10

Polysaccharide-Specific Isoperoxidases as an Important Component of the Plant Defence System

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1. Introduction

The plant cell wall is a very complex and dynamic system, similar in importance to both the extracellular and intracellular processes which are recognised nowadays. The cell wall is a "vanguard" - an effective barrier in the way of different negative chemical and biotic factors, including pathogens and wounding. The defence functions of plant cell walls are associated with the construction of physical barriers consisting of lignin- and suberin-containing polymers on the path of pathogens inside a plant. This reaction develops more or less automatically, and barriers are only formed in the zone of pathogen penetration during active pathogen expansion into the host plant's tissues. Yet the mechanisms of these events are still unclear. It is well-known that peroxidases (PO) are key enzymes involved in lignification (Cosio & Dunand, 2009) and one of the few proteins secreted into the plant cell wall. However, POs have numerous applications in industry and one of the most important of these is the use of POs for lignin degradation. Therefore, both analytics and industry require a great volume of stable PO preparations of a high quality and at a low price, and the search for new methods and substrates for their extraction and purification has great commercial importance. Thus, the ability of plant oxidoreductases to interact with the biopolymers of the cell walls of plants and fungi has been studied for several decades (Siegel, 1957; McDougall, 2001). Moreover, it has been shown that plant POs can bind electrostatically with calcium pectate (Dunand et al., 2002) and chitin (Khairullin et al., 2000). Plants are likely to contain a whole subclass of these "polysaccharide-specific" isoPOs and their encoding genes. This subclass should be characterised by the ability to bind with polysaccharides and the defence function focused on strengthening the cell wall of the host and isolating the non-infected host tissues from the pathogen with the help of lignin. We suppose that the ability of plant POs to interact with some biopolymers without losing their activity can be applied for the isolation and purification of these enzymes. The possibility of the application of chitin in agriculture, biomedicine, biotechnology and the food industry has received much attention due to its biocompactibility, biodegradability and bioactivity. The low price and the ecological safety of this biopolymer define it as an available matrix for technological processes. As such, it may be possible to produce the high-quality preparations of POs that are needed for various fields of industry and analytical methods with the use of chitin (or other

polysaccharide biopolymers) as a matrix. This article is focused on the biochemical and molecular features of POs binding with polysaccharides and their functions in plant defence reactions, and it covers some aspects of the application of polysaccharides for the purification of POs.

2. Molecular and biochemical features of plant peroxidases

PO (donor: H_2O_2 oxidoreductase, EC 1.11.1.7) belongs to a class of widespread and vital enzymes. These enzymes are used in enzyme immunoassays, diagnostic assays and industrial enzymatic reactions. The application of POs in the area of organic chemistry - especially when regio- and enantioselective oxidations are sought - are both numerous and appealing (Yoshida et al., 2003). Therefore, both analytics and industry require a great volume of stable PO preparations of a high quality and at a low price. Presently, the basic source of commercial POs is the horseradish roots (*Armoracia rusticana*). However, this PO has a poor stability under the different conditions required for industrial processes. Besides this, commercial POs often contain admixtures of other enzymes. One of the problems is purification of different isoPOs with different catalytic activity, since one of specific features of POs is the multiplicity of their molecular isoforms exhibiting various functions. Numerous of the genes encoding them are controlled by various *cis*-elements and *trans*-factors which are responsible for their different expression activity, depending on the environmental conditions and the stages of ontogenesis.

The rather high variety of PO genes together with the almost 90% homology of the functionally important sites responsible for their enzymatic activity was demonstrated by gene comparison, even for one plant species (Welinder et al., 2002; Duroux & Welinder, 2003; Cosio & Dunand, 2009). The inability of antibodies raised against the cationic isozymes of peanut PO to bind to the anionic PO of the same species indicates the differences in the structures of even those peroxidases belonging to a single species. At the same time, a high immune cross reactivity between the cationic isoPOs of horseradish, radish and carrot has been demonstrated (Conroy et al., 1982; Maksimov et al., 2010). POs are localised in cytoplasm, the plasma membrane or else secreted outside of the cell. In the cell wall, POs are present in the ionic-bound or covalent-bound fraction of proteins, and even freely circulate in apoplast. However, it is unclear which polysaccharides participate in PO anchoring within a plant cell wall.

PO performs vitally important functions in the plant cell and is mainly associated with the oxidation of phenolic compounds and with the formation and strengthening of the cell wall (Passardi et al., 2004). PO is involved in the oxidative transformation of molecules in growth-regulating or signalling activities and - as a result - can also perform regulatory functions in the cell. Plant POs are represented by genetically different proteins with the same enzymatic activity (Welinder et al., 2002).

These physiological functions of the enzyme are especially important in the case of cell damage due to exposure to various stress factors, including infection with pathogens (Almagro et al., 2009; Choi et al., 2007). The molecular and functional heterogeneity of isoPOs makes it possible to change the activity of different isoforms to the advantage of those that are most appropriate to the specific stress conditions of the environment, and an increase in PO activity can be suggested as a protective function of the organism. The

functions of PO can be associated not only with the synthetic processes during cell differentiation and organogenesis, but also with the regulation of plant cell metabolism and the control of plant growth and development (Tian et al., 2003). However, it is still difficult to understand why the same isoPOs can be responsible both for normal physiological processes (Cosio et al., 2009) and for the oxidative burst during pathogenesis.

The available information points to the need for the determination of the role of oxidoreductases in the resistance of plants to adverse environmental factors and consideration of their role in the concentration, generation, and utilisation of ROS in the infection zone (Bindschedler et al., 2006). It is suggested that both quantitative and qualitative changes in the level and activity of oxidoreductases can lead to changes in the reactions of free radical oxidation. Therefore, ROS and the oxidoreductases involved in the system of their generation and degradation can be combined into the pro-/antioxidant system (Maksimov & Cherepanova, 2006). Nonetheless, one of the most common and widely discussed functions of POs, currently, is their participation in lignin synthesis in the cell wall (Marjamaa et al., 2009).

3. Polysaccharide-specific plant peroxidases

Cell walls have a complicated polysaccharide structure which serves as a "stumbling block" for plant biologists. Thus, about 15% of the genes of *Arabidopsis thaliana* take part in the formation and functioning of its cell wall (Carpita et al., 2001). Cellulose is the major polysaccharide of the plant cell wall, but there are also many other polysaccharides once called "hemicelluloses". Hemicelluloses include connecting glycans (xyloglycans, xylans, mannans and glucans (callose)) and pectins (polygalacturonic acid, rhamnogalacturonan, xylogalacturonan, arabinan and arabinogalactan) (Scheller et al., 2009). The rigidity and water-impermeability of cell walls is determined by the presence of phenolic polymer lignin. Lignin is considered to be a specific barrier in the way of fungi penetrating into a plant. It is believed that the main mechanism of plant defence against fungal plant pathogens is the synthesis and accumulation of lignin at the sites of penetration. Obviously, lignin molecules interact with the polysaccharide skeleton of cell walls. As such, a number of researchers agree that oxycinnamic acids bound with polysaccharides serve as primers for lignin polymerisation. Consequently, the lignin formation begins at the specific sites of polysaccharide molecules containing their oxycinnamic acids (Gorskova, 2007). Next, it was shown that the side chains of sugar beet (*Beta vulgaris*) pectins - which are mainly composed of arabinose and galactose residues - are esterified by ferulic acid units. Feruloyl esters may also be involved in the cross-linking of polysaccharides to lignins (Levigne et al., 2004). Recently, the genes involved in the feruloylation of arabinoxylan have been identified in rice (Piston et al., 2010). So, pectins can be an "anchor" for the initial step of lignin formation. The next step – polymerisation - requires the presence of PO for the oxidation of monolignols (Boerjan et al., 2003). Thus, researchers from Geneva University have shown the sorption of PO isolated from zucchini and *Arabidopsis* by pectate in the presence of Ca^{2+} (Dunand et al., 2002). The hypothesis about the ionic interaction of these POs with pectates was proved using PO with a deletion of the nucleotide sequence responsible for the translation of the polysaccharide-binding amino acid sequence and the subsequent transgenesis of the mutated genes of the protein into tobacco plants (Dunand et al., 2003). Zones with high electrostatic activity were found on the surface of some POs, and these

zones can interact with cell wall pectins in the presence of calcium. Such isoPOs genes are activated in the zones of the formation of meristematic tissues and they display a strict tissue transcriptional activity (Carpin et al., 2001). It has been shown - with the help of electron microscopy - that in apical meristem of *Spinaceae* the majority of extracellular PO activity is found in middle lamella and cell corners (Crevecoeur et al., 1987), which coincides with the localisation of pectates in the cell wall.

In our investigations, we also detected the sorption of isoPO from potato, *Arabidopsis* and wheat, by calcium pectate. Moreover, we observed the binding with calcium pectate of potato PO from the fraction of proteins ionically bound with cell walls. It is likely that the ability of some PO isoforms to bind with pectin ensures the spatial proximity of these enzymes to the sites of the initiation of lignin synthesis and that these "pectin-specific" isoforms take part in this process.

The possible involvement of polygalacturonic acid-containing molecules in the defence reactions of tomato root cells against *Fusarium oxysporum* was suggested about 20 years ago by their accumulation at penetration sites. Since papillae are held to serve as a resistance mechanism to fungal penetration, it was assumed that the interrelation between pectin and other polymers - such as lignin - may contribute to enhancing the hardness of these newly formed structures (Benhamou et al., 1990).

Next, we observed that the activity of this isoPO was higher in calluses than in intact plants. Moreover, the significant activation of this isoform (pI~ 8.5) was shown in potato calluses infected by *Phytophthora infestans* (Fig. 1).

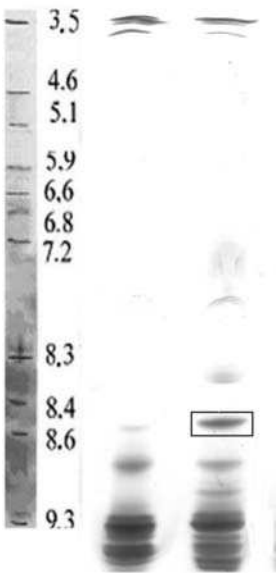

Fig. 1. Spectrum of potato calluses isoperoxidases: (a) non-infected plants protein extract, (b) protein extract from plants, infected by *P. infestans*. M - protein markers.

It may be that the presence of this isoform promotes the strengthening of calli cell walls through a special mechanism, since cultured cells have mainly undifferentiated cell walls missing lignification and suberin deposition, similar to meristematic cells in plants.

4. Peroxidases specific to the pathogen polysaccharides

It is interesting that Hammerschmidt and Kuc' (1982) observed the formation of lignin on the *Colletotrichum lagenarium and C. cucumerinum* fungal mycelium in the presence of PO extracted from cucumber plants, eugenol or coniferyl alcohol and hydrogen peroxide. Consequently, on the surface of these pathogens, a hypothetical "anchor" molecule for lignin synthesis initiation should be present, similar to pectin in the plant cell wall. Both plant tissues and pathogen hyphae may be lignified. The mechanisms of targeted lignification in the vicinity of infecting fungal structures, as well as the structures of plant cell walls, are poorly understood.

It has been shown that the cell walls of the majority of fungi contain chitin (Bowman, Free 2006). Chitin can be extracted in great quantities by industrial processes from crab and shrimp shells or the larvae cuticles of insects such as *Musca domestica* or *Galleria mellonella* (Ostanina et al., 2007). Some authors suggest a scheme of chitin extraction from the fungal mycelium of moulds (*Aspergillus sp., Mucor sp*.) (Wu et al., 2005). The low price and ecological safety of this biopolymer define it as an available matrix for technological processes. In this section, we would like to demonstrate the possibility of using chitin for the isolation of certain isoPOs from a great number of plants.

In 1958 Siegel showed that the addition of chitin to the reaction mixture for oxidation and polymerisation, with the help of the plant PO of the phenol compound eugenol present in lignin, increased the output of the reaction products and changed their structural quality as compared with the reaction without chitin. Based on these observations, it was proposed that chitin can be considered as a matrix for plant lignin formation. Using chitin as a biological matrix, we have developed an approach for isolating PO from the protein extracts of plants (Khairullin et al., 2000). Initially, we found that a fraction from wheat extract absorbed on chitin contained a dark-brown pigment which could be eluted with 1 M NaCl and which manifested the enzymatic activity of PO in the presence of H_2O_2. For the first time proteins with p*I* ~ 3.5 and a molecular weight of 37 kDa were found in plants, and these proteins could be absorbed by chitin due to ion exchange and manifest PO activity (Maksimov et al., 2005). Next, we detected POs with similar properties in a great number of plant species (Tab. 1) (Maksimov et al., 2003).

After this, we demonstrated the ability of wheat anionic POs to bind to the chitin of the cell walls of fungal pathogens. We called these POs "chitin-binding POs" (Maksimov et al., 2003). We were the first to demonstrate the binding of the anionic PO of wheat root to chitin (Maksimov et al., 1994). Besides this, we observed that in some species the activity of POs was increased in the unbound (*Armoracia rusticana, Lagenaria siceraria)* or eluted (*Pisum sativum, Galega orientalis, Brassica oleraceae)* fractions of proteins after interaction with chitin.

So, the sorption of PO on polysaccharides was not a classic ion-exchange interaction because the proteins were different in both isoelectric points and the molecular weights exhibited affinity for them. This conclusion was confirmed by the fact that the desorption of PO was facilitated by increasing NaCl concentrations as well as that isoPOs with a different p*I* can

bind chitin, as has been shown earlier (Maksimov et al., 2003). Additionally, it was shown that the ion-exchange affinity of POs for polysaccharides is determined by the presence of zones with high electrostatic potential in the enzyme molecule (Dunand et al., 2002). Therefore, it can be assumed that the wheat POs adsorbed on chitin contain sites that can specifically interact with their acetyl residues.

N	Plant species	PO activity (U/mg protein)			isoforms in plant tissue	pI of chitin-specific POs	Chitin-specific isoPOs
		Crude extract	Unbound with chitin fraction	Binding with chitin fraction			
1	*Triticum aestivum*	10.5±1.2	4.3±0.5	11.0±3.0	9	3.5	1
2	*Avena sativa*	3.6±0.5	3.1±0.2	11.1±2.5	11	3.5; 3.6	2
3	*Oryza sativum*	1.7±0.2	4.9±0.6	0.5±0.2	15	3.5; 4.8; 6.2	3
4	*Zea mays*	1.5±0.3	2.9±0.7	0.3±0.1	7	3.6	1
5	*Allium cepa*	1.7±0.2	0.5±0.2	0.4±0.1	10	4.8; 5.8; 6.8	3
6	*A. porrum*	1.4±0.2	1.2±0.2	-	6	-	-
7	*A. sativum*	2.3±0.5	0.4±0.1	1.8±0.4	7	3.5; 5.8; 6.5; 7.0; 9.8	6
8	*Aloe vera*	1.0±0.2	1.6±0.2	0.7±0.1	4	5.5; 6.2; 6.5	3
9	*Lilium regale*	2.5±0.5	2.2±0.3	-	8	3.6; 7.0	3
10	*Hosta glauca*	2.6±0.5	2.8±0.3	0.3±0.1	11	7.2	1
11	*Phoenix dactylifera*	2.0±0.3	1.25±0.2	0.8±0.4	4	3.5; 5.8	2
12	*Armoracia rusticana*	7.0±1.0	19.2±2.3	2.0±0.4	8	3.5; 3.8	2
13	*Raphanus sativus*	9.0±0.7	11.3±0.9	2.9±0.3	6	3.6	1
14	*Brassica oleraceae*	5.6±0.2	6.2±0.3	15.5±0.8	6	8.0; 9.0	2
15	*Solanum tuberosum*	1.9±0.2	6.8±1.1	1.6±0.3	19	3.5; 3.7; 8,8; 9.1	4
16	*Nicotiana tabacum*	24.0±0.9	9.0±0.9	13.7±2.0	6	3.7	1
17	*Petunia hybrida*	5.2±0.5	3.7±0.6	23.9±2.2	6	3.5; 4.3; 8.0; 8.6	4
18	*Cucumis sativus*	9.8±0.5	1.9±0.1	13.5±0.9	11	3.4; 3.8; 7.2; 8.3; 8.5	7
19	*Cucurbita pepo*	6.2±0.6	1.7±0.2	5.1±0.8	4	3.5; 3.8; 9.8; 10	2 4
20	*Lagenaria siceraria*	2.4±0.3	4.6±0.4	-	8	-	-
21	*Arachis hypogaea*	8.2±1.1	1.3±0.1	6.1±0.9	13	from 3.4 to 5.5	9
22	*Pisum sativum*	3.8±0.3	2.9±0.5	26.1±3.9	4	3.5; 8.3; 10.2	3
23	*Galega orientalis*	3±0.2	3.9±0.3	44.2±0.2	8	3.5; 7.8; 9.0; 10.2; 10.8	6

Table 1. Analysis of chitin-specific isoperoxidases in plant species

We found that the PO activity in all the plants tested increased in the presence of chitin as compared with the activity in crude extracts. This activation was not only found in chitin-eluted fraction but also in that fraction not adsorbed on chitin. This data confirms the

possibility of PO activation in the presence of polysaccharides (Gulsen et al., 2007). This increase in the activity is probably due to the conformational change of the enzyme molecules (Liu et al., 2005) or the clearing out from the extracts of various inhibitory factors possessing a higher affinity with chitin and remaining bound to it even after elution with 1 M NaCl (Maksimov et al., 2003).

In addition, such an increase in enzymatic activity could result from changes in the conformation of the enzymatic molecules due to the high electrostatic activity of chitin (Dunand et al., 2002; Ozeretskovskaya et al., 2002). It can be proposed that the PO sorption on chitin could not be considered to be a classic ion exchange process because both the anionic and cationic isoforms of the plant POs interact with chitin. Additionally, it contains 3 high anionic POs (3.5, 3.7, 4.0) but only 2 of them (3.5 and 3.7) adsorbed on chitin alongside with some cationic isoforms (Fig. 2).

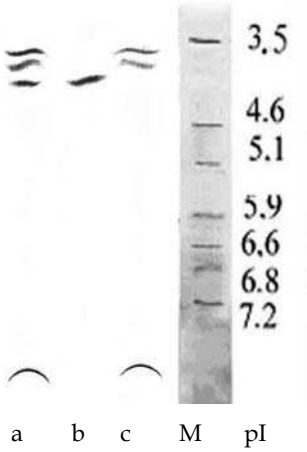

Fig. 2. Spectrum of anionic isoperoxidases isolated from potato: (a) crude protein extract, (b) protein fraction not adsorbed on chitin, and (c) chitin-specific peroxidises. M - protein markers.

In some cases, the anionic POs adsorbed on chitin have similar antigenic determinants, but the plants belonging to different families - and even members of the same family - could have polysaccharide-specific POs with different structures. Thus, the majority of investigated species had anionic chitin-specific peroxidises, and these isoforms from potato (*Solanaceae*) and horseradish (*Brassicaceae*) formed lines of precipitation with antibodies to wheat chitin-bound PO but not to anionic isoPO (Maksimov et al., 2000). However, protein extracts from several plants of *Brassicaceae*, *Cucurbitaceae* and *Fabaceae* formed precipitate with both the chitin-specific and anionic PO of wheat (Fig. 3). It was found that the greatest homology showed in plants and formed precipitation lines with the anionic PO of wheat (Tab. 2).

The analysis of the data on the presence of gene sequences encoding the polysaccharide-specific sites of PO in different plants was carried out. The fragment of the gene encoding amino-acid sequence 243-269 of wheat anionic isoPO TC151917 (tab. 2) homologous to the fragment of *Arabidopsis* gene encoding pectin-bound site of PO - was detected (Dunand et al., 2002).

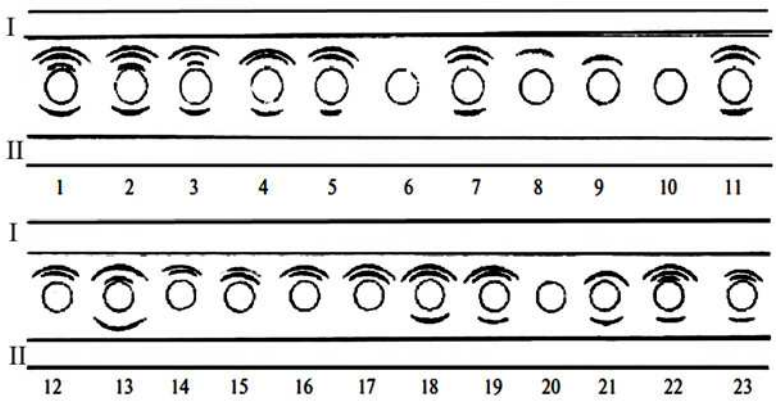

Fig. 3. The scheme of the precipitates formed by the crude protein extracts of plants of the groups monocotyledons (1-11, table 1) and dicotyledons (12-23 table 1) with antibodies against wheat chitin-binding proteins (I) and with antibodies against wheat anionic PO (II).

The characteristics of the POs to be activated by chitin and adsorbed suggest their involvement in the processes underlying two reaction-types which provide the plant with protection against pathogens. The first type includes the fast activation of the enzyme upon its contact with pathogen cell structures, as observed – most notably - with the rice, potato and horseradish POs interacting with chitin. The second reaction-type is comparable with the gradual accumulation of the enzyme molecules within a region of fungus location associated with the appearance of a specific "attracting" centre in the form of chitin-containing pathogen structures. Thus, it may be that due to these functions, lignification and the synthesis of other phenolic polymers resistant to the enzymatic attack of pathogenic fungi usually occur at the site where parasites are located (Simonetti et al., 2009; Kupriyanova, 2006).

The attachment of a pathogen to the surface of plant cells is the first step in formation of a multistep infection process. Unfortunately, it is difficult to observe these steps when fungi's growth and development are studied, and it is easier to study these steps using unicellular symbiotic bacteria. The A 75-kDa protein found in carrot is immunochemically similar to human vitronectin and the elongation factor eEF-1a and it is able to bind bacteria (Wagner & Matthysse, 1992). The authors indicated that these plant cells lost the ability to bind with bacterial cells after treatment with ionic detergents and that this proved the ionic character of these bonds. Proteins with a similar molecular weight and a high binding affinity with chitin were isolated from the microsomal fraction of rice cells (Ito et al., 2000; Yamaguchi et al, 2000). A vitronectin-like protein is involved in the adhesion of the plasma membrane to the cell wall and in extension of the fertilisation tube. In this connection, it is interesting that the PO activity was manifested by the animal protein peroxinectin, which is similar to vitronectin. The 76-kDa protein peroxinectin can bind phage particles and thus it is suggested as having lectin-like features. Both fibronectin and apolipoprotein E are found to possess a C-terminal heparin-binding domain (Kim et al., 2001). Unfortunately, the gene encoding the vitronectin-like protein has not been detected in plants, and this leaves open the degree of its molecular similarity to human vitronectin. The antibodies to the

vitronectin-like protein and to the protein binding with bacterial rhicadhesin did not cross-react; however, they could competitively suppress the binding of bacterial cells with plant cells (e.g. with pea cells). Consequently, the above-mentioned proteins are different in molecular structure but their biochemical features are somewhat similar.

Plant species	Locus	Analyzed fragment	Reference
Triticum aestivum	TC151917 X56011	243fd**K**qyyhnlln**KK**glltsdq269 238fdnayytnlmsq**K**gllhsdq257	(http:// PO. isb-sib.ch/) (Rebmann et al., 1991)
Avena sativa	AF078872	249fdnsyynnllsq**K**gllhsdq259	(Cheng et al., 1997)
Oryza sativum	X66125 D84400 OS378734	243fdnayysnllsn**K**gllhsdq262 249fdnryyqnllnq**K**gllssdq268 255fdlgyf**K**nva**KRR**glfhsdg280	(Riemann et al., 1992) (Ito et al., 2000) (Chittoor et al., 1997)
Zea mays	AF037033 AY500792 ZM004710	175fdndyy**K**nllte**R**gllssdq194 265fdn**K**yyvgltnnlglf**K**sdv285 256fdn**K**yyfdlia**K**qglf**K**sdq279	(Padegimas et al., 2004) (Gullet-Claude et al., 2004) (Teichman et al., 1997)
Allium cepa	TC6261	240fdn**K**yyvdlln**R**qtlftsdq259	(http:// PO. isb-sib.ch/)
Armoracia rusticana	D90115	253fdn**K**yyvnl**K**en**K**gliqsdq273	(Fujiyama et al., 1990)
Raphanus sativus	X91172	258fdnsyf**K**nlmaq**R**gllhsdq277	(http:// PO. isb-sib.ch/)
Brassica oleraceae	75974310	260fdn**K**yyvnl**K**eh**K**gliqtdq280	(http:// PO. isb-sib.ch/)
Arabidopsis thaliana	ATg08770 NM101321	217fdn**K**yyvnl**K**en**K**gliqsd250 241fdnnyy**R**nlmq**KK**gllesdq261	(Dunand et al., 2002) (http: // ncbi.nlm.nih.gov)
Solanum tuberosum	M21334	271fd**K**vyydnlnnnqgimfsdq290	(Roberts et al., 1988)
Nicotiana tabacum	L02124	221fdndyftnlqsnqgllqtdq240	(Diaz-De-Leon et al., 1993)
Petunia hybrida	CV299755	22fdnmyf**K**nlq**R**g**R**glftsdq41	(http:// PO. isb-sib.ch/)
Cucumis sativus	DQ124871	183fdnnyf**K**nlvq**RK**glletdq203	(http: // ncbi.nlm.nih.gov)
Cucurbita pepo	DQ518906	239fd**K**nyytnlqan**R**glltsdq59	(Carpin et al., 1999)
Arachis hypogaea	71040666	252ldnnyy**R**nildn**K**glllvdh272	(Yan et al., 2003)
Pisum sativum	AF396465	16fdvgyf**K**qvv**KRR**glfesda36	(http:// PO. isb-sib.ch/)

Note: The amino acid residues of lysine (K) and arginine (R) which may be responsible for the binding of POs to polysaccharides are in bold. According to Dunand et al. (2002), the mutual substitution of these amino acids has no influence on the sorption properties of the ATg08770 PO of *Arabidopsis* with pectins, and the deletion of the fragment results in the loss of this function.

Table 2. Search and comparison of the regions of plant peroxidases homologous to the polysaccharide-binding site

So, among diversified plant proteins, we found POs which could be adsorbed on chitin, thereby preserving their enzymatic activity. An analysis of the isoenzymatic range and activity of chitin-binding POs revealed considerable differences between plant species. In particular, anionic isoPOs of practically all the examined species were adsorbed on chitin. This fact is of great importance because investigators accentuated some remarkable properties of anionic POs – high stability, resistance to temperature and pH changes and activity under high-

oxidative conditions (Gazaryan & Lagrimini, 1996; Sacharov, 2004). Thus, the isolation of anionic POs with chitin matrices is likely to be very promising. Besides, practically every another polysaccharide-binding protein (such as chitinases) are cationic, and this fact makes chitin a more suitable matrix for POs isolation than - for example - pectates. However, in some species the constitutive activity of anionic POs is insignificant; therefore, one of the problems is how "to force" plants to synthesise more anionic isoPOs. The study of the physiological role of these proteins can help to solve this question.

5. Possible function of polysaccharide-binding plant peroxidases

We observed the activation of chitin-specific PO during infection with the causative agents of a number of diseases: in wheat under the influence of *Bipolaris sorokiniana* and the elicitors (Fig. 4), *Septoria nodorum* (Yusupova et al., 2006) and *Tilletia caries* (Khairullin et al., 2000); in potato infected by *Phytophthora infestans* (Maksimov et al., 2011), and in *Aegilops umbellulata* infected by *Septoria nodorum* (Maksimov et al., 2006).

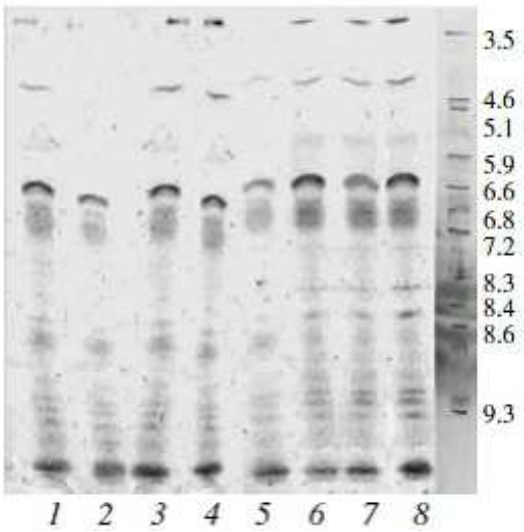

Fig. 4. PO pattern in a water-soluble protein fraction from the roots of wheat seedlings, contrasting in their resistance to the causal agent of the root rot *Bipolaris sorokiniana*, 48 h after infection (Burchanova et al., 2007). (1-4) – cultivar Znitsa (susceptible), (5-8) – cultivar Zarya (resistant). (1,5) control seedlings; (2,6) seedlings infected with *B. sorokiniana*; (3,7) seedlings treated with chitooligosaccharides; (4,8) – seedlings treated with chitooligosaccharides and infected with *B. sorokiniana*.

Akhunov et al. (2008) purified chitin-specific PO with fungicidal activity from cotton and observed the increase of its activity in plants, penetrated by *Verticillium dahliae*. Golubenco et al. (2007) showed the presence of the chitin-binding PO isozyme in *Hibiscus trionum*, which activated dramatically after inoculation by *V. dahliae*. The plants of *Nicotiana tabacum* overexpressing the anionic PO (chitin-specific according to our data) were more resistant to *Helicoverpa zea* and *Lasioderma serricorne* as compared with the wild-type (Dowd et al., 2006).

In this way, chitin-specific POs play an important role in the defence reactions of plants to microbial invasion.

These mechanisms are regulated substantially by the signalling molecule, inducing systemic resistance which is a form of long-lasting immunity to a broad spectrum of pathogens. For example, the accumulation of salicylic acid (SA) is often parallel to or else precedes the increase in the expression of PR genes and ROS accumulation needed for lignification (An & Mou, 2011). In our investigation, SA promoted the activity of wheat "chitin-specific" isoPOs, and SA-treated plants were more resistant to *Septoria nodorum* (Maksimov & Yarullina, 2007).

Thus, in wheat calli infected by *S. nodorum*, the activity of chitin-specific anionic PO with pI ~ 3.5 increased (Fig. 5). When the infected calli were treated with salicylic acid, the cytoplasmic anionic PO with pI ~ 3.5 (no. 1) was greatly activated as well as the one with pI~ 9.8 (no. 14), and was capable of interacting with the cell walls of fungi (presumably, "glucan-specific"). It is worth mentioning that all of the detected POs are involved in plant defence responses against pathogenic fungi.

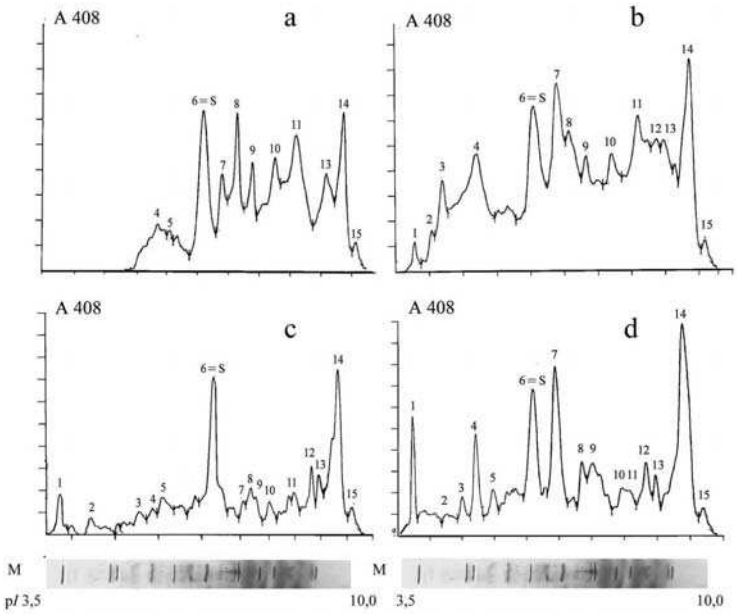

Fig. 5. Densitograms of the PAAG after IEF of water-soluble isoPOs isolated from wheat calli 10 days after infection. (a) – control, (b) calli, infected with *Tilletia caries*; (c) calli on the MS medium supplemented with 0.05 mM salicylic acid; (d) calli infected with *T. caries* on the MS medium supplemented with 0.05 mM salicylic acid; (M) marker (Maksimov & Yarullina 2007).

Therefore, the ability of certain POs to bind with chitin is a widespread phenomenon and - possibly - connected with the defence reactions of the organisms to pathogen attacks. Since it was shown that some biogenic molecules - such as chitooligosaccharides or salicylic acid - can activate an anionic POs, we might suggest that an application of these compounds optimises the process of anionic PO isolation with a chitin.

6. The degree of influence of the acetylation of polysaccharides on the sorption of peroxidases

Numerous polysaccharides in the cell walls of plants and fungi are subjected to cross-linking and are modified by methylation and acetylation in the cell wall (Scheller & Ulvskov, 2010). However, the functional role of the degree of the acetylation of polysaccharides is still unclear. According to our results, we suggest that the acetylation of polysaccharides promotes their binding with some PO isoforms, similarly to that of the chitin from fungal pathogens (Maksimov et al., 2005).

As such, the analysis of matrices capable of PO sorption revealed that PO interaction with chitin decreased with the deacetylation of the latter. It was suggested that the anionic PO of wheat should more actively interact with the acetylated derivatives of cellulose. In fact, acetyl cellulose adsorbed anionic PO, whereas cellulose did not adsorb it. This fact suggested the importance of the degree of polysaccharide acetylation for binding with PO. The binding coefficient of these oxidoreductases with acetyl cellulose was much higher relative to its sorption onto chitin. It is significant that acetylated chitooligosaccharides elicitors enhanced the defence reactions in *Arabidopsis* and wheat more effectively than the deacetylated ones (Cabrera et al., 2006). Besides, the effective concentration of deacetylated derivatives for triggering the defence reaction of soybean was higher than in those cases using highly acetylated oligomers (Shibuya, Minami, 2001). In our investigation, the ability of high-acetylated chitooligosaccharides to promote the transcription of wheat (Burchanova, 2007) and potato anionic PO was more significant than that of deacetylated ones. The investigations of El Guedarri et al. (2002) showed that the penetrating structures of the pathogens contained chitosan rather than chitin. This fact is argued by the absence of the interaction of the monoclonal antibodies specific to chitin following penetration and the appearance of specific reactions with chitosan antibodies. It should be noted that the aggressive race of phytopathogenic fungi has more active chitin deacetylase (Maksimov, Valeev, 2007). Our results show that microorganisms containing chitin (or its oligomers) are the targets of chitin-specific anionic plant POs performing a protective role. Thus, the defence mechanism in plants is specifically targeted and evolves where and when required. When chitin is deacetylated and transformed into chitosan, its ability to bind anionic PO declines (Fig. 6).

The ability of PO to interact with the acetyl residues of chitin allows us to compare them with monovalent lectins (i.e. extensins) which when binding with hemicellulose are only affected in a medium with a high ionic strength (Brownleader et al., 2006). As a rule, POs are bound with the plant cell wall and act as its modifiers. Some POs can form complexes with an extensin of cell walls (Brownleader et al., 2006). Consequently, chitin-specific sites that are capable of interacting with polysaccharides exist in the molecules of PO, and these sites can resemble the membrane receptor binding sites or else be similar to the domains of heparin-binding proteins (Kim et al., 2001).

As follows, during the plant-pathogen interaction a process of elicitor deacetylation takes place and it is possible that the effective penetration and colonisation of plant tissues in this case occurs due to avoiding "meeting" a specific plant PO in addition to the defence of fungus structures against plant chitinases. However, numerous of the polysaccharides of plants are also acetylated and it is not easy to suggest the cause for this fact. Thus, pectin acetylation with acetyl-CoA as the acetate donor has also been demonstrated *in vitro*. Recently, Scheller & Manabe (2010) have found that knockout mutants in one of the corresponding genes -

At3g06550 - are deficient in wall-bound acetate. It would be interesting to test their resistance, lignin depositions and other physiological parameters under the influence of pathogens. It may be that these experiments will make clear the role of polysaccharide acetylation.

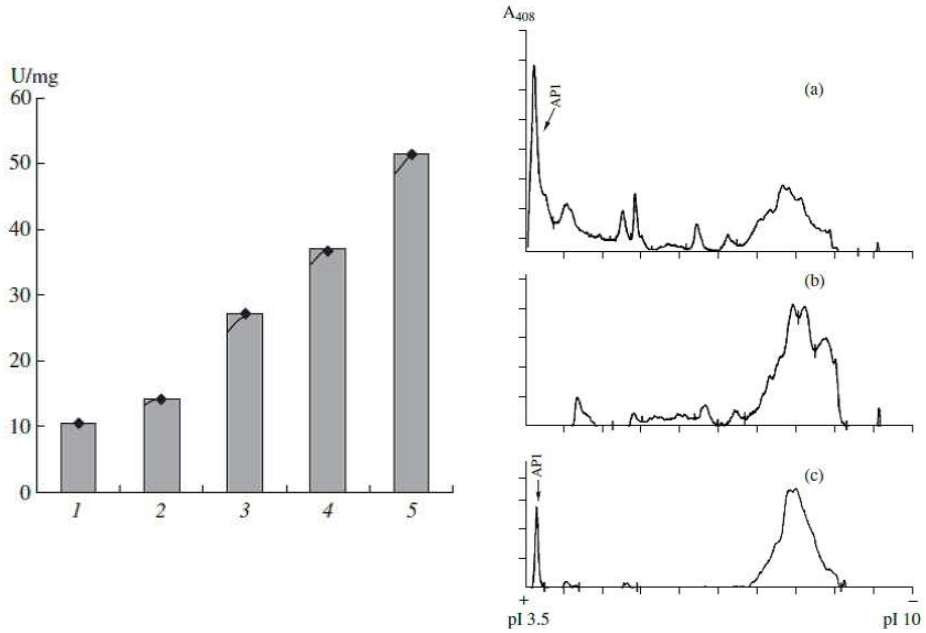

Fig. 6. Effect of the degree of chitin acetylation (%) on the interaction between chitin and chitin-specific wheat POs (A) (U/mg protein) (Maksimov et al., 2005); (B) PAAG after IEF of PO fractions from wheat roots (a) not bound to high-acetylated (b) and low-acetylated chitosan (c) (Khairullin et al., 2000). Designations: (1) 12%; (2) 23%; (3) 37%; (4) 45%; (5) 65%.

7. Can peroxidases bind with glucan-containing structures?

Earlier, we noted the ability of wheat PO to adsorb on purified chitin and suggested the possibility of the application of this polymer for POs' (in particular, for highly-stable anionic POs) purification. Chitin is a major component of fungi cell walls, but they also contain other polymers. Thus, using the spores of the bunt agent *Tilletia caries* as a sorbent for affinity chromatography we showed - as we predicted - the sorption on spores of the "chitin-specific" isoPO. However, in addition to this, significant content of PO with pI ~ 8.8 was observed (Fig.7) (Khairullin et al., 2000). Subsequently, through the chromatography of wheat POs on the cell walls of *Septoria nodorum* (Berk) we showed that only two cationic isoPOs bound with the cell walls of this pathogen and we didn't observe sorption of anionic PO.

It may be that this fact was associated with the rather complex composition of fungal mycelium, consisting of chitin enclosed in a glucan matrix (Bowman, Free, 2006). Therefore, mature saprophyte mycelium are completely covered by difficult-soluble glucans and the fraction of chitin in the apical cell wall is not sufficient. As such, we supposed that these cationic isoforms bound with another major component of the fungi

cell wall – glucans. To check this hypothesis, we carried out an investigation of the sorption of the potato PO using the cell walls of the late blight pathogen *Phytophthora infestans* as a sorbent. Strictly speaking, *P. infestans* is not a fungal pathogen as it belongs to the class of *Oomycota* and its cell walls do not contain chitin, consisting of glucans, cellulose and some another components (Gaulin et al., 2006).

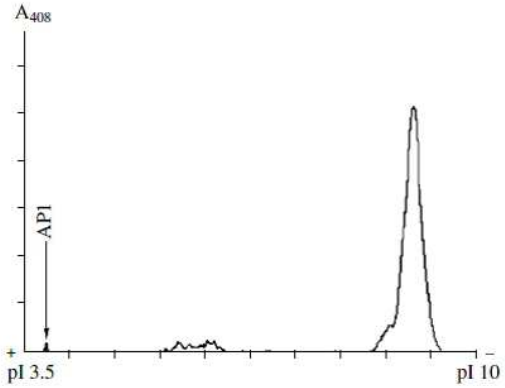

Fig. 7. PAAG densitogram after IEF of the PO fraction of wheat roots absorbed by the growing spores of *Tilletia caries*.

In fact, we observed the ability of cationic PO (pI 9.3) to bind with the purified cell walls of this pathogen (Fig. 8, A). This isoform was activated in the infected plants and under the influence of the stress hormone jasmonic acid, both individually and in combination with salicylic acid.

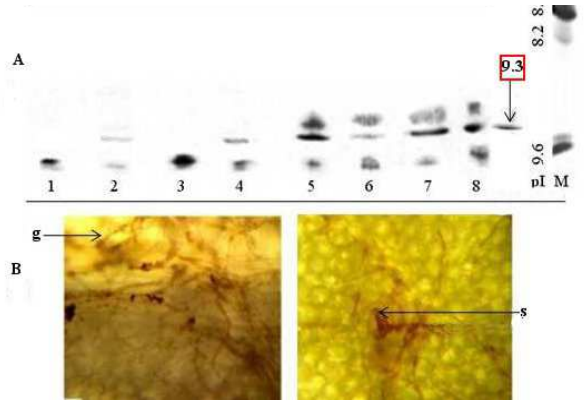

Fig. 8. Activation of the PO binding with *P. infestans* cell walls (glucan-specific?) under pathogen inoculation and treatment with salicylic (SA) and jasmonic (JA) acids (A); Peroxidase activity in stomata guard cells and intercellular spaces of adjoining epidermal leaf cells and on the surface of mycelium contacting with the stomata (B). (*1*) Non-treated control; (2) infection; (3) treatment with SA; (*4*) treatment with SA + infection; (*5*) treatment with JA; (*6*) treatment with JA + infection; (*7*) treatment with SA + JA; (*8*) treatment with SA + JA + infection; g – gifs of *P. infestans*; s – stomata guard cell. Specific to *P. infestans* cell walls, PO is highlighted.

Cytological experiments also demonstrated 2,2-diaminobensidine (DAB)-colouring of the infecting gifs on the surface of the leaves (Maksimov et al., 2011). Because DAB is intensively produced just on the surface of infectious products of a pathogen, this suggests focusing on them as ROS generators and their users, which are also likely to include POs (Fig. 8, B).

It is of interest that the activation of POs often takes place in stomata guard cells, since *P. infestans* mainly penetrates into plant tissues through stomata slits. The localisation of phenolic compounds - some of them seemingly being used by POs as a substrate - and PO activity was visible in guard cells. (Maksimov et al., 2011). As such, the immune reaction occurred in close proximity to pathogen structures.

8. Conclusion

It is well-known that POs can generate hydrogen peroxide and that it can act as a secondary messenger in defensive responses; besides this, they can oxidise numerous compounds - including phenols - and therefore catalyse a reaction that is directly associated with the lignification of the cell walls of plants and infectious fungal structures. These important physiological features of POs are found in the application of analytics and medical assays and industrial biocatalytic processes. Our results and our published data allow us to propose that a separate subclass of polysaccharide-binding isoPOs is present in plants and which take part in defence reactions against biotic stresses, including pathogen attacks and wounding (Carpin et al., 2001; Dunand et al., 2002). Unfortunately, currently, this subclass has yet to be characterised and its unique properties are not used in practice. It is possible that some of the POs of this class are functionally associated with the plant cell wall and contribute to its modification due to a high affinity for hemicellulose (most likely, to pectin). Some isoPOs can electrostatically bind with the components of the cell wall of pathogenic fungi and plants. These peroxidases probably facilitate the direct formation of lignin due to their ability to interact with polysaccharides. Therefore, it is possible that these isoenzymes play an important role in the defence reactions of plants against pathogens and wounding. The results obtained allow us to suggest the possibility of using polysaccharide biopolymers - chitin in particular - for some manipulations with POs.

Thus, the ability of PO to adsorb on chitin while preserving its enzymatic activity suggests the cooperative function of these enzymes in the defensive responses of wheat against chitin-containing pathogens and it opens up possibilities for using this biopolymer for the primary purification of chitin-specific proteins. It is worth noting that in the majority of cases the anionic POs have the ability to bind with chitin and according to some data (Gazaryan & Lagrimini 1996; Sacharov, 2004) these POs have higher thermal stability than cationic ones. Using these highly-stable, readily-produced POs can increase the quality of immunoblotting kits and stimulate the elaboration of new analytical methods.

It was shown that POs' binding with polysaccharides serves as a protective function in plants due to its immediate involvement in the action of the prooxidant and antioxidant systems. The possibility of regulating the PO-encoding genes' expression by the different regulators of plant resistance - including oligosaccharides - allows the determination of the role of the enzyme in plant immunity and it may also stimulate the production of POs (including anionic isoforms) by optimising their extraction. Besides this, we observed a unique feature of chitin in stimulating the POs activity. We suppose that this effect may be used for increasing the efficiency of obtaining PO preparations.

9. Acknowledgment

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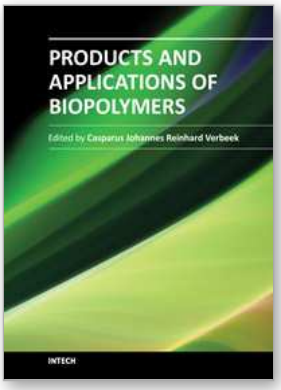

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It is interesting to consider that biopolymers are by no means new to this world. It is only because of our fascination with petrochemical products that these wonderful materials have been neglected for so long. Today we face a different challenge. Environmental pressure is pushing away from synthetic or petro-chemically derived products, while economic factors are pulling back from often more expensive "green" options. This book presents two aspects of biopolymers; potential products and some applications of biopolymers covering the current relevance of biopolymers.

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