FIR BARK (*ABIES ALBA* MILL.) LECTIN IS AN INHIBITOR OF FIR PEROXIDASE "IN VITRO"

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Abies (*Abies alba* Mill*)* bark agglutinin, AbA2 inhibited the peroxidase from the same tissue up to 35% with an IC_{50} of 0.763×10^{-2}M. The Km of free Fir peroxidase was 0.249×10^{-2}M., while by interaction with 2.1 μg/ml of AbA2 the binding efficiency increases 2 times. The kinetic data of the enzymatic activity of Fir bark peroxidase incubated with the AbA2 lectin (0.6–2.1 μg/ml) showed a mixed type of inhibition (competitive/uncompetitive). At relatively high concentration of AbA2 lectin (2.1 μg/ml) the mixed inhibition type of Fir peroxidase changed to fairly competitive. Hydrolysis of AbA2 lectin and Fir peroxidase with 5% H_2SO_4, and subsequent dyeing for Fe ions on TLC silicagel plates revealed the existence of Fe^{3+} in the structure of both AbA2 lectin and Fir peroxidase. Incubation of AbA2 lectin with EDTA, as possible chelator of Fe^{3+} increases the peroxidase inhibition changing the behavior into highly competitive. No modifications in peroxidase activity were shown after incubation of AbA2 lectin (1.2 μg/ml) with 40 mM of GlN, GalN, GlNAc, GalNAc and Fuc The interaction of AbA2 lectin with Fir peroxidase at the protein level through Fe^{3+} was discussed.

Key words: Abies bark Agglutinin 2/ Fir peroxidase; ROS, haem containing enzyme, iron containing lecitin.
Abbreviations: AbA2-Abies bark Agglutinin 2, EDTA-ethylen diamino tetraacetic acid ROS-Reactive Oxygen Species, GlcN-glucosamine, GalN-galactosamine, GalNAc-N acetyl galactosamine, GlcNAc-N acetyl glucosamine and Fuc-fucose.

INTRODUCTION

While carbohydrate-recognition by lectins is a functionally significant property, there is substantial evidence that these glycoproteins may exhibit other, physiologically relevant interactions. Recently, it has been shown the interaction of porphyrins with lectins (jacalin) and their important physiological role[1–3].

It has also been suggested that this binding process is mediated by hydrophobic forces[3]. Some other lectins, such as, *Trichosantes cucumerina* seed, was found to bind different porphyrins with comparable binding strength indicating that porphyrin binding takes place at a site different from the sugar binding site[4].

Porphyrins are a class of biologically important molecules, with aromatic structure, which contain different ions such as: Cu^{2+}, Mn^{2+}, Zn^{2+}, Fe^{3+}. For example, haem and chlorophyll have in their structure Fe^{3+} and Mn^{2+} porphyrins respectively.

Peroxidases, (class III) Prxc (EC 1.11.1.7) are well known in plant kingdom. Prxs are of a glycoprotein nature and are located in vacuoles and the cell wall[12]. Plant Prxs are haem-containing enzymes which catalyze the single one-electron oxidation of several "phenolic like" substrates using as secondary substrate H_2O_2: The physiological roles of peroxidases have been extensively investigated and it has been demonstrated that they catalyze a variety of important reactions such as auxin metabolism[5] lignin biosynthesis[6, 7], suberization of cell wall[8] and detoxification of H_2O_2[9].

Recently, it has been shown the role of haemin in the generation of ROS[10].

Our previous paper was devoted to the isolation of two Fir (*Abies Alba* Mill.) bark lectins using immobilized horseradish peroxidase on Sepharose 4B- BrCN activated[11]. In the unabsorbed material collected from the column some peaks exhibited peroxidase activity. The lectins AbA1 and AbA2 absorbed to the column were desorbed by using a salt low pH buffer. These lectins were found to have the same molecular weight as Fir peroxidase of 69kDa in SDS electrophoresis in unreduced and reduced conditions. None of these lectins presented peroxidase activity. The main differences were found to be in the percentage of total sugars (1.23% and 3.38% for AbA1 and AbA2 respectively against 2.31% of Fir peroxidase) and hemagglutination properties. Other properties such as sugars and glycoproteins affinities, RIP like activity will be exposed in other article (Budu, unpublished).

The aim of the present paper is to evaluate the inhibitory effect of AbA2 lectin towards Fir peroxidase using kinetic studies. The binding possibilities at the protein level of AbA2 lectin to Fir peroxidase will be discussed.

MATERIAL AND METHODS

Separation of the two Fir bark lectins and peroxidase from the same tissue

Fir peroxidase and the two Fir lectins were separated in the same one step procedure using immobilized horseradish peroxidase on Sepharose 4B, BrCN activated[11]. In brief, the active peroxidase fraction was found in the protein fraction eluted from the column with 0.04M Tris-0.15 M Boric acid, pH=7.4. The absorbed lectins fractions from the column were eluted using a low salt pH solution 0.1N HCl, 2M KCl, pH<2. The first eluted lectin was named AbA1, the second AbA2. The purity of the fractions was tested by SDS polyacrylamide electrophoresis following the method of Laemmli, 1970[13]. Proteins concentrations were determined by Bradford method, 1976[14] using serum albumin bovine as control.

Detection of Fe^{3+} ions in the structure of AbA2 lectin and Fir peroxidase

Both, AbA2 lectin and Fir peroxidase (400 μg) were subjected to hydrolysis for 12 hrs with 5% H_2SO_4. The hydrolysates were then dialyzed for 24 hrs against H_2O. An easy estimation of Fe^{3+} was performed by TLC silicagel plate. Thus, 10μl of peroxidase and AbA2 lectin was spotted against $FeCl_3$. The solvent mixture used was butanol: acetic acid: H_2O, (5:3:2). 5% K Ferrocyanide solution was used as specific dye for Fe ions.

Kinetic studies of enzymatic oxido-reduction

All kinetic experiments of peroxidase oxido-reduction activity were performed at different concentrations of H_2O_2 and the same concentration of hydroquinone (0.36 mg/ml)[15]. The enzyme activity was calculated according to the relation of enzyme activity ΔA/min ×2.1/18.18×0.1 μmoles pBQ/min/ml where: 2.1 is the volume expressed in ml in which determination was done; 18.18–extinction coefficient of 1×10^{-3} M pBQ sol. (1μmol/ml) (p benzoquinone resulted in the reaction; 0.1 volume of the sample used in assay (ml). According to the method presented, the enzyme activity is expressed in μmoles pBQ /min/ml or μmoles pBQ/min/mg protein.

Inhibition studies

All binding experiments were carried out in PBS. Fir peroxidase (40 μg/ml) was incubated with AbA2 lectin (0.6, 1.2 and 2.1 μg/ml), for 10 min at the room temperature prior to the peroxidase activity determination. The Fe^{3+} ion from AbA2 lectin was chelated by incubation with EDTA 1 mM for 10 min[16] and the excess of EDTA was removed by dialysis against PBS prior Fir peroxidase incubation. The possible implication of specific saccharides of AbA2 lectin upon Fir peroxidase activity was determined by the incubation of AbA2 lectin with 40 mM of GlN, GalN, GalnAc, GlNAc and Fuc for 10 min prior to the incubation with Fir peroxidase.

RESULTS

The existence of the Fe^{3+} in the structure of AbA2 lectin and Fir peroxidase was easily evaluated by hydrolysis and subsequent separation by silicagel TLC using $FeCl_3$ as standard (Fig. 1).

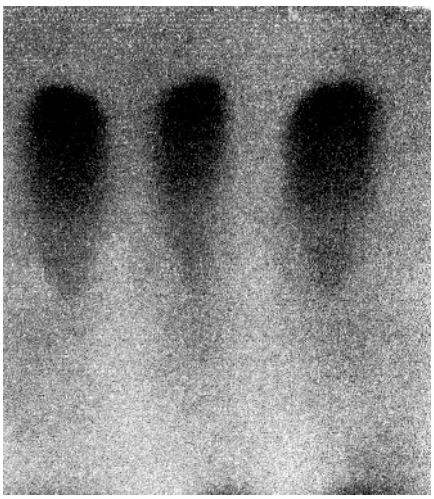

Fig. 1. Silica gel chromatography of AbA2 lectin and Fir peroxidase hydrolysates.
Both AbA2 lectin and Fir peroxidase (400 μg/ml) were hydrolysed with 5% H_2SO_4, for 12h. After hydrolysis the excess of EDTA was removed by dialysis against H_2O. 10 μl of samples were subjected to thin layer chromatography on silica gel. Solvent system: butanol: acetic acid: H_2O, (5:3:2). The spots were colored with 5% K ferricyanide. A. (AbA2 hydrolysate) B. ($FeCl_3$), C. (Fir peroxidase hydrolysate).

From our knowledge, this is one of the unique lectins evaluated so far, exhibiting a Fe^{3+} in its structure. This feature emphasizes new hypothesis about AbA2 lectin and its binding sites.

Different concentration of AbA2 lectin (0.5–36μg/ml) inhibited Fir peroxidase up to 34% (Fig. 2A) and an IC_{50} of 2.9μg/ml (Fig. 2B).

Michaelis Menten plot of free Fir peroxidase presented a substrate inhibition of 38% at 2×10^{-2}M H_2O_2 (Fig. 3). However, by incubating with different concentrations of AbA2 lectin, the substrate inhibition is reduced to 7%. at 2.1μg/ml of AbA2. The resulted kinetic parameters were presented in Table 1. As it was shown in Table 1, Km for free Fir peroxidase was 0.249×10^{-2}M. By incubation with 2.1 μg/ml AbA2 binding efficiency was increased 2.08 times, while dissociation constant, Ki decreased 5 times. As it was shown, the AbA2 lectin inhibited Fir peroxidase activity, while the affinity for H_2O_2 substrate was increased similarly with an uncompetitive type of interaction. Lineweaver Burk plot showed the pattern of mixed inhibition, (competitive/uncompetitive), to the respect of H_2O_2. At high concentration of AbA2 lectin (2.1 μg/ml), the interaction with Fir peroxidase changes the type of inhibition to fairly competitive.

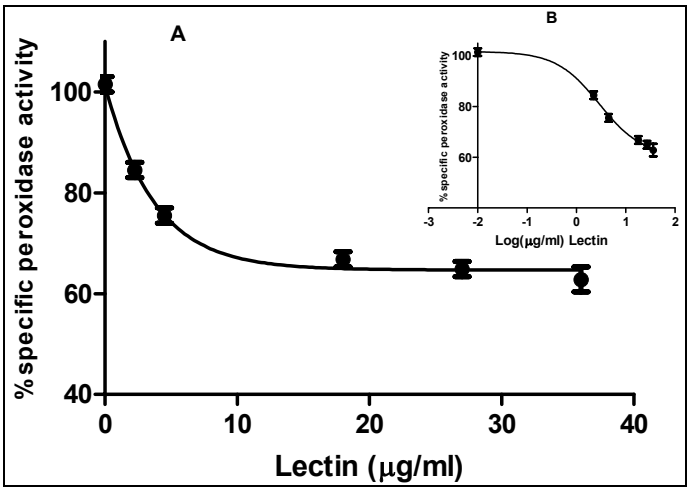

Fig. 2. The effect of AbA2 lectin on Fir peroxidase activity.

A. Fir peroxidase (40 μg/ml) was incubated with different concentrations of AbA2 lectin (0.5-36 μg/ml) for 10 min at room temperature prior Fir peroxidase activity evaluation. The activity was measured spectrophotometrically following p- benzoquinone formed at 250 nm from hydroquinone (0.36 mg/ml) and 0.05% H_2O_2. B. Log of the AbA2 lectin was plotted against % of specific peroxidase activity to give IC_{50} value. The results represent the average of three experiments ±SD.

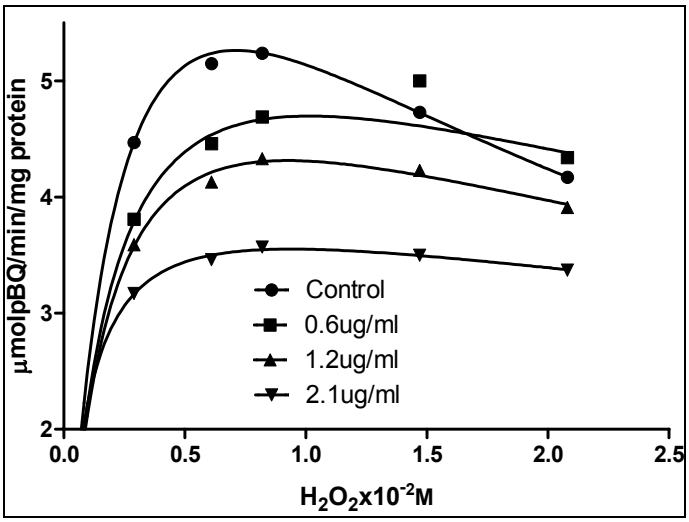

Fig. 3. Michaelis-Menten curve of Fir peroxidase activity.

Fir peroxidase (40μg/ml) was incubated with AbA2 lectin for 10min at room temperature prior peroxidase activity determination. Fir peroxidase alone (●), or in presence of AbA2 lectin : 0.6 μg/ml (■), 1.2 μg/ml (▲), 2.1 μg/ml, (◆).

Table 1

Kinetic parameters of the interaction between Fir peroxidase and ABA2 lectin

Parameters	Control (Per)	0.6 ug/AbA2	1.2 ug/AbA2	2.1 ug/AbA2
Vmax	0.96	6.42	5.9	4.21
Km	2.2498	0.1866	0.1175	0.088
Ki	2.025	5.52	4.85	10.01

The prior treatment of lectin with EDTA led to a marked decrease of Fir peroxidase activity (Fig. 5) and changed the known behavior of inhibition pattern[17], to highly competitive, which in our interpretation might result in possible conformational change in the AbA2 lectin structure, which favours the competition with peroxidase for the same H_2O_2 substrate. The existence of Fe^{3+} in the structure of AbA2 lectin (Fig. 1) can lead to the assumption that EDTA can act as a chelator of Fe^{3+} ions[16] at the lectin level. This might suggest the involvement of the proteic part of the lectin, via Fe^{3+} in the interaction with Fir peroxidase.

The incubation of the AbA2 lectin (1.2 ug/ml) with different concentrations of carbohydrates didn't significantly change the peroxidase activity (Fig. 6). Therefore, in this particular case the carbohydrate moieties of AbA2 lectin are not involved in the interaction of the lectin with peroxidase.

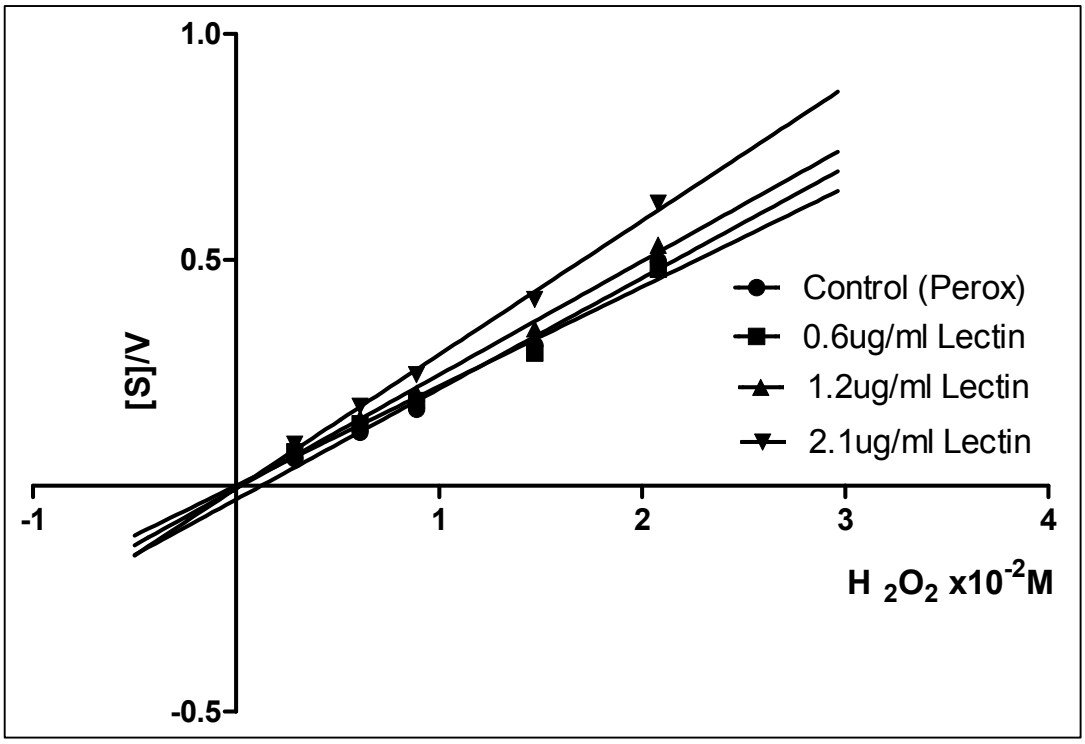

Fig. 4. Lineweaver Burk plot of Fir peroxidase activity after AbA2 lectin incubation.
Fir peroxidase (40 μg/ml) was incubated with AbA2 lectin for 10min at room temperature prior peroxidase activity determination.
Fir peroxidase alone (●), or in presence of AbA2 lectin: 0.6μg/ml (■), 1.2 μg/ml (▲), 2.1 μg/ml (◆).

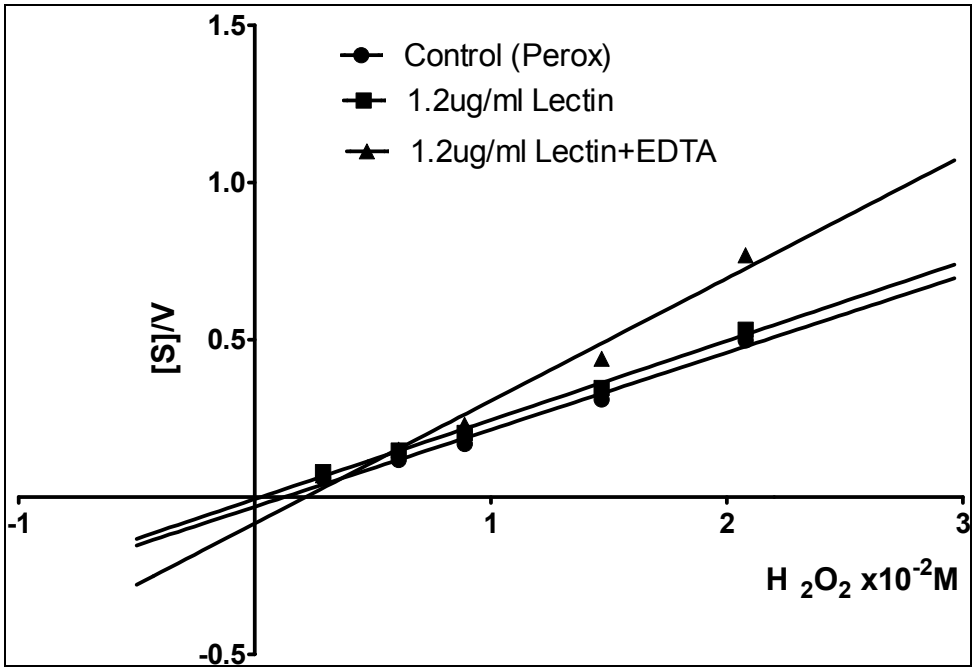

Fig. 5. The effect of chelation of Fe^{3+} of AbA2 lectin on Fir peroxidase activity.
AbA2 lectin (1.2 μg/ml) was treated with 1mM EDTA final concentration for 10min at room temperature. The excess of EDTA was removed by dialysis against PBS prior incubation with Fir peroxidase (40 μg/ml). Fir peroxidase alone, (●) or in presence of AbA2 lectin, 1.2 μg/ml (▲), or in presence of EDTA (1mM) treated AbA2 lectin (1.2 μg/ml) (■).

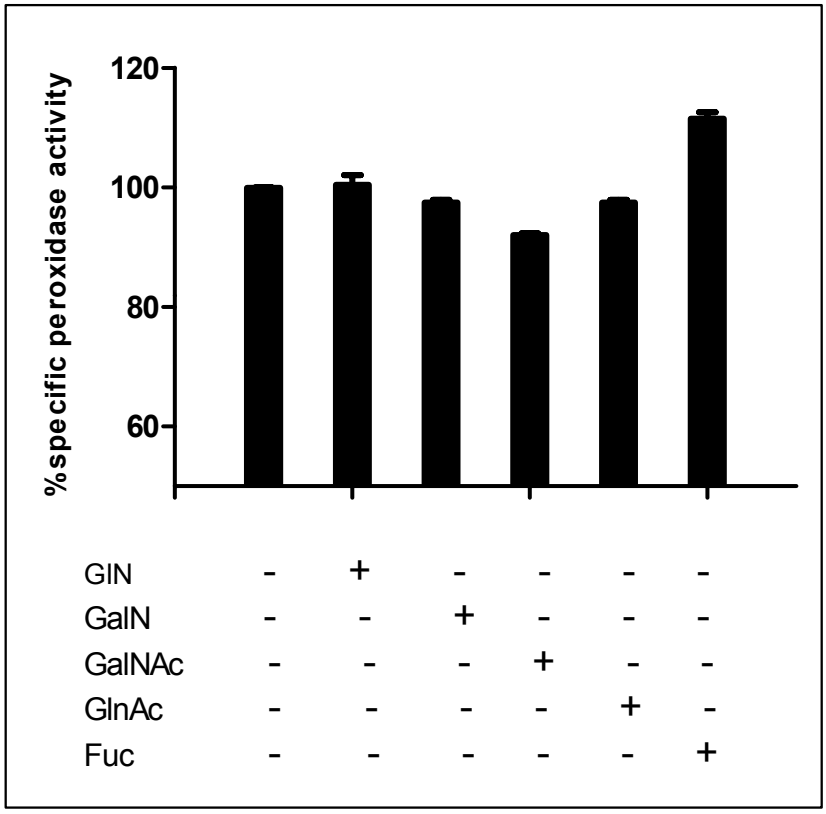

Fig. 6

DISCUSSION

The kinetic studies of the effect of AbA2 lectin on Fir peroxidase activity clear showed the interaction between AbA2 bark lectin and Fir bark peroxidase. The low value of IC_{50} 2.9μg/ml (Fig. 2B) indicated the high effectiveness of AbA2 lectin in inhibiting Fir peroxidase.

This result might have an important physiological role in correcting H_2O_2 imbalance resulted from biotic or abiotic stress. Hydrogen peroxide is highly reactive, and the activity of enzymes utilizing this compound (peroxidase, in particular) is very sensitive to changes in the physiological state of the plant tissue[18]. It is also believed that the inhibition of hydrogen peroxide synthesis by lectins may be a defense factor protecting infected plants from autologous damage in the course of extensive production of reactive oxygen species[19].

The Km of free Fir peroxidase was in agreement with other peroxidases, such as horseradish peroxidase with Km 1.2 mM for 1, 2, 4, 5 tetrametoxy benzene and 7.5 mM for pentamethoxybenzene as a substrate respectively. Lignin peroxidase has been shown to have an Km of 0.12 mM for Pentamethoxy substrate[21] See Km related to h2o2. In our case, the interaction of Fir peroxidase with 2.1 μg/ml, AbA2 lectin, changes the affinity for H_2O_2 substrate from 2.4 to 0.88 mM. The behavior of mixed type (competitive/ uncompetitive) at the Fir peroxidase level might be explained by possible existence of two different sites more likely with different affinities for H_2O_2 (index) ??? The higher level of AbA2 lectin (2.1 μg/ml) switched the mixed type (competitive/ uncompetitive) to fairly competitive type of inhibition (Fig. 4). The change in the inhibition pattern of AbA2 lectin by incubation with EDTA (Fig. 5) led to the assumption that the protein moieties might have an important role in this interaction being modulated by Fe^{3+}. There are different theories related to the protein part binding at the lectin level. It has been shown that the linkage between enzymes and lectins at the protein level can be made by ionic forces. Therefore, it was suggested that *Canavalia ensiformis* and *Phaseolus vulgaris* enzymes and their own lectins might be linked through ionic strength[22]. Moreover, it was shown that Fe^{3+} alters the reactivity of lactoferrin and other related proteins towards lectins such as: *Sambuccus nigra* agglutinin, Wheat germ agglutinin, *Datura stramonium* agglutinin, Peanut agglutinin[23]. Further, similar to the $Fe^{2+/3+}$ catalyzed Haber-Weiss reaction, horseradish peroxidase, for example, can reduce hydrogen peroxide to hydroxyl radicals[24]. Thus, one expects that whenever cell wall–bound peroxidases come into contact with suitable concentrations of superoxide and H_2O_2, originating from the oxidative cycle of peroxidase or other sources, hydroxyl radicals should form within the cell[25].

Taking into consideration the existence of Fe ions in both discussed glycoproteins AbA2 lectin and Fir peroxidase, we can also assume the possibility of Fe-Fe binding with a first coordination sphere constituted possible by nitrogen atoms, carboxylate like ligand or oxo bridges[28]. On the other hand, the existence of Fe-Fe complex has been reported in red kidney bean purple acid phosphatase which seems to react with oxygen, forming a Fe(III)-Fe(II)-O-$O^{\cdot}$ complex[29]. This complex is unbalanced with the second complex, resulting oxygen and H_2O_2 and 2 [Fe (III)-Fe (III)] enzymes[29]. This step might be the reason for the subsequent formation of OH radicals from H_2O_2 observed by Aisen[30].

Our results showing that sugar moieties of AbA2 lectin, was not be implicated in Fir peroxidase binding (Fig. 6) was in agreement with the finding that, in case of apolactoferrin for example, glycan was not implicated in Fe binding or determined the overall structure of the protein[23].

Taking into consideration that AbA2 lectin interacted with Fir peroxidase, inhibiting enzyme activity might have implications “in vivo”. It has already been shown that in case of sessile oak seedlings, a floem glycoprotein, G2, can interact with peroxidase from the same tissue “in vivo” and change its activity[20]. Moreover, this formed complex might have an influence in the regulation of H_2O_2 concentration at the cellular level.

We can conclude that AbA2 lectin inhibited Fir peroxidase activity, which more likely implicates Fe^{3+} at the protein level of both glycoproteins.

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