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Partial characterization of bean and maize root peroxidases and their ability to crosslink potato protein

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Abstract: Two fractions of Class III peroxidases (POX; EC 1.11.1.7), soluble and ionically bound to the cell wall, were partially purified from bean and maize roots and characterized. According to the measured K_m, both the soluble and ionically bound to the cell wall fractions of POX had high affinity for H_2O_2 and the high specificity for caffeic acid. Approximate molecular weights of POX in their tertiary (native) structure were determined by modified sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). Proteomic analysis resolved the identity and pI of different enzyme bands. The ability of maize and bean soluble peroxidase to crosslink native potato proteins was evaluated. The results obtained by SDS-PAGE showed that both POX enzymes were capable of crosslinking potato protein, in particular patatin, a globular protein, with and without the presence of H_2O_2. To investigate the possible role of phenolic compounds in facilitating crosslinking, commercial horseradish peroxidase (HRP) with/without the addition of caffeic acid was used to crosslink potato protein. Information provided here could be useful for the purification of POX from maize and bean roots and for examination of protein-protein interactions.

Keywords: soluble peroxidase; ionic cell wall-bound peroxidase; bean and maize root; potato protein; protein crosslinking; phenolic compounds

Abbreviations: cPOX – covalent bound peroxidase; iPOX – ionically cell wall-bound peroxidase; iPOXb – ionically cell wall-bound bean peroxidase; iPOXc – ionically cell wall-bound maize peroxidase; sPOX – soluble peroxidase; sPOXb – soluble bean peroxidase; sPOXc – soluble maize peroxidase

INTRODUCTION

Peroxidases (POX; EC 1.11.1.7) are monomeric glycosylated hemoproteins and a subclass of oxidoreductases that catalyze the oxidation of different substrates using hydrogen peroxide [1, 2]. At all stages of plant development, from germination to aging, peroxidases participate in a broad range of plant physiological processes (lignification, suberization, auxin catabolism, antioxidative reactions) [2-6]. Plants possess a large number of peroxidase isoenzymes differing in molecular weight, thermal stability, pH optimum, substrate specificity and physiological role [2, 6-8]. Plant peroxidases are localized intracellularly (vacuoles and cytoplasm) and extracellularly (apoplast and cell wall) [9-11]. In addition, membrane-bound plant peroxidases were found and described [12]. Intracellular and apoplastic peroxidases are either soluble or bound to the cell wall via covalent (covalently bound POX, cPOX) or electrostatic (ionically bound POX, iPOX) forces [2, 11]. The POXs are capable of oxidizing a broad range of organic compounds, including phenols, aromatic amines, indoles and sulfonates, using hydrogen peroxide as the oxidant [13]. It has been shown that the kinetics of peroxidase enzymes involve several consecutive reactions and several enzyme states according to the Chance-George mechanism [14-16]. In addition to utilizing H_2O_2 for oxidative cross-linking of wall components, class III POX also possess the capacity to produce H_2O_2 via one-electron reduction of oxygen, thereby oxidizing NAD(P)H, ascorbate or auxin *in vitro* in the presence of trace amounts of Mn^{2+} and phenolic compounds and hydroxyl radicals

[17-19]. Another source of H_2O_2 for POX in vacuoles and apoplast can be from the process of autooxidation of phenolic compounds [9].

Enzymatic crosslinking of proteins results in modifications of their mechanical and functional properties as a result of the introduction of intra- and intermolecular covalent bonds in the protein network. Different oxidative enzymes such as laccase, tyrosinase and peroxidase have been used to crosslink mainly food proteins [20-22]. These oxidative enzymes are known to induce oxidation and crosslinks on tyrosine, cysteine or tryptophan residues [22, 23]. A recent study has highlighted the importance of tyrosine location in tyrosinase-mediated crosslinking of soy glycinin-derived peptides [24]. It has been shown that horseradish POX could induce crosslinking of some proteins in the presence of hydrogen peroxide and a low molecular weight hydrogen donor such as ferulic acid [25, 26]; the enzyme has been used to crosslink whey proteins and caseins [21, 22, 27]. Potato protein was recently reported to undergo crosslinking with tyrosinase from a bacterial source [28].

In the present study, POX was used to examine the ability to crosslink potato protein with a different type of an oxidoreductase. In this context, the aim of the work was partial purification of soluble and ionically cell wall-bound POX enzymes from maize and bean roots, and determination of their ability to crosslink potato protein. The role of phenolic compounds in potato protein crosslinking was also investigated and discussed.

MATERIALS AND METHODS

Plant materials and growth conditions

Bean (*Phaseolus vulgaris* L.) and maize (*Zea mays* L.) seeds were washed under tap water and germinated at 18°C in the dark for 3 days. Seedlings were then placed in tap water, which was changed after a week and grown hydroponically for 14 days in a growth chamber with a photoperiod of 16h/8h (light/darkness) at 24°C and 18°C. Irradiance of 80 $\mu mol \cdot m^{-1}s^{-1}$ was provided by white fluorescent tubes. After two weeks, the roots were stored at -18°C until analysis.

Peroxidase purification

The roots were cut and placed in sodium phosphate buffer (SPB) [100 mM SPB pH 6.4, 1 mM phenylmethylsulphonyl fluoride (PMSF)] at 4°C and immediately homogenized in a blender for 3 min (at a ratio of 1:3=plant:buffer). The homogenate was filtered through four layers of cloth and centrifuged at 17000 x *g* for 20 min. The obtained supernatant was further used for purification of soluble POX, while the pellet with cell wall fragments was used in further steps for purification of iPOX.

Protein precipitation from the supernatant was carried out at 70% $(NH_4)_2SO_4$ saturation. The mixtures were incubated for 2 h at 4°C. The recovered precipitates were centrifuged at 17000 x *g* for 20 min at 4°C and suspended in SPB, followed by dialysis against SPB to remove salt. The dialysate was concentrated 20-fold using ultrafiltration through regenerated cellulose filters with a MW cut-off of 3 kDa (Amicon Ultra-4 Centrifugal Filter Device) and used for analysis of the soluble POX fraction (sPOX), from bean root (sPOXb) and from maize root (sPOXc). The pellet with cell wall fragments was washed four times in 100 mM SPB (pH 6.4). To extract the ionically bound protein fraction, the pellet was suspended in 1M NaCl, incubated for 1 h at 4°C, and then centrifuged at 17000 x *g* for 20 min. The obtained supernatant was dialyzed against SPB for 24 h, concentrated about 20-fold using an Amicon Ultra-4 Centrifugal Filter Device and used for analysis of (iPOX) from bean root (iPOXb) and from maize root (iPOXc).

Peroxidase activity

The sPOX and iPOX activities of bean and maize were measured spectrophotometrically in a reaction mixture consisting of 100 mM SPB pH 6.4, 0.01 M pyrogallol and aliquots (2 µL) of soluble or ionically cell wall-bound fractions. The reaction was started by the addition of 3.3 mM H_2O_2 and the increase in absorbance at 430 nm was followed. Peroxidase activity was calculated using the extinction coefficient for purpurogallin (ε=12 $mM^{-1}cm^{-1}$) [29].

Oxidation of hydroxycinnamic acids (*p*-coumaric, chlorogenic (CGA), or caffeic acid) by iPOX or sPOX was measured in a reaction mixture (3 mL) containing 2 µL of soluble or ionically cell wall-bound fraction,

3.3 mM H_2O_2 and 0.04 mM *p*-coumaric acid, or 4mM chlorogenic or caffeic acid in 100 mM SPB (pH 6.4). The increase in absorbance was followed at 410 nm for CGA and caffeic acid, and the decrease at 286 nm for *p*-coumaric acid [30]. K_m was determined for pyrogallol at a concentration ranging from 0.33-10 mM, and for H_2O_2 in the range from 0.083-6.67 mM. Protein concentrations were determined according to Bradford [31].

Modified SDS-PAGE

Modified SDS-PAGE at a final concentration of 0.1% (w/v) SDS in all solutions and gels was used to separate peroxidase isoforms [12]. Samples were diluted in loading buffer to a final concentration of 50 mM Tris-HCl (pH 6.8), 0.1% (w/v) SDS, 10% (w/v) glycerol and 0.002% (w/v) bromophenol blue, without reducing compounds, and loaded onto the gels without heating. POX isoforms remained active after separation on the gel by staining with 1 mM 3,3-diaminobenzidine (DAB) and 0.03% H_2O_2 in 100 mM SPB pH 6.4. This enabled determination of the apparent molecular weights of peroxidase isoforms with a preserved tertiary structure, using molecular weight standards (Broad Range, Bio-Rad, Munich, Germany) according to Laemmli [32]. The R_f values of POX isoforms were determined on the scanned gel by the program Image Master Total Lab TL 120 software (Nonlinear Dynamics Ltd., Durham, USA).

Enzymatic crosslinking of potato protein

Potato protein (1%) was suspended in 100 mM SPB (pH 6.4) and stirred for 30 min at ambient temperature. The crosslinking reaction was carried out with soluble potato protein and the addition of sPOX or iPOX fractions from bean and maize. sPOX or iPOX were added at a 1:25 enzyme to protein ratio, while the non-crosslinked samples were treated similarly but without enzyme addition. Every 5 min, 2 µL of a 50 mM H_2O_2 solution was added to the reaction mixture of crosslinked and non-crosslinked samples [21], and a control without addition of H_2O_2 to the reaction mixture was used. The same crosslinking reaction was carried out with commercial horseradish peroxidase (HRP) (Sigma-Aldrich, Steinheim, Germany) and the addition of 2 mM caffeic acid [33], followed by gradual addition of H_2O_2. The reaction mixtures were incubated at 37°C with shaking at 250 rpm in an incubator shaker (TU-400 Orbital Shaker Incubator, MRC, Holon) for 5 h. Samples were taken at various time points (0, 60, 180 and 300 min, respectively) and the reaction was stopped by directly mixing the reaction mixture with electrophoresis sample buffer (x4) at a 1:1 ratio (v/v). The samples were analyzed by SDS-PAGE.

SDS-PAGE analysis

Electrophoresis was performed on a discontinuous buffered system [32] using a 15% separation gel and a 4% stacking gel. The samples were heated for 10 min at 95°C after addition of sample buffer (4x), 1:1 (v/v). Samples were mixed with reducing (5% β-mercaptoethanol) sample buffer. The gels were stained with 0.25% Coomassie brilliant blue (R-250) in 50% ethanol and 10% acetic acid and destained in 10% acetic acid [methanol:acetic acid:water, 20:10:70 (v/v/v)].

In-gel proteolysis and mass spectrometry analysis

Clean SDS-PAGE gels were prepared taking care to avoid keratin contamination (gloves and hair nets were used). Lanes with crude enzyme extract were used for extraction. The pieces of gel were cut using a clean razor blade and placed in a microfuge tube. The proteins in the gel were reduced with 3 mM DTT (at 60°C for 30 min), modified with 10 mM iodoacetamide in 100 mM ammonium bicarbonate (in the dark at room temperature for 30 min), and digested in 10% acetonitrile and 10 mM ammonium bicarbonate with modified trypsin (Promega) overnight at 37°C. The resulting tryptic peptides were resolved by reverse-phase chromatography on 0.075 X 200-mm fused silica capillaries packed with Reprosil reversed phase material (Dr Maisch GmbH, Germany). The peptides were eluted with linear 65-min gradients of 5 to 45% and 15 min with 95% acetonitrile and 0.1% formic acid in water at flow rates of 0.25 µL/min. Mass spectrometry was performed by an ion-trap mass spectrometer (OrbitrapXL, ThermoFisher) in positive mode using a repetitively full MS scan followed by collision-induced dissociation (CID) of the 5 most dominant ions selected from the first MS scan. The mass spectrometry data was analyzed using Protein Discoverer 1.4 (ThermoFisher Inc.) and Sequest search engine, searching against bean (*Phaseolus vulgaris* L.) and maize (*Zea mays* L.)

databases (Uniprot). Peptide- and protein-level false discovery rates (FDRs) were filtered to 1% using the target-decoy strategy.

Statistical analysis

All experiments were conducted in triplicate, and the results are expressed as the mean±standard deviation. The data were subjected to analysis of variance (ANOVA), and the means were compared by the Holm-Sidak test (SigmaPlot 11.0, Systat Software, Inc., USA). The level of significance was set at $P<0.05$.

RESULTS

Peroxidase purification and relative molecular weight

The soluble and ionically bound fractions of POX were partly purified from maize and bean roots and characterized. SDS-PAGE with a low SDS concentration and gel staining with 3,3-diaminobenzidine (DAB) revealed the presence of isoforms at a wide range of molecular weights (Fig. 1). Common peroxidase isoforms of about 45 and 38 kDa were present in all isolates as well as isoforms with molecular weights higher than 130 kDa. In Fig.1 it can be seen that isoforms with molecular weights around 50 and 78 kDa were present in all POX fractions except in bean iPOX. A 35 kDa isoform was observed in maize iPOX and in bean iPOX and sPOX, while the soluble and bound bean POXs had an isoform of about 36 kDa. A ~55 kDa isoform was observed in maize sPOX, and a ~57 kDa isoform was detected in bean iPOX.

The results of liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) presented on Table 1 confirmed the presence of the maize sPOX isoform with a molecular weight of 38 kDa (pI 6.05, 6.96, 7.14 and 7.9), as well as the presence of bean sPOX isoforms with molecular weights of 35 kDa (pI 5.08 and 6.84), 36 (pI 8.43 and 8.75) and 38 kDa (pI 7.87).

Peroxidase activity and kinetic studies

The K_m values for all enzymes for pyrogallol were determined in the presence of 3.3 mM H_2O_2, while the values for H_2O_2 were determined in the presence of 3.3 mM pyrogallol as substrate (Table 2). For pyrogallol, K_m values were in the millimolar range for all POX except for sPOXc. According to the K_m, all fractions exhibited a lower affinity for pyrogallol than for H_2O_2, especially bean POX. The maize iPOX fraction had a 1.5-fold lower affinity for pyrogallol compared to the sPOX fraction. Bean sPOX and iPOX had about 4-fold lower affinity for pyrogallol than maize sPOX/iPOX. For H_2O_2 as a substrate, the K_m did not differ between the soluble and bound fractions. Three-fold higher affinity was found for the maize and bean ionic fractions compared to the soluble fractions in the presence of H_2O_2. Bean POX (soluble and bound) had lower affinity (higher K_m) for both substrates than maize POX under the investigated conditions (Table 2).

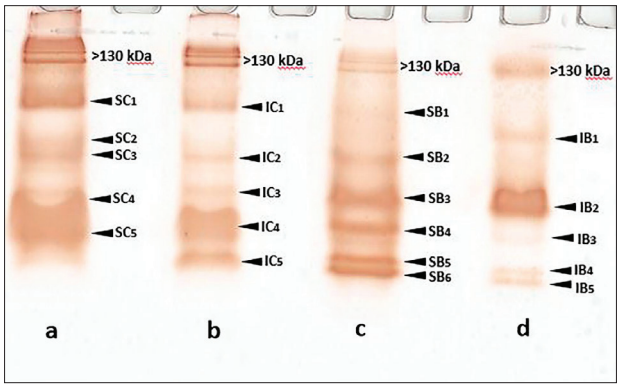

Fig. 1. Modified SDS-PAGE of POX isoforms: a – maize soluble fraction; b – maize ionically cell wall-bound fractions; c – bean soluble fraction; d – bean ionically cell wall-bound fractions. M – protein standards with their corresponding molecular weights. Gels were stained with 1 mM DAB. Arrows indicate different POX isoforms with approximate molecular weights: maize soluble (SC_1 78 kDa, SC_2 55 kDa, SC_3 50 kDa, SC_4 45 kDa and SC_5 38 kDa) and cell wall bound fractions (IC_1 78 kDa, IC_2 50 kDa, IC_3 45 kDa, IC_4 38 kDa and IC_5 35 kDa); and bean soluble (SB_1 78 kDa, SB_2 50 kDa, SB_3 45 kDa, SB_4 38 kDa, SB_5 36 kDa and SB_6 35 kDa) and cell wall bound fractions (IB_1 57 kDa, IB_2 45 kDa, IB_3 38 kDa, IB_4 36 kDa and IB_5 35 kDa).

The activities of different POX crude enzymes were determined with several substrates in addition to pyrogallol. Higher peroxidase activity was detected in ionically bound bean fractions compared to soluble bean fractions, while the opposite trend was observed for maize POX, independent of the used substrate (Table 3). The highest peroxidase activity of the soluble and bound bean POX and ionically bound maize was observed with caffeic acid as a substrate, while for the

Table 1. List of soluble POX from maize and bean root extracts detected by LC-MS/MS.

Accession	Description	Score[1]	Coverage[2]	Proteins[3]	Amino acid seq. length	MW [kDa]	calc. pI
Maize (*Zea mays* L.)							
B4FG39	Peroxidase	413.48	62.50	2	344	36.6	7.85
B4FHG3	Peroxidase	471.42	71.15	5	364	38.7	6.05
B4FH68	Peroxidase	373.16	70.14	5	355	37.5	6.67
A0A1D6N0K3	Peroxidase	227.57	59.00	1	361	38.1	7.90
A0A1D6LYW3	Peroxidase	432.04	58.89	4	360	38.6	6.96
A0A1D6LYW3	Peroxidase	432.04	58.89	4	360	38.6	6.96
A0A1D6LYW3	Peroxidase	432.04	58.89	4	360	38.6	6.96
A5H8G4	Peroxidase	377.18	50.14	14	367	38.3	7.14
A0A1D6MSC0	Peroxidase	332.32	50.14	14	367	38.3	7.14
B6THU9	Peroxidase	169.75	53.35	2	328	35.4	7.66
A0A1D6F4C8	Peroxidase	377.73	62.81	12	320	33.5	8.07
B4FSW5	Peroxidase	130.40	55.59	7	340	37.0	6.06
A0A1D6F4C8	Peroxidase	377.73	62.81	12	320	33.5	8.07
B4FSW5	Peroxidase	130.40	55.59	7	340	37.0	6.06
A5H452	Peroxidase	243.74	63.86	2	321	33.5	8.90
A0A1D6IMZ9	Peroxidase	268.91	78.44	3	320	33.2	6.65
B6T3V1	Peroxidase	122.62	55.56	3	333	35.7	8.18
A0A1D6H658	Peroxidase	198.28	58.26	2	321	33.1	8.07
Bean (*Phaseolus vulgaris* L.)							
V7C6Q8	Peroxidase	413.03	74.85	3	330	35.2	5.08
V7BY15	Peroxidase	238.22	57.72	3	324	35.8	6.84
V7CNV0	Peroxidase	215.50	72.51	5	331	36.6	8.43
V7BR14	Peroxidase	317.53	56.03	1	348	38.0	7.87
V7CCH8	Peroxidase	268.76	51.98	3	329	36.0	8.75

[1]Score – displays the protein score, which is the sum of the scores of the individual peptides.
[2]Coverage – displays the percentage of the protein sequence covered by identified peptides.
[3]Proteins – displays the number of identified proteins in the protein group of a master protein.

Table 2. K_m values of soluble bean (sPOXb) and maize (sPOXc) root peroxidase and ionically cell wall-bound bean (iPOXb) and maize (iPOXc) root peroxidase for pyrogallol in the presence of 3.3 mM H_2O_2. K_m values for H_2O_2 were obtained with 3.3 mM pyrogallol as a substrate.

	K_m (mM)			
Substrate	sPOXb	iPOXb	sPOXc	iPOXc
H_2O_2	0.339 ± 0.009a	0.111 ± 0.008b	0.319 ± 0.020a	0.110 ± 0.030b
Pyrogallol	4.371 ± 0.110c	4.076 ± 0.120c	0.833 ± 0.02d	1.319 ± 0.110e

a, b, c, d, e – different letters indicate significant differences at p<0.05 according to the t-test. Comparison was made between the same substrates and different POX and between different substrates for the same POX.

Table 3. POX activities of soluble bean (sPOXb) and maize (sPOXc) root peroxidase and ionically bound bean (iPOXb) and maize (iPOXc) root peroxidase for different substrates.

	POX activity (μmol mg^{-1} protein min^{-1})			
Substrate	sPOXb	iPOXb	sPOXc	iPOXc
Pyrogallol	34.84 ± 8.17a	108.99 ± 0.75b	73.19 ± 5.70c	10.41 ± 1.55d
p-CA	10.72 ± 0.38a	116.94 ± 30.70b	6.41 ± 1.65c	2.14 ± 0.53d
CGA	15.06 ± 1.18a	77.94 ± 9.66b	12.10 ± 0.96c	4.62 ± 0.32d
CA	67.50 ± 5.33a	240.88 ± 49.69b	56.79 ± 8.99a	19.11 ± 1.44c

Peroxidase activities were determined by absorbance change at 430 nm for 6.67 mM pyrogallol (PG), at 286 nm for 0.04 mM p-coumaric acid (p-CA), and at 410 nm for 4 mM chlorogenic (CGA) and caffeic (CA) acid.

a, b, c, d, e – comparison was made between the same substrates and different POX; different letters indicate significant differences at p<0.05 according to the t-test.

soluble maize POX, pyrogallol was the best substrate. The lowest activity for soluble bean and maize POX was measured with *p*-coumaric acid as a substrate. The lowest activity for iPOXb was with chlorogenic acid as a substrate, while for iPOXc it was with *p*-coumaric acid.

Crosslinking of potato protein catalyzed by peroxidase

The ability of maize and bean sPOX to crosslink potato protein was examined using SDS-PAGE analysis which demonstrated the formation of high-molecular-weight

fragments after 60 min from the addition of bean and maize sPOX, followed by a concomitant decline of the protein monomer bands, especially patatin (Figs. 2 and 3).

The globular potato protein patatin was crosslinked with maize and bean sPOX after the addition of H_2O_2. The activity observed in the absence of H_2O_2 could be due to the presence of other enzymes in the crude enzyme extract. Formation of a new protein band at about 25 kDa was observed after 1 h of incubation with both maize and bean sPOX. Figs. 2 and 3 show crosslinking with maize and bean sPOX and H_2O_2, however the same pattern on the SDS-PAGE gel was observed even without H_2O_2. The ability of maize and bean iPOX to crosslink potato protein was examined and the results revealed a weaker crosslinking ability of iPOX as compared to sPOX. This could be explained by the lower amount of phenols present in iPOX crude enzyme extract, which is in accordance with the higher K_m and substrate specificity (Tables 2 and 3) as well as with the results obtained after SDS-PAGE analysis (Fig. 1).

To compare the abilities of maize and bean sPOX to crosslink potato protein, as well as the role of phenolic mediators, HRP was used to crosslink potato protein with (Fig. 4) or without (Fig. 5) caffeic acid . Crosslinking of potato protein with HRP and gradual addition of H_2O_2 (Fig. 4) led to polymerization after 3 h of incubation, but with a less significant decrease in the patatin band, as observed in the case of maize and bean sPOX (Figs. 2 and 3). When caffeic acid was added, extended polymerization was observed, followed by the formation of a new band (slightly above 25 kDa) after 3 h of incubation, but without the decline in patatin band intensity (Fig. 5). Self-polymerization of HRP was evident in the presence of caffeic acid, as well as the formation of high-molecular-weight polymer bands of potato protein that were not capable of entering the stacking gel (Fig. 5). In addition, pigmented formations at the end of the incubation time were observed in dispersions with caffeic acid and non-crosslinked potato protein, crosslinked potato protein, as well as in HRP with caffeic acid samples (Supplementary Fig. S1D). The suspension with HRP and without caffeic acid remained unchanged (Supplementary Fig. S1C). When crosslinking was performed without a phenolic mediator, insignificant pigmentation was observed only in the crosslinked suspension, and was followed by precipitation of the crosslinked products (Supplementary Fig. S1A,B).

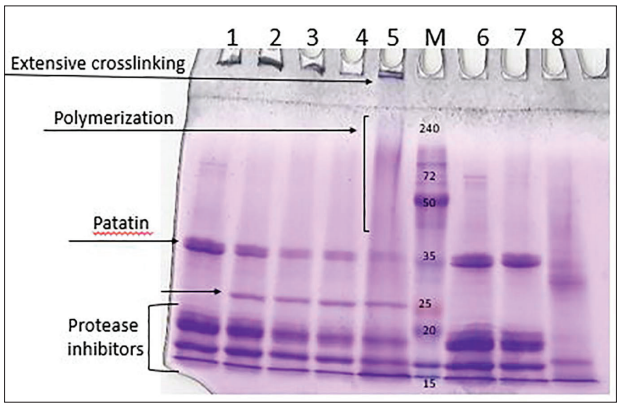

Fig. 2. SDS-PAGE patterns of bean sPOX-polymerized potato protein. M – molecular weight markers; lanes 1-5 – potato protein with sPOX and 50 mM H_2O_2 at 0, 60, 300 min and 12h, respectively; lanes 6-7 – potato protein without bean sPOX at 0 and 300 min, respectively; lane 8 – bean sPOX extract.

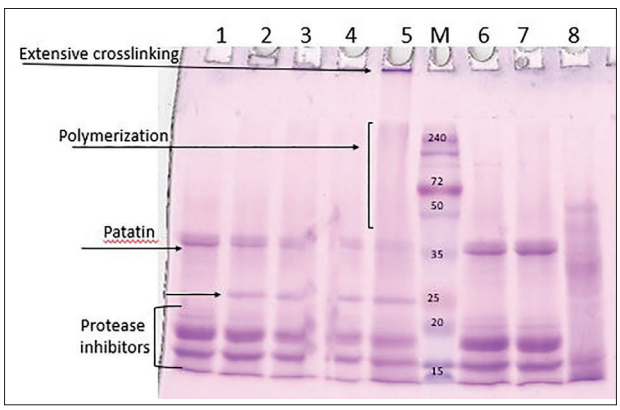

Fig. 3. SDS-PAGE patterns of maize sPOX crosslinked potato protein. M – molecular weight markers; lanes 1-5: potato protein with sPOX and H_2O_2 at 0, 60, 300 min and 12h, respectively; lanes 6-7 – potato protein without maize sPOX at 0 and 300 min, respectively; lane 8 – maize sPOX extract.

DISCUSSION

The aim of the present research was to isolate and partially characterize POX from maize and bean roots. The crude extract rich with soluble and ionically bound POX showed differences in the number of POX isoforms and in the molecular weights of the isoforms. A high molecular weight fraction (130 kDa) was found in all bean and maize fractions, as

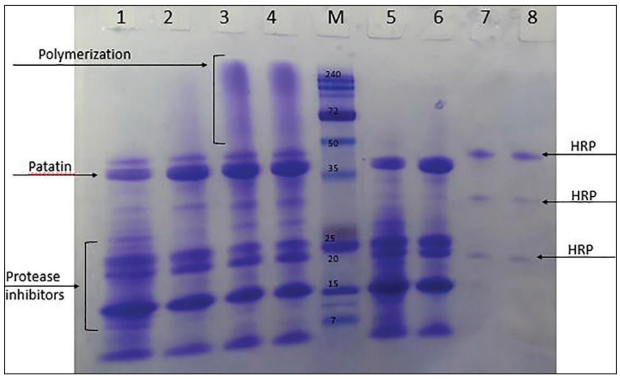

Fig. 4. SDS-PAGE patterns of HRP-crosslinked potato protein. M – molecular weight markers; lanes 1-4 – potato protein with HRP and H_2O_2 at 0, 60, 180 min and 300 min, respectively; lanes 5-6 – potato protein without HRP at 0 and 300 min, respectively; lanes 7-8 – HRP at 0 and 300 min, respectively.

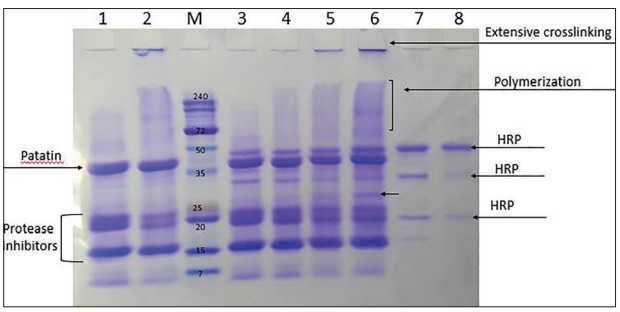

Fig. 5. SDS-PAGE patterns of HRP-crosslinked potato protein with caffeic acid (2 mM). M - molecular weight markers, lanes 1-2 – potato protein with caffeic acid, but without HRP at 0 and 300 min, respectively; lanes 3-6 – potato protein with HRP and caffeic acid at 0, 60, 180 min and 300 min, respectively; lanes 7-8 – HRP with caffeic acid at 0 and 300 min, respectively.

previously shown for maize cell wall POX fractions [34, 35]. High-molecular-weight proteins were also detected among peroxidases tightly bound to the plasma membrane of maize roots and other soluble plant POX, and were ascribed to oligomeric forms of 63 kDa units [12, 36]. With the exception of the 78 kDa band, typical isoforms in the range of 28-60 kDa [34] were identified in all maize and bean fractions. Differences in the molecular weight of POX enzymes could be due to differences in amino acid sequence or the degree of glycosylation [37, 38]. Maize peroxidase, ionically bound to the cell wall with a molecular weight of 45 kDa was described and considered as a monomer [35]; the soluble bean POX with the same molecular weight was also previously described [39]. The amino acid sequence length of bean and maize sPOX obtained by LC-MS/MS was in the range of 305-370, which is the recorded length of polypeptide chains of secretory peroxidase class III [37].

Examination of the effect of pyrogallol concentration on enzymatic activity showed that enzyme activity increased with increasing concentrations of pyrogallol and that it followed typical Michaelis-Menten kinetics with K_m values similar for bean soluble and ionically bound POX. Higher affinities for all POX fractions were observed when H_2O_2 was used as a substrate. Even in the same plant, different isoforms of peroxidase showed variations in K_m values between soluble peroxidase and cell wall-bound peroxidase [40]. Low K_m values for substrates indicate high affinity for the active site of enzymes. Thus, variations in K_m values for the same substrate may depend on subtle variations in the tertiary structure of the active site. Also, according to differences in kinetic parameters between solubilized and ionically bound enzymes, it can be assumed that binding of peroxidase to the cell wall results in conformational changes that influence its substrate affinity [35]. Thus, association with cell walls increased enzyme affinity for H_2O_2, suggesting increased accessibility of the enzymes to the substrate [35]. The higher peroxidase activity in ionically bound bean POX compared to soluble bean fractions also pointed to increased accessibility of the iPOX to different phenolic substrates. When caffeic acid was used as a substrate, compared to chlorogenic acid, pyrogallol and p-coumaric acid, the activities of soluble and ionic maize and bean POX fractions, were the highest. The difference in substrate affinity could be explained as a consequence of substrate structure, and probably each substrate binds differently to the active center of soluble and ionic POX from maize and bean. Furthermore, covalent, ionic and apoplastic POX from pea had the highest affinity with caffeic acid as a substrate [11]. It was shown that naturally occurring phenolic compounds could be substrates for cell wall-bound and apoplastic peroxidases [9, 41-43]. The monolignols, ferulic and *p*-coumaric acid esters were substrates for the peroxidase responsible for the process of lignification and crosslinking of cell wall polymeric constituents in grasses [44].

Crosslinking by peroxidase has different and important applications, with the main aim of improving protein functional properties in such a way so as to enhance the final product, comprised of the

crosslinked protein. To date, it has been reported that peroxidase-mediated crosslinking was successfully used to produce protein nanoparticles of controlled size and mesostructure [45], to produce α-lactalbumin nanoparticles with increased foam stability [46], to create food emulsions with improved stability [47], to create physical hydrogels made of proteins [48], and to decrease peanut allergy [49].

In nature, enzymes from the oxidoreductases family, such as laccases, peroxidases and tyrosinases, are not used for protein crosslinking and only initiate the first chemical reaction, forming a reactive species that spontaneously polymerizes with other functional groups, leading to covalent crosslinks [20]. Furthermore, while the enzyme showed good crosslinking ability on proteins with a low degree of complexity, such as casein, proteins with a well-folded and complex structure were not crosslinked and required the addition of a small-molecular-weight molecule (phenol or caffeic acid) as a mediator [20]. The results obtained herein showed that the globular potato protein patatin was crosslinked with maize and bean sPOX with and without the addition of H_2O_2. Additionally, protease inhibitors were also crosslinked and the formation of a new band around 25 kDa was observed on SDS-PAGE. Polymerization without H_2O_2 can be the consequence of the presence of other oxidizing enzymes in the crude enzyme extract comprising the soluble fraction of bean and maize. It can be assumed that peroxidases produce H_2O_2 in the presence of a reducing agent [17, 45, 46] in a sufficient quantity to initiate polymerization. Furthermore, a gradual supply of H_2O_2 could improve enzyme efficiency and oligomerization due to changes in the surrounding conditions which affect protein conformation [47]. Certain proteins can polymerize to some extent in the presence of H_2O_2 alone [22]; however, no polymerization was observed when potato protein and H_2O_2 were mixed. In addition, when bacterial tyrosinase from *Bacillus megaterium* (TyrBm) was used to crosslink potato protein, it was found that patatin was a poor substrate, while protease inhibitors with a molecular weight of ~20 kDa were rapidly crosslinked by TyrBm [28]. Among ionically and soluble bean and maize POX fractions, the highest specificity was found when caffeic acid was used as a substrate, as compared to pyrogallol, *p*-coumaric acid and CGA. This could explain crosslinking in the absence of H_2O_2, which in turn could be generated through the oxidative activity of peroxidase stimulated by a phenolic compound [48]. In addition, it has been shown that POX also possess the capacity to produce H_2O_2while oxidizing different types of reductants, including phenolics, in the presence of trace amounts of metal ions [17, 35, 48, 49]. Since some phenolics are auto-oxidizable and can function as reductants, phenolics in the apoplast, including the cell wall, may contribute to the generation of H_2O_2 by reducing O_2 [9, 17]. The dark pigmentation of crosslinked suspension at the end of incubation points to the presence of phenolic compounds that form melanoidic molecules [50]. To examine the role of phenolic mediators in crosslinking, HRP with and without the addition of caffeic acid was used to crosslink potato protein, with the gradual addition of H_2O_2. Polymerization of protease inhibitors by HRP was observed with and without caffeic acid, while the patatin band remained unchanged. When caffeic acid was added, auto-polymerization of HRP was observed, as well as polymerization of potato protein in the absence of enzyme. This could be explained by the fact that potato protein is more susceptible to oxidation with H_2O_2 in the presence of caffeic acid, which leads to polymer formation. Caffeic acid was easily oxidized to a quinone derivatives, which led to crosslinking of peanut proteins in the absence of the enzyme [51]. In addition, phenolic compounds are known to react under oxidizing conditions and form reactive quinones that interact with hydroxyl, sulfhydryl or amine groups in the protein chain, producing covalent bonds [20, 23, 52, 53] and protein crosslinks [54, 55], which can explain the crosslinking of potato protein in the absence of enzyme. When caffeic acid was added along with HRP, a new 25 kDa band was observed. In the presence of caffeic acid, tyrosinase induced cross-linking of α-lactalbumin, β-lactoglobulin and lysozyme, while in the absence of caffeic acid only polymerization of α-lactalbumin was observed [53]. Similar results were found for crosslinking of soy glycinin by bacterial tyrosinase after the addition of different phenolic compounds [33]. According to Isaschar-Ovdat et al. [33], caffeic acid led to the highest crosslinking rate, presumably because of the high diphenolase activity of tyrosinase on caffeic acid as compared to its monophenolase activity on *p*-coumaric acid. Beside caffeic acid, ferulic acid can be converted into a semiquinone radical after one-electron oxidation by peroxidase and H_2O_2, leading to the formation of polymers [26]. It was

previously reported that introducing a phenolic mediator in the crosslinking of soy glycinin mediated by tyrosinase led to the formation of undesired products in the form of large crosslinked complexes and dark pigmentation of crosslinked dispersions [33].

Considering the possible presence of phenolic compounds in the crude enzyme extract, sPOX from maize and bean roots were capable of facilitating crosslinking by the oxidized phenolic molecule and an amine residue in the globular potato patatin protein. However, in the absence of phenolic mediators, sPOX hydroxylated the peptide-bound tyrosine residue to the reactive quinone and created a reactive group for potato protein crosslinking.

CONCLUSION

The presented results support the use of a cheap source of peroxidase for enzymatic protein crosslinking and for modulating the functional properties of protein matrices. For the first time it has been shown that crude enzyme extracts of soluble peroxidase from maize and bean roots were capable of crosslinking the globular potato protein patatin. The crosslinking is most likely facilitated by the presence of phenolic compounds in the crude enzyme extract. Information provided here could be useful for further research into the structure of patatin via crosslinking catalyzed by partially purified peroxidases from maize and bean roots. In addition, the same modification using crude enzyme extracts could be useful for potential applications in food structuring.

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Supplementary Data

Supplementary Table S1.
Available at: http://serbiosoc.org.rs/NewUploads/Uploads/000ABS_Glusac%20et%20al_3531_Supplementary%20Fig.%20S1.pdf