Planta (2005) 220: 708–716
DOI 10.1007/s00425-004-1390-7

ORIGINAL ARTICLE

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A rapid response of β-amylase to nitric oxide but not gibberellin in wheat seeds during the early stage of germination

Received: 13 July 2004 / Accepted: 25 August 2004 / Published online: 23 October 2004

Abstract The effects of nitric oxide (NO) and gibberellic acid (GA_3) on the responses of amylases in wheat (*Triticum aestivum* L.) seeds (caryopses) were investigated during the first 12 h of germination. GA_3 had no effects on the activities of α-amylase (EC 3.2.1.1) or β-amylase (EC 3.2.1.2), either in intact seeds or embryoless halves within 12 h. In contrast, addition of sodium nitroprusside (SNP), an NO donor, was able to induce a rapid increase in β-amylase activity without affecting α-amylase. Furthermore, the rapid response of β-amylase to SNP in wheat seeds could be attributed to NO and was approximately dose-dependent. Some other aspects of SNP induction of amylase isozymes were also characterized. Further investigations showed that SNP might play an interesting role in the dissociation of free β-amylase from small homopolymers or heteropolymers. Furthermore, SNP also directly induced the release of bound β-amylase from glutenin and its crude enzyme preparation. However, the slight increase in protease also induced by SNP might not be responsible for this action. Interestingly, based on the fact that the rapid response of β-amylase to NO also existed in seeds of other species, such as barley, soybean, rice and watermelon, it might be a universal event in early seed germination.

Keywords β-Amylase · Germination · Gibberellin · Nitric oxide · Seed · *Triticum*

Abbreviations cPTIO: 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide · GA: Gibberellin · GA3: Gibberellic acid · IEF: Isoelectric focusing · 2-ME: 2-Mercaptoethanol · NO: Nitric oxide · PAGE: Polyacrylamide gel electrophoresis PCMB: *p*-Chloromercuribenzoate · SNP: Sodium nitroprusside

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Introduction

During germination of wheat seeds (caryopses), various hydrolytic enzymes are synthesized and secreted to mobilize reserves. Production of these enzymes is mostly regulated by gibberellin (GA) produced by the embryo (Lovegrove and Hooley 2000). Among these hydrolytic enzymes, α-amylase is synthesized de novo in the aleurone layer. This synthesis is dependent on GA and is regulated mainly at the transcriptional step. Moreover, α-amylase is synthesized according to a time sequence, and appears later than β-amylase in germinating wheat and barley seeds (Daussant and Corvazier 1970; Hardie 1975). Although de novo β-amylase synthesis during rice seed germination has been reported (Okamoto and Akazawa 1980; Wang et al. 1996; Yamaguchi et al. 1999), in germinating wheat and barley, unlike other hydrolytic enzymes, it is not synthesized de novo, nor is it released from either the scutellum or the aleurone layers (Daussant and Corvazier 1970; Hardie 1975). Instead, it accumulates during grain development in two forms. One is a soluble or free form in ungerminated wheat kernels, which can form high-molecular-weight homopolymers or heteropolymers (Forsyth and Koebner 1992). Alternatively, a large proportion (about 80%) of the β-amylase in the starchy endosperm occurs as an inactive, latent form bound by disulphide linkages to protein bodies such as glutenins, or their remnants (Ziegler 1999; Rowsell and Goad 1962a, 1962b). Bound β-amylase is deposited on the periphery of the starch granules during grain development and is probably synthesized as a mature protein in the cytosol. It is thus a component of the protein matrix, which covers starch

and might protect it from premature attack by α-amylases (Ziegler 1999). Extracts of the enzyme can be activated through release from its bound form, either by digestion of glutenin with proteolytic enzyme (Guerin et al. 1992), or by disrupting the binding linkages with some active compounds secreted from the embryo or the aleurone layer (Rowsell and Goad 1962a, 1962b). Also in the grain, GA stimulates protease synthesis and release from the aleurone layer, which might in turn affect the release of bound β-enzyme. However, a mounting body of evidence has confirmed that GAs play a key role only in the late stage of seed germination (Richards et al. 2001; Gallardo et al. 2002). Therefore, it is possible that another regulatory mechanism is active in the early stage of seed germination.

Nitric oxide (NO), which can be produced enzymatically by nitric oxide synthase (NOS; EC 1.14.13.39) or nitrate reductase (NR; EC 1.6.6.1/2) or non-enzymatically from nitrite at low pH in plants, has been implicated in a number of diverse physiological processes, including seed germination (Beligni and Lamattina 2000; Zhang et al. 2003), root organogenesis (Pagnussat et al. 2002), stomatal closure (Garcia-Mata and Lamattina 2003; Guo et al. 2003; Neill et al. 2003), maturation and senescence (Leshem et al. 1998; Tu et al. 2003), and programmed cell death (Beligni et al. 2002; Pedroso et al. 2000). For example, Beligni and Lamattina (2000) provided evidence that NO is involved in light-dependent seed germination. Further study indicated that NO enhanced α-amylase accumulation slightly and prolonged the life of barley aleurone cells incubated in GA_3 compared with GA_3 treatment alone (Beligni et al. 2002). More recently, Simontacchi et al. (2004) found a rapid burst of NO induced by 1×10^{-3} mol l^{-1} NO_2^- and 0.1×10^{-3} mol l^{-1}NADH during early germination of sorghum seeds, indicating a potential role for NO as a signal molecule in the early germination of seeds. Also, we discovered that NO not only stimulated the germination of wheat seeds under osmotic stress but also increased the activities of amylase in embryoless halves during early imbibition (for 6 h) under normal conditions (Zhang et al. 2003). However, whether the increase in amylase activities was derived from α- or β-amylase, or both, is not fully understood. In this report, we provide evidence that NO, but not GA, is able to induce a rapid response of β-amylase in wheat seeds during the early stage of germination (within 12 h). The mechanism of action of NO was preliminarily investigated, and its potential role is also discussed.

Materials and methods

Materials

Seeds of wheat (*Triticum aestivum* L., Yangmai 158), barley (*Hordeum vulgare* L.), soybean (*Glycine max* L.), rice (*Oryza sativa* L.), maize (*Zea mays* L.), *Arabidopsis thaliana* (L.) Heynh., watermelon (*Citrullus vulgaris* Schräd. ex Ecklon & Zeyh.), oilseed rape (*Brassica napus* L.), and Indian mustard (*Brassica juncea* L.), used in this research, were kindly supplied by Jiangsu Academy of Agricultural Sciences, Jiangsu Province, P.R. China. SNP ($[Na_2Fe(CN)_5]\cdot NO$, purchased from Sigma) was used as NO donor. cPTIO [2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide] was chosen as an NO scavenger and $NaNO_3$, $NaNO_2$ and $K_3Fe(CN)_6$ as additional controls for SNP decomposition. Casein was used as the substrate of proteases, leupeptin and antipain (Sigma) as protease inhibitors. Gibberellic acid (GA_3) was purchased from Sigma. $CuSO_4$ and PCMB (*p*-chloromercuribenzoate; Sigma) were used as β-amylase inhibitors. Na_2EDTA and $CaCl_2$ were chosen as α-amylase inhibitor and activator, respectively.

Preparation of extracts for amylase analysis

Selected seeds of wheat (*Triticum aestivum* L., Yangmai 158) were bisected transversely, the embryoless half-seeds and intact seeds were surface-sterilized with 0.1% $HgCl_2$ for 3 min and washed six times with sterile distilled water, then dried. Batches of 20 endosperm halves or 10 intact seeds, 0.500 ± 0.001 g (net weight), were shaken at room temperature in 6.0 ml of 50×10^{-3} mol l^{-1} Tris–HCl (pH 7.5) containing 1% polyvinylpyrrolidone (PVP), 15×10^{-3} mol l^{-1} 2-ME, and SNP, GA_3 or other chemicals at the specified concentrations (mentioned in results or figures). Seeds were ground in mortars with their own treatment solutions. The homogenate was centrifuged at 10,000 *g* for 30 min and the supernatant was used as total amylase preparations for further assay. To inhibit α-amylase, the preparations were incubated for 16 h at 20°C with 0.025 mol l^{-1}EDTA and 0.1 mol l^{-1}NaCl according to the method reported by Guerin et al. (1992). To inactivate β-amylase, the preparations were heated to 70°C for 20 min and centrifuged at 5,000 *g* for 10 min.

Assays of α/β-amylase activities and electrophoretic analysis

The amylase activities were determined using the starch–iodine method according to Collins et al. (1972). One unit of activity (U) was calculated by taking the quantity of the enzyme to reach 50% of the original color intensity. Amylases were analyzed in 10% vertical PAGE gels. Aliquots (15–30 μl) of the crude α-/β-amylase preparations mentioned above were applied. To visualize the bands of α-/βamylase activities, the gel was incubated at 25°C for 30 min in 50×10^{-3} mol l^{-1} PBS (pH 7.0) containing 1% boiled soluble starch. After washing three times with distilled water, the gel was stained with 0.6% I_2 and 6% KI solution. To demonstrate the dissociation of free β-amylase from its polymers, crude preparations were subjected to various treatments followed by 5–25% vertical gradient PAGE.

Preparation of glutenin and crude enzymes of free and latent β-amylase

Dry half-seeds were ground into powder. Then glutenin was isolated according to the procedure of Bilinski and McConnell (1958). Crude extracts of free and bound β-amylase were prepared according to the method of Guerin et al. (1992) with some modification. Halves (0.500 ± 0.001 g) were homogenized with 10 ml Tris–HCl (50×10^{-3} mol l^{-1}, pH 7.5). Then, the homogenate was pass through three layers of cheesecloth, and centrifuged for 30 min at 10,000 *g*. This extraction was repeated at least three times. The re-suspension of the residue using the above Tris–HCl buffer, was regarded as the bound β-amylase crude enzyme preparation, and the supernatant was collected as free β-amylase crude enzyme for further assay.

Characterization of amylases induced by SNP

After electrophoresis of crude enzyme preparations from seeds treated with SNP for 8 h, the gel was split into slices to characterize some properties of amylases induced by SNP. To distinguish the types of iso-amylase, the gel slices were pre-incubated at 25°C for 30 min in 50×10^{-3} mol l^{-1} PBS (pH 7.0) containing $CuSO_4$, PCMB, $HgCl_2$, Na_2EDTA or $CaCl_2$. Then the slices were transferred into the same buffers containing 1% boiled soluble starch at 25°C. After another 30 min, the slices were stained with iodine.

To examine the isoelectric point (pI) of the amylases induced by SNP, isoelectric focussing (IEF) was performed as described by Yamaguchi et al. (1999), using Pharmacia broad-range ampholines (pH 3.0–10.0).

The effect of temperature on amylase activities was examined by incubating the gel slices in 50×10^{-3} mol l^{-1} PBS (pH 7.0) containing 1% boiled soluble starch for 30 min at 0, 20, 30, 40, 50, 60 or 70°C. Temperature stability was checked by incubating the gel slices in 50×10^{-3} mol l^{-1} PBS (pH 7.0) at 0, 20, 30, 40, 50, 60, 70, 80, 90 or 95°C for 15 min, transferring them to buffer containing 1% boiled soluble starch at 25°C for 30 min and staining with iodine.

To determinate the optimum pH of the amylases, the gel slices were incubated at 25°C for 30 min in buffers containing 1% boiled soluble starch at pH 3.5, 4.5, 5.5, 6.5, 7.0, 8.0 or 9.0. To determinate the pH stability of amylases, the gel slices were incubated at 25°C for 30 min in different buffers at pH 3.5, 4.5, 5.5, 6.5, 7.0, 8.0 or 9.0, then transferred to 50×10^{-3} mol l^{-1} PBS (pH 7.0) containing 1% boiled soluble starch at 25°C for another 30 min. After washing three times with distilled water, the gel slices were stained.

Determination of protease activity

The method used was a modified version of the technique reported by Zhang et al. (2001). The crude enzyme preparation used for determining protease activity was the same as the total amylase extract mentioned above. An aliquot of the crude enzyme preparation was added to an equal volume of 2.5% casein substrate, which was prepared in 200×10^{-3} mol l^{-1} acetic acid–sodium acetate buffer (pH 5.2). This mixture was incubated at 37°C for 1 h, and 3 vol. of 7.5%(w/v) trichloroacetic acid (TCA) was added. As a blank control, TCA was added to the mixture prior to incubation. After equilibration at 4°C for 30 min, the mixture was centrifuged at 10,000 *g* for 10 min and the absorption of the supernatant was measured at 278 nm. Protease activity is presented as ΔA_{278} (g DW)$^{-1}$ min^{-1}.

Statistical analysis

Significances were tested by one-way ANOVA, and the results are expressed as the mean values ± SD of three independent experiments. Each experiment was repeated at least three times.

Results

Rapid response of β-amylase rather than α-amylase to the NO donor SNP but not to GA_3

In the previous work, we preliminarily demonstrated that SNP, an NO donor, was capable of stimulating amylase activities in embryoless halves of wheat imbibed for 6 h under normal conditions (Zhang et al. 2003). However, whether α- or β-amylase, or both, in wheat seeds had these intriguing responses to NO during the early stage of germination (within 12 h) was not fully understood. Figure 1 shows that during the early germination of intact wheat seeds, there were no apparent differences in β-amylase digestion bands between GA_3 and the control treatments, and the activities of the enzyme remained similar to that of the control at 0 h (CK_0, Fig. 1a). However, addition of SNP (0.5×10^{-3} mol l^{-1}), an NO donor, resulted in a more rapid increase in β-amylase activity compared with the control (CK) or GA_3 alone (G), without affecting the activity of α-amylase (Fig. 1a). In the gels, two isozyme types, I and II, were observed, and most of the β-amylase activity could be attributed to type I. There was apparently no difference between induction of type-I β-amylase in the control dry seeds (CK_0) and any of the treated seeds (Fig. 1a). In contrast, type II was induced in only small amounts in control seeds and in seeds treated with GA_3, while it appeared in large quantities in the presence of SNP. These results suggest that the transparent bands, where isozyme type I is located, might be due to the digestion of starch by intrinsically active/free β-amylases accumulated during kernel development. Type II, in this report, is therefore ascribed to the specific isozymes susceptible to SNP. When intact seeds were incubated in Tris buffer containing

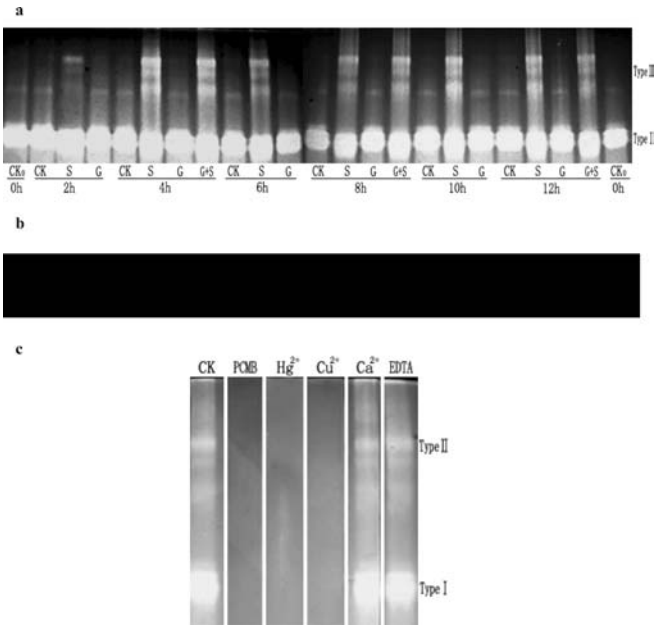

Fig. 1 a,b Rapid response of β-amylase but not α-amylase to SNP but not GA during the early stage of wheat (*Triticum aestivum*) seed germination (within 12 h). Ten intact wheat seeds (0.500 ± 0.001 g) were incubated at room temperature (25°C) in Tris buffer [50×10^{-3} mol l^{-1} Tris–HCl (pH 7.5) containing 1% polyvinylpyrrolidone (PVP) and 15×10^{-3} mol l^{-1} 2-ME] plus: no additions (control; *CK*), 0.5×10^{-3} mol l^{-1} SNP (*S*), 10 µmol l^{-1} GA_3 (*G*), and 10 µmol l^{-1} GA_3 plus 0.5×10^{-3} mol l^{-1} SNP ($G+S$). At 2-h intervals (0, 2, 4, 6, 8, 10 and 12 h), samples of incubated seeds were ground in a mortar with their own treatment solutions. The homogenates were centrifuged and the supernatants of the crude extracts were used as total amylase preparations. To inhibit α-amylase activity, the crude extracts were incubated for 16 h at 20°C with 0.025 mol l^{-1} EDTA plus 0.1 mol l^{-1} NaCl. To inactivate β-amylase, the crude extracts were heated to 70°C for 15 min and centrifuged at 5,000 rpm for 10 min. **a** Native PAGE of β-amylase (20 µl of sample per well). **b** Native PAGE of α-amylase (30 µl of sample per well). After electrophoresis, the gels were incubated for 20 min in 50×10^{-3} mol l^{-1} PBS buffer (pH 7.0), containing 1% boiled soluble starch. After washing three times with distilled water the gels were stained with iodine solution. **c** Effects of inhibitors on total amylase in crude extracts of wheat seeds treated with 0.5×10^{-3} mol l^{-1} SNP for 8 h. 20-µl samples were applied to the gel. After electrophoresis, the gel was split into several slices, and the slices were pre-incubated in 50×10^{-3} mol l^{-1}PBS (pH 7.0) containing 10×10^{-3} mol l^{-1} Na_2EDTA, 10×10^{-3} mol l^{-1} $CaCl_2$, 100 µmol l^{-1} $CuSO_4$, 50 µmol l^{-1} PCMB or 0.05% $HgCl_2$, at 25°C for 30 min. Then the slices were transferred into 50×10^{-3} mol l^{-1} PBS (pH 7.0) containing these different chemicals and 1% boiled soluble starch at 25°C. After another 30 min, the slices were stained with iodine. Each experiment was repeated at least three times

SNP or GA_3 mixed with SNP for 4, 8 or 12 h, there was no obvious difference between SNP and GA_3 + SNP lanes in the gel, indicating that SNP and GA_3 do not act additively (Fig. 1a), and that the rapid response of β-amylase to the NO donor is independent of GA_3.

α-Amylase was determined at the same time as β-amylase (Fig. 1b). As expected, GA_3 was not able to stimulate the synthesis and secretion of α-amylase in the intact seeds at the first stage of germination (within 12 h); moreover, the NO donor did not possess this capability, either. This apparent lack of induction might be due to the fact that α-amylase activities in wheat seeds incubated in GA_3, SNP or water were too weak to be detected in the gels (Fig. 1b).

To further confirm the types of amylase, total amylase preparations from seeds treated with SNP for 8 h were applied to a PAGE gel. After electrophoresis, the gel was split into several slices, and the slices were pre-incubated at 25°C for 30 min in 50×10^{-3} mol l^{-1} PBS (pH 7.0) containing PCMB, $HgCl_2$, $CuSO_4$, $CaCl_2$ or Na_2-EDTA. Then the slices were transferred into the same buffers containing 1% boiled soluble starch at 25°C. After another 30 min, the slices were stained with iodine. Figure 1c shows that PCMB and Hg^{2+} completely inhibited activities of these enzymes even at low concentrations of 50 µmol $l^{-1}$and 0.05%, respectively. 5.0×10^{-3} mol l^{-1} Cu^{2+} reduced the activities by 90% or more. However, 10×10^{-3} mol l^{-1} Na_2EDTA and 10×10^{-3} mol l^{-1} $CaCl_2$ had no apparent effects on these enzymes. From the above results, it was concluded that it was β-amylase but not α-amylase that was induced in response to the NO donor in intact wheat seeds in the early stage of germination (12 h), while GA_3 had no role in this action.

To further confirm the relationship between NO and GA_3 in the induction of amylase in intact seeds during the first 12 h of germination, embryoless half-seeds, in which the endogenous production of GA_3 was disrupted, were utilized. Figure 2 shows that GA_3 played no apparent role in the induction of either β-amylase (Fig. 2a,b) or α-amylase (Fig. 2c) during the first 12 h of germination, while the NO donor SNP significantly induced the activities of β-amylase (Fig. 2a,b) but not that of α-amylase (Fig. 2c). Moreover, the results of inhibitor/activator experiments showed that the specific amylases induced by SNP were β-forms rather than α-forms (Fig. 2d). The absence of additive effects between SNP and GA_3 (Fig. 2e,f) is further proof that the responses of β-amylase to NO and GA_3 are in different pathways.

The response of wheat seeds to SNP can be attributed to NO and is dose-dependent

To verify the role of NO in inducing β-amylase, an NO scavenger, cPTIO, was also used in the same experimental system, as were NO_3^-/NO_2^- and $K_3Fe(CN)_6$ (as controls for SNP decomposition). Figure 3a,b shows that cPTIO treatment alone at 8 h did not have any apparent effects on the response of β-amylase compared with the control. However, when combined with SNP, cPTIO, as an NO scavenger, was able to abolish the SNP-mediated increase in enzyme activity. Furthermore, neither NO_3^-/NO_2^- nor $K_3Fe(CN)_6$ was able to induce β-amylase (Fig. 3a). Further analysis using PAGE confirmed that it was NO, but not other derived compounds or GA_3, that was responsible for the induction of type-II β-amylase (Fig. 3b). Moreover, treatment with different SNP concentrations (0.0, 0.005, 0.01, 0.05, 0.1, 0.5, 1.0,

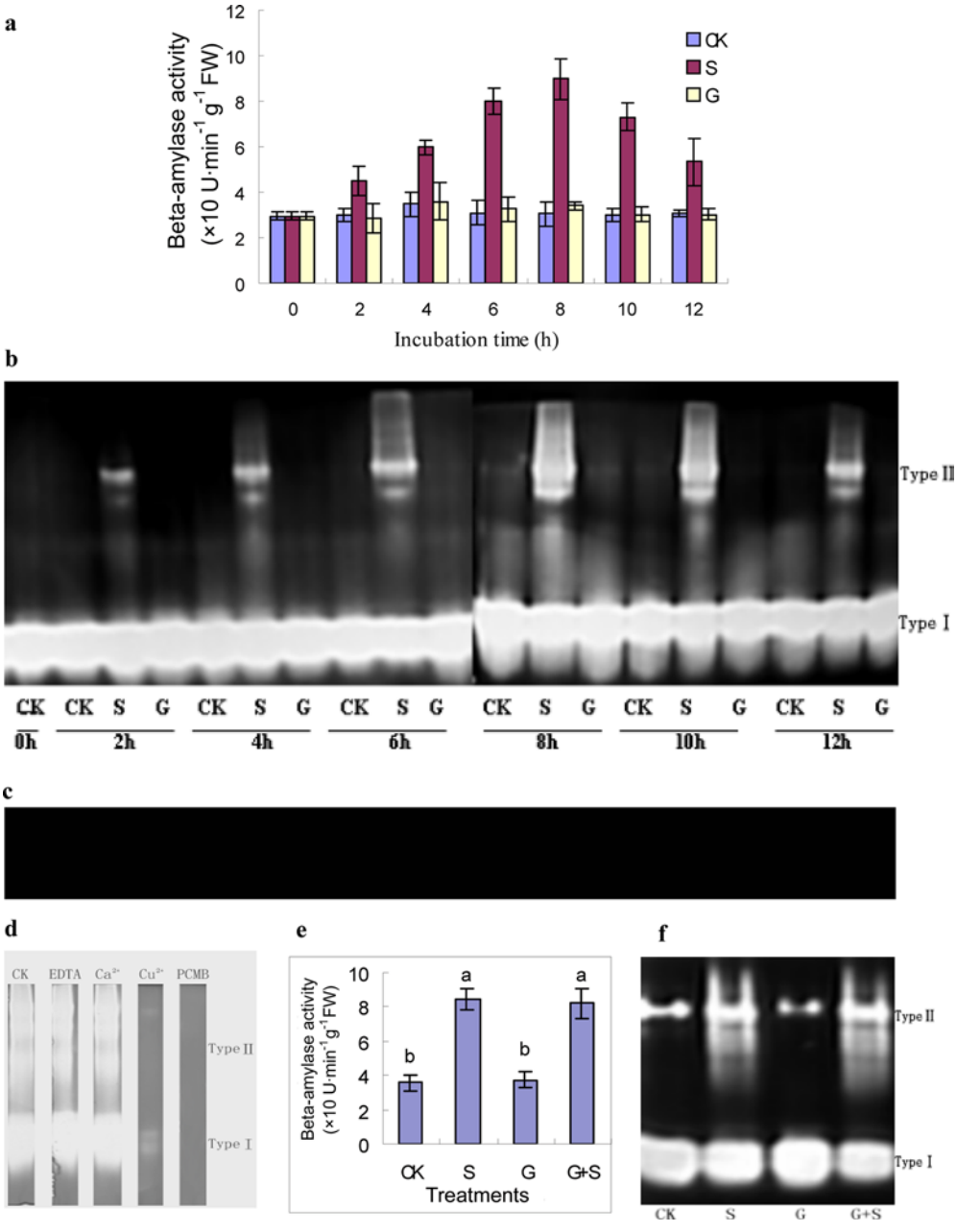

Fig. 2a–f Effects of SNP and GA_3 on amylases in embryoless half-seeds of wheat. Twenty embryoless halves (0.500 ± 0.001 g) were treated for 12 h in the same way as the intact wheat seeds in Fig. 1. **a** Changes in β-amylase activities. **b** Native PAGE of β-amylase (20-µl samples). **c** Native PAGE of α-amylase (30-µl samples). **d**. Effects of inhibitors on total amylase in halves treated with 0.5×10^{-3} mol l^{-1} SNP for 8 h. **e,f** Effects of SNP and GA_3, alone and in combination, on β-amylase activity in halves treated for 8 h. **e** Quantification of β-amylase activity. **f** Native PAGE of β-amylase (20-µl samples). The abbreviations are the same as those in Fig. 1. Each experiment was repeated at least three times. Values in **a** and **e** are the means ± SE of three independent sets of 20 halves, 0.500 ± 0.001 g, each. One-way ANOVA was used for comparisons between the means. Different letters in **e** show significant differences at $P < 0.01$

5.0×10^{-3} mol l^{-1}) confirmed that the induction of β-amylase at 8 h was approximately dose-dependent (data not shown), with a gradual increase to a maximum at 0.5×10^{-3} mol l^{-1} SNP, but no further increase above this concentration. In fact, the transparent bands of the intrinsic type-I isozyme were at a constant level regardless of the SNP concentration, while those of the NO-inducible isozymes, type II, increased in an approximately dose-dependent manner, which was consistent with the data of a β-amylase activity assay (data not shown). α-Amylase could not be detected in either of these two experiments (data not shown).

The β-amylase isozymes induced by NO were further characterized. The pI of the SNP-induced β-amylase, type II, as determined by IEF, was in the range 6.0–7.0, and that of the intrinsically active enzyme, type I, in the range 4.8–6.0. The optimum pH of these amylases was found to be 4.5–6.5, and they were stable in the pH range 3.0–9.0. The optimum temperature of these enzymes was 40°C, but they were active even up to 55°C.

Effects of SNP on the free and bound forms of β-amylase

Induction of β-amylase by SNP might be due to the activation of the free form or the release of the bound form. To obtain monomers of β-amylase, free β-amylase preparations were treated with 2-mercaptoethanol (2-ME), which is a strong de-polymerization reagent and

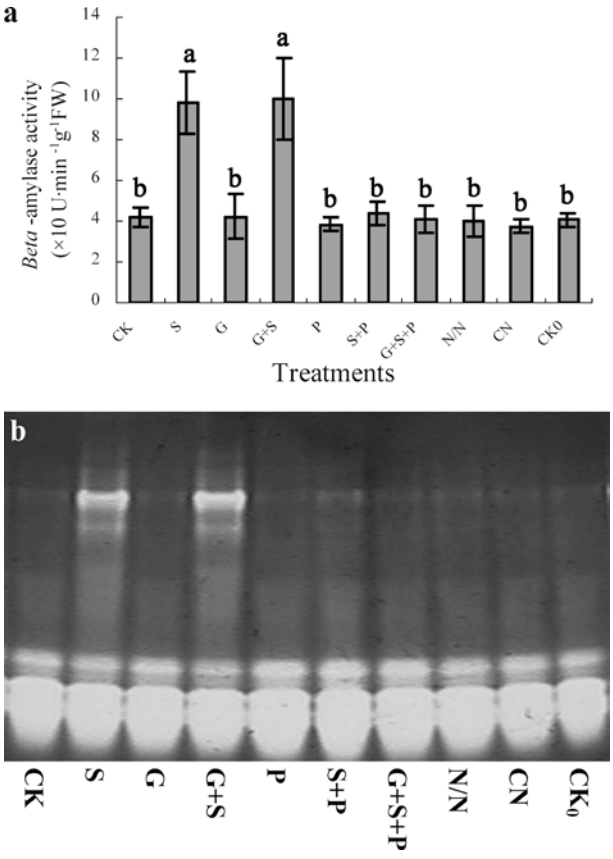

Fig. 3a,b The response of β-amylase to SNP can be attributed to NO. Wheat seeds were treated for 8 h in Tris buffer (see legend to Fig. 1) containing: no additions (*CK*), 0.5×10^{-3} mol l^{-1} SNP (*S*), 10 µmol $l^{-1}GA_3$ (*G*), 10 µmol $l^{-1}GA_3$ plus 0.5×10^{-3} mol l^{-1} SNP (*G*+*S*), 1.0×10^{-3} mol l^{-1}cPTIO (*P*), 0.5×10^{-3} mol l^{-1} SNP plus 1.0×10^{-3} mol l^{-1} cPTIO (*S*+*P*), 10 µmol l^{-1} GA_3 plus 0.5×10^{-3} mol l^{-1} SNP and 1.0×10^{-3} mol l^{-1} cPTIO (*G*+*S*+*P*), 0.5×10^{-3} mol $l^{-1}NO_3^-$ plus 0.5×10^{-3} mol l^{-1} NO_2^- (*N*/*N*), 0.5×10^{-3} mol l^{-1} $K_3Fe(CN)_6$ (*CN*), dry seeds (*CK0*). **a** Changes in β-amylase activities. Values are means ± SE. One-way ANOVA was used for comparisons between the means. Different letters show significant differences at $P<0.01$. **b** Native PAGE of β-amylase (20-µl samples)

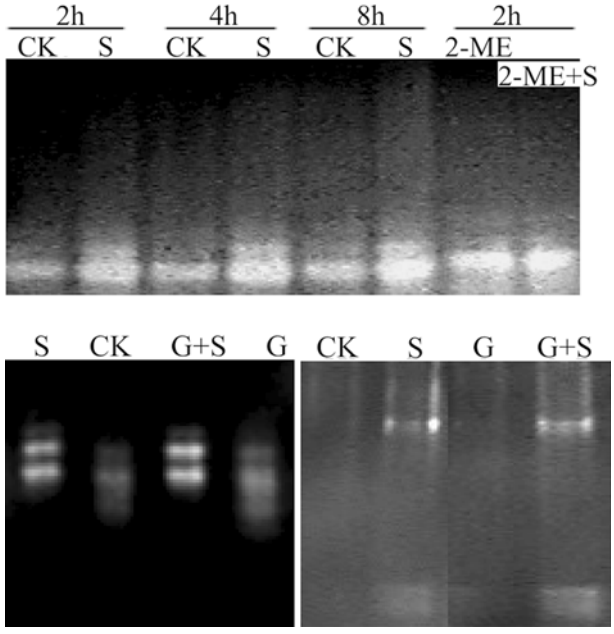

Fig. 4 Effects of SNP on the free and bound forms of β-amylase. *Upper panel* Effects of SNP on monomers and polymers of free β-amylase. Free β-amylase preparations were treated with water (control; *CK*) or 0.5×10^{-3} mol l^{-1} SNP (*S*) for 2, 4 or 8 h. Other samples of the free β-amylase preparation were pre-treated with 15×10^{-3} mol l^{-1} 2-ME for 2 h, and then treated with water (control; *2-ME*) or 0.5×10^{-3} mol l^{-1} SNP (*2-ME*+*S*) for another 2 h. Then 20-µl aliquots of these different preparations were subjected to 5–25% gradient PAGE. *Lower panels* Effects of SNP and GA_3 on the release of latent β-amylase from glutenin (*left*) and its crude enzyme (*right*). Glutenin and latent β-amylase crude extract were treated for 8 h with: water (control; *CK*), 0.5×10^{-3} mol l^{-1} SNP (*S*), 10 µmol l^{-1} GA_3 (*G*), 10 µmol l^{-1} GA_3 plus 0.5×10^{-3} mol l^{-1} SNP (*G*+*S*). After centrifuging, 30 µl of the supernatant was subjected to PAGE

can thoroughly split homopolymers or heteropolymers of the enzyme into monomers. After 2 h pretreatment, the solutions were further treated for 2 h with or without SNP. After gradient PAGE, no differences between 2-ME and 2-ME + SNP treatments could be found (Fig. 4a), demonstrating that SNP plays no role in the activation of β-amylase monomers. However, after treatment of free β-amylase preparations with SNP alone for 2, 4 and 8 h, low-activity polymers with a high molecular weight gradually dissociated, leading to the formation of high-activity monomers with a lower molecular weight, compared with no-SNP treatment samples (Fig. 4, upper panel). For example, within 8 h of SNP treatment bands of β-amylase with a lower molecular weight (monomers) were significantly enhanced, suggesting that NO can promote β-amylase activities through de-polymerizing the aggregates containing the free enzyme.

Normally, the liberation of bound β-amylase is considered to be partially dependent on the breaking of disulphide bands. So we further tested the possibility of whether NO, as an active/reductive molecule, could directly release bound β-amylase from glutenin or its crude enzyme preparation. The observations in Fig. 4 (lower panels) confirm this possibility. When the crude preparation was treated with NO donor for 8 h (lanes S, G + S), the bands were much more obvious than in the two treatments without the NO donor (lanes CK, G), indicating that NO might directly release β-amylase from its bound form.

SNP-induced protease is not responsible for the liberation of bound β-amylase

Aside from disulphide bands broken, the liberation of bound β-amylase was considered to be partially dependent on proteolytic cleavage. Therefore, the changes in protease activities of intact wheat seeds in response to different treatments were also investigated during the early stage of germination. Figure 5a shows that protease activity in the seeds increased slightly in response to

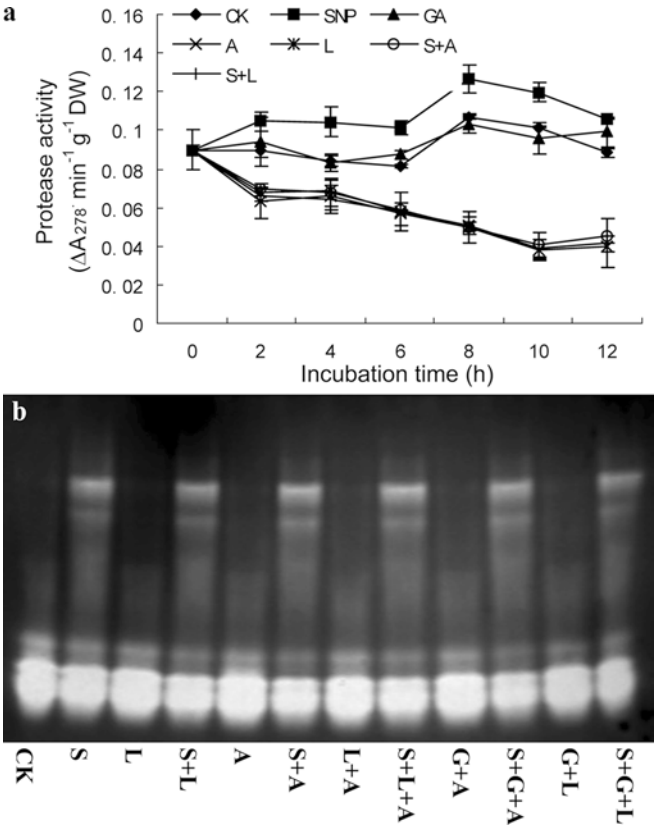

Fig. 5a,b The slight increase in protease activity induced by SNP does not contribute to the increase in β-amylase. **a** Protease activities. Ten intact wheat seeds (0.500 ± 0.001 g) were incubated in Tris buffer (see legend to Fig. 1) containing: water (control; *CK*), 0.5×10^{-3} mol l^{-1} SNP (*SNP*), 10 µmol l^{-1} GA_3 (*GA*), 10×10^{-3} mol l^{-1} antipain (*A*), 50 µmol l^{-1} leupeptin (*L*), 0.5×10^{-3} mol l^{-1} SNP plus 10×10^{-3} mol l^{-1} antipain (*S*+*A*) and 0.5×10^{-3} mol l^{-1} SNP plus 50 µmol l^{-1} leupeptin (*S*+*L*). At intervals (0, 2, 4, 6, 8, 10, 12 h), wheat seeds were homogenized in their own treatment solutions. The supernatants were used for determination of protease activity. **b** Native PAGE of β-amylase. Seeds were treated for 8 h in 50 mmol l^{-1} Tris buffer (see part **a**) containing water (control; *CK*), 0.5×10^{-3} mol l^{-1} SNP (*S*), 50 µmol l^{-1} leupeptin (*L*), 0.5×10^{-3} mol l^{-1} SNP plus 50 µmol l^{-1} leupeptin (*S*+*L*), 10×10^{-3} mol l^{-1} antipain (*A*), 0.5×10^{-3} mol l^{-1} SNP plus 10×10^{-3} mol l^{-1} antipain (*S*+*A*), 50 µmol l^{-1} leupeptin plus 10×10^{-3} mol l^{-1} antipain (*L*+*A*), 0.5×10^{-3} mol l^{-1} SNP plus 50 µmol l^{-1} leupeptin and 10×10^{-3} mol l^{-1} antipain (*S*+*L*+*A*), 10 µmol l^{-1} GA_3 plus 10×10^{-3} mol l^{-1} antipain (*G*+*A*), 0.5×10^{-3} mol l^{-1} SNP plus 10 µmol l^{-1} GA_3 and 10×10^{-3} mol l^{-1} antipain (*S*+*G*+*A*), 10 µmol l^{-1} GA_3 plus 50 µmol l^{-1} leupeptin (*G*+*L*), 0.5×10^{-3} mol l^{-1} SNP plus 10 µmol l^{-1} GA_3 and 50 µmol l^{-1} leupeptin (*S*+*G*+*L*). After homogenization and centrifugation of seeds, samples (15 µl of β-amylase crude extract) were subjected to PAGE. After electrophoresis, the gel was incubated in starch solution for 20 min, then washed with water and stained with iodine

treatment with 0.5×10^{-3} mol l^{-1} SNP (S), then began to decline after 10 h, but remained slightly higher than for the treatments with GA_3 alone (G) and distilled water (CK), both of which had no significant effect. To test whether the slight increase in protease was responsible for the induction of β-amylase, two inhibitors of protease (leupeptin and antipain) were used alone and in combination with SNP. Both of them strongly counteracted the slight increase in protease activity induced by SNP, even reducing it to below the level of the control treatment. The electrophoretic analysis in Fig. 5b shows that there were no differences between the control (lane CK) and inhibitor or inhibitor plus GA_3 treatments at 8 h (lanes L, A, L + A, G + A, G + L), and no differences among SNP treatments whether or not protease inhibitors and GA_3 were added (lanes S, S + L; S + A; S + L + A; S + G + A, S + G + L). However, there were highly significant differences between the treatments with and without SNP. These results indicate that the NO-induced increase in β-amylase was not facilitated by the slight increment in protease caused by SNP.

The rapid response of β-amylase to SNP might be a universal event in a wider spectrum of botanical species

In order to detect whether the rapid response of amylase to NO was a universal event during early germination of other species, seeds of barley, soybean, rice, maize, watermelon, Indian mustard, oilseed rape and *Arabidopsis* were used as research materials in further experiments. When these seeds were treated with the NO donor for 8 h (Fig. 6), there was a rapid induction of β-amylase. β-Amylase activities in all SNP-treated seeds were significantly higher than those in non-SNP-treated samples. In fact, these phenomena could be classified into two types. In the first type, some new β-amylase isozymes were induced, as shown for barley (Fig. 6, upper left panel), rice (Fig. 6, lower panel: 1, 1'), maize (Fig. 6, lower panel: 2, 2') and watermelon (Fig. 6, lower panel: 3, 3'). In the second type, some existing isozyme activities were enhanced, as shown for soybean (Fig. 6, upper right panel), Indian mustard (Fig. 6, lower panel: 4, 4'), oilseed rape (Fig. 6, lower panel: 5, 5') and *Arabidopsis* (Fig. 6, lower panel: 6, 6'). Therefore, we speculate that β-amylase induction by NO might be a universal event in early seed germination. Furthermore, GA_3 played no such role in barley or soybean seeds (Fig. 6, upper panels).

Discussion

In this work, we have provided evidence for an involvement of NO in the rapid induction of β-amylase, but not α-amylase, in both intact seeds (Fig. 1) and embryoless half-seeds of wheat (Fig. 2) during the first 12 h of germination, and have shown that GA_3 has no such role (Figs. 1, 2).

It has been demonstrated that GA regulates the initiation of germination and the mobilization of storage products. However, previous studies on a wide variety of species, including mutants, have shown that GAs play a key role only in the late stages of seed germination (Bewley 1997; Gallardo 2002). Moreover, α-amylase,

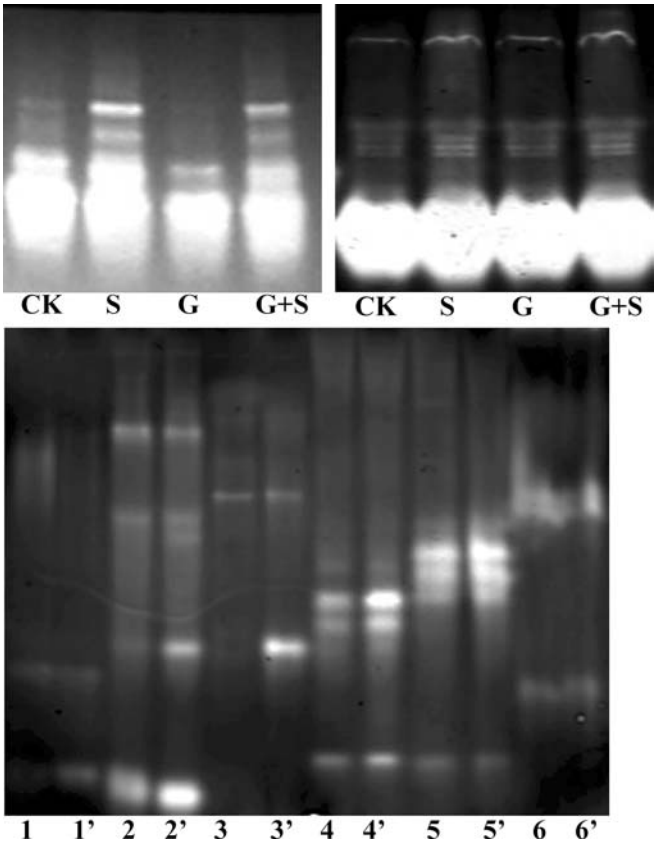

Fig. 6 The rapid response of *β*-amylase to SNP in seeds of other species. *Upper panels* Seeds of barley (*Hordeum vulgare*; *left*) and soybean (*Glycine max*; *right*) were treated for 8 h in Tris buffer (see legend to Fig. 1) containing: water (control; *CK*), 0.5×10^{-3} mol l^{-1} SNP (*S*), 10 μmol l^{-1} GA_3 (*G*), and 10 μmol l^{-1} GA_3 plus 0.5×10^{-3} mol l^{-1} SNP (*G* + *S*). *Lower panel* Seeds of rice (*Oryza sativa*; *1*, *1'*), maize (*Zea mays*; *2*, *2'*) watermelon (*Citrullus vulgaris*; *3*, *3'*), Indian mustard (*Brassica juncea*; *4*, *4'*), oilseed rape (*Brassica napus*; *5*, *5'*), and *Arabidopsis thaliana* (*6*, *6'*) were treated for 8 h with 50×10^{-3} mol l^{-1} Tris–HCl (pH 7.5) containing water (controls; *lanes 1–6*) or 0.5×10^{-3} mol l^{-1} SNP (*lanes 1'–6'*)

synthesized de novo in the aleurone layers in response to GA, is also not involved in the early stage of germination (Collins 1972). Unlike α-amylase, *β*-amylase has not attracted much attention until now. It is well known that *β*-amylase is distinct from α-amylase with respect to its manner of expression during seed germination in cereals. For example, it is already present in the dry seeds, as a result of accumulation during the process of grain development (Ziegler 1999). As mentioned by Nandi et al. (1995), the long-lived *β*-amylase degrades starch during cereal germination until such time as α-amylase might be synthesized de novo in the aleurone; thus *β*-amylase helps to initiate germination and support early seedling growth. Moreover, their results suggested that *β*-amylase activity could be used as an index for cereal germination potential, due to the fact that it was associated with initiation of germination whereas α-amylase affected the rate of seedling growth at a later stage of germination (Nandi et al. 1995).

Our previous work has shown that the NO donor SNP was able to stimulate the germination of wheat seeds under osmotic stress, and even induce the activities of amylase in embryoless half-seeds during early imbibition (for 6 h) under normal conditions, a process which might be independent on GA_3 (Zhang et al. 2003). Beligni and Lamattina (2000) found that NO donors effectively stimulated germination of lettuce seeds. Recent research also illustrated that NO could stimulate germination of *Lupinus luteus* seeds and counteract the inhibitory effect of heavy metals and salinity on root growth (Kopyra and Gwozdz 2003). In this paper, we further showed that employment of an NO donor was able to induce a rapid increase in *β*-amylase, but not α-amylase, during the early germination of intact wheat seeds (12 h, Fig. 1). In contrast, when GA-treated barley aleurone layers were incubated with NO donors, the rate of α-amylase secretion and the total amount of α-amylase produced were more than from layers treated with GA alone; also, α-amylase accumulation in the medium was enhanced slightly from 12 to 60 h of treatment by different NO donors (Beligni et al. 2002). However, Leshem et al. (1998) have testified that NO occurs in a wide spectrum of botanical species ubiquitously, and endogenous generation of NO from either enzymatic or non-enzymatic pathways was recently reported in different tissues and organs, especially aleurone layers and roots (Chandok et al. 2003; Beligni et al. 2002; Bethke et al. 2004). Recently, Simontacchi et al. (2004) reported a rapid burst of NO during early germination of sorghum seeds. Figure 6 also illustrates that the rapid response of *β*-amylase to SNP treatment for 8 h exists in a wide spectrum of botanical species seeds, including barley, soybean, rice and watermelon. The above results indicate that the NO-induced enhancement of *β*-amylase within the first 12 h of treatment (Fig. 1) might be an important issue in relation to early germination, and the burst of endogenous NO production during seed germination might also act as a signal molecule (Simontacchi et al. 2004).

In fact, as a small, highly diffusible and redox molecule, NO could rapidly cross biological membranes and trigger diverse biological response in a short period of time. It might provide some possible explanation for the complexities of the rapid response of *β*-amylase (Figs. 1, 2, 6) and the stimulation of wheat seed germination (Zhang et al. 2003). It is well known that application of 0.15×10^{-3} mol l^{-1}SNP produces less than 0.2×10^{-6} mol $l^{-1}$of NO in plant tissues (García-Mata and Lamattina 2001), which is approximately consistent with the level of endogenously generated NO reported by other researchers (Leshem et al. 1998; Simontacchi et al. 2004). In our experiment, application of 0.5×10^{-3} mol l^{-1} of the NO donor SNP could directly de-polymerize *β*-amylase aggregates (Fig. 4a) and release the enzyme from its bound form (Fig. 4b,c), as could high concentrations (1×10^{-3} to approx. 15×10^{-3} mol l^{-1}) of reductive substances such as 2-ME (Guerin et al. 1992). In the early stages of seed germi-

nation, however, it would certainly be impossible to produce the effects of such high concentrations of 2-ME, DTT or even cysteine etc. in vivo. The results of enzyme activity determination and PAGE analysis using SNP and two protease inhibitors (leupeptin and antipain) illustrated that the slight enhancement of protease activity was not responsible for the induction of β-amylase (Fig. 5). Moreover, all of our results indicate that there are no additive effects between GA_3 and NO, and that the two active molecules might function in different signalling cascades.

As early as 1962, Rowsell and Goad (1962a, 1962b) predicted that developing wheat embryos secreted an active substance that released glutenin-bound β-amylase. In 1979, Okamoto and Akazawa also postulated that at the onset of germination, the activation and subsequent release of β-amylase from starch granules, which might be activated by some unknown molecule(s) secreted from the epithelium, was the primary step of starch digestion. In view of recent research (Chandok et al. 2003; Simontacchi et al. 2004), we speculate that this active substance might be NO or its derivatives, which could act as an early stimulator of β-amylase (through splitting and aggregating bound forms) to initiate the germination, while GA plays a key role in the later stage of germination. It must be mentioned, however, that germination involves mobilization of reserves as a whole, rather than being an event associated with one or two enzymes. It would be interesting to study the further regulatory mechanisms of early germination controlled by NO in vivo.

Acknowledgement This work was supported by the National Natural Science Foundation of China (No. 30471049), by a grant to Innovative Young Scholars of Jiangsu Province in China (No. BK2004417), and by Undergraduate's Creative Projects in Colleges of Jiangsu Province (China; fellowship to H. Zhang).

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