Asian Journal of Chemistry Vol. 20, No. 1 (2008), 477-482

Purification of Peroxidase from Latex of Euphorbia (*Euphorbia amygdaloides*) and Investigation of Kinetic Properties

AZIZE ALAYLI GÜNGÖR, YASAR DEMIR† and NAZAN DEMIR*
College of Arts and Sciences, Atatürk University, 25240, Erzurum, Turkey
E-mail: nazdemir@atauni.edu.tr

The enzyme peroxidase was isolated from the latex of the Euphorbia (*Euphorbia amygdaloides*) and partially purified. The original extract (the supernatant) was purified by ammonium sulfate fraction, by CM-cellulose ion exchange chromatography and by Sepacryl S-100 gel filtration chromatography. The optimum pH value and the optimum temperature were determined pH: 6 and 40 ºC. The ν_{max} and Km values were found 0.079 µmol/L/min and 0.26 mM, respectively. The purification degree and molecular weight were controlled by using SDS-PAGE. The enzyme was dimmer and composed with 23 kDa monomers. Then, the molecular weight of the enzyme was determined as 46 kDa using the gel filtration chromatography. It was investigated whether or not the purified enzyme can be used in the synthesis reaction. The activity of enzyme was measured in the different solvent. The results showed that methanol can be used to be a solvent for enzymatic reaction.

Key Words: Euphorbia (*Euphorbia amygdaloides*), Ethanol, Methanol, Peroxidase, Tetrahydrofurane (THF).

INTRODUCTION

Peroxidases (EC 1.11.1.7; donor:hydrogen-peroxide oxidoreductase) catalyze the oxidation of various electron donor substrates (*e.g.*, phenols, aromatic amines) by hydrogen peroxide. They are extensively used in clinical biochemistry and immunoassay. Other applications include synthesis of various aromatic compounds and removal of peroxide from foodstuff and industrial wastes.

Currently, peroxidases are used also in organic synthesis for the production of polymers and for the biotransformation of various drugs and chemicals. Peroxidases are used commercially as catalysts for phenolic resin synthesis, as indicators for reactive oxygen species formed during

†College of Education, Atatürk University, 25240, Erzurum, Turkey.

food processing and as components of kits for research, medical diagnosis and bioremediation. More than 90 % of immunoassay kits utilize peroxidase and antibody conjugates[1].

In addition, the peroxidase is one of the key enzymes controlling plant differentiation and development. It is known that this enzyme participates in the construction, rigidification and eventual lignifications of cell walls, in the biosynthesis of H_2O_2, in the protection of plant tissues from damage and infection by pathogenic microorganisms and in wound healing.

Some of plant source for peroxidases have been investigated in American palm oil (*Elaies guineensis*), *Cucumis sativus*, *Raphanus sativus*, horseradish rots, ipomeas, sweet potato tubers, fresh flowers of *Cyzara scolymus* L., Turnip, Grape, Orange fruit flavedo and *Oryza sativa* L.[2-5].

In this study, an alternative enzyme, for using in the synthesis reaction was researched. For this reason, the peroxidase was first purified and characterized from Euphorbia (*Euphorbia amygdaloides*)'s latex and investigated its kinetic properties.

EXPERIMENTAL

Sephacryl S-100, Coomassie Brillant Blue G-250, CM-Cellulose, ammonium sulfate, 2,2'-azino-*bis*(3-etilbenztiazolin-6-sulfonic acid)(ABTS), Na_2HPO_4, Triton X-100, KCl, methanol, ethanol, tetrahydrofuran (THF) was obtained from Sigma Corp.

Purification steps

Homogenization: Euphorbia (*Euphorbia amygdaloides*) was used as raw materials for peroxidase. They were collected from the flora of Erzurum in August. The latex was drawn from cut branches of (*Euphorbia Amygdaloides*) and collected in glass containers. Its gummy was removed centrifugation and supernatant was used. Latex homogenization, centrifugation and dialysis were carried out between 4 and 8ºC.

Ammonium sulfate precipitation: This supernatant was brought to 30-60 % saturation with solid ammonium sulfate under continuous stirring. After 1 h the solution was centrifuged at 35000 rpm for 0.5 h and the pellet discarded. Additional ammonium sulfate was added to the clear supernatant to give 60-75 % saturation and stirred for 1 h. Significant activity was not observed at a range 30-60 %. After doing activity assay, it was determined that the enzyme was in the pellet (60-75 %). After centrifugation, the precipitate was suspended in 10 mL, 10 mM phosphate buffer (pH:6).

Ion exchange chromatography: The enzymes present in the extracts were purified with ion exchange chromatography on a 3 × 50 cm column that contained CM-cellulose. The supernatant was loaded on a CM-cellulose column, which had been pre-equilibrated with 10 mM phosphate buffer (pH:6). The column was washed with 100 mL of the same buffer. After this

washing, the peroxidase was eluted with a 300 mL gradient from 10 mM phosphate buffer (pH:6), to 200 mM phosphate buffer (pH:6). Fractions corresponding to the peroxidase were pooled.

Sephacryl S-100 gel filtration chromatography: Concentrated enzyme (5 mL samples) was loaded onto a 3 × 70 cm gel filtration column (Sephacryl S-100), equilibrated with 10 mM phosphate buffer (pH:6) and eluted at 0.5 mL/min in the same buffer.

Determination of molecular weight

Gel filtration: Molecular weight of purified peroxidase enzymes of latex of Euphorbia (euphorbia amg.) was determined by using Sephadex-G 100. Mixture of standard proteins, which had a concentration of 0.2 mg/mL, was applied on this column. Then, purified peroxidase enzyme was added on to equilibrated on columns and they were eluted with 0.05 M sodium phosphate/l mM ditihioeritritol, pH 7.0 buffer. Purified peroxidase and calibration proteins [Lipase (50 kDa), Celulase (31kDa)] were applied on the column and eluted at a flow-rate 0.5 mL/min[6].

SDS-PAGE electrophoresis: SDS polyacrylamide gel electrophoresis was performed after the purification of the enzyme. It was carried out in 3 and 10 % acrylamide concentrations for the running and the stacking gel, respectively, containing 0.1 % SDS according to Laemmli. 20 µg of the sample was applied to the electrophoresis medium. Gels were stained for 1.5 h in 0.1 % Coomassie Brilliant Blue R-250 in 50 % methanol, 10 % acetic acid and 40 % distilled water. It was destained by washing with 50 % methanol, 10% acetic acid and 40% distilled water several times[7]. The electrophoretic pattern was photographed (Fig. 2).

Protein determination: In subsequent steps, the specific activity and purification rate were determined. The protein concentration was determined according to Bradford methods which a uses bovine serum albumin as a standard[8]. The absorbance at 280 nm was used to monitor protein in the column eluents.

Peroxidase activity assay: Peroxidase activity assay was carried out following the procedure of Shindler, with some modifications. This method is base on oxidation of 2,2'-azino-*bis*(3-ethylbenzthiazoline-sulfonic acid) diammonium salt (ABTS) as a chromogenic substrate, by means of H_2O_2 and resultant colored compounds were measured at 412 nm. For this purpose, 2.8 mL of 1 mM ABTS in 0.1 M phosphate buffer pH: 6.8 was mixed with 0.1 mL of enzyme in 1 mM phosphate buffer, pH: 6.8 and 0.1mL of 3.2 mM H_2O_2 solution. The absorbance was measured at 412 nm as a function of time for 1-2 min[9].

$$\underset{\text{(Reductive form)}}{H_2O_2 + ABTS} \xrightarrow{\text{peroxidase}} \underset{\text{(Oxidative form)}}{H_2O + ABTS}$$

During the calculation of the purification rate of PO, one unit of activity was defined as amount of enzyme catalyzing the oxidation of 1 mmol of ABTS min^{-1} at 293 K (molar absorption coefficient 32400 $M^{-1} \times cm^{-1}$).

Effect of organic solvents on the enzyme activity: The effect of added organic solvents (methanol, ethanol and THF) on peroxidase activity was investigated using ABTS as substrate. When peroxidase enzyme was incubated the presence of methanol, ethanol or THF (100, 400, 600, 800, 1100 mL) chancing of the activity was observed.

RESULTS AND DISCUSSION

Although peroxidases are ubiquitous in the plant kingdom, at the present the major source of commercially available peroxidase is roots of horseradish (*Armoracia rusticana*). However, the availability of peroxidases with elevated stability and different specificity allows the improvement of already existing analytical procedures and/or the development of new ones. Therefore, numerous investigations on purification of alternative plant peroxidases have been carried out.

For this reason, peroxidase enzyme was purified from euphorbia. At the result of precipitation of ammonium sulfate, ion exchange chromatography and gel filtration chromatography enzyme was purified 328.8 times and result of purification was shown in Table-1.

TABLE-1
THE PURIFICATION OF PEROXIDASE FROM EUPHORBIA (*Euphorbia amygdaloides*)

Enzyme fraction	Volume (mL)	Activity (EU/mL)	Total activity EU (%)	Protein (mg/mL)	Specific activity (EU/mg)	Purification fold
Crude extract	20	0.043	0.874 (100)	1.610	0.027	_
$(NH_4)_2SO_4$ 75 %	10	0.045	0.45 (51.60)	0.260	0.170	6.4
After CM-cellulose column	15	0.050	0.75 (85.80)	0.100	0.500	18.5
After sephacryl S-100 column	5	0.080	0.40 (45.76)	0.009	8.880	328.8

The activity of Euphorbia's peroxidase was produced at high level of purification by making SDS-PAGE as shown in electrophoresis photo. Molecular weight was found 46 kDa by using gel filtration chromatography. The molecular weight of the peroxidase purified from euphorbia amg. is almost the same *Euphorbia characias* (48 kDa)[10].

Optimum temperature's measurement of enzyme was determined at between 5 and 80 ºC by increasing 5 ºC at each step and optimum temperature was found 40 ºC (Fig. 1).

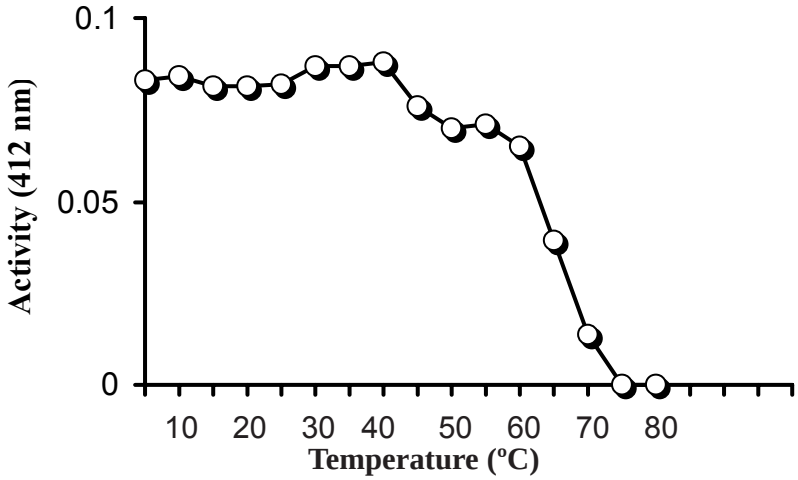

Fig. 1. Effect of temperature on the purified peroxidase

In addition, activity of enzyme was examined for finding optimum pH between 4 and 8. Optimum pH for peroxidase was found as pH: 6 (Fig. 2). This result is nearly one founded for similar spaces. PH value of euphorbia characias latex is 5.57.

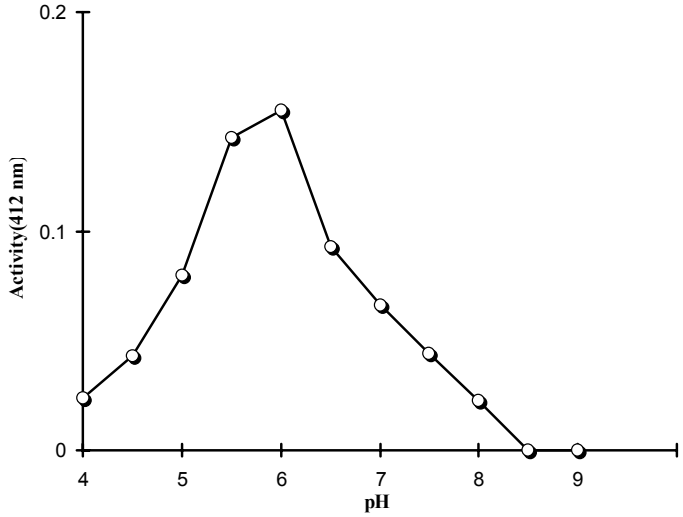

Fig. 2. Effect of pH on the purified peroxidase

ν_{max} and Km values were calculated by drawing Lineweaver-Burk graphics. ν_{max} and Km values of peroxidase enzymes are 0.079 mmol/L/min and 0.26 mM, respectively. The effect of methanol, ethanol and THF solvents

on enzyme activity was determined. Using methanol as a solvent is more proper than ethanol and THF. Ethanol and THF is not seen relevant solvent for biosynthesis reaction (Fig. 3).

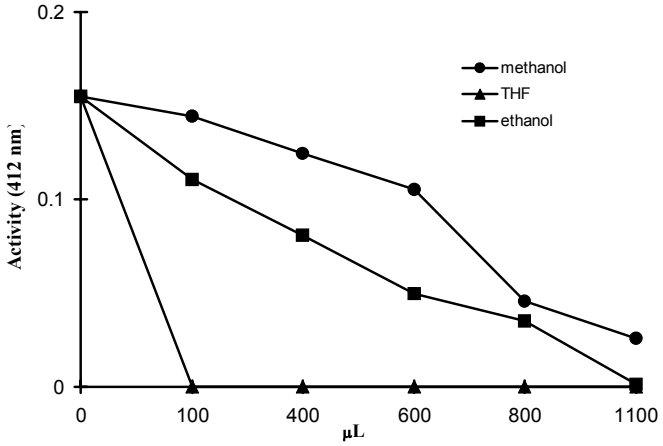

Fig. 3. Effects of methanol, ethanol and THF on the peroxidase

At the results of the study, peroxidase enzyme was characterized and purified from Euphorbia (*Euphorbia amygdaloides*) plant, grown in Anatolia and whether this enzyme can be used in clinic, cosmetic and biosynthesis purposes are examined.

REFERENCES

1. L.D. Molina, H.A. Heering, G. Smulevich, J. Tudela, R.N.F. Thorneley, F. García-Cánovas and J.N. Rodríguez-López, *J. Inorg. Biochem.*, **94**, 243 (2003).
2. N, Aruna and A. Lali, *Process Biochem.*, **37**, 431 (2001).
3. G. Battistuzzi, M. D'Onofrio, L. Loschi and M. Sola, *Anal. Biochem.*, **293**, 96 (2001).
4. A. Henriksen, O. Mirza, C. Indiani, K. Teilum, G. Smulevich, K.G. Welinder and M. Gajhede, *Protein Sci.*, **10**, 108 (2001).
5. N.D. Srinivas, R.S. Barhate and M.S. Raghavarao, *J. Food Eng.*, **54**, 1 (2002).
6. J.R. Whitaker, *Anal. Chem.*, **35**, 1950 (1963).
7. U.K. Laemmli, *Nature*, **227**, 680 (1970).
8. M.M. Bradford, *Anal. Biochem.*, **72**, 248 (1976).
9. J. Keesey, Biochemica Information, Boehringer Mannheim Biochemicals, Indianapolis, IN, edn. 1, p. 58 (1987).
10. G. Floris, R. Medda and A. Rinaldi, *Photochemistry*, **23**, 1527 (1984).

(*Received*: 19 November 2006; *Accepted*: 14 September 2007) AJC-5848