Annals of Tropical Research 32[1]:1-16(2010)

Purification and partial characterization of white radish (*Raphanus sativus* L. var. Long white) peroxidase from cell suspension culture extract

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ABSTRACT

Peroxidase mainly Horseradish peroxidase (HRP) has been widely used as a component of clinical diagnostic reagent for Enzyme Linked Immunosorbent Assay (ELISA) technique. White radish (*Raphanus sativus* L.) was found as another source of peroxidase . In this study, white radish was used for the production of peroxidase by cell suspension culture technique. Isolation of the enzyme was conducted by ammonium sulfate precipitation followed by purification using DEAE-Cellulose column chromatography eluted with 0.01 M phosphate buffer, pH 7.5 and 0-0.5 M NaCl gradient. A major peak of protein having the highest activity and purity 25 folds compared to the crude enzyme was observed. This protein was partially characterized. SDS-Polyacrilamide gel electrophoresis showed one main band with molecular weight of 47.000 Da. This white radish peroxidase (WRP) is a very efficient enzyme with demonstrated maximum activity at temperature 55^{o}C and pH 7.5 as well as a K_m 76.6 µg/mL and V_{max} 275 µg/mL/ minute toward hydrogen peroxide as substrate and pyrogallol as hydrogen donor.

Key words: Peroxidase, white radish, cell suspension culture, purification, characterization

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DOI: 10.32945/atr3211.2010

INTRODUCTION

Peroxidase is an oxidoreductase that catalyzes oxidation of several organic substrates of aromatic compounds such as fenol, hydroquinone, amine hydroquinone and benzidine derivatives by reducing hydrogen peroxide. Horseradish peroxidase has been widely used as a component of Enzyme-Linked Immunosorbent Assay (ELISA) reagent for the detection of several hepatitis diseases B, C and G as well as HIV diagnose .

Peroxidase has also been suggested to be used for several purposes such as treatment of waste water containing fenolic compounds , synthesis of aromatic compounds , elimination of peroxides from foods and industrial waste, biotransformation of isosafrole into piperonal as well as chalcone into biflavanones compound.

Commercial production of peroxidase was carried out by extracting the root of horseradish plant (*Armorachia lapathifolia*) that only grows in countries having relatively cool climate as agriculture gulma. To determine other sources of peroxidase having similar characteristic to HRP, screening on peroxidase activity of several local vegetables under the same family with horseradish (*Brassicaceae* family) was carried out. White radish was found to have the highest enzyme activity among the tested Brassicaceae family members.

Studies on the production of HRP by cell culture technique had been conducted by some researchers. Activity of the enzyme produced was much higher than the activity of enzyme isolated from horseradish plant. Therefore, it had also been reported the possibility of producing HRP by cell culture technique. However, only little amount of peroxidase production by cell suspension culture of white radish was reported.

In our previous study, we have shown the effect of naphtalene acetic acid (NAA) and kinetin on the production of WRP through cell suspension culture technique. WRP was found as intracelluler enzyme (in the callus) and extracellular (secreted into the medium) with the enzyme activity higher than that of white radish tuber.In this study, WRP from cell suspension culture supernatant was purified and the enzyme was characterized.

MATERIALS AND METHODS

White radish seed

White radish seed of white, long and cylindrical shape variety was a product of China which was purchased from a local market in Bandung.

Sterilisation of seed

White radish seed was soaked in subsequently 70% (v/v) ethanol for three minutes and 20% (w/v) sodium hyphochlorite in 0.1% (v/v) tween-80 for 15 minutes. The seed was then washed five times with sterilized water for 15 minutes each to remove the excess of hyphochlorite.

Seed germination

Germination of seed was conducted on a medium of wet sterilized filter paper placed in a petridish incubated in the dark in a germination cabinet at 25oC for four days.

Callus induction

The cotyledon of white radish sprout was grown on Murashige-Skoog (MS) agar medium containing 5.73.10-7 M NAA and 4.64.10-6 M kinetin, and incubated in the dark in a germination cabinet at 25^{o}C for three weeks (10). The callus was then subcultured on the same medium incubated at the same condition.

Preparation of WRP by cell suspesion culture

The friable calluses were transferred into 25 mL MS liquid medium containing various concentrations of NAA and kinetin (10). Incubation was done in a shaking incubator at dark condition with agitation speed of 120 rpm for four weeks. Subculture was carried out every week by replacing one fourth portion of the old medium with three fourth portion of the same fresh medium. Peroxidase crude extract was obtained by filtering the culture to

separate the biomass using filter paper. The filtrate was used as enzyme crude extract.

Precipitation of WRP by ammonium sulfate fractionation

Enzyme crude extract was saturated with ammonium sulfate with gentle stirring and the mixture was left overnight at 4°C after the salt was completely dissolved. To separate the supernatant from the precipitate, the mixture was centrifugated at 12.500 rpm, 4°C for 20 minutes. The supernatant was then treated for further fractionation, while the precipitate was diluted with 0.01 M phosphate buffer pH 6.0 to determine the enzyme activity and protein concentration. The precipitate from ammonium sulphate fractionation with 30-80% saturation was diluted with 0.01 M phosphate buffer pH 6.0 and dialysed in cellofan tube with gentle stirring against 0.01 M phosphate buffer pH 6.0 at 4°C until all salts were removed from the solution.

Purification of WRP by anion-exchange column chromatography

The dialysed enzyme was then loaded on DEAE-Cellulose (1cm x 25cm) column equilibrated with 0.01 M phosphate buffer pH 7.5. The column was eluted with the same buffer and continued with a NaCl gradient from 0-0.5 M at a flow rate of 1 mL/minute, collecting fractions of 5.0 ml at 4°C. The active fraction was then used for characterization.

Analysis of enzyme activity

Peroxidase activity was determined by spectrophotometric method using pyrogallol as hydrogen donor (14). Pyrogallol was converted by peroxidase in the presence of H_2O_2 at 20°C and pH 6.0 to purpurogallin. The orange color of purpurogallin was measured at 300 nm. Concentration of purpurogallin was calculated from calibration curve of purpurogallin standard. One unit of enzyme activity was defined as mg purpurogallin formed from pyrogallol at 20°C, pH 6.0 in five minutes. Protein enzyme concentration was analyzed by spectrophotometric method using Lowry reagent, and the blue-purple color

resulted in the reaction was measured at 500 nm. Concentration of the protein enzyme was calculated from the calibration curve of Bovine Serum Albumin standard which was determined using the same method.

Characterization of enzyme

Optimum temperature and pH of enzyme activity

To determine the optimal temperature of enzyme activity, the active fraction was assayed for its peroxidase activity at various temperatures (30°, 35°, 40°, 45°, 55°, and 60°C) in 0.01 M phosphate buffer pH 7.5. For optimal pH, enzyme activity of the active fraction was determined at optimum temperature in 0.01 M phosphate buffer with various pH ranging from 5.0 to 8.0.

Measurement of molecular weight

Analytical SDS-PAGE was performed generally according to the method of Laemmli (16). The sample, 50 µl, which had been treated with 1% SDS + 1% 2-mercaptoethanol at 80-90°C was loaded into the gels (upper gel: 4 % polyacrylamide and lower gel: 15 % polyacrylamide). Protein standards, with low molecular weights in the range of 14,000 to 94,000 were used to calibrate the gels. They were run at 20 mA and 30 mA for the upper and the lower gels respectively. After electrophoresis, the proteins were stained with Comassie Blue.

Estimation of Michaelis-Menten Constant (K_m)

K_m value of the enzyme was determined by measuring the peroxidase activity of the active fraction at optimal temperature and pH using various concentrations of hydrogen peroxide as substrate (b/v) namely 0.004 % ; 0.006 %; 0.008 %; 0.01 %; 0.012 %; 0.014 % and 0.016 %.

Table 1. Precipitation of WRP by eight steps ammonium sulfate fractionation.

(NH4)2SO4 Fraction	Precipitation
0-10 %	No precipitate
10-20 %	No precipitate
20-30 %	Precipitate
30-40 %	Precipitate
40-50 %	Precipitate
50-60 %	Precipitate
60-70 %	Precipitate
70-80 %	Precipitate

RESULTS AND DISCUSSION

Precipitation of WRP by ammonium sulfate fractionation

WRP crude extract used for purification was obtained from the earlier experiment (10). Various concentrations of growth hormones such as NAA and kinetin were applied to MS medium for white radish cell suspension culture in order to find out the optimal composition for maximum enzyme activity. The culture of each composition was separated from the biomass using filter paper. The filtrate of all cultures were collected and used as crude enzyme. To determine the optimum concentration of ammonium sulfate giving the highest enzyme purity and yield, a portion of crude extract was fractionated using increasing levels of ammonium sulfate saturation with 10 % interval. The result is presented in Table 1.

Since there was no precipitation observed at 0-20% saturation, six steps of fractionation were then applied starting from 0-30% ammonium sulfate saturation. Low enzyme purity and yield was observed at the first and second step of fractionation (Table 2), Increasing level of saturation from 0-30 % to 30-80 % and 0-40% to 40-80 % were further carried out and the results are shown in Table 3. At 0-30 % followed by 30-80 % fractionation the highest enzyme purity and yield of 22.93 and 49.93 % were attained respectively. To obtain sufficient amount of enzyme for the purpose of purification and characterization, 100 ml of crude extract was fractionated by two steps of fractionation, 0-30% and 30-80 %, and dialyzed thereafter. As shown in Table

Table 2. Precipitation of WRP by six steps fractionation using ammonium sulfate

Fraction (NH4)2SO4	Volume (mL)	Unit Activity (Unit/mL)	Total Activity (Unit)	Protein (mg/mL)	Total Protein (mg)	Specific Activity (Unit/mg Protein)	Purity	Yield (%)
Crude extract	10	16.011	160.11	1.3	13	12.316	1	100
0-30 %	2.5	4.921	12.303	0.152	0.380	32.376	2.628	7.684
30-40 %	2.5	2.631	6.576	0.069	0.172	38.134	3.096	4.108
40-50 %	2.5	8.481	21.203	0.109	0.272	77.809	6.317	13.242
50-60 %	2.5	16.759	41.898	0.213	0.532	78.681	6.388	26.218
60-70 %	2.5	13.198	32.995	0.166	0.415	79.506	6.455	20.607
70-80 %	2.5	6.576	16.440	0.079	0.197	83.240	6.758	10.267

Table 3. Fractionation of WRP by two steps fractionation using ammonium sulfate

Fraction (NH4)2SO4	Volume (mL)	Unit Activity (Unit/mL)	Total Activity (Unit)	Protein (mg/mL)	Total Protein (mg)	Specific Activity (Unit/mg Protein)	Purity	Yield (%)
Crude extract	10	1.067	10.67	0.147	0.147	7.26	1	100
0-30 %	1	2.970	2.970	0.041	0.041	72.44	9.97	27.83
30-80 %	1	5.328	5.328	0.032	0.032	166.50	22.93	49.93
0-40 %	1	3.016	3.016	0.045	0.045	67.02	9.23	28.26
40-80 %	1	5.238	5.238	0.034	0.034	154.06	21.22	49.09

4, enzyme purity was increased by 17 folds compared to the crude extract. According to Wilkinson, et.al the good yield for enzyme purification is higher than 50 %. The low yield obtained in this study might be due to the low enzyme concentration in the crude extract enzyme, so that precipitation by ammonium salt became less effective. When collecting the enzyme crude extract, all of the medium broth of white radish cell suspension cultures having either high or low peroxidase activity were collected together in order to obtain an appropriate volume of enzyme for optimization of the fractionation process. After dialysis, the protein content of the crude extract decreased from 0.147 to 0.04 mg/mL.

Purification of WRP by anion-exchange column chromatography

The dialysed enzyme was then subjected to DEAE-Cellulose column chromatography equilibrated with 0.01 M phosphate buffer pH 7.5 and eluted with the same buffer followed by 0-0.5 M NaCl gradient in the same buffer. Sixty seven fractions containing five mL eluate were collected and tested for peroxidase activity as well as protein absorbance at 280 nm. At this wavelength, amino acids with aromatic ring (fenil alanine, serine, tyrosine and tryptophan) that are present in enzyme were absorbed at its maximum. The pattern of protein absorbance and enzyme activity of all fractions are summarized in Figure 1. It can be shown that one major protein peak with the highest peroxidase activity was observed at fraction number 9. Based on its ionic properties, DEAE-Cellulose which has cationic functional group, diethylaminoethyl, would act as anion exchanger bounds anionic materials from the samples. This result suggests that the peroxidase is a cationic enzyme that was bounded weakly by the cationic matrix DEAE-Cellulose and detached at the beginning of elution. Purification with column chromatography increased enzyme purity 25 times higher than that of the crude enzyme (Table 4). Crude enzyme with relatively high protein concentration should be used in order to obtain higher yield. Membrane filtration to concentrate the enzyme might also be applied.

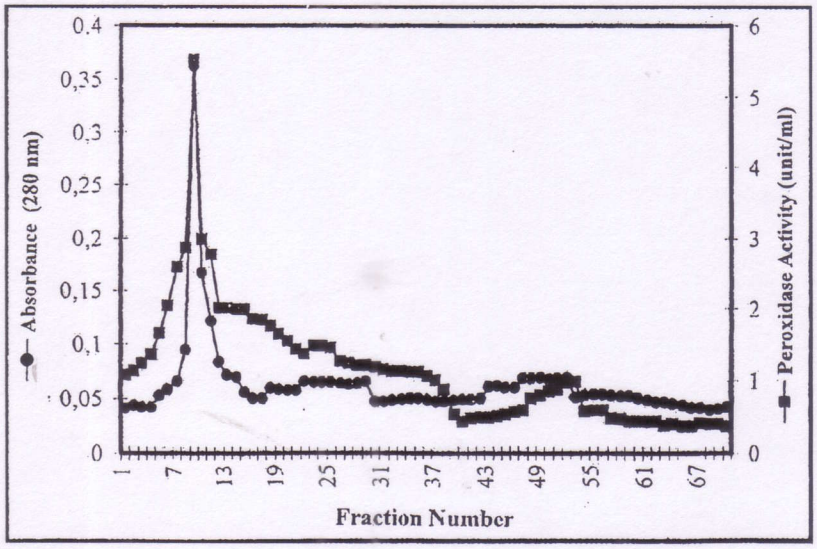

Figure 1. WRP elution profile on DEAE-Cellulose Anion Exchange Column Chromatography using 0.05M phosphate buffer pH 7.5, 0-1.5 M NaCl linear gradient in the same buffer. Fractions were assayed for protein absorbance (280 nm) and peroxidase activity.

Table 4. Purification of WRP by ammonium sulfate precipitation followed by Anion-Exchange Chromatography using DEAE-Cellulose.

Fraction	Volume (mL)	Unit Activity (Unit/mL)	Total Activity (Unit)	Protein (mg/mL)	Total Protein (mg)	Specific Activity (Unit/mg Protein)	Purity	Yield (%)
Medium broth of cell culture	100	1.06	106.7	0.14	14.7	7.26	1	100
(NH4)2SO4, precipitate, dialysis	5	5.42	27.09	0.04	0.21	129	17.81	25.46
DEAE-Cellulose, fraction	95	5.53	27.66	0.03	0.15	178.45	24.64	25.99

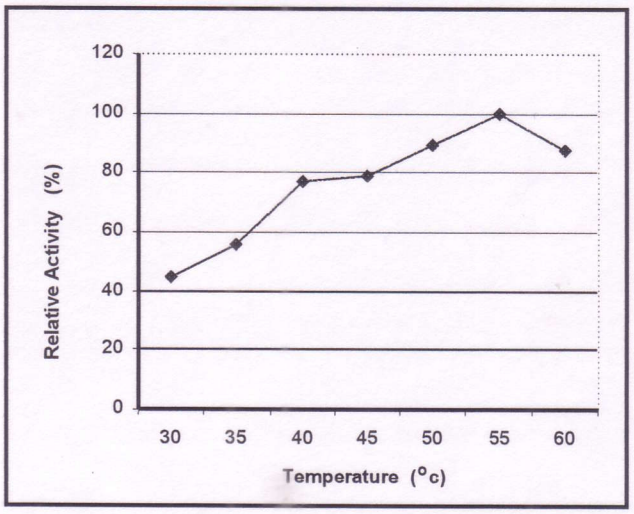

Figure 2. Relative activity of WRP peroxidase determined in phosphate buffer pH 7.5 at various temperatures toward H_2O_2 as substrate for 5 minutes

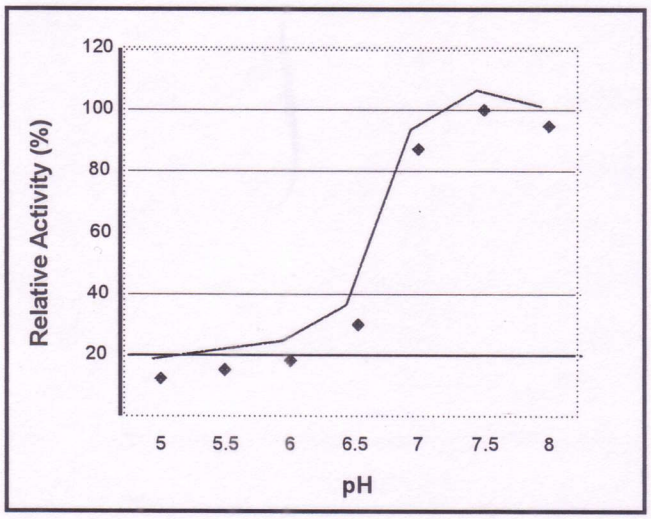

Figure 3. Relative activity of WRP determined at optimal temperature in phosphate buffer with various pH toward H_2O_2 as substrate for 5 minutes.

Properties of WRP

Optimum temperature and pH

The optimum temperature and pH for the activity of WRP is presented in Figures 2 and 3. Maximum enzyme activity was observed at temperature of 55°C and pH 7.5. These results suggest that WRP is a thermophylic and neutral enzyme.

Molecular weight of WRP

SDS-PAGE of fraction 9 showed one major band (Figure 4.), indicating that WRP has only one subunit. Molecular weight of the sub unit was calculated by comparing the mobility of fraction 9 against protein standards using regression linear equation derived from Figure 5. WRP has molecular weight of 47.100 Da which is almost similar to the molecular weight of HRP isoenzyme (HRPi) 1 standard which is 47.900 Da from SIGMA.

Michaelis-Menten Constant (K_m)

Michaelis-Menten Constant (K_m) was estimated from double-reciprocal plots of the reaction rates against hydrogen peroxide concentrations using Lineweaver-Burk equation resulting to $-1/K_m$ value of -13.05, giving K_m value of WRP of 76.6 µg/mL (Figure 6.). K_m value describes the binding affinity of enzyme toward a substrate, Low K_m values indicate higher enzyme reactvity to the substrate and favor more substrate-enzyme complex formation which in turn, produce more products. The low K_m value of WRP obtained in this study, indicates that WRP is a very reactive enzyme against hydrogen peroxide. It can also be derived from Figure 6, the V_{max} value was 1.3798 mg/mL/5 minutes.

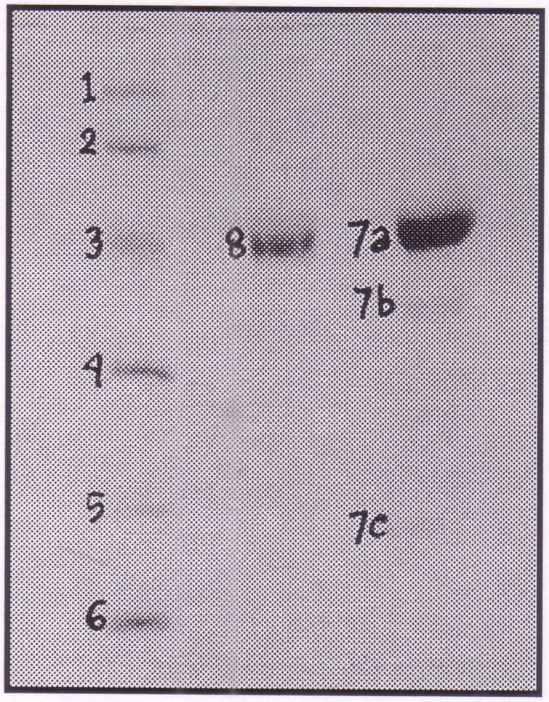

Figure 4. Subunit number of WRP by SDS-PAGE. Bands: (1) Fosforilase b, (2) Bovine Serum Albumin, (3) Ovalbumin, (4) Carbonate-Anhydrase, (5) Soybean Trypsine Inhibitor, (6) á-Lactalbumin, (7a) HRPi 1 (7b) HRPi 2, (7c) HRPi 3, and (8) Purified enzyme, fraction 9.

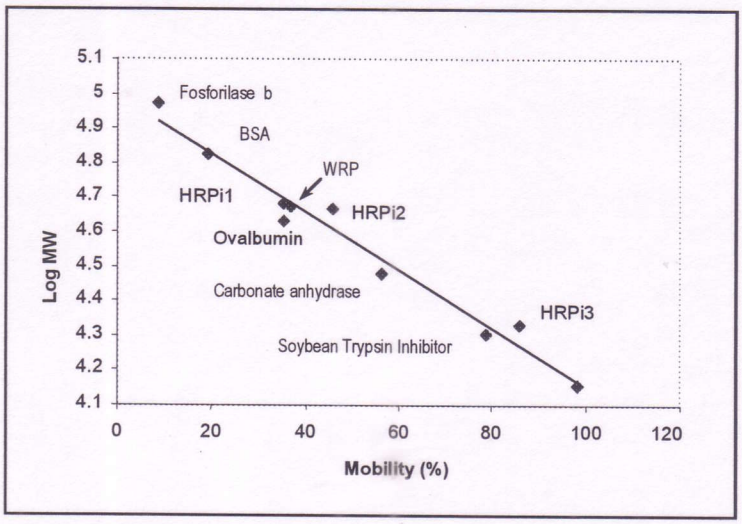

Figure 5. Molecular weight of WRP by SDS-PAGE. Log molecular weight is plotted as a function of mobility relative to the dye front. Protein standards: Fosforilase b, 94 kDa, Bovine Serum Albumin, 67 kDa, Ovalbumin, 43 kDa, Carbonate-Anhydrase, 30 kDa, Soybean Trypsine Inhibitor, 20.1 kDa, á Lactalbumin, 14.4 kDa, HRPi1, 47.9 kDa, HRPi 2, 46.3 kDa, HRPi 3, 21.4 kDa and WRP 47.1 kDa.

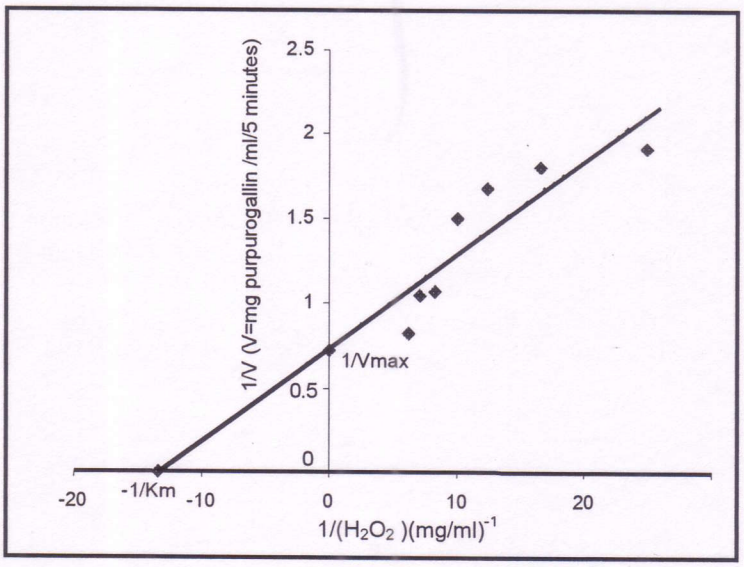

Figure 6. Lineweaver-Burk curve between reaction velocity of WRP against hydrogen peroxide concentration.

CONCLUSION

Peroxides was produced from white radish (*Raphanus sativus* L) through cell suspension culture technique. Ammonium sulfate precipitation using 0-30% saturation followed by 30-80% produced enzyme with high degree of purity and yield. This was followed by DEAE-Cellulose column chromatography eluted with 0.01 phosphate buffer, pH 7.5 and 0-0.5 M NaCl gradient which purified the enzyme 25 folds compared to the crude enzyme. The molecular weight of WRP was 47.000 Da, with maximum activity at temperature 55°C and pH 7.5. It has a K_m value of 76.6µg/mL and V_{max} of 275µg/mL/minute toward hydrogen peroxide and pyrogallol.

ACKNOWLEDGMENTS

This experiment was supported by the Government of Indonesia through DIPA Project of Indonesian Institute of Sciences (LIPI). The authors wish to thank Prof. A. Sidik for his very worthy ideas, Dr. Julie Diamante for the language corrections and R. Berliani, who had assisted this work.

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