.0)</td>
<td>0.0</td>
<td>0%</td>
</tr>
<tr>
<td>Microsomes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Membranes</td>
<td>2.1</td>
<td>1 (0.1)</td>
<td>0.4</td>
<td>0%</td>
</tr>
<tr>
<td>Matrix</td>
<td>5.9</td>
<td>19 (1.7)</td>
<td>6.1</td>
<td>0%</td>
</tr>
</tbody>
</table>

*Values in brackets: % of total.
At 6 h of culture, AR embryos presented a low specific activity of NADP$^+$ phosphatase in comparison with D embryos. The activity was markedly greater in the D embryos grown in the non-permissive condition of 30 °C. In addition, at 10 °C, the activity of D embryos measured after 6 h decreased, to reach, at 26 h, the same level of AR embryos at 30 °C and 6 h. At the end of the sensu stricto germination, i.e. 26 h at 10 °C for the D embryos and 6 h at 30 °C for the AR embryos, the activities were similar in both types of seed. It was noticed that, whatever the temperature, the activity measured at 26 h was augmented in germinated AR embryos whereas a decrease was observed in D embryos.

In order to compare the NADP$^+$ phosphatase activities from AR and D caryopses, the soluble fractions ($S_{105}$) of both types of embryo were fractionated by size-exclusion chromatography.

**Chromatographic analysis of NADP$^+$ phosphatase**

The $S_{105}$ from D and AR caryopses imbibed for 6 h at 30 °C were fractionated on a Sephadex G200 column (Fig. 4). The chromatographic patterns revealed two isoforms of NADP$^+$ phosphatase characterized by different molecular weights. The heavier isoform presenting a molecular weight of 160 kDa, was at least five times more active in D embryos than in AR ones (Fig. 4A). In contrast, the lower molecular weight isoform (37 kDa) displayed an activity only twice as strong in D embryos. Similar chromatographic patterns were obtained with three different extracts of both types of seed.

$S_{105}$ from D embryos harvested after 26 h of culture at 10 °C was also analysed on a G200 column. Reproducible patterns were obtained (Fig. 4B) and compared to those from D embryos cultured for 6 h at 30 °C (Fig. 4A). Whatever the conditions of culture, the activity of the 37 kDa isoform was relatively constant, in contrast to that of the 160 kDa NADP$^+$ phosphatase. As a matter of fact, a weak activity of the 160 kDa isoform was measured at 10 °C, whereas this activity was markedly enhanced in seeds imbibed at 30 °C.

From these results, only the 160 kDa NADP$^+$ phosphatase isoform appeared to be characteristic of non-germinating D caryopses, at 30 °C.

**Properties of the NADP$^+$ phosphatases**

The effect of pH was tested on phosphatase activities separated by G200 chromatography (Fig. 5). For these experiments, assay conditions were similar to those used was a mix of 50 mM acetic acid, 50 mM MES, and 50 mM Bicine. Experiment reproduced three times with similar results.
described in the Materials and methods, except that the incubation buffer was substituted by a mix of 50 mM acetic acid, 50 mM MES and 50 mM Bicine buffered from pH 5 to 10 with NaOH. Different optimum pH values for maximum activity were noticed: around pH 6.2 for the dormancy-specific isof orm of 160 kDa, and pH 5.7 for the 37 kDa isof orm. It was seen that the activity of the 37 kDa isof orm displayed a shoulder at pH 7.5, suggesting a heterogeneous protein.

Substrate specificities of each phosphatases isolated from Sephadex G200 were investigated at their optimum pH (Table 2). The two isof orms differently dephosphorylated a variety of phosphate esters. The 160 kDa enzyme efficiently hydrolysed NADP\(^+\), and weakly hydrolysed some sugar-phosphoesters (such as G6P) and \(\alpha\)-phosphate amino acids (such as P-Ser). The 37 kDa isof orm was characterized by a strong inorganic pyrophosphatase activity, and a lower activity towards tri- and di phosphate nucleosides, carbamyl phosphate, phosphoenol pyruvate, \(\alpha\)-phosphotyrosine, and pNPP. By comparison with the 160 kDa isof orm, the 37 kDa phosphatase was only weakly active towards NADP\(^+\).

### Discussion

Little is known about the complex metabolic mechanisms underlying cereal dormancy. Although several enzyme activities are specific for AR imbibed caryopses, most of them appear after the visible protrusion of the radicle, and therefore are correlated with growth and not with dormancy (Kovacs and Simpson, 1976). For example, in aleurone cells of barley caryopses stimulated by GA\(_3\) for 72 h, the time after which the grain germinates, the acid phosphatase is active (Ashford and Jacobsen, 1974). Conversely, dormancy-specific peptides were reported in *Avena fatua* seeds (Johnson et al., 1995; Dyer, 1993).

This study clearly demonstrates the presence of two NADP\(^+\) phosphatase isof orms in *Avena sativa* embryos, one of which, with an apparent native molecular weight of 160 kDa, is specific to the D embryos during the *sensu stricto* germination preceding radicle protrusion.

Cereal embryos contain numerous phosphatases: seven and nine acid phosphatase isozymes correlated to changes during germination were observed in barley seeds and wheat germs, respectively (Papageorgakopoulou and Georgatos, 1977; Kawarasaki et al., 1996). The 160 kDa NADP\(^+\) phosphatase of oat described in this report displayed the same optimum pH (about pH 6.2) as six of the wheat acid phosphatases, but a very different molecular mass (12 and 35–47 kDa for barley and wheat). The second phosphatase isof orm of oat was active towards di- and tri phosphate nucleosides, and possessed an acidic pH optimum and molecular weight similar to those of wheat and barley phosphatases (between 35 and 47 kDa). It must be noticed that NADP\(^+\) was not tested as a substrate for barley and wheat phosphatases. The oat 160 kDa isof orm, able to dephosphorylate a few types of phosphate monoester compounds, has been reported here for the first time as an acid phosphatase using the NADP\(^+\) as a specific substrate.

Most of the studies on various plants suggest a role of phosphatases in the mobilization of phosphorus reserves during germination (Yamagata et al., 1980; Laidman, 1982; Tamura et al., 1982; Kawarasaki et al., 1996). The physiological role of each acid phosphatase could not be thoroughly investigated because of the small quantities, the instability and the denaturation of the enzymes during purification. In *Avena sativa* seeds, a role for the 160 kDa phosphatase could participate in germination inhibition. As a matter of fact, the NAD\(^+\) kinase activity, forming the NADP\(^+\), was previously found to be comparable in AR and D embryos (Gallais and Laval-Martin, 1999).

However, during imbibition, the concentration of NAD(P)(H) increased in AR embryos whereas it remained constant in D embryos (Gallais et al., 1998). This could be explained by the high level of dormancy-specific NADP\(^+\) phosphatase activity shown in this study. As a consequence of the NADP\(^+\) hydrolysis, the pentose phosphate pathway would be slowed down, the NADPH level reduced, and the new biosyntheses necessary for growth impaired. This type of metabolic regulation was described in an achlorophyllous ZC mutant of *Euglena gracilis*, in which NADP\(^+\) phosphatase and NAD\(^+\) kinase activities varied circadianly in opposite phases (Laval-Martin et al., 1990a, b). Furthermore, due to NADP\(^+\) phosphatase and complex regulation, most of the enzymes detoxifying the

<table>
<thead>
<tr>
<th>Substrates (1 mM)</th>
<th>Isoforms</th>
<th>160 kDa</th>
<th>37 kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine triphosphate</td>
<td>2 ± 1</td>
<td>72 ± 10</td>
<td></td>
</tr>
<tr>
<td>Adenosine diphosphate</td>
<td>nd</td>
<td>80 ± 15</td>
<td></td>
</tr>
<tr>
<td>Adenosine monophosphate</td>
<td>nd</td>
<td>16 ± 5</td>
<td></td>
</tr>
<tr>
<td>Cytidine triphosphate</td>
<td>nd</td>
<td>39 ± 12</td>
<td></td>
</tr>
<tr>
<td>Inosine triphosphate</td>
<td>nd</td>
<td>71 ± 3</td>
<td></td>
</tr>
<tr>
<td>Inorganic pyrophosphate</td>
<td>2 ± 1</td>
<td>100 ± 9</td>
<td></td>
</tr>
<tr>
<td>NADP(^+)</td>
<td>100 ± 5*</td>
<td>6 ± 3</td>
<td></td>
</tr>
<tr>
<td>Glucose-6-phosphate</td>
<td>25 ± 3</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>6-Phosphogluconic acid</td>
<td>2 ± 1</td>
<td>11 ± 3</td>
<td></td>
</tr>
<tr>
<td>Phosphoenol pyruvate</td>
<td>nd</td>
<td>65 ± 12</td>
<td></td>
</tr>
<tr>
<td>Carbamyl phosphate</td>
<td>nd</td>
<td>84 ± 7</td>
<td></td>
</tr>
<tr>
<td>p-Nitrophenyl phosphate</td>
<td>nd</td>
<td>41 ± 16</td>
<td></td>
</tr>
<tr>
<td>Phytic acid</td>
<td>nd</td>
<td>5 ± 1</td>
<td></td>
</tr>
<tr>
<td>Naphthyl phosphate</td>
<td>nd</td>
<td>6 ± 2</td>
<td></td>
</tr>
<tr>
<td>o-Phosphoesterine</td>
<td>31 ± 10</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>o-Phosphothreonyl</td>
<td>7 ± 2</td>
<td>8 ± 3</td>
<td></td>
</tr>
<tr>
<td>o-Phosphotyrosine</td>
<td>nd</td>
<td>42 ± 10</td>
<td></td>
</tr>
</tbody>
</table>

The relative activity of each isof orm is respectively expressed as a percentage of activity toward *A* NADP\(^+\) and *b* inorganic pyrophosphate ± SD. The enzyme activity was performed by measuring the inorganic phosphate liberated from the denoted substrates. nd: not detectable.

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reactive oxygen species requiring NADPH as a substrate, such as glutathione reductase and monodehydroascorbate reductase, would be less effective (Cakmak et al., 1993). Therefore, the oxidative cell damage being less efficiently compensated for, the germination of the D caryopses of oat would fail.

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References


