Title:

Evaluation of the anti-nitrative effect of plant antioxidants using a cowpea Fe-superoxide dismutase as a target

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Running title: Search for anti-nitrative compounds in plants

Abstract

Nitric oxide cytotoxicity arises from its rapid conversion to peroxynitrite ($ONOO^-$) in the presence of superoxide, provoking functional changes in proteins by nitration of tyrosine residues. The physiological significance of this post-translational modification is associated to tissue injury in animals, but has not been yet clarified in plants. The objective of this study was to establish new approaches that could help to understand $ONOO^-$ reactivity in plants. A recombinant Fe-superoxide dismutase from cowpea (*Vigna unguiculata* (L.) Walp.), r*Vu*FeSOD, was the target of the $ONOO^-$-generator SIN-1, and the anti-nitrative effect of plant antioxidants and haemoglobins was tested in vitro. Nitration on r*Vu*FeSOD was evaluated immunochemically or as the loss of its enzymatic activity. This assay proved to be useful to test a variety of plant compounds for anti-nitrative capacity. Experimental data confirmed that rice (*Oryza sativa* L.) haemoglobin-1 (r*Os*HbI) and cowpea leghaemoglobin-2 exerted a protective function against $ONOO^-$ by diminishing nitration on r*Vu*FeSOD. Both plant haemoglobins were nitrated by SIN-1. The chelator desferrioxamine suppressed nitration in r*Os*HbI, indicating that Fe plays a key role in the reaction. The removal of the haem moiety in r*Os*HbI importantly suppressed nitration, evidencing that this reaction may be self-catalyzed. Among small antioxidants, ascorbate remarkably decreased nitration in all tests. The phenolic compounds caffeic acid, gallic acid, pyrogallol, 4-hydroxybenzoic acid and the flavonoid gossypin also diminished tyrosine nitration and protected r*Vu*FeSOD to different extents. It is concluded that small plant antioxidants, especially ascorbate and haemoglobins may well play key roles in $ONOO^-$ homeostasis in vivo.

Abbreviations

Hbs, haemoglobins; NADH, nicotinamide adenine dinucleotide; ·NO, nitric oxide; NO_2-Tyr, nitrotyrosine; $ONOO^-$, peroxynitrite; RNS, reactive nitrogen species; ROS, reactive oxygen species; r*Os*HbI, recombinant rice haemoglobin-1; r*Vu*LbII, recombinant cowpea leghaemoglobin-2; r*Vu*FeSOD, recombinant cowpea iron-superoxide dismutase; SODs, superoxide dismutases; Tyr, tyrosine

Keywords

Antioxidants; Haemoglobins; Peroxynitrite; RNS; SODs; Tyrosine Nitration; *Vigna unguiculata*

1. Introduction

Nitric oxide (·NO) is considered a fundamental signalling molecule in many organisms, including plants, where its implication has been addressed in numerous physiological processes (Besson-Bard et al., 2008). Also, it may be a potent mediator of cellular damage in a wide range of conditions (Pacher et al., 2007). Recent evidence indicates that most of the cytotoxicity attributed to ·NO is rather due to free radical peroxynitrite ($ONOO^-$), produced from the diffusion-controlled reaction between ·NO and superoxide radical ($O_2^{\cdot-}$) (Pacher et al., 2007; Radi, 2013). Thus, NO may regulate a signalling pathway through the modification of tyrosine-kinases, or even induce cell death, either apoptotic or necrotic (Pacher et al., 2007).

Due to the short half-life of $ONOO^-$ (ca. 5-20 ms) (Radi, 2013), it is rather difficult to be directly measured. It is more usual to detect the biochemical modifications arising after its oxidative attack. Currently, the main analytical assay for $ONOO^-$ effect is the immunochemical assay for protein tyrosine nitration. Tyrosine (Tyr) nitration often occurs when $ONOO^-$ reacts with protein tyrosine residues originating negatively charged hydrophilic nitrotyrosines (NO_2-Tyr) (Radi, 2013). In animals, this alteration is often related to certain pathologies (MacMillan-Crow et al., 1996; Ischiropoulos, 1998). Although tyrosine nitration has been less studied in plants, it is currently gaining considerable interest and there are descriptions of nitration targets in a wide variety of species (Chaki et al., 2009; Melo et al., 2011; Serrano et al., 2012; Begara-Morales et al., 2013).

Superoxide dismutases (SODs; EC 1.15.1.1) catalyze the dismutation of $O_2^{\cdot-}$ into hydrogen peroxide (H_2O_2) and molecular oxygen, as a part of the cell antioxidant machinery. It is known that ·NO and ·NO-derived molecules interact with several Fe-containing proteins including the mentioned SODs (Jackson *et al*., 2003) or haemoglobins (Hbs) (Kalinga, 2006). Our group reported an assay using a recombinant FeSOD from the legume cowpea (*Vigna unguiculata* (L.) Walp.) as a target of $ONOO^-$ attack, which could be employed as a marker of nitrative stress in vitro (Larrainzar et al., 2008). It was acknowledged that certain Tyr residues were nitrated by $ONOO^-$ in recombinant cowpea Fe-superoxide dismutase (rVuFeSOD), and such modification was detected either immunochemically or as the estimation of the loss of enzymatic activity (Larrainzar et al., 2008). The native cowpea FeSOD is an unusual plant FeSOD for it exhibits a cytosolic localization (Moran et al., 2003). Moreover, this Fe- and some other

cambialistic SODs have been located in the nucleus of plant cells (Rubio et al., 2009; Asensio et al., 2011), evidencing the signaling role of free radicals within this compartment. The fact that SODs are targets of $ONOO^-$ points out that these enzymes might play a role in the response to environmental stresses as modulators of the essential reactive oxygen and nitrogen species (ROS/RNS) production in plants (Asensio et al., 2012).

In the last decades, a wide range of molecules have been somehow related to ·NO homeostasis in biological systems. Haemoglobins have emerged as ·NO modulators in plants and in their presence, ·NO half-life is significantly reduced, as ·NO is rapidly oxidized originating nitrate and met-Hb (Dordas et al., 2003). Hence, Hbs, as well as FeSODs, may be modulating the contents or regulating the action of ROS and RNS in biological systems. Additionally, in vitro studies with ascorbate have shown that it is an effective agent against $ONOO^-$ attack (Kirsch and de Groot, 2000), and consequently its role in nitration reactions deserves further studies. Also, plant phenolic compounds have been considered as antioxidants against ROS (Moran et al., 1997) but few studies have provided evidence on the antioxidant role of phenolic and reducing compounds as anti-nitrative molecules. Additionally, it has already been stated that Hbs, ascorbate and phenolic compounds can control the levels of ROS/RNS (Igamberdiev et al., 2006).

The present work evaluates the anti-nitrative effect of some small plant antioxidants, using assays with r*Vu*FeSOD as a target in order to estimate their $ONOO^-$ scavenging capacity. Besides, the role of two cytosolic Hbs, rice (*Oryza sativa* L.) haemoglobin-1 (*Os*HbI) and cowpea leghaemoglobin-2 (*Vu*LbII) is studied in relation to r*Vu*FeSOD Tyr nitration. Our data suggest that some plant ferroproteins and small antioxidant molecules interact to modify the degree of protein nitration, which may eventually have important consequences in ROS/RNS signalling.

2. Materials and Methods

2.1. Chemicals and biological material

All reagents were purchased from Sigma (St. Louis, MO, USA), except where indicated. Nickel-HisTrap HP and Q Sepharose FF chromatography columns were supplied by GE Healthcare (Uppsala, Sweden).

The bacterial strain used to over-express cytosolic cowpea Fe-superoxide dismutase contained the construct pET28a(+)::Vu*FeSOD* (Moran et al., 2003). *Escherichia coli* strain used in the over-expression of cowpea Lb-2 was kindly provided by Dr. Raúl Arredondo-Peter (Arredondo-Peter et al., 1997). Rice Hb-1 was cloned from vector pEMBL-19-Os*HbI* construct. PCR was performed with *Pfu* polymerase (Biotools B&M Labs) following the manufacturer protocols. The amplicon was digested and cloned into pET28b(+) between NdeI and BamHI restriction sites. The vector was introduced into *E. coli* XL-1 blue cell line. Resultant colonies were screened by PCR and positive ones were sequenced in order to check the lack of errors. Selected constructions were cloned into Novablue cells (Novagen), which was the line used for the over-expression.

2.2. Production and purification of recombinant proteins

In order to obtain the recombinant proteins a self-inducible method was employed. This option was chosen as the normal IPTG induction method generates proteins too fast, causing an impairment between the prosthetic group and the apo-protein. In addition, this method does not generate inclusion bodies (Studier, 2005). Cells containing the constructs were spread at 37 ºC over Petri dishes containing Luria-Bertani broth supplemented with kanamycin (100 mg · ml^{-1}). A colony from the plate was picked and 5 ml of ZYP-0.8G medium were inoculated, and the culture was maintained overnight in gentle stirring at 37 ºC and used for the inoculation of 500 ml of ZYP-5052 in 2-l flask at final absorbance A_{600}=0.05. The recombinant proteins were purified using a 5 ml-nickel column (HisTrap HP) and subsequently an anion exchange chromatography step was performed according to Urarte et al. (2008). Unlike r*Vu*FeSOD, that was dialyzed overnight after the first purification step against 10 mM sodium phosphate buffer (pH 7.0) containing 1 mg thrombin, both haemoglobins were dialyzed against 50 mM Tris-HCl and 0.05 % Triton X-100 (pH 7.5).

Apo-r*Os*HbI was generated by incubation of protein aliquots at 80 ºC for 30 min with 100 mM carbonate buffer pH 9.6. Subsequently, samples were centrifuged using centricons (5,000 MWCO PES; Vivaspin, Sartorius, Göttingen, Germany) for 15 min at 4,000 *g*. The haem group visibly precipitated and the supernatant was sterilized through a 0.22 μm filter syringe. An aliquot of the supernatant was electrophoresed in 12.5 % (w/v) SDS-PAA gels in order to monitor the integrity of the apo-protein.

2.3. Protein nitration system

The nitration system included SIN-1 (3-morpholinosydnonimine-*N*-ethylcarbamide, Invitrogen) as $ONOO^-$ generator. SIN-1 simultaneously generates equimolar quantities of ·NO and $O_2^{\cdot-}$, which originates $ONOO^-$ and nitrates r*Vu*FeSOD (Feelisch et al., 1989). The nitration assay was performed as described in Larrainzar et al. (2008). Briefly, recombinant *Vu*FeSOD aliquots (Moran et al., 2003) were incubated with 1 mM SIN-1 in a reaction buffer (50 mM sodium phosphate, pH 7.4) at 37 ºC for 2 hours. Some samples were additionally incubated with r*Os*HbI and r*Vu*LbII and the effect of NADH, desferrioxamine and ascorbate was also assessed. All samples were bubbled for a few seconds with air before incubation.

For assaying the influence of different plant antioxidant molecules on tyrosine nitration, r*Vu*FeSOD was further incubated with the following compounds at a final concentration of 0.3 mM (Moran et al., 1997): gallic acid, caffeic acid, pyrogallol, 4-hydroxybenzoic acid, and gossypin (Indofine Chemicals Inc., USA). The chemical composition of these molecules is illustrated in Supplementary Fig. 2.

All these assays were performed at pH 7.4, a physiological cell pH (Moran et al., 1997). The same tests were made at acidic pH (5.8) but the SIN-1-mediated nitration system was not effective in this case, and it did not provoke a decrease in r*Vu*FeSOD activity when it was added to the mixture (*not shown*).

2.4. Detection of 3-nitrotyrosine by immunoblot analysis

After incubation, proteins (1 μg per lane) were separated on 12.5 % (w/v) SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes (PDVF, Pall Corporation, Pensacola, USA). Membranes were blocked with 1 % (w/v) BSA in Tris-Buffered Saline. Protein tyrosine nitration was immunochemically detected using polyclonal anti-

3-nitrotyrosine antibody (1: 3,000, v/v) purchased from Upstate Biotechnology (Lake Placid, NY). As secondary antibody, goat anti-rabbit IgG alkaline phosphatase conjugate (1: 5,000, v/v) was used. Cross-reacting 3-nitrotyrosine bands were visualized using NBT/BCIP mix as a substrate. To confirm equal protein loading, replica gels were run in parallel and stained using Gel-Code Blue Stain reagent (Pierce Biotechnology, Inc, Rockford, USA).

2.5. In-gel and spectrophotometric SOD activity assays

In-gel SOD activity assays were performed as described in (Beauchamp and Fridovich, 1971). Recombinant *Vu*FeSOD was separated on 15 % (w/v) native PAGE in the cold room and the electrophoresis was run for 2 hours at 200 V. As a complement to the in-gel activity assay, SOD activity was measured by the spectrophotometric method (McCord and Fridovich, 1981). Briefly, reduction of ferricytochrome-*c* was monitored at 25 ºC by following the increase in absorbance at 550 nm for 2 min (Larrainzar et al., 2008). The reaction was started by the addition of 5 µl of diluted xanthine oxidase. Commercial milk xanthine oxidase (Sigma) was 7-fold diluted in reaction buffer in order to reach 0.080-0.100 units of absorbance variation within the blank mix.

2.6. Statistical analysis

The quantitative data obtained from these studies were analyzed with the program IBM SPSS Statistics (21.0). The mean was used as a measure of central tendency and the standard error (S.E.) as a measure of dispersion. Student's t-test was employed in order to compare the means of the samples. In all cases, statistical analyzes were conducted at a significance level of 5 % ($P \leq 0.05$).

3. Results

3.1. Recombinant Vu*FeSOD and plant haemoglobins rice Hb-1 and cowpea Lb-2 are nitrated by SIN-1*

Recombinant cowpea FeSOD (r*Vu*FeSOD) was over-expressed and purified to near homogeneity as evidenced by SDS-PAGE electrophoresis (Suppl. Fig. 1). This protein has been described as a target for Tyr nitration and it is used as a nitration model by SIN-1 along the study. Rice Hb-1 (r*Os*HbI), which possesses a hexacoordinate haem conformation, and cowpea Lb-2 (r*Vu*LbII), which is a pentacoordinate haemoglobin, were employed here to study their influence on the r*Vu*FeSOD nitration pattern.

The immunoassay, that implied detection with antibody against 3-nitrotyrosine, showed that r*Vu*FeSOD was visibly nitrated by SIN-1, but r*Os*HbI and r*Vu*LbII were nitrated as well (Fig. 1A). When reduced nicotinamide adenine dinucleotide (NADH) was added, a subtle but consistent decrease on r*Vu*FeSOD nitration was observed (Fig. 1A, lane 3 and Fig. 1B). When r*Vu*FeSOD was incubated along with r*Os*HbI or/and r*Vu*LbII, a thinner 3-nitrotyrosine band for r*Vu*FeSOD was detected on the immunoblot (Fig. 1A, lanes 4 to 8) and densitometry also showed decreasing nitration values. This effect was enhanced by NADH and when both globins were present together in the reaction mix. When BSA was present in the system instead of Hbs, r*Vu*FeSOD nitration values remained the same (Fig. 2), excluding a non-specific protein effect. The subtle anti-nitrative effect of NADH was additionally confirmed in Fig. 2.

To further characterize the interaction of r*Os*HbI and $ONOO^-$, the r*Os*HbI-containing nitrating mix was also incubated with sodium ascorbate, r*Vu*LbII, NADH or desferrioxamine. It was yet again observed that both Hbs possessed nitrated residues after exposition to $ONOO^-$ (Fig. 3A, lanes 2 and 3). NADH originated a slight but consistent decrease of the NO_2-Tyr signal in r*Os*HbI and r*Vu*LbII (Fig. 3A, lanes 4 and 7; Fig. 3B). More remarkable was the effect of ascorbate and desferrioxamine, which practically suppressed Tyr nitration in r*Os*HbI (Fig. 3A, lanes 5 and 6; Fig. 3B).

3.2. The haem group plays a role in Hb self-nitration

In order to analyze the implication of the haem group on Tyr nitration, the haem moiety was removed from the protein and the effect of SIN-1 in apo-r*Os*HbI was tested (Fig. 4). Our results showed that SIN-1-mediated nitration was importantly suppressed when

the haem moiety was removed from r*Os*HbI, while desferrioxamine fully inhibited both r*Os*HbI and apo-globin Tyr nitration. When r*Vu*FeSOD and r*Os*HbI were incubated together, desferrioxamine seemed to protect r*V*uFeSOD to a higher extent (Fig. 4).

A 3D crystallographic study of the Hbs revealed Tyr residues close to the prosthetic group with a probable role in nitration (Fig. 5). Rice Hb-1 (Hargrove et al., 2000) contains a tyrosine located at 8.03 Å of the Fe at the haem group (Tyr151) which may well be subject for nitration, and soybean Lb*a* (Hargrove et al., 1997) has two residues (Tyr31 and Tyr134) very close to the catalytic centre as well (Fig. 5). It has been proved that *Vu*LbII conserves the same two tyrosines near the catalytic centre (Arredondo-Peter et al., 1997).

3.3. Evaluation of small antioxidants as protectors against tyrosine nitration

By using some of the compounds tested in previous assays on free radical scavenging (Moran et al., 1997), we found that some of them also mitigated NO_2-Tyr production (Fig. 6). All antioxidants employed in the immunoassay (ascorbate, gossypin, gallic acid, caffeic acid, pyrogallol and 4-hydroxybenzoic acid) decreased r*Vu*FeSOD Tyr nitration in comparison to the control nitrated sample (Fig. 6, lane 2), being the aromatic compounds gossypin, gallic acid and caffeic acid those with the highest capacity to inhibit Tyr nitration (Fig. 6, lanes 4 to 6). Again, the presence of rice Hb-1 in the mix resulted in a decrease in the levels of nitrotyrosine (Fig. 6, lane 9).

In order to confirm the antioxidant role of some of the molecules tested, SOD enzymatic activity was also evaluated in-gel and by the spectrophotometric assay (Larrainzar *et al*., 2008). Gallic and caffeic acids slightly decreased r*Vu*FeSOD enzymatic activity in the absence of SIN-1, while the other phenolics did not produce any modifications (Fig. 7A, left panel). In the nitration assay, SIN-1 induced a remarkable decrease in SOD activity. Desferrioxamine, ascorbate, gossypin, caffeic acid and r*Os*HbI proved to be the molecules that best preserved the enzymatic activity in the in-gel assay (Fig. 7, right panel). Gallic acid, pyrogallol and 4-hydroxybenzoic acid had a lower anti-nitrative effect, but they were still able to maintain SOD activity to a detectable level.

The absence of nitration on either r*Vu*FeSOD or BSA after incubation with SIN-1 at a lower pH (5.8 instead of 7.4) was observed using immunodetection with anti-NO_2Tyr antibody and confirmed for r*Vu*FeSOD activity in the in-gel assay (*not shown*). This pH

effect may be related to a much slower decomposition rate of SIN-1 at pH 5.8 than at neutral pH (Asahi et al., 1971).

Additionally, a second assay, which detected SOD activity by following the inhibition of cytochrome-*c* (cyt c) reduction by $O_2^{\cdot -}$ was employed (Larrainzar et al., 2008). The spectrophotometric assay confirmed the general antioxidant role of rice Hb-1 (Fig. 8), and of ascorbate (Figs. 8 and 9) and desferrioxamine (Fig. 9). Hydroxybenzoic acid, on the contrary, did not seem to preserve r*Vu*FeSOD activity as it did in-gel. The spectrophotometric assay proved ineffective to evaluate most of the phenolic compounds, as some of them strongly interfered with cytochrome-*c* reduction (Suppl. Fig. 3). Thus, gossypin (+ 37.5 %), gallic acid (+ 110 %) and pyrogallol (+ 125 %) directly enhanced cyt c reduction in a significant way in the absence of an incubation period for SIN-1 analysis. It is also feasible that some of the compounds, such as gossypin, which exhibited a yellowish colour in solution, and pyrogallol, which showed a brownish colour after incubation with SIN-1, may be masking cyt c absorbance at 550 nm. Therefore, we concluded that the spectrophotometric assay was not suitable to examine these three antioxidants (gossypin, gallic acid and pyrogallol) and the corresponding results in Fig. 8 must be interpreted in the light of these considerations. Both r*Os*HbI and ascorbate slightly enhanced reduction of cyt c in the absence of SIN-1 with respect to the control, but never above 10 % (Figs. 8 and 9 respectively). However, when we included SIN-1 in the mixture, r*Os*HbI preserved the enzymatic activity up to 30 % (Fig. 8). Ascorbate also showed a highly protective function so it was further analyzed to study the dose effect. For this purpose, the enzyme was exposed to a range of concentrations (from 0.1 mM to 1 mM) of ascorbate along with SIN-1, and its enzymatic activity was followed using both SOD activity methods (Fig. 9). Results showed a dose-dependent effect on ascorbate protective role up to 0.7 mM ascorbate. From that point on, enzymatic activity decreased, although not in a prominent manner.

4. Discussion

4.1. Self-nitration of plant haemoglobins and mechanism of Fe-mediated nitration of rVuFeSOD

Plant haemoglobins have already shown to cope with the ·NO molecule (Dordas et al., 2003), which in our nitration model is produced by decomposition of SIN-1. While Tyr

nitration of a hexacoordinate Hb has been previously reported for *Arabidopsis* Hb-1 (Sakamoto et al., 2004), we show here that both penta- and hexacoordinate Hbs from plants became tyrosine-nitrated as a result of their interaction with $ONOO^-$ (Figs. 1A and 3A). The Fe atom in the haem group is implicated in the self-nitration reaction of Hbs, since the removal of the haem from r*Os*HbI fairly suppressed Tyr nitration (Fig. 4). The fact that some residual nitration was still detectable was probably due to non-haem-Fe loosely bound to the Hb, as evidenced by the full suppression of nitration after the treatment of the apo-protein with desferrioxamine, which once again confirms that Fe is the active molecule. Deeb and cols. (2006) already showed the implication of the haem in the self-nitration of a non-plant haem-protein. The prostaglandin H_2 synthase holoenzyme was fully inactivated when exposed to $ONOO^-$ but the apo-enzyme was still able to metabolize arachidonic acid after incubation with the nitrating agent. Nitration of catalytic residue Tyr385 did not occur in the absence of the haem, pointing out that the prosthetic group was essential to trigger the inactivation of the protein (Deeb et al., 2006).

The 3D crystallographic models of *Os*HbI and *Gm*Lb*a* (the latter related to *Vu*LbII), showed several Tyr residues in the vicinity of the haem (Fig. 5). The initial hypothesis was that coordination of the haem was a relevant factor in tyrosine nitration, as hexacoordination allows a higher affinity for ligands than pentacoordination (Kundu et al., 2003). Nevertheless, in our data, both Hbs showed similar nitration levels despite the structural differences in their haem pockets (Figs. 1 and 3).

In plants, class 1 Hbs scavenge ·NO and generate NO_3^-, having an effect in growth, development and several stress responses (Groβ et al., 2013). Human Hbs are known to oxidize $ONOO^-$ to NO_3- *in vitro*, action mediated by thiol-dependent peroxidases in plants (Sakamoto et al., 2004). In the last decades, several haem-proteins have been reported to become nitrated, both in animal and plant phyla. Nitration of several Tyr residues of the haem moiety in human myoglobin was described (Nicolis et al., 2006) and horseradish peroxidase appeared to be nitrated on the haem 4-vinyl groups (Wojciechowski and de Montellano, 2007). Nitration mechanisms in plant leghaemoglobins have been recently elucidated, where the nitro group ($-NO_2$) is added to the 4-vinyl of the prosthetic group (Navascues et al., 2012). In legumes, the formation of nitrated green Lbs is predominantly observed during the senescence of the nodule (Jun et al., 1994). All this data substantiates the importance of haem-mediated nitration mechanisms among different groups of organisms. Nevertheless, little

information has been obtained to date concerning the fate of nitrated Hbs in biological systems. In a recent work, nitration of human Hb-Tyr residues at a low level resulted in a conformational change in the vicinity of the haem, which decreases Hb affinity for the ligand. However, an excess of $ONOO^-$ was capable of opening the haem pocket enhancing peroxidase activity, which evidenced the implication of these reactions in the prevention of nitrative damage to other proteins (Xiao et al., 2009). It has also been suggested that red blood Hb can be "sacrificed" in order to detoxify free radicals, as erythrocytes are constantly renovated and dame to other proteins would result in more significant effects (Barbieri et al., 2013). In plants, a peroxidase-like function for non-symbiotic Hbs has been evidenced in *Arabidopsis* (Sakamoto et al., 2004), for rice Hb-1 (Violante-Mota et al., 2010) and for the non-symbiotic Hb of the legume *Medicago sativa* (Maasen and Hennig, 2011). However, kinetic data suggest that rice Hb-1 is unlikely to physiologically function as a peroxidase in planta (Violante-Mota et al., 2010), and further experimentation will be necessary to understand the role of nitrated Hbs.

Regarding r*Vu*FeSOD, Fe-mediated nitration had been addressed some years ago by its suppression with the chelator desferrioxamine (Larrainzar et al., 2008). As it has been observed in non-Fe proteins such as BSA, nitration may well be exerted by trace Fe loosely bound to the protein (Larrainzar et al., 2008). Cowpea FeSOD shows 9 Tyr residues per polypeptide, and two of them are located within the 10 Å radio of the Fe at the active center: Tyr51, whose hydroxyl group is located at a distance of 5.6 Å and Tyr212, which is 7.5 Å from the catalytic Fe in the 3D model (Muñoz et al., 2005). Nitration of residues in the vicinity of the active center was evidenced in r*Vu*FeSOD by the loss of enzymatic activity. In our nitration system, the anti-nitrative effect of desferrioxamine could be explained by its capacity to chelate loosely bound-Fe and also by its interaction with Fe at the active site, as nitration is fully suppressed when this compound is present in the system.

4.2. Tyrosine nitration on rVuFeSOD and the loss of enzymatic activity can be used to evaluate the effect of plant antioxidants

In Larrainzar et al. (2008), anti-NO_2Tyr immunoblots and activity gels provided an indirect quantitation of the effective Tyr nitration in r*Vu*FeSOD. Based on these tests,

we have evaluated the capacity of certain antioxidant compounds of plant origin (Suppl. Fig. 2) to scavenge $ONOO^-$ or to avoid its production.

The role of ascorbate as a $ONOO^-$ scavenger has been reported in vitro in kinetic (Kurz et al., 2003) and in antioxidant-capacity evaluating assays (Moran et al., 1997), while several aromatic compounds have been described to modulate production of ROS in legume plants (Moran et al., 1997). In our study, the compounds tested exhibited a predominantly high anti-nitrative effect on the immunoblot with anti-NO_2-Tyr antibody (Fig. 6), and protected in general terms r*Vu*FeSOD enzymatic activity in-gel (Fig. 7). Caffeic and gallic acids, and the flavonoid gossypin are known to exert an antioxidant and reducing action even at neutral pH (Cai et al., 1999; Moran et al., 1997) and they were very efficient in preventing Tyr nitration on r*Vu*FeSOD (Fig. 6). The in-gel assay confirmed the results obtained in the immunoblot, although some specific differences could be observed regarding aromatic compounds with respect to ascorbate and r*Os*HbI. Thus, aromatic compounds promoted a higher protection than ascorbate and r*Os*HbI in the immunoblot assay, but, in general, they all maintained similar SOD activities. This effect could be explained if ascorbate and the globin specifically protected Tyr residues near the r*Vu*FeSOD active centre, a suggestion that deserves further research. To our surprise, pyrogallol exhibited an important anti-nitrative effect after detection with antibody (Fig. 6) but this was not consistent with the SOD activity observed in the in-gel assay (Fig. 7), where the activity band was perceptibly weakened in the presence of this compound.

Regarding the antioxidant mechanisms of the aromatic compounds tested, some of them may act as antioxidants by scavenging ROS and delocalizing the unpaired electron within the aromatic rings (Halliwell and Gutteridge, 2007). Other aromatic compounds, like for instance gossypin and caffeic and gallic acids, may exert a reducing action even at neutral pH (Moran et al., 1997), or act as metal chelators suppressing their initial effects and the subsequent chain reactions, as it is the case of gallic acid and gossypin (Moran et al., 1997; Paganga et al., 1996). The existence of hydroxyl groups in the ring structure of the phenolics tested may be a competition mechanism towards the hydroxyl of the Tyr molecule (Suppl. Fig. 2).

4.3. Haemoglobins, ascorbate and antioxidant metabolism in plants

The relation between Hbs and antioxidant metabolism has already been evidenced in plants. Using alfalfa root mutants it was proved that Hb-over-expressing tissues had an increased antioxidant metabolism with higher ascorbate contents and monodehydroascorbate reductase and peroxidase activities (Igamberdiev et al., 2006). Transcriptomic and metabolomic studies in *Arabidopsis* revealed that over-expression of non-symbiotic *At*Hb1 improved the general redox status of the hypoxic cell (Thiel et al., 2011).

The anti-nitrative effect of NADH was consistently observed in experimental replicates as appreciated on the standard error on densitometries (Figs. 1B, 2B and 3B). Haemoglobins can be directly reduced by NADH, which may exert a synergic protective effect towards r*Vu*FeSOD. The data on Fig. 1B supported this effect, as the combined action of r*Vu*LbII plus NADH resulted in a visible decrease of r*Vu*FeSOD nitration, whereas both molecules had a lower anti-nitrative effect when studied in independent assays.

In our experiments, both r*Os*HbI and r*Vu*LbII prevented r*Vu*FeSOD from nitration as observed by immunoblot detection and this was consistent with the results obtained in the in-gel and spectrophotometrical assays, where inactivation of r*Vu*FeSOD by $ONOO^-$ was diminished by r*Os*HbI (Figs. 7 and 8). On the other hand, Hbs could interfere with the system by initially binding and scavenging $ONOO^-$ (Fig. 1 lanes 4, 5 and 8), but we cannot exclude that Hbs may bind the SIN-1-derived ·NO, as it has already been stated in plant systems (Dordas et al., 2003; Herold and Puppo, 2005).

The observed role of ascorbate is physiologically relevant as it is present in important concentrations inside the plant cells. In *Arabidopsis*, for instance, average ascorbate concentrations have been estimated on 5 mM, while higher concentrations up to 50 mM can be detected in chloroplasts (Wang and Hargrove, 2013). This fact, together with the protective function illustrated in this work, suggest that ascorbate may exert an essential role against nitration under stressful conditions. Moreover, ascorbate may both interact with Hbs and further have a protective function, as it has been established that met-*Os*HbI can be directly reduced by ascorbate in order to be recycled and continue with ·NO scavenging under stress conditions (Wang and Hargrove, 2013). Ascorbate has been described to modulate the contents of other RNS such as the ·NO-donor S-nitrosoglutathione, by reducing the molecule with concomitant release of ·NO (Smith and Dasgupta, 2000). Diverse proteins have been proved to be selectively nitrosylated in plants, including non-symbiotic Hbs and SODs (Astier et al., 2012). Thus, the

ascorbate content in plants is key in molecular processes inherent to RNS-mediated signal transductions, as ascorbate can modulate post-translational modifications of proteins by ·NO-derived products.

5. Conclusions

Ferroproteins like FeSODs and Hbs are crucial in their interactions with signalling ROS and RNS such as ·NO and $ONOO^-$. In this work, we show that activity assays and immunochemical detection of nitrotyrosines in r*Vu*FeSOD can be used to trace for nitrative attack and select anti-nitrative agents. Our results evidence that FeSODs and Hbs may be endogenous nitration targets. However, ascorbate, NADH and some aromatic molecules of plant origin strongly protected both ferroproteins according to several tests. We also found that the two Hbs, alone or synergically with ascorbate, exhibited a dual role: on one side as $ONOO^-$ targets and on the other side as protectors against nitrative attack to other proteins that may be essential in plants in order to cope with $ONOO^-$.

Supplementary Data

Supplementary data can be found in the online version of this article.

Acknowledgements

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Captions to Figures

Fig. 1. Effect of two plant haemoglobins and NADH on r*Vu*FeSOD Tyr nitration. (A) Immunoassay for 3-nitrotyrosine detection in different Fe-containing plant recombinant proteins: cowpea Fe-superoxide dismutase (r*Vu*FeSOD), rice haemoglobin-1 (r*Os*HbI) and cowpea leghaemoglobin-2 (r*Vu*LbII). Lane 9 contained nitrated BSA (Sigma) as a positive control. Incubations were held for 2 hours at 37º C. SIN-1 was employed at a final concentration of 1 mM and NADH at 0.2 mM. 1µg of protein was loaded into each lane. (B) Densitometries of the nitrotyrosine bands, expressed as percentages relative to the corresponding control band (lane 2). Values represent mean ± S.E. (n=3).

Fig. 2. Study of the specificity of the anti-nitrative effect. (A) Immunoassay for 3-nitrotyrosine detection in cowpea Fe-superoxide dismutase (r*Vu*FeSOD) and BSA. Lane 7 contained nitrated BSA (Sigma) as a positive control. Assay conditions as in Fig. 1. 1µg of protein was loaded into each lane. (B) Densitometries of the nitrotyrosine bands, expressed as percentages relative to the corresponding control band (lane 2). Values represent mean ± S.E. (n=3).

Fig. 3. Effect of reductants and Fe in tyrosine nitration of two plant haemoglobins. (A) Immunoassay for 3-nitrotyrosine detection in rice haemoglobin-1 (r*Os*HbI) and cowpea leghaemoglobin-2 (r*Vu*LbII). Assay conditions as in Fig. 1. Lane 8 contained nitrated BSA (Sigma) as a positive control. NADH (0.2 mM), sodium ascorbate (Asc, 0.3 mM) and desferrioxamine (Desf, 1 mM) were used where indicated. 1µg of protein was loaded into each lane. (B) Densitometries of the bands are expressed as percentages relative to the corresponding control band (lane 2, SIN-1). Values represent mean ± S.E. (n=3).

Fig. 4. Effect of the haem group on rice haemoglobin-1 (r*Os*HbI) tyrosine nitration. (A) Replica SDS-PAGE, 12.5 % (w/v) PAA, stained with Coomassie blue brilliant. (B) Representative immunoblot against 3-nitrotyrosine. Assay conditions as in Fig. 1. The conditions for haem removal (apo-r*Os*HbI) are fully described in section 5. 1µg of protein was loaded into each lane.

Fig. 5. View of the 3D structures of (A) rice haemoglobin-1 (*Os*HbI) and (B) soybean leghaemoglobin-*a* (*Gm*Lb*a*) around the haem moiety. Potential targets for tyrosine nitration (Tyr151 in *Os*HbI and Tyr31 and Tyr131 in *Gm*Lba) are situated near the catalytic center (haem).

Fig. 6. Effect of plant antioxidants on recombinant cowpea Fe-superoxide dismutase (r*Vu*FeSOD) nitration. (A) Representative immunoblot against 3-nitrotyrosine. Assay conditions as in Fig. 1. Sodium ascorbate (Asc) and plant antioxidant compounds were used where indicated at a final concentration of 0.3 mM. 1µg of protein was loaded into each lane. (B) Densitometries of the bands are expressed as percentages relative to the corresponding control band (lane 2, SIN-1). Values represent mean ± S.E. (n=5).

Fig. 7. Effect of plant antioxidants on cowpea Fe-superoxide dismutase (r*Vu*FeSOD) activity in-gel. (A) Representative SOD activity assay. Where indicated, SIN-1 was employed at a final concentration of 1 mM. Sodium ascorbate (Asc), desferrioxamine (Desf), plant antioxidant compounds (0.3 mM) and rice Hb-1 (r*Os*HbI, 0.05 $\mu g \cdot \mu l^{-1}$) were used where indicated. Incubations were held for 2 hours at 37 ºC. 1 µg of protein was loaded into each lane. (B) Replica native gel, 15 % (w/v) PAA, stained with Coomassie blue brilliant.

Fig. 8. Effect of plant antioxidants on cowpea Fe-superoxide dismutase (r*Vu*FeSOD) activity measured by the spectrophotometric assay. Activity of control samples (r*Vu*FeSOD) were taken as 100 % and corresponded to 1.5 units of SOD activity. Incubation conditions as in Fig. 7. Data represent average values ± S.E. (n= 8-16). Letters (a, b) and (A, B) show significant differences at $P \leq 0.05$ for SOD activities between the control and the rest of the treatments for (-)SIN-1 samples and (+)SIN-1 samples respectively.

Fig. 9. Effect of ascorbate on nitrated r*Vu*FeSOD activity. (A) Representative SOD activity assay in-gel along with the Coomassie-stained replica gel (below). Lanes 1 to 10: treatments as in Fig. 9B. (B) Spectrophotometric SOD activity assay. Cowpea Fe-superoxide dismutase (r*Vu*FeSOD) was incubated with 1 mM SIN-1. Desferrioxamine (Desf, 0.3 mM) and increasing concentrations of sodium ascorbate (Asc, mM) were

added where indicated. Ascorbate as a control (lane 9) was added at a concentration of 0.3 mM. More assay conditions as in Fig. 8.

Supplementary Material

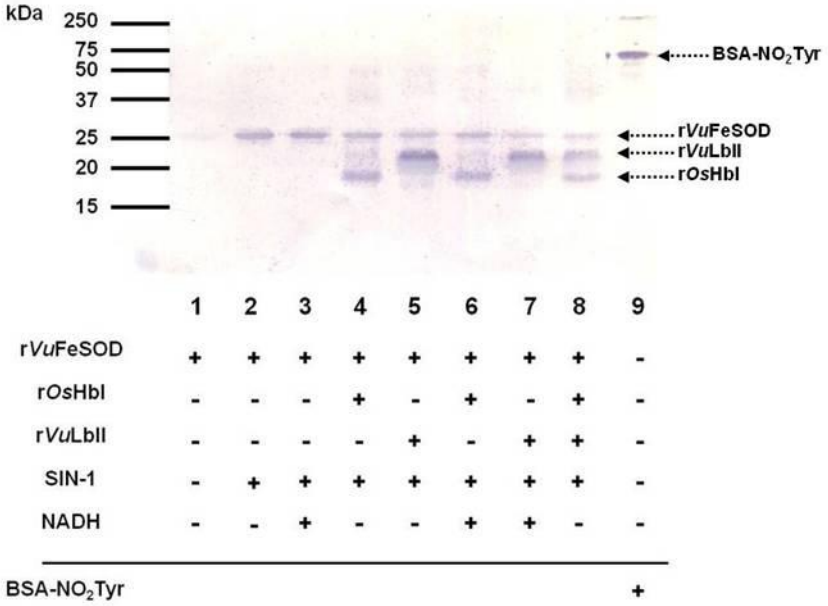
Suppl. Fig. 1. Migration of purified recombinant cowpea Fe-superoxide dismutase (r*Vu*FeSOD) in 20 % (w/v) SDS-PAGE. Lane 1, BSA (1 µg); lane 2, r*Vu*FeSOD (30 µg); lane 3, r*Vu*FeSOD (60 µg).

Suppl. Fig. 2. Chemical structures of the plant molecules employed in this study. (A) 4-hydroxybenzoic acid, R_2= OH. Gallic acid, R_1, R_2, R_3= OH. (B) Caffeic acid. (C) Gossypin, R_4= O-glucose. (D) Pyrogallol.

Suppl. Fig. 3. Direct effect of compounds tested on the reduction of cytochrome-*c* in the presence of the superoxide-producing system xanthine/XOD (SOD spectrophotometric assay). The 2-hour incubation step was omitted as no SIN-1 was used in the analysis. Increase of absorbance at 550 nm was measured within 2 min (same conditions as in Fig. 8.). Antioxidants were employed at 0.3 mM. Data represent average values ± S.E. (n= 3-7). Letters (a, b) show significant differences at $P \leq 0.05$ for cytochrome-c reduction rates before and after adding the compound to the reaction mixture.

Figure 1

A



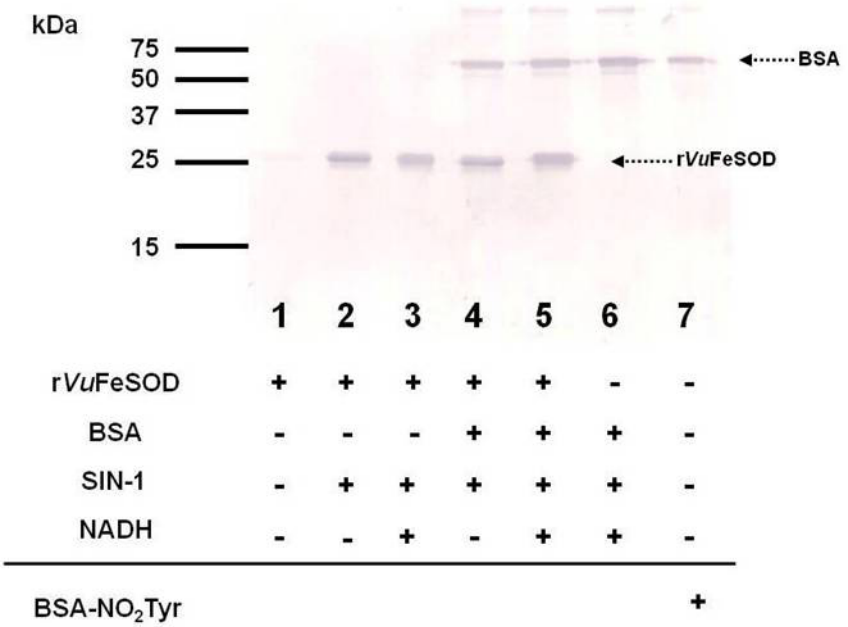
	1	2	3	4	5	6	7	8	9
r*Vu*FeSOD	+	+	+	+	+	+	+	+	-
r*Os*HbI	-	-	-	+	-	+	-	+	-
r*Vu*LbII	-	-	-	-	+	-	+	+	-
SIN-1	-	+	+	+	+	+	+	+	-
NADH	-	-	+	-	-	+	+	-	-
$BSA\text{-}NO_2Tyr$									+

B

Lane	Treatment	Units (%)
2	SIN-1	100
3	SIN-1+NADH	92 ± 8
4	SIN-1+r*Os*HbI	69 ± 10
5	SIN-1+r*Vu*LbII	59 ± 3
6	SIN-1+NADH+r*Os*HbI	68 ± 7
7	SIN-1+NADH+r*Vu*LbII	42 ± 6
8	SIN-1+r*Os*HbI+r*Vu*LbII	32 ± 3

Figure 2

A

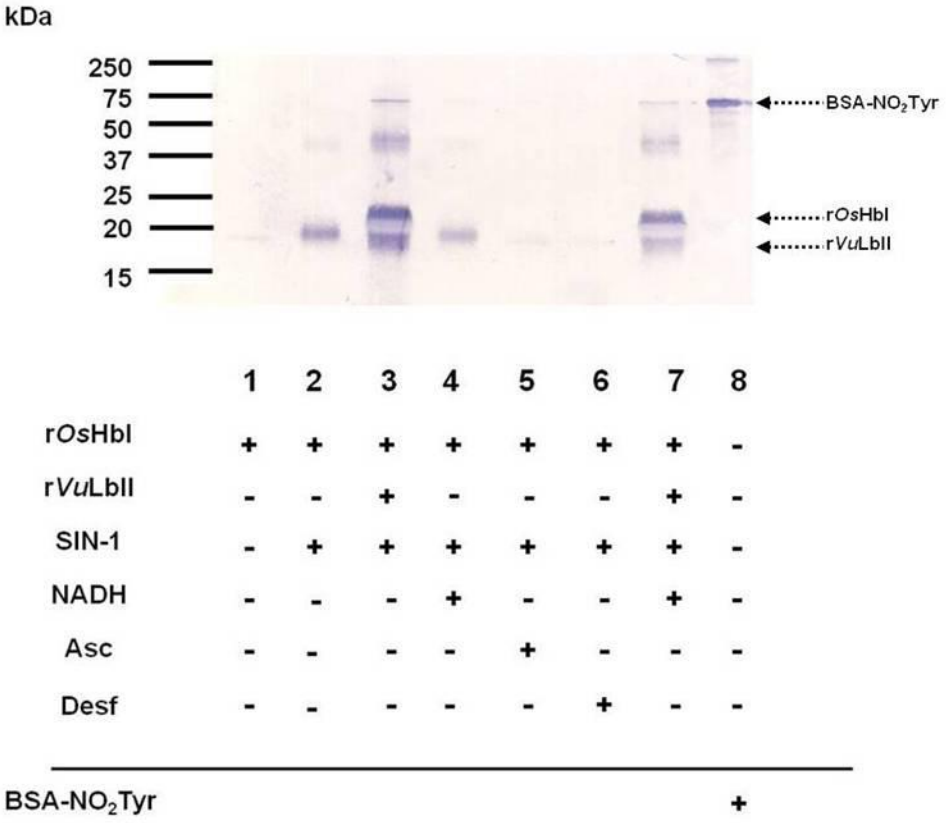


B

Lane	Treatment	Units (%)
2	SIN-1	100
3	SIN-1+NADH	78 ± 6
4	SIN-1+BSA	105 ± 5
5	SIN-1+BSA+NADH	102 ± 7

Figure 3

A



B

Lane	Treatment	Units (%)
2	SIN-1	100
3	SIN-1+r*Vu*LbII	143 ± 1
4	SIN-1+NADH	65 ± 1
5	SIN-1+Ascorbate	13 ± 0.1
6	SIN-1+Desferrioxamine	7 ± 0.2
7	SIN-1+r*Vu*LbII+NADH	66 ± 0.3

Figure 4

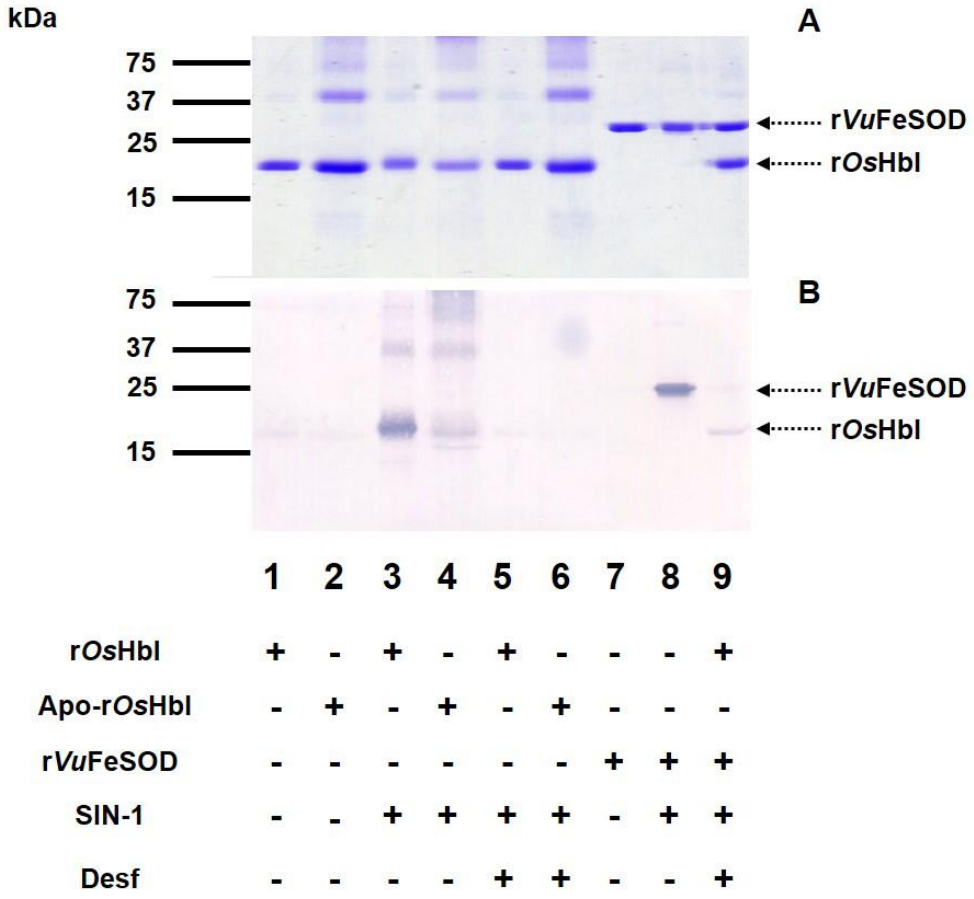

kDa
A
75
37
25
15
rVuFeSOD
rOsHbl
B
75
37
25
15
rVuFeSOD
rOsHbl
1 2 3 4 5 6 7 8 9
rOsHbl + - + - + - - - +
Apo-rOsHbl - + - + - + - - -
rVuFeSOD - - - - - - + + +
SIN-1 - - + + + + - + +
Desf - - - - + + - - +

Figure 5

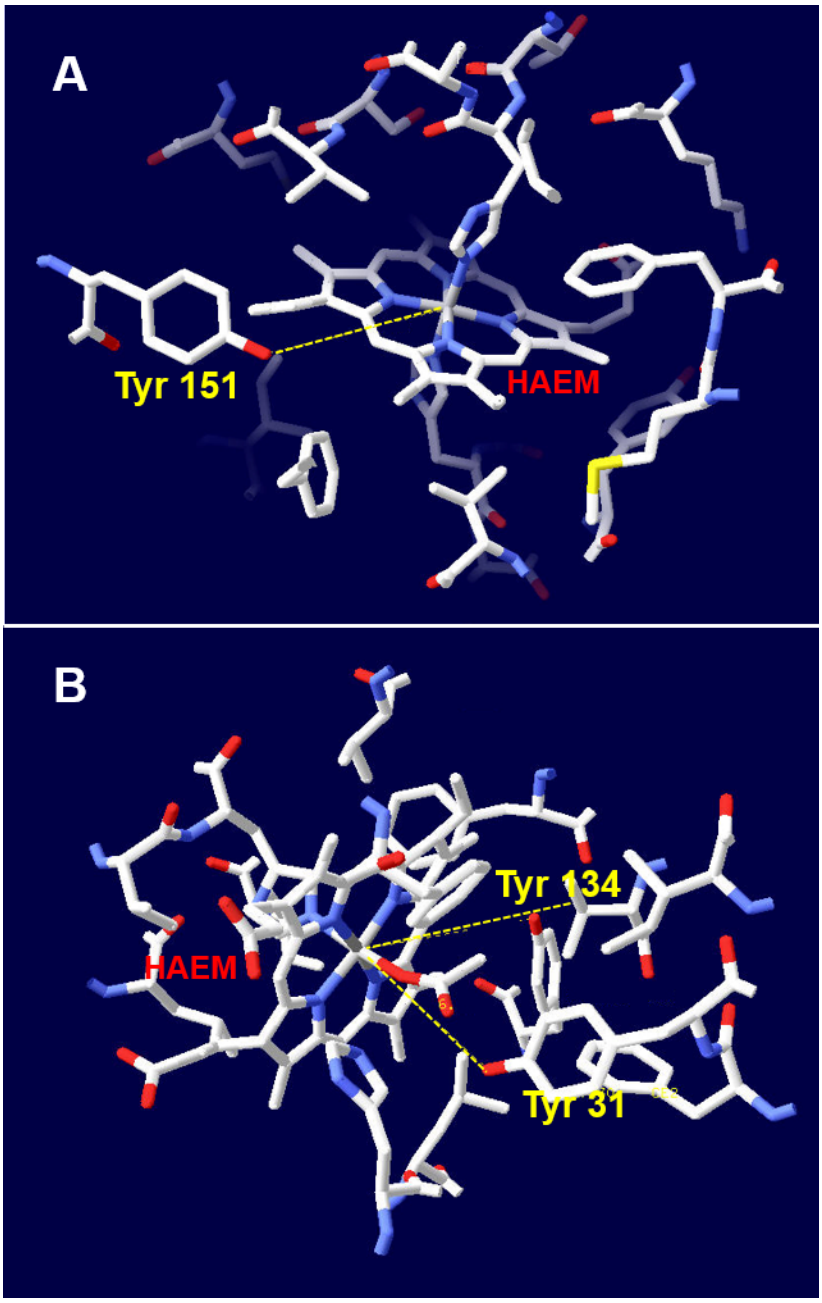
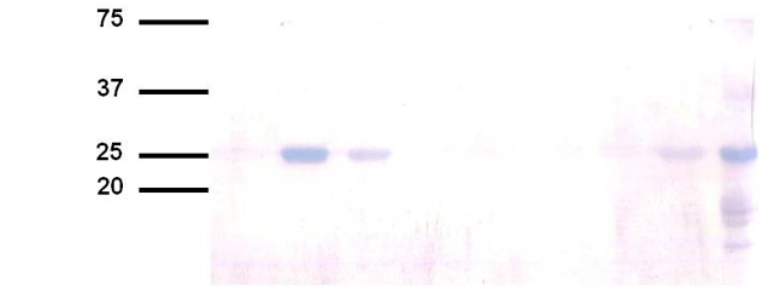


Figure 6

A



	1	2	3	4	5	6	7	8	9
r*Vu*FeSOD	+	+	+	+	+	+	+	+	+
SIN-1	-	+	+	+	+	+	+	+	+
Asc	-	-	+	-	-	-	-	-	-
Gossypin	-	-	-	+	-	-	-	-	-
Gallic acid	-	-	-	-	+	-	-	-	-
Caffeic acid	-	-	-	-	-	+	-	-	-
Pyrogallol	-	-	-	-	-	-	+	-	-
4-Hydroxyb.	-	-	-	-	-	-	-	+	-
r*Os*Hbl	-	-	-	-	-	-	-	-	+

B

Lane	Treatment	Units (%)
2	SIN-1	100
3	SIN-1 + Asc	35 ± 1
4	SIN-1 + Gossypin	0
5	SIN-1 + Gallic acid	0
6	SIN-1 + Caffeic acid	0
7	SIN-1 + Pyrogallol	20 ± 2
8	SIN-1 + 4-Hydroxyb.	51 ± 5
9	SIN-1 + r*Os*Hbl	72 ± 4

Figure 7

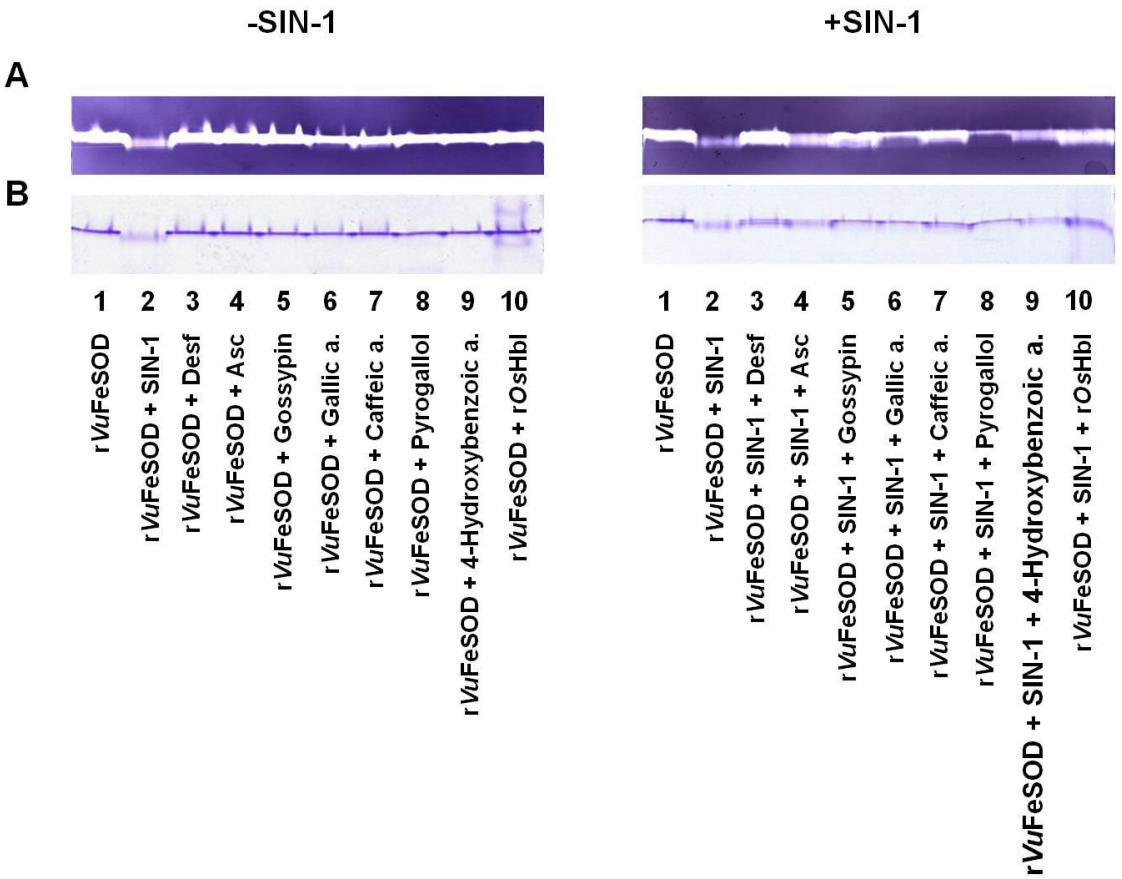

-SIN-1
+SIN-1
A
B
1 rVuFeSOD
2 rVuFeSOD + SIN-1
3 rVuFeSOD + Desf
4 rVuFeSOD + Asc
5 rVuFeSOD + Gossypin
6 rVuFeSOD + Gallic a.
7 rVuFeSOD + Caffeic a.
8 rVuFeSOD + Pyrogallol
9 rVuFeSOD + 4-Hydroxybenzoic a.
10 rVuFeSOD + rOsHbl
1 rVuFeSOD
2 rVuFeSOD + SIN-1
3 rVuFeSOD + SIN-1 + Desf
4 rVuFeSOD + SIN-1 + Asc
5 rVuFeSOD + SIN-1 + Gossypin
6 rVuFeSOD + SIN-1 + Gallic a.
7 rVuFeSOD + SIN-1 + Caffeic a.
8 rVuFeSOD + SIN-1 + Pyrogallol
9 rVuFeSOD + SIN-1 + 4-Hydroxybenzoic a.
10 rVuFeSOD + SIN-1 + rOsHbl

Figure 8

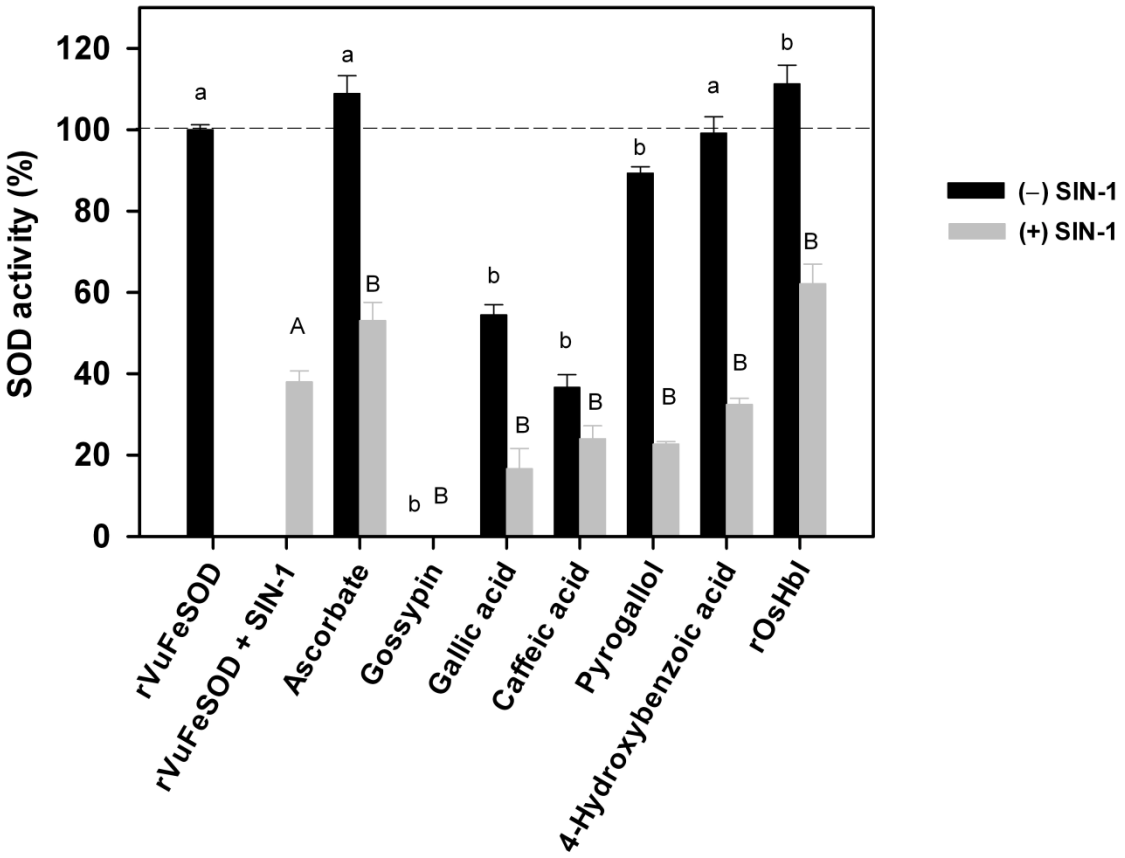
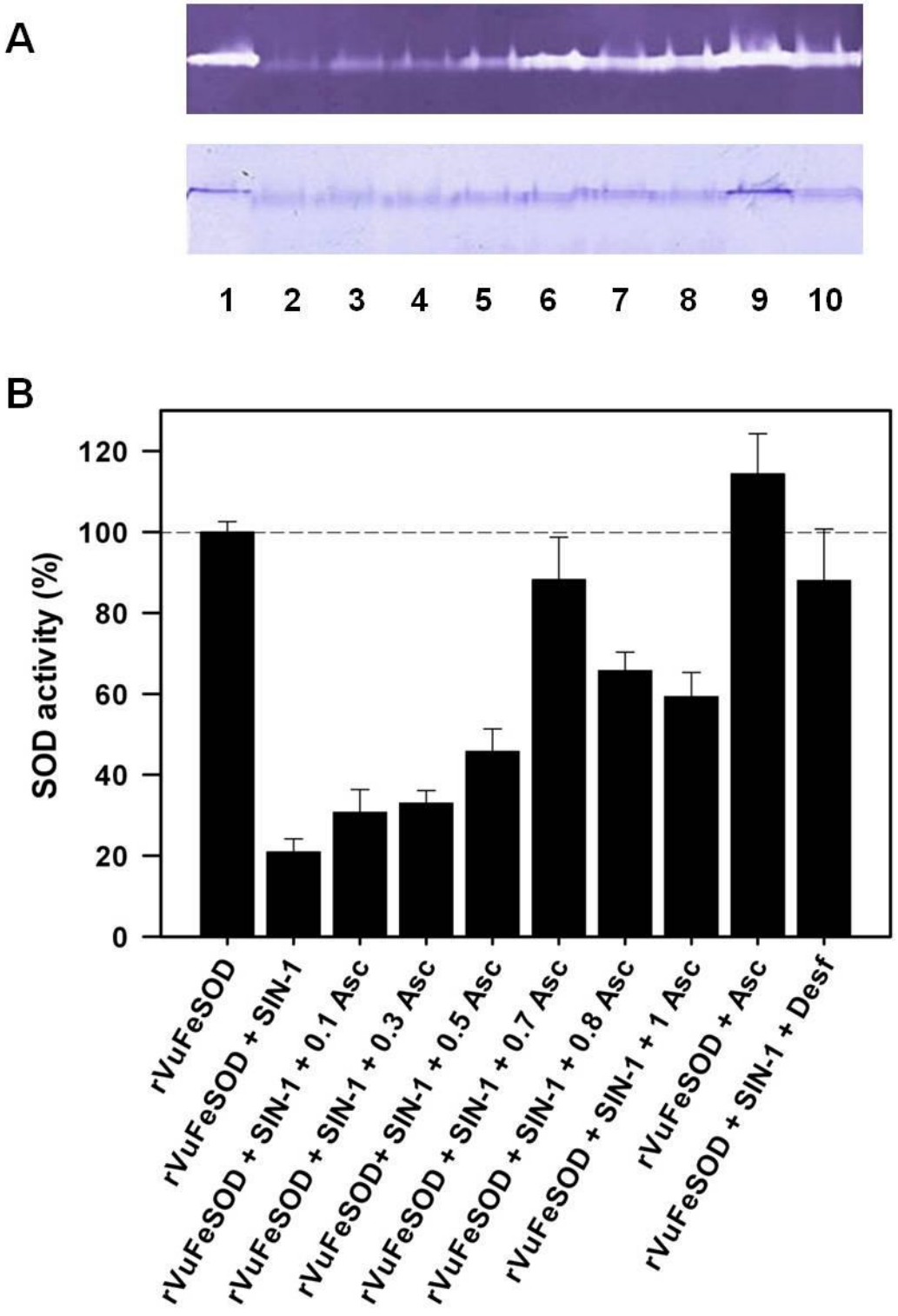
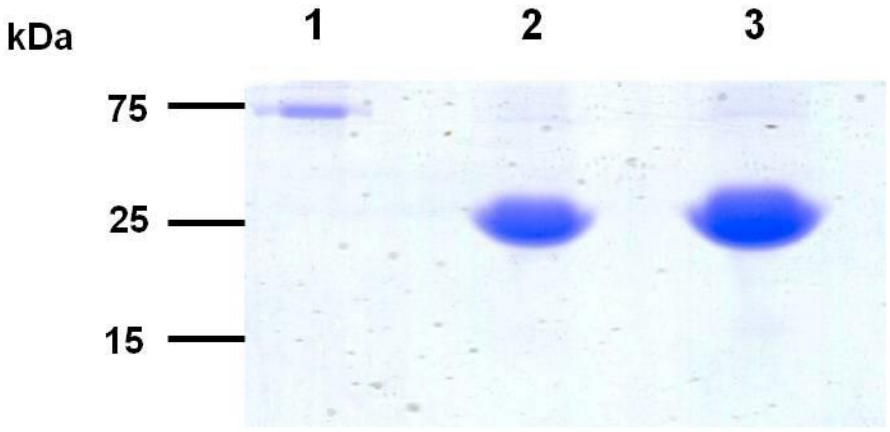

SOD activity (%)
120
100
80
60
40
20
0
a
A
a
B
b
B
b
B
b
B
b
B
a
B
b
B
rVuFeSOD
rVuFeSOD + SIN-1
Ascorbate
Gossypin
Gallic acid
Caffeic acid
Pyrogallol
4-Hydroxybenzoic acid
rOsHbI
(–) SIN-1
(+) SIN-1

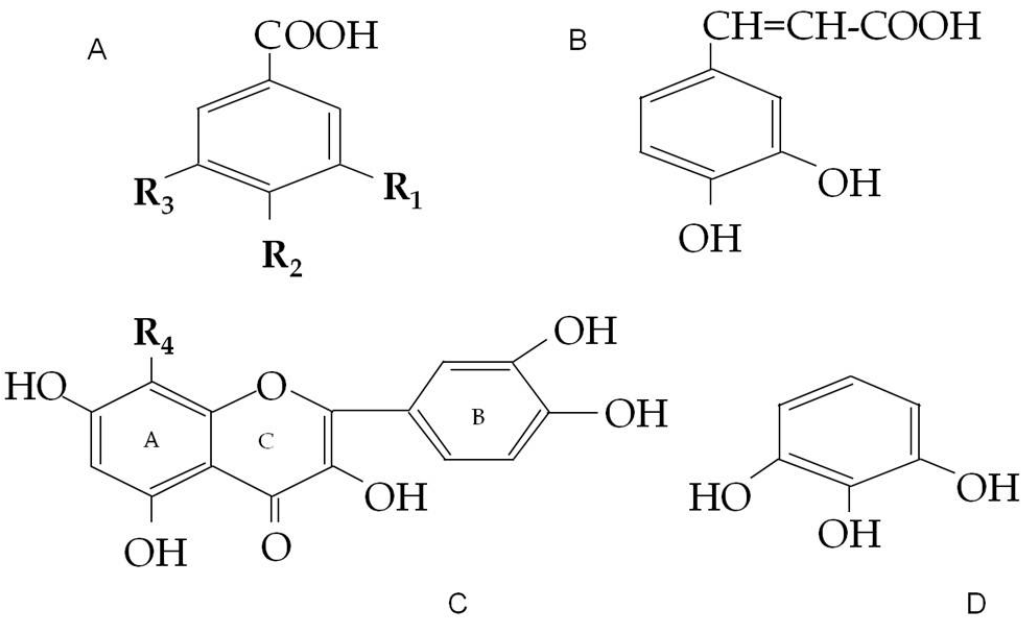
Figure 9


A
1 2 3 4 5 6 7 8 9 10
B
SOD activity (%)
0
20
40
60
80
100
120
rVuFeSOD
rVuFeSOD + SIN-1
rVuFeSOD + SIN-1 + 0.1 Asc
rVuFeSOD + SIN-1 + 0.3 Asc
rVuFeSOD+ SIN-1 + 0.5 Asc
rVuFeSOD + SIN-1 + 0.7 Asc
rVuFeSOD + SIN-1 + 0.8 Asc
rVuFeSOD + SIN-1 + 1 Asc
rVuFeSOD + Asc
rVuFeSOD + SIN-1 + Desf

Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3