



DIFFERENTIAL EXTRACTION AND VARIATION IN SUBSTRATE SPECIFICITY IN ISOPEROXIDASES OF ANNONA SQUAMOSA L

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ABSTRACT

The present study was aimed to explore role of pH on extraction of isoforms, activity and substrate preference of isoperoxidase (EC 1.11.1.7). Variation in pH of extraction buffer from 4.5 to 8.8 has shown considerable variation in extraction of isoperoxidases. In total 18 isoperoxidases have been visualized through activity staining in native PAGE. These isoperoxidases also exhibited differential substrate selectivity with *o*-dianisidine and guaiacol. Through in gel activity staining with guaiacol, high molecular weight isoforms were found active while with *o*-dianisidine, low molecular weight isoforms were found active (especially in samples extracted at pH 6.8 and 7.0). Very prominent effect of extraction pH on isoperoxidase was visible at pH 6.8 and 7.0. More than 9 isoforms were noticed at pH 6.8 while the number reduced to 3 at pH 7.0. Maximum peroxidase activity, with guaiacol as substrate, was observed with enzyme isolated at pH 6.8 and activity taken at pH 8.8 (0.0322 ± 0.0070 nanokatal), while with *o*-dianisidine as substrate, maximum activity was observed with enzyme isolated at pH 4.5 and activity taken at pH 7.0 (0.9409 ± 0.1123 nanokatal). Two ways ANNOVA revealed no significant variation between extraction and activity pH with *p*-value > 0.05 using guaiacol as substrate. However, Significant variation was observed in enzymes isolated at different pH with *p*-value < 0.05 with *o*-dianisidine as substrate. It can be concluded that in *A. squamosa* different pH of extraction buffer influence isolation of different isoperoxidases, their activity and substrate preference as well. It is worth mention here that role the isoforms detected at different extraction and activity pH with two different substrates would be of immense academic and mechanistic significance.

KEYWORDS: Annona, assay pH, extraction pH, enzyme, peroxidase.

INTRODUCTION

A. squamosa L., belongs to the family Annonaceae, and is known as 'custard apple'. It is an inhabitant of West Indies, and commonly grows in deciduous forests, including India. The available literature verifies that each part of *A. squamosa* possesses remedial properties and useful for diabetes, hyperthyroidism, cardiac disorders and cancer.^[1] Leaves are considered to be effective in prolapse in children. The crushed leaves are inhaled to overcome fainting spells and hysteria. Bark is known to be a potent astringent.^[2] In Ayurveda, fruits are believed to be a good tonic; enriches blood, boost muscle power, used as expectorant, reduces blazing sensation and propensity to biliousness. It also acts as tranquilizer to heart and reduce vomiting. The seeds are supposed to be abortifacient and an excellent source to obliterate lice in hair in *Yunani* medicine. Oil and resin obtained from seeds of *A. squamosa* is used in detergents. Seeds are potent nuisance of conjunctiva and make ulcers in the eye.^[3] *A. squamosa* is also used as an hypolipidemic,

insecticidal, antioxidant, anti-diabetic, antitumor, and anti-inflammatory agent that is due to the existence of the cyclic peptides. In addition, the crushed leaves have been found useful on the ulcers and wounds and leaf extracts have been used in case of dysentery.^[4] According to Jayshree et al. and Dinesh et al.^[5,6] leaves contains many phytochemicals, viz., Anonaine, 4-(2-nitroethyl)-1-((6-O- β -D-xylopyranosyl)- β -D-glucopyranosyl) oxy) benzene, Benzyltetrahydroisoquinoline, Carvone, Camphor, Borneol, Camphene, car-3-ene, Eugenol, Methylsalicylate, β -Caryophyllene, n-Triacontanol Methylanthranilate, Farnesol, Geraniol, Higenamine, 1,6-Hentriacontanone, Hexacontanol, Isocorydine, Limonine, Linalool, Menthone, Linalool acetate, Methylheptenone, n-Octacosanol, α -Pinene, β -Pinene, Rutin, Stigmasterol, *p*-(hydroxybenzyl)-6,7-(2-hydroxy,4-hydro) isoquinoline, Thymol, and β -Sitosterol. Many of these are phenolic derivatives which are substrate for plant peroxidases (EC 1.11.1.7). Since peroxidases are present as variable isoforms and their

expression is spatiotemporally regulated, they might influence the metabolism of these phenolic derivatives. Within the pH limit, which could be a narrow or wide range, there is an optimum at which the highest activity is exhibited by the enzyme. Not only the activity but its extraction is also influenced by pH of extraction medium and thus can be influenced by the nature of the buffer used during extraction. There could also be a discontinuity in activity of enzyme in a broad pH range because of the use of various buffers.^[7] For standard enzymatic assays, a pH is taken, which is near to the optimum, unless some other constituent of the assay mixture adversely affect the reaction kinetics.^[8]

Looking at importance of *A. squamosa* as a medicinal plant, the present study was conducted which focuses on pH dependent extraction of isoperoxidases and their validation for substrate selectivity. The study would help the investigators in future to see the effect of various stress stimuli on antioxidants as respondent or as protective mean.

MATERIAL AND METHODS

Protein isolation and separation

To isolate enzymatic antioxidant 0.5 gram of tissue was homogenized in 5 ml of extraction buffer having 50mM Sodium acetate pH 4.5 and 50 mM Tris HCl pH 6.8, 7.0, and 8.8. The homogenization was done in presence of 50 mg PVP (insoluble) and homogenate was incubated at 4°C for 20 minutes. The homogenate was centrifuged at 12000 x g at 4°C for 15 minutes. Supernatant collected after centrifugation was used as source of enzymatic antioxidant during enzymatic assay. The activity staining of peroxidase was done using specific substrate of the enzyme isolated as mentioned below.

Activity assay and in gel activity detection of isoperoxidases

Peroxidase activity assay was carried out following the protocol of Singh et al^[9] with some modifications. The reaction mixture consisted of 50 mM buffer, pH (4.5/ 6.8/ 7.0/ 8.8 as per requirement), 3 mM H₂O₂, and 3 mM guaiacol ($\epsilon_{470\text{nm}} = 2.66 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$) or 3 mM *o*-dianisidine ($\epsilon_{470\text{nm}} = 11.3 \text{ mM}^{-1} \text{ cm}^{-1}$) and suitable aliquot of enzyme. The activity was observed by taking absorbance at 470 nm (double beam UV- visible spectrophotometer, Shimadzu UV- 1800). Enzymatic kinetics in the form of increase in absorbance for 2 minutes was noted and change in absorbance was calculated accordingly taking nanokatal as unit (1 nanokatal is defined as enzyme activity by which 1 nanomoles of substrates are converted to product per minute). An isolated protein was separated on native polyacrylamide gel electrophoresis (PAGE) using 10% resolving and 3% stacking gel. After electrophoresis the native gel was incubated in activity reaction mixture as mentioned above (guaiacol and *o*-dianisidine as substrate, respectively). The gel was then placed in incubator at 37°C with shaking at 40 rpm, for 15-30

minutes until the bands appeared. Gel was photographed using Nikon 5300 digital camera.

RESULT AND DISCUSSIONS

The present study shows the effect of variation in pH on the extraction, activity and substrate specificity of peroxidase. Extraction pH and assay pH exhibited influence on activity of peroxidase which is given in figure 1. It was observed that maximum activity of peroxidase with guaiacol as substrate was observed with extraction at pH 6.8 and activity assay at pH 8.8 (0.0322± 0.0070 nanokatal). While minimum was observed (with guaiacol as substrate) with extraction pH 8.8 and activity assay pH 4.5 with (0.0023±0.0008 nanokatal). When *o*-dianisidine was taken as substrate it was observed that maximum activity was observed with extraction pH 4.5 and activity assay pH 7.0 with (0.9409± 0.1123 nanokatal) While minimum was observed (with *o*-dianisidine as substrate) with extraction pH 7.0 and activity assay pH 7.0 (0.0805±0.01730 nanokatal), much higher than that with guaiacol. This indicates substrate suitability in favor of *o*-dianisidine.

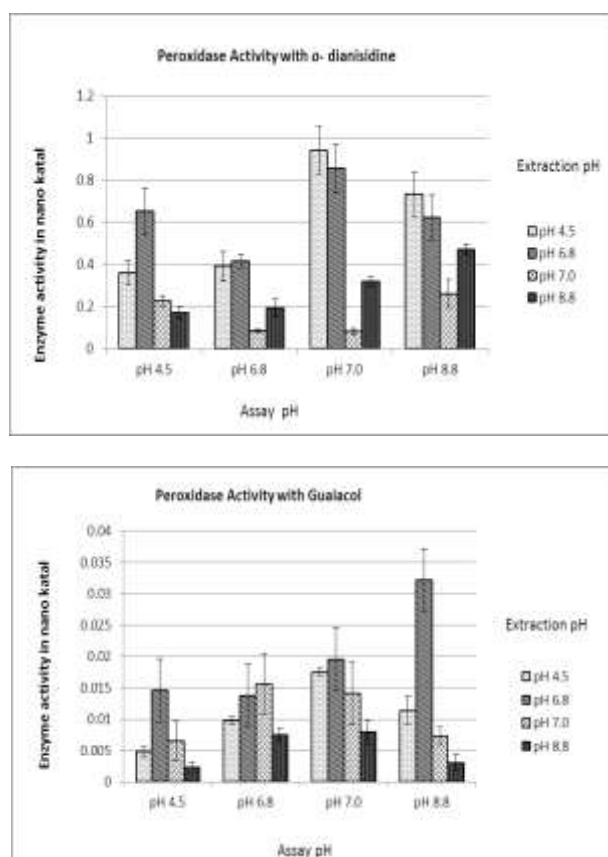


Figure 1: Effect of extraction and assay pH on activity of peroxidases. Upper panel presents result with *o*-dianisidine and lower panel with guaiacol as substrate.

Similar studies reveal that the activity of the enzyme is affected by pH of buffer used. It means that the rate of oxidation fluctuates with the acidity or alkalinity of the

buffer. This may be a subcellular tool to regulate expression of isoforms in response to pH or metabolites affecting pH. This regulation of differential expression and activity is involved in different metabolic pathways involved in generating responses against stimuli (both internal and external). This is supported by study for silk peroxidase where a wide range of pH (5.0 to 7.0) was determined by Deepak et al.^[10] which reveal that plant peroxidases assume to arbitrate modifications in the mechanical properties of plant cell walls. For peroxidases, acidic pH optima were reported from vegetable sources,^[11,12] *Cassia didymobotrya*^[13] and soyabean.^[14,15] It was reported by Kamal and Behere,^[14] that maximum decrease of β - strands and β - turns at pH 5.5, seems to be responsible for the heme to be extra exposed to the solvent and rising the overall conformational elasticity of the enzyme. Peroxidases oxidize numerous substrates in the presence of H_2O_2 and generally have a protoporphyrin IX prosthetic group.^[16,17] The substrate diversity seems to be correlated with isoform diversity in this study.

When activity staining of gel was done using guaiacol and as substrate for isoperoxidase, it was observed that different isoforms were found at different pH and the number of isoforms varied in accordance to the variation in pH. It is evident from Figure 2 that at pH 4.5 and 8.8 relating large molecular weight isoperoxidase show activity with *o*-dianisidine a clear difference is observed at pH 6.8 and 7.0 when small molecular weight isoperoxidase show active bands. While with guaiacol relatively less isoform show activity bands. pH 4.5, 7.0 and 8.8 show predominant large molecular weight bands while at extraction pH 6.8 high moderate size bands are visible. A surprising effect of minor change in pH was observed in the staining where 3 bands are visible at pH 7.0, while more than 9 distinct bands are visible at extraction pH 6.8. Ario de Marco et al.^[18] has shown pH dependence on activity and substrate specificity of seven isoperoxidases in tobacco. Their specific activities measured in the presence of different substrates, as monolignols and NAD (P)H, indicated possible specialized functions in cell wall metabolism. However, effect of pH on extraction and substrate specificity together finds no reference in published literature.

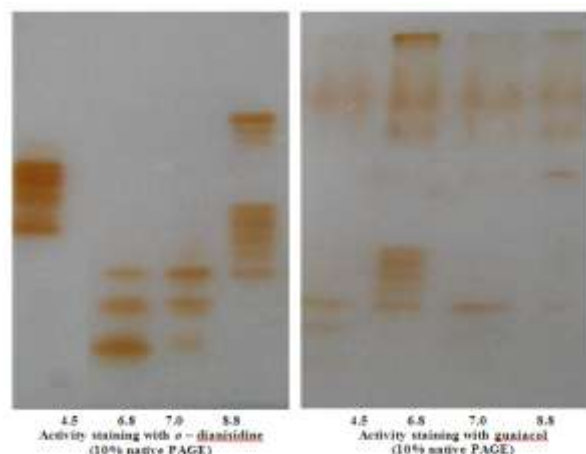


Figure 2: In-gel activity staining for peroxidases showing pH dependent (4.5 to 8.8) difference in isoperoxidase activity profiling with different substrates, *o*-dianisidine and guaiacol.

It has been studied by De Marco et al.^[19] that, variation in pH can modify the relative part of various isoperoxidases to total activity and this is obvious with the appearance of different pH optima in pH activity curves. The peroxidase system of higher plants exists in several isoforms that are developmentally synchronized and extremely reactive in response to exogenous stimuli.^[20-22] In higher plants up to 40 genes corresponding to isoperoxidases have been reported. Post-transcriptional modifications may further add up to the number of isoforms of peroxidases.^[23,24]

To justify the significance of the observed facts two way ANNOVA was done. It was found that peroxidase activity using guaiacol as substrate did not show significant variation with *p*- value > 0.05 with enzyme isolated at different pH or activity taken at different pH values (0.0982 and 0.8964 respectively). When it was calculated for peroxidase activity using *o* – dianisidine as substrate no significant variation is shown in activity taken with different pH values with *p* – value > 0.05 (0.0763), but it shows significant variation in enzyme isolated at different pH with *p* – value < 0.05 (0.0033).

CONCLUSION

Any change in pH above or below the optimum will quickly cause an alteration in extraction, the rate of reaction and probably conformational changes. Existence of isoforms make it more complex. Since more of the enzyme molecules will have active sites whose shapes are not (or at least are less) complementary to their alternative/ different substrate. Small changes in pH above or below the optimum may cause physiological change to the enzyme, thereby exhibiting above mentioned alterations. Enzymes in different locations have different optimum pH values since their environmental conditions may be different and same may be responsible for their altered behavior against pH stimuli.

Author's Contribution: Mohammed Shariq Iqbal has worked for data generation and compilation under guidance and planning of Brijesh Pandey.

Conflict of Interests: Authors do not find any conflict of interest with anyone.

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