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Research Article

Investigating on the Stability of Peroxidase Extracted from Soybean (*Glycine max* var. Williams) and Effects of Na^+ and K^+ Ions on its Activity

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Abstract

In the present study, some techniques were used for Soybean peroxidase (SBP) purification including: ammonium sulfate fractionation, DEAE Sephadex anion exchange chromatography and Concanavalin A Sepharose 4B affinity chromatography. Molecular weight of purified SBP was estimated about 44 kDa by SDS-PAGE as a single polypeptide band. The optimal pH and temperature for enzyme activity were found to be 4.5 and 70°C, respectively. The enzyme was more stable in alkaline pH than acidic ones and could tolerate 10 minutes heating in 40-50°C without any loss of its activity. Both NaCl and KCl were found to have significant effects on the enzyme stability, but presence of NaCl was more effective than KCl. Our results showed that after 24 hours incubation of the enzyme in the presence of 20 mM NaCl, more than 60٪ of the enzyme activity was remained while it would fall to 3٪ if incubation was not accompanied by NaCl. Purified peroxidase from seed hull of soybean relative to the other identified peroxidases was more stable, for this reason a lot of benefit will be considered by use of this enzyme in different industry.

***Keywords*:** Enzyme stability, *Glycine max*, Peroxidase, Purification, Soybean

Introduction

Peroxidases are important heme-containing enzymes that have been studied for more than a century. Few enzymes are represented so widely in the scientific and patent literature as peroxidases. These enzymes utilize hydrogen peroxide to oxidize a wide variety of organic and inorganic compounds. Peroxidases are used as reagents for diagnostic test kits, immunoassays and treatment of waste waters (Penel et al., 1992; Vianello et al., 1997). An ideal peroxidase for large-scale biocatalysis as discussed by Liu et al. (1999) would be one that is readily abundant, possesses wide substrate specificity and remains stable over a wide range of pH and temperature. Peroxidase from soybean coat satisfies these criteria. In addition to the vast resource of soybean coats as a major by-product of the food industry, the enzyme is easy to isolate. Soybean seed coat peroxidase (SBP, EC. 1.11.1.7) belongs to class III of the plant peroxidases superfamily and has a wide range of potential applications (Chanwun et al., 2013; Daengkanit and Suvachittanont, 2005). Its structure is a particular interest for engineering purposes and as a model for stable heme peroxidases. There are reports in literature commenting on the purification, activity and application of SBP (Nissum et al., 1998). However, after McEldoon and Dordick (1996) first showed that SBP is substantially more thermo stable than HRP C, considerable interest was shown in this enzyme. The crystal structure of SBP shows that its three-dimensional structure is very similar to HRP C. There are several common features between these two enzymes although SBP has not been studied as thoroughly as HRP C.

In addition to substrates and buffer properties, distinct enzyme tests need a variety of special additives, such as dissociable cofactors or metal ions, which may be directly involved in the catalytic reaction, stabilize the native enzyme structure, or protect it against denaturation. The ions interfere with the weak ionic bonds of proteins. Typical enzymes are active in salt concentrations of 1-500 mM. mono- or divalent cations (M^+ and M^{2+} respectively) neutralize surplus charges at the protein surface and mediate bonds such as salt bridges.

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On the other hand, heavy metal ions promote oxidative processes, especially with thiol groups, and can affect the native structure. Therefore, addition of the ions is advantageous for some types of enzymes although it may be harmful for others (Bisswanger, 2004). Molecular mechanisms of metal ion coordination and their effects are an important aspect in the characterization of biological macromolecules. Over one-third of known proteins are metalloproteins (Castagnetto et al., 2002). Conceptual associations with protein-metal complexes tend to favor divalent metals of which significance in protein structure and function has been reviewed in detail (Armstrong, 2000) however, several evidence suggest that group I alkali metals (such as Na^+ and K^+) play important role other than nonspecific ionic buffering agents or mediators of solute exchange and transport.

Because of the considerable treatment of peroxidases in different industry and key role of its stability, a lot of interests have been shown by study on this enzyme. In this paper, we explain about purification of a peroxidase enzyme from seed coats of soybean using anion exchange chromatography, affinity chromatography and SDS-polyacrylamide gel electrophoresis technique (SDS-PAGE). Another goal of this research was investigation on stability and activity changes of the isolated fraction during incubation of the purified enzyme under various conditions such as different pH, temperatures, time storage and effect of the presence of Na^+ and K^+ ions in enzyme reaction mixture.

Materials and Methods

Enzyme Extraction and Purification

Soybean seeds (*Glycine max* var. Williams) were supplied from Oil Seed Company (Tehran, Iran) and its coats were obtained by soaking whole seeds in distilled water for approximately one hour. The coats were, then, removed from the seeds, grinded by blender and homogenized in 0.02 M sodium phosphate buffer (SPB, pH=6.8) containing 2% polyvinyl polypyrolidon (PVPP) in a cold room. Homogenate was centrifuged (20,000 *g*, 45 min) and the supernatant was passed through 8 layers of gauze. The 30-80% ammonium sulfate precipitate was prepared based on Hejri and Saboora (2009) and then the pellet dispersed in 0.02 M sodium phosphate buffer (pH 6.8), dialyzed against the same buffer, and applied to a DEAE-Sephadex A-50 ion exchange column (4×17 cm) previously equilibrated with the same buffer. Peroxidase was eluted with a linear salt gradient from 0 to 1.5 M NaCl in 0.02 M sodium phosphate buffer (pH 6.8). Protein fractions which showed both absorbance at 403 nm and peroxidase activity were mixed and pooled as peroxidase extract. This extract was applied to a Concanavalin A-Sepharose 4B column (1.6 cm × 13 cm, Merck) and then equilibrated with a solution containing 0.5 M NaCl, in 0.02 M SPB (pH 6.8). Fraction with peroxidase activity was retained on the column and eluted with a linear gradient from 0 to 1.75 M glucose in 2.5 M NaCl and 0.02 M SPB (pH 6.8). Elutes were pooled, concentrated and then assessed for physical and chemical properties.

Electrophoresis

Purity and molecular weight of the fractions were examined using analytical SDS-polyacrylamide gel electrophoresis techniques (Laemmli, 1970). Protein fractions were run on 7.5% polyacrylamide gel (PAGE) in a cold room (40 mV, 6 h). peroxidase bands were detected on the gel by submerging the gel in a staining solution containing 80 ml of acetate buffer (0.1 M, pH 5), 8 ml of H_2O_2 3% and 4 ml of benzidine 0.04 M in methanol 50% (Van Loon, 1971). Purity and molecular weight of the enzyme was examined on the 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) (Hames and Rickwood, 1990). Proteins were visualized by staining with Coomassie brilliant blue R250. Molecular weight markers were obtained from Fermentas (Lithuania; Mw= 11-170 kDa).

Protein and Peroxidase Assay

Protein concentration was determined according to the method proposed by Bradford (1976) using bovine serum albumin as a standard. Peroxidase activity was determined at 25ºC following the formation of tetraguaiacol (A_{max} = 470 nm, ε = 26.6 $mM^{-1}cm^{-1}$) in a 3 ml reaction mixture containing 950 µl of citrate buffer (0.05 M, pH 4.6), 1 ml of 15 mM guaiacol, 1 ml of H_2O_2 1.6 mM and 50 µl enzyme extract. One unit of peroxidase activity represents the amount of enzyme catalyzing the oxidation of 1µM of guaiacol in one minute (Liu et al., 1999).

Enzyme Stability

The effect of pH on activity and stability of SBP was determined at 25˚C with the use of two buffer systems, 0.05 M phosphate-citrate buffer ranging from pH 2.5 to 8 and 0.05 M glycine-NaOH buffer ranging from pH 8.5 to 9.5.

For determination of enzyme thermostability, 50 µl of enzyme extract was preincubated in citrate buffer

pH 4.5 for 10, 20 and 30 minutes at different temperatures (30–90°C). Then, remaining activity was determined with guaiacol as substrate. The activity was expressed as the percentage of initial enzyme activity (Saboora et al., 2012).
In order to determine the role of monovalent metal ions on the SBP activity, distinct concentrations of NaCl and KCl (2 and 20 mM) were applied within assay mixture. The remained enzyme activity was measured after 1 minute, 2 and 24 hours of incubation.

Statistical Analysis

All experiments were done at least three times. Data were subjected to ANOVA (one way variance analysis) using statistical software SPSS 11 (SPSS Inc, Chicago, USA). Means values were compared by post hoc Tukey test and significant differences determined among the treatments at $p<0.05$.

Results

The result showed that there was 4 mg protein in each gram of soybean coats (*Glycine Max* var. williams). The specific activity of the purified peroxidase was 110.94 U/mg proteins in crude extract which increased to about 1550 U/mg proteins after eradication of impurities and developing of the purification procedure (Table I). The purification method after chromatography on DEAE-Sephadex A-50 and Concanavalin A-Sepharose 4B, respectively, led to 3.8 and 14 fold purification of the peroxidase fraction (Table 1). Finally, 98% of the protein fraction were removed from crud extract and fraction numbed between 30 to70 were collected (Fig. 1, 2).

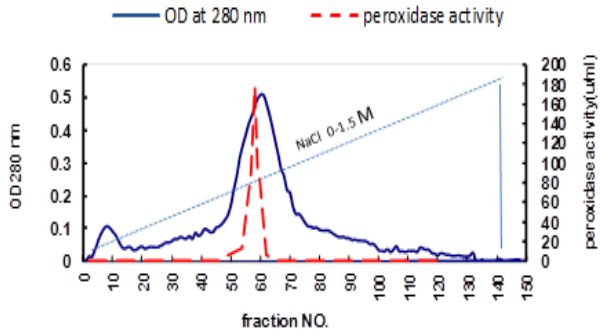

Figure 1. Purification spectra of the soybean seed coat peroxidase. Gradient elution profile from DEAE-sephadex A-50 ion exchange chromaograph column.

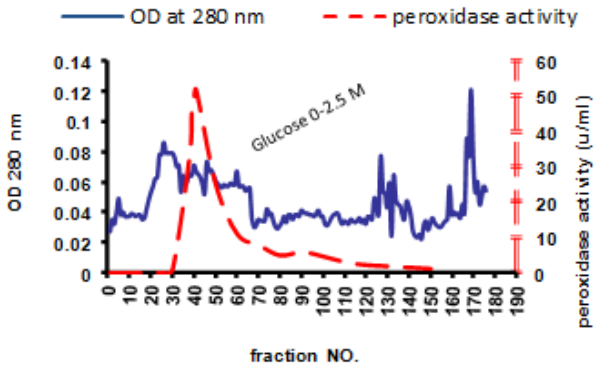

Figure 2. Chromatography of active fractions from spectra of the soybean seed coat peroxidase. Gradient elution profile was obtained from concanavalin A-sepharose 4B affinity chromatography column.

Isolated peroxidase revealed as a single band on SDS-PAGE and had a low molecular weight (44 kDa) according to the migration of the molecular weight markers (Fig. 3). Also, the purity of the peroxidase preparations was determined by measuring the ratio of the heme absorbance absorbance (at 403 nm) to the protein absorbance (at 280 nm). This ratio is denoted the RZ (Reinheistszahl) value. As shown in the Table 1, RZ value increased from 0.3 in crude extract to 0.5 in final preparation of the enzyme.

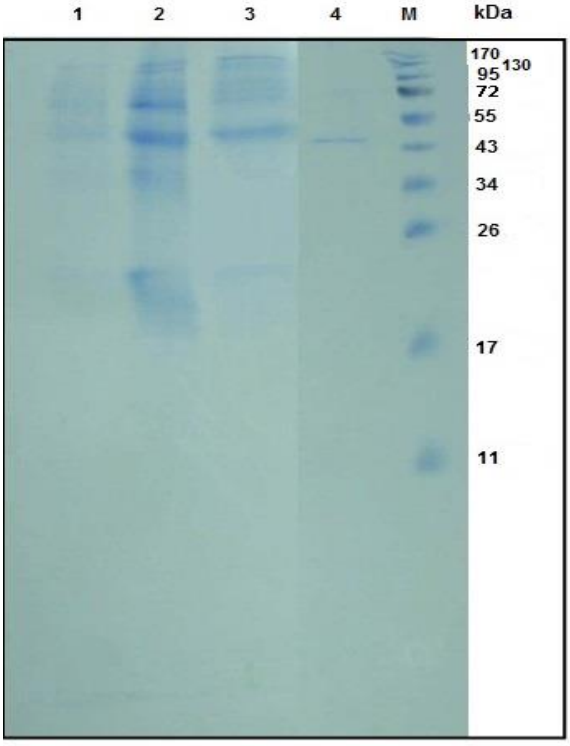

Figure 3. SDS PAGE analysis of soybean peroxidase. Lane M: protein marker, Lane 1: protein extract, lane 2: Amonium sulfate precipitate, lane 3: Elutes from DEAE sephadex chromatography column, lane 4: Elutes from Con A sepharose 4B chromatography column.

Table 1: Summary of the purification of a peroxidase isoform isolated from seed hull soybean

Purification procedure	Total protein (mg/ml)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Recovery (%)	RZ value
Crude extract	168.48	18690.90	110.94	1	100	0.297
DEAE sephadex column	17.46	7314.23	418.82	3.78	39.13	0.376
Con A sepharose 4B column	0.25	389.05	1549.89	13.97	2.08	0.500

*Units (U) are expressed as µmol of guaiacol oxidized per min and specific activity is expressed as units (U) per mg of protein. Data presented are average of three replicates

The effect of pH on the activity of the purified peroxidase was determined over the range of pH between 2.5 and 9.5. The optimum pH of the purified enzyme was 7. For pH stability, the enzyme activity started to lose at pH below 5.5. Peroxidase activity of the purified fraction was completely abolished after 1h incubation at pH 2.5–3.5 or after 24 h incubation at pH 2.5-5.0 (Fig. 4). Soybean peroxidase was more stable in alkaline pHs than acidic ones.

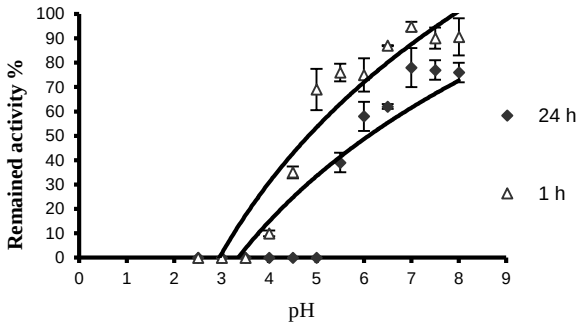

Figure 4. Characterization of the soybean seed coat peroxidase, pH stability profile.

The results of the effect of temperature, 30–80°C, on the enzyme activity demonstrated that the purified enzyme was tolerate for 10 min by warm up at temperatures 40ºC and 50ºC, it exhibited a little inactivation (Fig. 5).

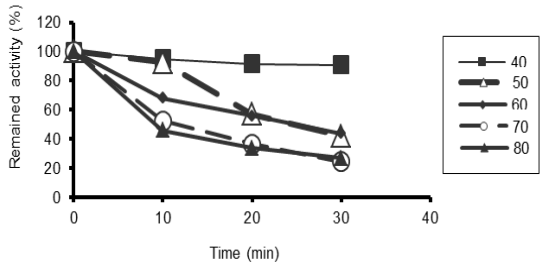

Figure 5. Characterization of soybean seed coat peroxidase, thermostability profile

The purified peroxidase preserved about 50% of the relative activity after 10 min incubation at 70-80°C but the activity was lost more than 67% within 30 minutes (Fig. 5). The enzyme was heat stable at 40°C over a wide range of the experiments and its activity was very low down comparative to 25°C as standard condition. At standard condition, peroxidase activity usually assay under low concentration of the salt (0.05 M sodium citrate or acetate buffer). Compared to the control, our results showed that only one minute incubation of the purified enzyme in the presence of 20 mM concentration of KCl or NaCl led to decrease in peroxidase activity by 4% and 10%, respectively. However, as can be seen in the Table 2, fall in the enzyme activity after 2 hours incubation of the purified enzyme in the presence of sodium chlorid was less noticeable (17.4% for KCl against 11.4% for NaCl). At the same time, peroxidase activity was reduced about 64.6% in the control reaction (Fig 6). Thus, it seems that NaCl is more effective in support of the enzyme stability. Since, the ability of NaCl in keeping the enzyme stable was a bit more than KCl, overnight incubation of Soybean peroxidase was examined in the presence of NaCl. According to the result shown in Fig. 6, it was led to enzyme activity remained up to ٪60 of the control without any impact on pH of assay mixture.

Table 2. Effects of metal ions on the activity and stability of SBP. Both of KCl and NaCl decreased the activity of enzyme in the first minute, but after incubation they had a positive effect in keeping the enzyme stable.

	% Remained Activity	
	after 1 min	**after 2 h**
Control	100 a	35.41 ± 2.46 b
KCl	95.91 ± 4.75 a	82.62 ± 5.14 a
NaCl	89.95 ± 7.38 ab	88.61 a

Data presented are average of three replicates. Different letters denote statistically significant differences by Tukey test at $P < 0.05$

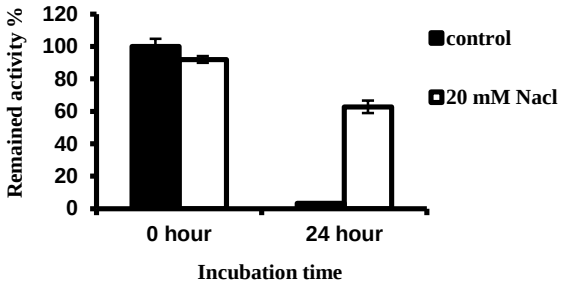

Figure 6. The effect of overnight incubation of the purified SBP in the presence of NaCl.

Discussion

In this study purification was done through three steps, ammonium sulfate precipitation, anion exchange chromatography (DEAE-Sepharose A-50), affinity chromatography (Con A-sepharose 4B) and then followed by SDS-PAGE. The binding of the enzyme to the Con A-sepharose column indicated that the purified peroxidase was a glycoprotein compatible with the previous studies reporting that most of the plant peroxidases were glycoproteins (Joansson et al., 1992; Kvaratskhelia et al., 1997; Passardi et al., 2005). Our purified peroxidase had a molecular weight of 44 kDa as determined by SDS-PAGE while the molecular weights of various peroxidases have been reported to be in the range of 30–150 kDa (Regalado et al., 2004). A similar molecular weight for the purified peroxidase has been previously reported from *Moringa oleifera* leaves and *Ipomoea Batatas* (44 kDa), *Brassica napus* (34-39 kDa) and avocado (40 kDa) (Khatun et al., 2012; Diao et al., 2014; saboora et al., 2012; Singh et al., 2002, Rojas-Reyes et al., 2014). These data are lower than peroxidase from cabbage leaves (67 kDa) and those of *Hevea brasiliensis* cell (70 kDa) (Kharatmol and Pandit, 2012; Chanwun et al., 2013).

Our results revealed that the extracted soybean peroxidase (SBP) enzyme was found to be optimally active at pH 4.5. This is similar to those obtained on POX from *Brassica oleracea capitata L.* (pH 4), *Glycin max* var HH2 (pH 4.6), *Withania somnifera* (AGB 002) and *Hevea brasiliensis* cell (pH 5) (Tabatabaie Yazdi et al., 2002; Liu et al., 1999; Johri et al., 2005, Chanwun et al., 2013). However, these results are lower than those of *Brassica oleracea gongylodes* (pH 8.5) and *Spinacia oleracea* (pH 6.5) (Manzoori et al, Köksal, 2011).

The extracted peroxidase from soybean was optimally active at 70°C. This optimum temperature was lower than those reported for hyperthermostable peroxidase from the *Solanum melongena* (84°C) (Vernwal et al., 2006) and was higher than POX of Turnip root (*Brassica napus* var. okapi) and *Hevea brasiliensis* cell (50 °C) (Saboora et al., 2012; Chanwun et al., 2013).

Also, our results showed that the purified enzyme from seed hull was a thermostable enzyme which had a broad pH-activity profile. Up to pH 8, relative activity retained about 76-78% of the original activity after 24 h incubation. Activity of the enzyme depends strongly on pH of the reaction media for two major reasons: (1) the presence of essential proton-accepting groups in the catalytic center, and (2) maintenance of overall structure of the enzyme. Also, pH dependence of its activity depends on the relative accessibility of reducing substrates and the subsequent electron transfer rate (Veitch, 2004). Kamal and Behere (2003) have reported that the enhanced conformational stability of SBP arises mainly due to the active site architecture in terms of the amino acid interactions with the heme. Therefore, any change around the heme active site brought about by external agents such as pH, solvent, concentration of special ions and a number of factors in reaction mixture can significantly affect on the magnitude of the conformational stability of the enzyme. In most cases, pH-dependent activity changes are reversible, and enzyme incubated in the weakly acid or alkaline range of the titration curve regains its maximum activity when shifted to the optimum pH. However, overall structural changes in the enzyme influenced by pH are often not reversible, and the enzyme does not recover its maximum activity when shifted back to neutral conditions.

The purified peroxidase exhibited high thermal stability in the inactivation profile; its relative activity retained 52-45% of the original activity after heating at 70-80 °C for 10 minutes while at 40 °C, the activity maintained approximately constant during 30 minutes. Most soluble peroxidases, apoplastic and cytosolic isoforms that extracted from several plant tissues have temperature optimas between 30°C and 60°C (Bernards et al., 1999; Loukili et al., 1999).A few number of peroxidases from plants and animals seem to have high temperature optima and show high thermal stabilities (Bakardjieva et al., 1996; Madhavan and Naidu, 2000). Thermal stability of peroxidases has been attributed to the presence of large number of cystein residues in the polypeptide chain (Johri et al., 2005).

Generally, incubation time plays an important role in SBP activity decrease since all treatments were kept in room temperature, but this drop in activity was not statistically significant when the incubation was in the presence of mono-cationic ions (M^+). Regulation of activity through metal ion complexes plays a key role in many enzyme-catalyzed reactions. M^+ lie at a conceptual crossroad between modifier of solvent and metal-cofactor assisting enzyme function. In terms of macromolecular stability, observed differences in valence parameters of M^+ lead to a host of macroscopic features based on hydration properties of these ions (Ahmad et al. 2001). Group IA alkali metals bear a single positive charge with different ionic radius which correspond with significant alteration of ionic volume, hence charge density, and downstream effects upon bonding parameters. Hydration shells of M^+ are dissimilar, and this extends to secondary and tertiary shells of the ion. Na^+ is small enough to bind three or four water molecules with reasonable affinity and result in a larger apparent size in aqueous solution. K^+ favors four or five water molecule coordinated with weaker strength (Page and Cera, 2006). The stability of the alkali ion complexes increased with decreasing ionic radii in aqueous solutions. The stability constant depends on the water activity of any given metal, thereby implicating the differential coordinating abilities of various metal ions at protein surfaces (Sigel et al., 1982).

Conclusion

In conclusion, peroxidase from seed hulls of the soybean grown in Iran can be extracted and purified by means of few classical purification steps. Our study has indicated that there is a single acidic isozyme. Since that last purification steps which were obtained by ion-exchange chromatography and affinity chromatography tend to one peak eluted peroxidase, it can conclude that, enzyme isolation from waste soybean seed coats is relatively easily and cheaply without last stage. Biochemical parameters with respect to enzyme activity and stability revealed its wide possible utilization in biotechnological process involving peroxidases

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