Journal of Materials and Environmental Sciences
ISSN : 2028-2508
CODEN : JMESCN

J. Mater. Environ. Sci., 2018, Volume 9, Issue 5, Page 1398-1404

https://doi.org/10.26872/jmes.2018.9.5.152

http://www.jmaterenvironsci.com

Extraction and comparison of two new peroxidases from leaves and roots of *Brassica oleraceae* var. ramosa

Touda ZAHIDI[1], Abdnnebi LEKCHIRI[2], Titrite ZAHIDI[3], Wafae LEKCHIRI[1], Abdelbasset BERRICHI[3], Mostafa MIMOUNI[4] and Hassan EL HALOUANI[1]

[1]*Laboratoryof 1Laboratory of ecology, environment and sustainable development, University Mohamed I[st], Biology Department, Oujda, Morocco*
[2] *Laboratory of Genetics and Biotechnology, University Mohamed I[st], Biology Department, Oujda, Morocco*
[3] *Laboratory of Plant Biology and Microorganisms, University Mohamed I[st], Biology Department, Oujda, Morocco*
[4] *Laboratory of Materials Chemistry, Department of Chemistry, University Mohamed I[st], Biology Department, Oujda, Morocco*

Received 22 Jun 2016,
Revised 21 Oct 2016,
Accepted 25 Oct 2016

Keywords
- ✓ *peroxidases,*
- ✓ *Brassica oleraceae var. ramosa,*
- ✓ *extraction,*
- ✓ *enzyme.*

Touda.zahidi@gmail.com ;
Phone: +212674876755;

Abstract

*Brassica oleraceae var. ramosa*is a leafy vegetable commonly used in southeast of Morocco in population's food. So far, no study has been made on Brassica oleraceae var. ramosa's. In this study we extract and partially purify new peroxidases from leaves and roots of Brassica oleraceavar.ramosa. A simple purification method was performed by: precipitation technique, gel filtration on Sephadex G-25 and dialysis. The molecular weight of this enzyme was found by SDS-PAGE to be around 62.34kDa for roots, and 70.35 kDa for leaves. The purified enzymes had affinity with guaiacol as substrate. The optimal activity of the enzymes was at 40°C and pH 6.00. Moreover, the reactions with different ions and salt were studied (NaCl, Fe^{3+}, Cu^{2+}…). The enzymes were inhibited by β-mercaptoethanol and sodium azide. In conclusion, those new peroxidases are stable at different conditions, and show an interesting result with some metal ions, which makes it a good candidate for biotechnological applications.

1. Introduction

The peroxidases (E.C.I.II.I.7) are a family of enzymes able to oxidize different substrates using H_2O_2. The plant's peroxidase are class III of this enzyme, POD's involved to many plant's reactions such as pathogen-defense, wound healing [1], and cell development[2].The peroxidases enzymes are widely used on many research areas and biotechnological tools[3]. So peroxidases have a commercial importance, for example, in diagnostics tool, such as enzyme immunoassays, biosensors… etc[4].

A recent study proves the value of peroxidase. It's used as an ecological alternative on polymerization of some polymer [5]. It is also used on new immunoassays techniques using nanostructures. In addition, peroxidases are used in wastewater treatment, and dye contamination [6].

The peroxidasesclass III are extracted from plants[7]. Currently, the main origin of plants' peroxidase is Horseradishof the Brassica family, and the most studied one[8]. However, many plants have peroxidase activity. *Brassica oleraceae* var. *ramosa* has never been studied, and is commonly consumed by Moroccan Southeast's population as a food. This family is consumed all over the world since centuries. Brassica is a family of diverse species which include horseradish, cabbage; broccoli, cauliflower, etc...[9, 10]

The species of Brassica are cultivated and consumed across the world [11]. This family represents an evolutionary example of genotypic and phenotypic diversity. In the southeast of Morocco, population breeds and cooks *Brassica oleraceae* var. *ramosa* (zegzaw).

Thus, brassicaceae vegetables have been widely studied for their nutritional value, and their bioactive compounds[12, 13]. *Brassica oleracea* var. *ramosa*, is commonly used in the southeast of Morocco as vegetables with couscous or soup. Only the aerial part is consumed, the roots are generally thrown away.

This study describes the extraction and comparison of two peroxidasesof leaves and roots of *Brassica oleracea* var. *ramosa*, and its characterization.

2. Material and Methods

2.1. Crude extracts preparation

Brassica oleraceae var. *ramosa* was collected from hothouse plant of Faculty of Sciences (University Mohamed Ist, Oujda, Morocco). Taxonomic identification was performed by Pr. A. Berrichi from the Department of Biology, Faculty of Sciences (University Mohamed Ist, Oujda, Morocco).

2 . 1 . 1 Leaves:

Brassica Oleracea var. *Ramosa* leaves (50 g) were cut into pieces and homogenized with 90 ml of 4°C phosphate buffer 50 mM pH 6. The homogenate was filtrated through Buchner funnel and centrifuged at 3000 rpm for 15 min. The supernatant was collected.

2 . 1 . 2 Roots:

Brassica Oleracea var. *Ramosa* leaves (50 g) were cut into pieces and homogenized with 60 ml. The same steps were followed for protein precipitation.

2.2. Protein precipitation

Several precipitations were preceded by ammonium sulfate in range of 30-75% saturation, and centrifuged again at 3000 rpm for 15 min. The pellets were collected and dissolved in phosphate buffer 50 mM pH=6.

2.3. Chromatography procedure and dialysis

Two Milliliters of the concentrated enzyme sample was loaded onto G-25 column. The column was equilibrated and eluted by 50 mM Phosphate buffer pH 6.0 using isocratic elution. Two milliliters fractions were collected throughout the elution. Absorbance at 280 nm and peroxidase activity was monitored. The fractions of the active peroxidase were pooled and dialyzed for 24 h against distilled water.

2 . 2 SDS-PAGE electrophoresis:

Molecular weight: SDS-PAGE was performed in gel 15%. Purified enzyme was loaded. gels were stained with Coomassie Brillant Blue R-250 [14]. Standard protein, such as lysozyme (14 kDa), trypsin (23.3 kDa), bovine serum albumin (67kDa), lactoperoxidase (77.5 kDa) were used for the calibration.

2 . 3 Protein Concentration

Protein concentration was measured according to the procedure described by Bradford. Bovin serum albumin (BSA) was used as standard protein[15].

2 . 4 Enzyme characterization:

Substrate specificity, optimum pH and peroxidase activity assay:

2 . 5 Substrate specificity:

Substrate specificity was studied by spectrophotometric assay, using H_2O_2 with tree different substrates: Guaiacol, Benzidine and amino antipyrine. To study the kinetic parameters, different concentrations of each substrate were added in 1.3 mL of reaction solution containing 50 mM of suitable buffer, 100 µL sample of enzyme and different concentrations of H_2O_2.

The following wavelengths and molar extinction coefficients were: Guaiacol λ 470 nm and ξ 26.6 $mM^{-1}.cm^{-1}$, Benzidine λ 652 nm and ξ 39 $mM^{-1}.cm^{-1}$, Amino antipyrine λ 510 nm and ξ 6.58 $mM^{-1}.cm^{-1}$. One unit of enzymatic activity was defined as the amount of enzyme that oxidizes 1µmol/min of hydrogen donors under assay conditions. The Michaelis constant (Km) and maximum catalytic rate (V_{max}) were calculated from Lineweaver-Burk plotting.

The Guaiacol was chosen as the substrate for the peroxidase activity assay. The following paragraph describes the enzymatic assay.

2 . 6 Enzymatic assay:

Enzymatic assay was determined by colorimetric reaction using spectrophotometer (Metash V-5600 Visible Spectrometer). The concentration of tetraguaiacol was followed at 470 nm during 5 minutes.[16] The changes of

470 nm wavelength absorbance were followed during 5 minutes. Enzymatic reaction was done in 1.3 mL volume reaction mixtures for root's enzyme (PrxR) and leave's enzyme (PrxL). The 100 µL sample of enzyme, 7.5 mM guaiacol with different concentrations of H_2O_2 in 50 mM phosphate buffer (pH 6.00).

2 . 7 Determination of optimum temperature:

The optimal temperature was determined by measuring enzyme activity under different temperature ranging from 10 to 80°C.

2 . 8 Determination of optimum pH:

The optimal pH was determined by measuring enzyme activity under different pH, using the following buffers: 50 mM citrate buffer (pH 2.5 to 5.5), phosphate buffer (pH 6.0 to 7.5) Tris-HCl (pH 8.0 to 8.5), and glycine-NaOH (pH 9.00 to 11.00).

2 . 9 Effect of various compounds and metals ions:

Enzyme was pre-incubated for 30 minutes at 40°C with various metal ions (Ca^{2+}, Cu^{2+}, Fe^{3+}, Mg^{2+}, Zn^{2+}). Enzyme activity was evaluated with different compounds: sodium azide, β-mercaptoethanol, and NaCl.

2 . 10 Statistical analysis:

All the experiments were repeated three times and expressed as means ± SD. The statistical significances of different factors were evaluated by analysis of variance (ANOVA), using Microsoft excel. P-value less than 0.05 ($p<0.05$) was considered to be statistically significant.

3. Results and discussion

3 . 1 Enzyme purification:

3 . 1 . 1 Leave's peroxidase:

From 50 g of fresh leaves and 50 g of fresh roots, we extracted 90 mL and 60 mL of protein mixture respectively. We followed protocol which includes three steps: ammonium sulfate precipitation, Sephadex G-25 desalting chromatography and dialysis. The results for each purification step are summarized in Table1.

Table 1: Summary of each purification step of peroxidase from *Brassica oleraceae* var. *Ramosa* leaves and roots.

Leaves	Total protein (µg/ml)	Enzymatic activity (U)	Specific activity (U/mg)	Purification fold	yield (%)
Crude extract	2353,9	85,65	36,39	1	100
Ammonium precipitation	4728,9	41,98	8,88	0,24	49,01
Sephadex G-25	4,69	24,67	5264,07	144,67	28,80
Roots					
Crude extract	549,24	240,18	437,30	1	100
Ammonium precipitation	2609,16	91,36	35,02	0,08	38,04
Sephadex G-25	3,39	2,82	833,13	1,91	1,18

At higher concentrations, protein solubility usually decreases, leading to precipitation: salting-out. This principle is used to precipitate the peroxidase. Crude extracts of leaves and roots of *Brassica oleraceae* var. *Ramosa*, were precipitated by ammonium sulfate in range of 30-75% saturation. For leave's and root's extract, the best results were obtained by 40% ammonium sulfate saturation. For leave's extract, we lost 51% with ammonium precipitation. Purification fold was 144.67 and yield 28.80 (table1.) After filtration chromatography with Sephadex G-25, we concentrate and dialyze active fractions against distilled water for 24 h. We reach a reasonable yield (28.8%), and leave's peroxidase (PrxL) was purified 144.67 time. As for root's extract, we followed the same steps, but we lost 98% of POD's activity. After the last step (Sephadex G-25) we have a low yield 1.18%. However, with the same quantity of plant material, roots had bigger enzymatic activity (2.8 times) than leave's extract.

a) b)

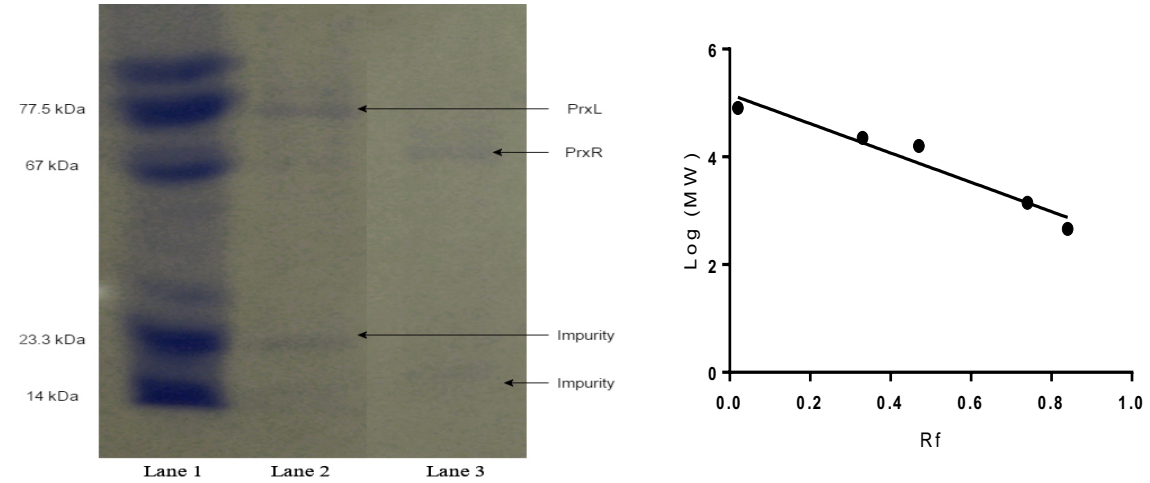

Figure1: a) SDS-PAGE analysis of peroxidases enzymes PrxL and PrxR with Coomassie Blue staining. Lane1: protein molecular weight marker; Lane 2: Purified PrxL; Lane 3: Purified PrxR. b) Standard graph log MW-Rf.

3 . 2 Molecular weight:

The molecular weight and purity were determined by SDS-PAGE, after Coomassie Blue staining. The molecular weightsare calculated from a plot of log Rf versus protein standard. Figure 1. a) shows lane 1: with proteins standards. Lane 2 shows Peroxidase extracts from leaves of *Brassica oleraceae* var. *Ramosa* (PrxL). PrxL lane shows two bands: an impurity band and peroxidase enzyme (PrxL), the molecular weight of PrxL was around 70.35 kDa. Lane 3 shows two bands: the first one is the peroxidase band (PrxR) (62.34 kDa), the second band is an impurity. SDS-PAGE analysis shows that the peroxidase in the leaves is different than the peroxidase in the roots. Molecular weights of most plants peroxidases varies from 30 to 60 kDa[17]. In comparison with Horseradish peroxidase's (40 – 46 kDa), PrxL and PrxR are greater [18].

Table 2: Substrate specificity of PrxL

	λ (nm)	**Km (mM)**	**Specific activity (µmol/min/mg)**
Guaiacol	470	6.34	24.62
Benzidine	652	4,7	4.59
Amino antipyrine	510	34	1.34

3 . 3 Enzyme characterization

3 . 3 . 1 Determination of substrate specificity:

For this purpose, we test three different substrates: Guaiacol, Benzidine and Aminoantipyrine. Only the PrxL were tested. PrxL were able to oxidize other substrates: hydroquinone, pyrocatechol (data not shown). The Km and Vmax of each substrate were determinate at 40°C under optimum pH. The results in Table 2. shows that guaiacol and benzidine have high affinity to PrxL, with Km value of 6.34 mM and 4.7mM respectively. For aminoantipyrine the Km value was 34 mM, which is 5.4 folds higher than that of guaiacol.

3 . 3 . 2 Determination of optimum pH and temperature:

As shown in Figure 3, the maximum activity for PrxL and PrxR was found to be at 40°C and pH 6.00. Both peroxidases showed no significant differences. Moreover, both PrxL and PrxR have their highest activity in acidic conditions. PrxR and PrxL had narrow pH optimum range at pH 6.00. As shown in Figure 3. a) relative activities decrease rapidly. This similarity in optimal pH suggests that peroxidases may be synthesized for the same aim (defense against pathogen, wound healing…) [19, 20]. For example, *Brassica rapa* has his optimal pH 6.00, as for *Raphanus sativus*[21, 22].

Optimum temperature was investigated. PrxL and PrxR were incubated with buffer at different temperature (10 – 80 °C). The maximum activity was found at 40°C for both peroxidases. Our results agrees with those reported for *Brassica oleraceae* var. *botrytis*, on the fact that optimum temperature was around 45 – 55 °C [23].

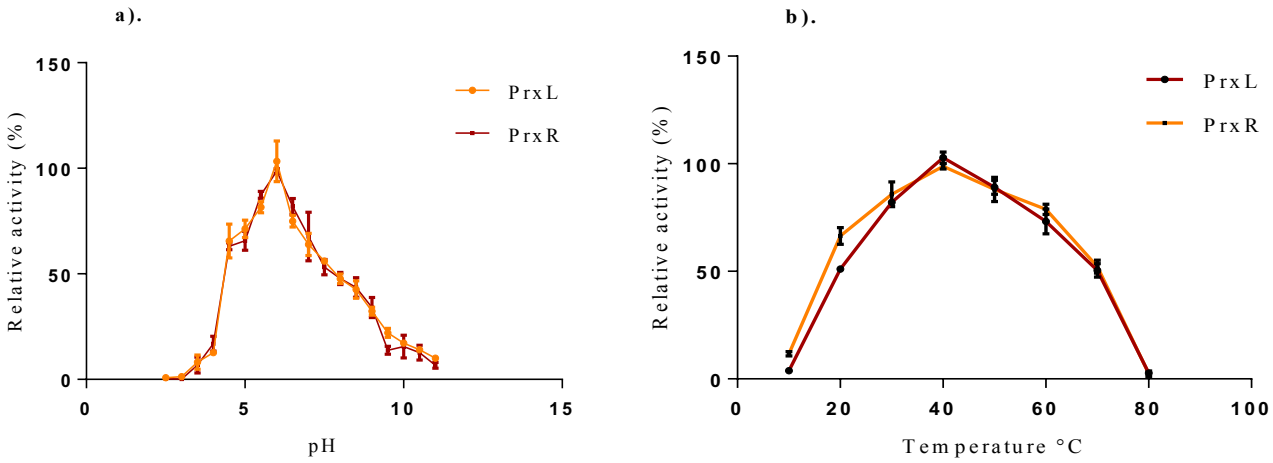

Figure 3: (a) Effect of pH on the PrxL activity. PrxL activity was incubated for 2h with various pH values (2.5 – 11), using guaiacol as a substrate. The highest activity was set as 100%. (b) Effect of temperature on the PrxR activity. PrxL was measured under different temperature (10 – 80°C) using guaiacol as substrate.

Table 3: Effect of metal on PrxL and PrxR. The enzymes were first incubated with 0.08, 0.15 and 0.77 g/L of various metal ions for 2h, and then the enzymatic activity was measured. Activity of the enzyme without metal ions incubation (control) was set as 100%. Results were shown as mean ± standard deviation of three replicas.

Compound		**Concentration (g/l)**		
		0,08	0,15	0,77
Control		100	100	100
CaCO3	PrxL	109,41 ± 17,13	115,96 ± 21,68	137,93 ± 26,79
	PrxR	103,69 ± 15,03	105,82 ±17,36	146,27 ± 18,48
CuCl2	PrxL	82,63 ± 7,56	117,91 ± 11,65	93 ± 9,21
	PrxR	65,17 ± 10,33	123,97 ± 13,01	75,7 ± 4,89
ZnSO4	PrxL	72,64 ± 6,45	111,81 ± 9,12	116,87 ± 12,45
	PrxR	131,01 ± 23,86	153,56 ± 18,37	158,46 ± 20,85
FeCl3	PrxL	90,59 ± 5,33	80,23 ± 6,48	76,2 ± 7,67
	PrxR	78,31 ± 7,00	43,62 ± 7,34	23,49 ± 6,61
MgCl2	PrxL	80,23 ± 7,24	91,13 ± 12,82	119,52 ± 18,23
	PrxR	142,02 ± 16,35	162,27 ± 12,45	193,82 ± 15,84
NaCl	PrxL	99,87 ± 7,21	124,5 ± 19,37	82,83 ± 6,79
	PrxR	68,17 ± 4,42	46,34 ± 3,72	13,94 ± 4,69
NaN_3	PrxL	1,6 ± 0,11	1,34 ± 0,21	0
	PrxR	10,34 ± 1,39	9,55 ± 1,14	0
β- mercaptoethanol	PrxL	90,98 ± 10,27	37,15 ± 4,54	10,93 ± 5,39
	PrxR	5,34 ± 0,25	0	0

3 . 3 . 3 Effect of various compounds and metals ions:

The enzymatic activities (PrxL and PrxR) were investigated in presence of different metal ions. Table 3 show the results obtained. We tested three ions concentrations on the enzymatic activities. PrxL and PrxR were inhibited by $FeCl^{3+}$. For PrxR the metal ions strongly decrease the enzymatic activity: we lost 76.51% of the activity with 0.77g/L of $FeCl^{3+}$. For PrxL, while 24% inhibition was caused by $FeCl^{3+}$. Inhibition with $FeCl^{3+}$ was also observed in peroxidases, which were extracted from chewing stick miswak and vanilla [24, 25].

On the other side, the enzymatic activities were increased by $CaCO_3$, $ZnSO_4$, $MgCl_2$ and some concentrations of $CuCl_2$. The largest increase was observed in PrxR by $MgCl_2$ at the three used concentrations (more 93.82% activity by 0.77g/L). Similar results were observed on peroxidase purified from *Jatropha curca*[17].

Moreover, we tested the effect of NaCl on enzyme activities. The activity of PrxL decreases with NaCl (0.08 and 0.77g/L). However, we obtained an increase of enzymatic activity at 0.15 g/L of NaCl. For root's peroxidase (PrxR), NaCl shows an inhibition with the three concentrations (Table 3).
Both enzymes (PrxR and PrxL) were inhibited by NaN_3, and β-mercaptoethanol. We obtained a total inhibition in some case.

Conclusion

Brassica oleraceae var. *Ramosa* is a leafy vegetable consumed by south east population of Morocco. In this study, we have extracted and partially purified the peroxidase enzyme from leaves and roots. The enzymes extracted have different molecular weight which means that two different peroxidases are synthetized. The roots synthetize a larger quantity of the peroxidase. The Enzymes extracted show stability at different conditions (temperature and pH), with various metals ions: PrxR was increased with some ions, such as $MgCl_2$ (the activity has increased almost twice (+98%)), which make them a good candidate for biotechnological tools. In addition, only leaves of *Brassica oleraceae* var. *ramosa* are consumed. Recycling roots may have an economical and environmental interest. Purification procedure needs to be further optimized. Also, this plant makes an interesting candidate for further studies in different fields.

References

1. S. Pandey, Fartyal, D., Agarwal, A., Shukla, T., James, D., Kaul, T., Negi, Y.K., Arora, S., and Reddy, M.K., Abiotic stress tolerance in plants: myriad roles of ascorbate peroxidase,*Frontiers in plant science,* 8 (2017) 581.
2. M.C. Criqui, Plesse, B., Durr, A., Marbach, J., Parmentier, Y., Jamet, E., and Fleck, J., Characterization of genes expressed in mesophyll protoplasts of Nicotiana sylvestris before the re-initiation of the DNA replicational activity,*Mech Dev,* 38 (1992) (2) 121-32.
3. Y. Lu, Ye, W., Yang, Q., Yu, J., Wang, Q., Zhou, P., Wang, C., Xue, D., and Zhao, S., Three-dimensional hierarchical porous PtCu dendrites: A highly efficient peroxidase nanozyme for colorimetric detection of H2O2,*Sensors and Actuators B: Chemical,* 230 (2016) 721-730.
4. J. Sonet, Bierla, K., Bulteau, A.-L., Lobinski, R., and Chavatte, L., Comparison of analytical methods using enzymatic activity, immunoaffinity and selenium-specific mass spectrometric detection for the quantitation of glutathione peroxidase 1,*Analytica Chimica Acta,* (2018).
5. S. Wang, Wang, Q., Fan, X., Xu, J., Zhang, Y., Yuan, J., Jin, H., and Cavaco-Paulo, A., Synthesis and characterization of starch-poly(methyl acrylate) graft copolymers using horseradish peroxidase,*Carbohydrate Polymers,* 136 (2016) 1010-1016.
6. T.-S. Xu, Bioconjugation of peroxidase-like nanostructures with natural enzyme for in-situ amplified conductometric immunoassay of tissue polypeptide antigen in biological fluids,*Biochemical Engineering Journal,* 105, Part A (2016) 36-43.
7. D. Schomburg. *BRENDA, AMENDA and FRENDA* 2017 [cited 1987 2017]; Available from: www.brenda-enzymes.org.
8. Y.Y. Lim, Ong, L., Loh, T.P., Sethi, S.K., Sng, A.A.J., Loke, K.Y., Halsall, D.J., Hughes, I.A., and Lee, Y.S., A diagnostic curiosity of isolated androstenedione elevation due to autoantibodies against horseradish peroxidase label of the immunoassay,*Clinica Chimica Acta,* 476 (2018) 103-106.
9. D.J. Shea, Itabashi, E., Takada, S., Fukai, E., Kakizaki, T., Fujimoto, R., and Okazaki, K., The role of FLOWERING LOCUS Cin vernalization of Brassica: the importance of vernalization research in the face of climate change,*Crop and Pasture Science,* 69 (2018) (1) 30-39.
10. B. Diyansah, Aini, L.Q., and Hadiastono, T., The effect of PGPR (plant growth promoting rhizobacteria) Pseudomonas fluorescens and Bacillus subtilis on leaf mustard plant (Brassica juncea L.) infected by TuMV (turnip mosaic virus),*Journal of Tropical Plant Protection,* 1 (2017) (1) 30-38.
11. G. R.Dixon, *VEGETABLE BRASSICAS AND RELATED CRUCIFERS* in *VEGETABLE BRASSICAS AND RELATED CRUCIFERS* C. International, Editor. 2007: Library of Congress Cataloging-in-Publication Data.

12. S.S. Ferreira, Passos, C.P., Cardoso, S.M., Wessel, D.F., and Coimbra, M.A., Microwave assisted dehydration of broccoli by-products and simultaneous extraction of bioactive compounds,*Food Chemistry,* 246 (2018) 386-393.
13. F.S. Hanschen, Kaufmann, M., Kupke, F., Hackl, T., Kroh, L.W., Rohn, S., and Schreiner, M., Brassica vegetables as sources of epithionitriles: Novel secondary products formed during cooking,*Food chemistry,* 245 (2018) 564-569.
14. T.H. Steinberg, *Chapter 31 Protein Gel Staining Methods: An Introduction and Overview*, in *Methods in Enzymology*, R.B. Richard and P.D. Murray, Editors. 2009, Academic Press. p. 541-563.
15. M.M. Bradford, A rapid and sensitive method of quantitation of microgram quantities of protein utilizing the principle of protein-dye binding,*Analytical Biochemistry* 72 (1976).
16. D.R. Doerge, Divi, R.L., and Churchwell, M.I., Identification of the Colored Guaiacol Oxidation Product Produced by Peroxidases,*Analytical Biochemistry,* 250 (1997) (1) 10-17.
17. C.O. Feng Cai, Peipei Duan, Shun Gao, Ying Xu, Fang Chen, Purification and characterization of a novel thermal stable peroxidase from Jatropha curcas leaves,*Journal of Molecular Catalysis B: Enzymatic,* 77 (2012).
18. K.G.S. Paul, T., Four isoperoxidases from horseradish root,*Acta Chem. Scand,* 24 (1970) 3607-3617.
19. Y. Wang, Wang, Q., Zhao, Y., Han, G., and Zhu, S., Systematic analysis of maize class III peroxidase gene family reveals a conserved subfamily involved in abiotic stress response,*Gene,* 566 (2015) (1) 95-108.
20. V. Sivakami, Ramachandran, B., Srivathsan, J., Kesavaperumal, G., Smily, B., and Kumar, D.M., Production and optimization of laccase and lignin peroxidase by newly isolated Pleurotus ostreatus LIG 19,*Journal of Microbiology and Biotechnology Research,* 2 (2017) (6) 875-881.
21. M.A. Duarte-Vazquez, Whitaker, J.R., Rojo-Dominguez, A., Garcia-Almendarez, B.E., and Regalado, C., Isolation and thermal characterization of an acidic isoperoxidase from turnip roots,*J Agric Food Chem,* 51 (2003) (17) 5096-102.
22. A. Omumi, Morshedi, D., and Samadi, A., Isolation of a novel stable peptide from cultivated Raphanus sativus with peroxidase activity,*World Journal of Microbiology and Biotechnology,* 17 (2001) (8) 827-828.
23. E. Koksal and Gulcin, I., Purification and characterization of peroxidase from cauliflower (Brassica oleracea L. var. botrytis) buds,*Protein Pept Lett,* 15 (2008) (4) 320-6.
24. A.M. Saleh, Properties of peroxidase from chewing stick miswak,*African Journal of Pharmacy and Pharmacology,* 6 (2012) (9).
25. O. Márquez, Waliszewski, K.N., Oliart, R.M., and Pardio, V.T., Purification and characterization of cell wall-bound peroxidase from vanilla bean,*LWT - Food Science and Technology,* 41 (2008) (8) 1372-1379.

(2018) ; http://www.jmaterenvironsci.com