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Full Length Research Paper

An optimized procedure for the purification of Zo peroxidase (ZoPrx), a low abundance peroxidase from Japanese radish roots

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Purification of low abundance enzymes for biochemical characterization is frequently labor and cost-intensive. The existence of co-expressing multigene families increases the complexity of the procedure even further as it occurs with plant peroxidases where only the most abundant species have been studied. In this paper we present an optimized purification method for Zo peroxidase, a low abundance isoenzyme with unusual tolerance to hydrogen peroxide. This protocol is straightforward, allowing the purification of the enzyme at milligram levels in 10 days. Furthermore, the protocol may be easily adapted for the direct purification of other non abundant peroxidase isoenzymes from plant tissues.

Key words: Peroxidase, hydrogen peroxide, desactivation, Japanese radish.

INTRODUCTION

Multigene families are common in organisms from all phyla: with this genetic strategy, individuals aim to fulfill different regulatory purposes. On one hand, it allows the differential regulation of otherwise similar activities in a time- or tissue-specific manner. Additionally, it facilitates the tagging of individual gene products, even when they are simultaneously expressed, to specific cellular compartments or to the extracellular space. Finally, the transitory co-expression of a multigene family enables the rapid accumulation of the encoded enzymatic activity through a gene-dosage effect (Taylor and Raes, 2004; Zhang, 2003).

Plants are known to present an unusual genomic architecture characterized by a high frequency of gene duplication events resulting in multigene families (Wendel, 2009). Among these, the heme peroxidases family is particularly abundant, containing as many as 73 isogenes in the model plant *Arabidopsis thaliana* (Tognolli et al., 2002; Valério et al., 2004). Similarly, radish roots have been demonstrated to harbor many isoenzymes with diverse properties (Veitch, 2004). Pioneer investigations by Morita et al. demonstrated that least eighteen different peroxidase isoenzymes could be isolated from roots of Japanese radish (*Raphanus sativus* L.) (Morita et al., 1970; Morita et al., 1971). However, only the most abundant species have been thoroughly characterized. Recently, we published the results of the systematic separation and characterization of all the peroxidase iso-enzymes from roots of commercially available Japanese radish (also known as Daikon radish) and the iden-tification of one of these isoforms, named Zo peroxidase (ZoPrx), as the first case of a naturally occurring peroxidase isoenzyme intrinsically stable to oxidative inactivation (Gil-Rodríguez et al., 2008).

One problem in studying the components of multigene families is the technical difficulty in isolating a particular isoform from a complex for selective characterization: this experimentation is labor and cost intensive and frequently precludes the deeper study of otherwise interesting model enzymes. The original protocol aimed to the purifi-

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fication of ZoPrx, an isoenzyme corresponding to less than two percent of the total peroxidase activity found in a crude extract, required six to eight weeks of experimentation. With the optimized protocol presented here, only two weeks are required to obtain a highly pure enzyme preparation.

EXPERIMENTAL PROCEDURES

Purification scheme

Fresh Japanese radish (50 kg; *R. sativus* cv Daikon) were purchased from local suppliers. The juice from the whole stock was obtained using a domestic extractor (Turmix, México) and immediately centrifuged on-line at 13,200 g. The supernatant was concentrated by ultrafiltration using a 10 kDa cutoff membrane to 2 L. The pH of the concentrate was adjusted to pH 4.5 by dialysis overnight at 4°C against 20 volumes of 20 mM sodium acetate buffer and centrifuged at 15,600 g for 20 min. The resultant solution was applied onto a 100 ml Macro-Prep CM (Bio- Rad) column equilibrated with the same buffer. The non-bound fraction was collected while the bound fraction was eluted using 1 M NaCl in the same buffer.

The non-bound fraction was dialysed overnight at 4°C against 20 volumes of 10 mM sodium phosphate buffer pH 6.1 (SPB) and equilibrated with ammonium sulfate to a final concentration of 2.5 M at room temperature. This preparation was applied onto a 200 ml Macro- Prep t-Butyl HIC (Bio-Rad) column equilibrated in the same buffer. The sample was eluted with a linear gradient of ammonium sulfate from 2.5 - 1 M in the same buffer. Individual fractions were tested for stability after incubation with hydrogen peroxide as described below. Fractions presenting a minimum recovery of 70% of the initial activity were pooled for further analysis.

Excess salt was removed from the pool by overnight dialysis at 4°C against 50 volumes of 10 mM SPB and concentrated by ultrafiltration using a 1 kDa cutoff membrane in an Amicon pressure vessel (Millipore). The concentrated sample was applied onto a 50 cm column containing 320 ml of Hi-Load 26/60 Superdex 200 equilibrated in 100 mM SPB pH 6.1, eluted with a flow rate of 2.5 ml/min and 5 ml fractions collected and analyzed. Fractions presenting a least 90% of the initial activity after incubation in the presence of hydrogen peroxide were pooled, concentrated and dialyzed against 0.1 mM SPB. Pure enzyme preparations preserved at 4°C remained fully active for at least one year.

Activity and stability assays through the purification

The peroxidase activity of each fraction during the purification (50 - 100 l) was estimated as the initial rate of the oxidation of 16 mM guaiacol in 1 ml of 60 mM SPB, pH 6.1. Reactions were started by the addition of H_2O_2 to 1 mM. The oxidative stability of the peroxidase activity in each fraction was evaluated as the activity remaining after two hours incubation in 60 mM SPB pH 6.1 containing 10 mM H_2O_2.

Electrophoretic analyses

Denaturing electrophoresis was performed as previously described (Gil-Rodríguez et al., 2008) using the Page Ruler prestained protein ladder from Fermentas International (Ontario, Canada). Isoelectric point determination was performed with the Phast System from Pharmacia (Uppsala, Sweden) using the Isoelectric focusing calibration kit Broad pI (pH 3-10) (Amersham Biosciences, UK).

RESULTS AND DISCUSSION

Peroxidase purification schemes are based on the physicochemical properties of the target isoenzyme. The most common property utilized has been the surface charge through the use of ionic exchangers. Alternative methods exploiting other properties have also been developed: differential solubility (Aruna and Lali, 2001; Berg and Huystee, 1984; Miranda et al, 1998; Szamos and Hoschke, 1992), thermal stability (Loprasert et al., 1990), ligand affinity (Brattain et al., 1976; Franco et al., 2004; Guo and Ruckenstein, 2003; Keeping and Lyttle, 1984; Miranda et al, 1998), hidrophobicity (Jen et al., 1980), chromatofocusing (Ollikka et al., 1995), metal affinity (Estela da Silva and Texeira F, 2000), and reverse micellar extraction (Regalado et al., 1996). However novel, all these methods have been optimized for the purification of the most abundant peroxidase species, comprising 60 to 90% of the initial activity. In contrast, in this paper we aimed the fast and efficient purification of a minor species, comprising less than 2% of the peroxidase activity found in the starting material.

The original purification scheme for ZoPrx initially separated all the peroxidase isoenzymes in the radish extract in two groups, anionic or cationic, and only afterwards performed the purification of the target species. To achieve this, we submitted the radish extract to successive anionic or cationic exchange chromatographies at pH 6.1, the optimal value for the guaiacol peroxidation activity. The more stable fraction happened to be the non-bound eluate suggesting that the peroxide tolerant isoenzyme had an isoelectric point around pH 6.1. After purification, ZoPrx presented a molecular mass of approximately 50 KDa and an isoelectric point value of 4. Evidently, the experimental value for the isoelectric point did not match the behavior of the protein during the purification. We have no clear explanation for this although we suspect that enzyme glycosylation might be involved.

With this knowledge, we developed an optimized protocol where a single weak cationic exchange step was performed at pH 4. At this pH value, ZoPrx did not bind to the chromatographic matrix and eluted with approximately 30% of the original peroxidase activity and a purification factor of 7 (Table 1). This fraction contained a predominant species of approximately 50 KDa, although at least other five protein bands could be detected (Figure 1). This result, along with the experimental value for stability towards hydrogen peroxide, which was lower than that obtained from the previous protocol (120 versus 42 min), indicate that at least one other protein in the mixture could be a conventional peroxidase.

In order to further separate these species, the recovered fraction was submitted to a hydrophobic interaction chromatography at pH 6.1. Peroxidase activity

Table 1. Summary of the purification stages of ZoPrx. The activity half-life was estimated after incubation at different times in the presence of 10 mM hydrogen peroxide and compared to the initial activity. Peroxidase activity and stability were estimated as previously described (Gil-Rodríguez et al., 2008).

Steps	Activity (U)	Protein content (mg)	Specific activity (U/mg)	Purification factor (fold)	Yield (% of starting Prx activity)	Stability (min)
Crude extract	540,000	2,097	257	1	100	35.00
Weak cationic exchange (CM Cellulose)	184,962	100	1,849	7	34	42.00
Hydrophobic interaction (*t*-butyl sepharose)	7,934	10	793	3	1.5	>160
Gel exclussion (Superdex 200)	6,714	3	746	3	1.5	>160

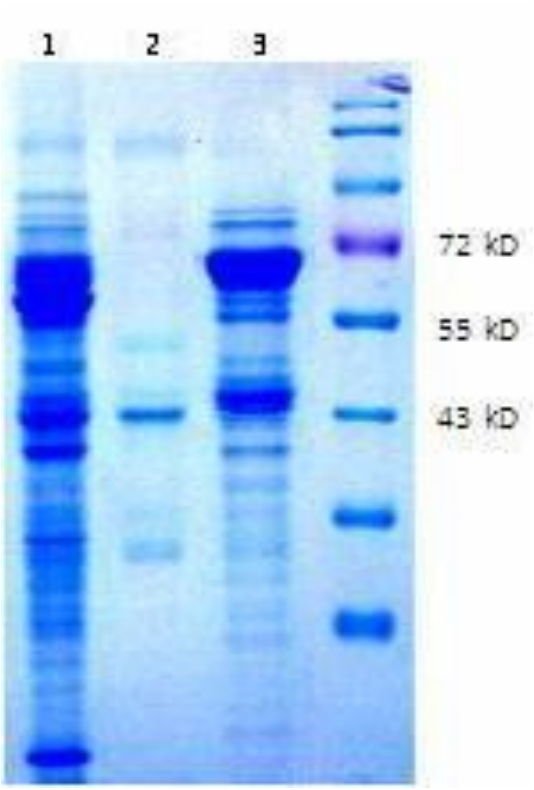

Figure 1. Denaturing polyacrylamide gel electrophoresis (PAGE) revealed by Coomassie brilliant blue staining before and after the first purification step (CM Cellulose chromatography). Lane 1 - Radish roots total extract; lane 2 - Non-bound fraction after weak cationic exchange (CM Cellulose); lane 3 - Bound and eluted fraction after weak cationic exchange (CM Cellulose); lane 4 - Molecular weight marker.

eluted throughout the ammonium sulfate gradient but at least four different protein species could be separated as observed by electrophoretic analysis. All eluted fractions were characterized by stability of the guaiacol peroxidation activity toward hydrogen peroxide. Those fractions which presented the highest stability values were pooled, concentrated and equilibrated with ammonium sulfate for a second chromatographic round.

After this step, we obtained a preparation containing a single protein band with a purification factor of 3. It is important to notice that the yield diminished at this step since a contaminating conventional peroxidase was removed, on the other hand, the stability value increased to a half-life of more than 160 min.

Selected fractions were pooled, concentrated and dialyzed against 100 mM SPB pH 6.1 before a polishing step through molecular exclusion. The resultant protein presented a molecular mass of 50 KDa, an isoelectric point value of 4 (Figure 2) and a significant tolerance toward hydrogen peroxide.

In conclusion, despite the abundance of peroxidase genes in plants, much of what we know about peroxidases comes from the study of the most abundant cationic (HRPC) and anionic (HRPA2) isoenzymes from horseradish roots (Veitch, 2004). The unusual properties of ZoPrx show the hidden potential of low abundance isoenzymes (Gil-Rodríguez et al., 2008), though complete disclosure requires the separation of the less abundant species in a cost- a labor-efficient manner. In this manuscript we describe a procedure for the purification of a targeted isoenzyme, which may be adapted to other low-abundance species by adjustments during the first chromatography, either by subtle changes in the pH of the buffer during the weak cationic exchange chromatography or by the use of weak anionic exchangers

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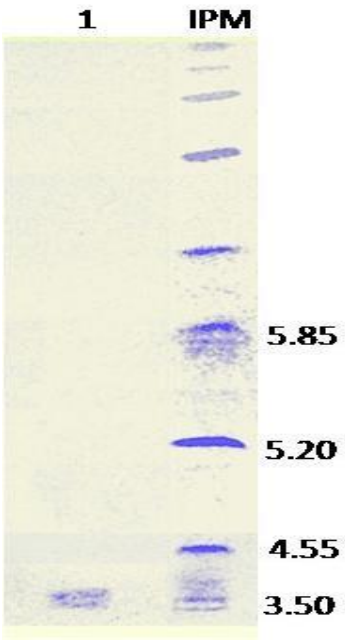

Figure 2. Non-denaturing isoelectric focussing gel revealed by Coomassie brilliant blue staining. Lane 1 - Pure ZoPrx preparation; lane 2 – Isoelectric point markers. Native and denaturing electrophoresis were performed as previously described (Gil-Rodríguez et al., 2008). Isoelectric point determination was performed using the Phast System from Pharmacia (Uppsala, Sweden).

REFERENCES

Aruna N, Lali A (2001). Purification of a plant peroxidase using reversibly soluble ion-exchange polymer. Process Biochem. 37: 431-437.

Berg BM, Huystee RB (1984). Rapid isolation of plant peroxidase. Purification of peroxidase A from Petunia. Physiol. Plantarum 60: 299-304.

Brattain MG, Marks ME, Pretlow II TG (1976). The purification of horseradish peroxidase by affinity chromatography on Sepharose-bound concanavalin A. Anal. Biochem. 72: 346-352.

Estela da Silva M, Teixeira FT (2000). Purification of soybean peroxidase (*Glycine max*) by metal affinity partitioning in aqueous two-phase systems. J. Chromatogr. B 743:287-294.

Franco FL, Batista-Viera F, Carlsson J (2004). Preparation of high-density Concanavalin A adsorbent and its use for rapid, high-yield purification of peroxidase from horseradish roots. J. Chromatogr. B 803: 237-241.

Gil-Rodríguez P, Ferreira-Batista CV, Vazquez-Duhalt R, Valderrama B (2008). A novel heme peroxidase from *Raphanus sativus* intrinsically resistant to hydrogen peroxide. Eng. Life Sci. 8: 286-296.

Guo W, Ruckenstein E (2003). Separation and purification of horseradish peroxidase by membrane affinity chromatography. J. Membrane Sci. 211: 101-111.

Jen JJ, Seo A, Flurkey WH (1980). Tomato peroxidase: purification via hydrophobic chromatography. J. Food Sci. 45: 60-63.

Keeping H S, Lyttle CR (1984). Monoclonal antibody to rat uterine peroxidase and its use in identification of the peroxidase as being of eosinophil origin. BBA-Gen subjects 802: 399-406.

Loprasert S, Urabe I, Okada H (1990). Overproduction and single-step purification of *Bacillus stearothermophilus* peroxidase in *Escherichia coli*. Appl. Microbiol. Biol. 32: 690-692.

Miranda MV, Fernández-Iahore HM, Cascone O, Dobrecky J (1998). The extractive purification of peroxidase from plant raw materials in aqueous two-phase systems. Acta Biotechnol. 18: 179-188.

Morita Y, Yoshida C, Kitamura I, Ida S (1970). Isoenzymes of Japanese-radish peroxidase. Agric. Biol. Chem. Tokyo 34: 1191-1197.

Morita Y, Yoshida C, Maeda Y (1971). Properties and structures of peroxidase isoenzymes of Japanes-radish. Agr. Biol. Chem. Tokyo 35: 1074-1083.

Ollikka P, Leppanen VM, Anttila T, Suominen I (1995). Purification of Major Lignin Peroxidase Isoenzymes from *Phanerochaete chrysosporium* by Chromatofocusing. Protein Expr. Purif. 6:337-342.

Regalado C, Asenjo JA, Pyle DL (1996). Studies on the purification of peroxidase from horseradish roots using reverse micelles. Enzyme Microb. Tech. 18: 332-339.

Szamos J, Hoschke A (1992). Purification of horseradish peroxidase by three-phase partitioning (TPP). Acta Aliment Hung. 21:253-260.

Taylor JS, Raes J (2004). Duplication and divergence: The evolution of new genes and old ideas. Annu. Rev. Genet. 38:615-643.

Tognolli M, Penel C, Greppin H, Simon P (2002). Analysis and expression of the class III peroxidase large gene family in Arabidopsis thaliana. Gene 288: 129-138.

Valério L, de Meyer M, Penel C, Dunand C (2004). Expression analysis of the *Arabidopsis peroxidase* multigenic family. Phytochemistry 65: 1331-1342.

Veitch NC (2004). Horseradish peroxidase: a modern view of a classic enzyme. Phytochemistry 65: 249-259.

Wendel JF (2009). Gene duplication and evolutionary novelty in plants. N. Phytol. 183: 557-564.

Zhang J (2003). Evolution by gene duplication: an update. Trends Ecol. Evol. 18:292-298.