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A cationic peroxidase from leaves of *Vitis pseudoreticulata*

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Abstract

A cationic peroxidase in leaves of *Vitis pseudoreticulata* was purified using ion exchange chromatography and on a zeolite column. Characteristics of the purified cationic peroxidase were partially analyzed. The results showed that the cationic peroxidase had a maximum absorbance at *ca.* 450 nm, a M_r *ca.* 39000, and a *p*I more than 8.0. The optimum catalytic pH value was *ca.* 5.76, when guaiacol was used as substrate. The apparent V_{max} and K_m was 637 μmol/mg protein/min and 1.02 mmol/l H_2O_2 respectively. The peroxidase was stimulated by high concentrations of inorganic salts.

Keywords: Vitis pseudoreticulata; Vitaceae; Grape; Cationic peroxidase; Inorganic salt

1. Introduction

Peroxidases (EC 1.11.1.7) are haemoproteins that catalyze the oxidation of a wide variety of substrates, using hydrogen peroxide. Their *in vitro* catalytic properties have been extensively studied, and are thought to be involved in lignification, auxin metabolism, and an increasing number of other functions (Asada 1987; Greppin, Penel and Gaspar 1986; Everse, Everse and Grisham 1991). Many environmental stresses result in the enhanced production of active oxygen species (AOS) in plants, including O_2^- radicals and H_2O_2. (Bowler, Van Montagu & Inze, 1992; Foyer, Lelandadais & Kunert, 1994; Inze & Van Montagu 1995).

Under salinity stress, accumulation of AOS are due to the reduction of activities of antioxidant enzymes (e.g. superoxide dismutase, catalase, and peroxidase) and enhancement of AOS production (Liao & Zhu 1996). Superoxide dismutase is associated with salt tolerance, (Hernandez, Corpus, Gomez, Del Rio & Sevilla 1993; Hernandez, Del Rio & Sevilla; Hernandez, Olmos, Corpas Sevilla & Del Rio, 1995), but less is known about peroxidase involvement (Olmos, Hernandez, Sevilla & Hellin 1994). In an investigation of salt tolerance in Chinese wild *Vitis*, we found a cationic peroxidase which is concerned in the response of *Vitis* leaf disks to salinity (Liao, 1996). In this paper, a cationic peroxidase was purified and partially characterized from leaves of *Vitis pseudoreticulata* W.T. Wang.

2. Results and discussion

2.1. Purification of the cationic peroxidase

The three purification steps are shown in Table 1. After the DEAE-cellulose column chromatography, the cationic activity represented *ca.* 65% of the total activity present in the crude extract. After adjustment of the pH of the cationic fraction to 8.0, about 87% of the cationic activity was bound to the synthesized zeolite column. The bound cationic fraction contained only one peroxidase, as shown in gels stained for peroxidase activity or with Coomassie Brilliant Blue

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Table 1
Activity of peroxidase during purification

	Total activity (U)	Specific activity (U/mg protein)
Crude extract	11200	44.4
Cationic fraction	7370	206
Cationic peroxidase	6420	928

(Fig. 1). Thus, the leaves of *V. pseudoreticulata* can be considered as a potential source of cationic peroxidase.

2.2. Molecular and spectroscopic properties

SDS–PAGE was performed with the bound cationic fraction after being eluted with 6.4% (w/v) NaCl, dialyzed against 20 mM Tris–acetate, and concentrated 10-fold. The M_r of the single band was *ca.* 39000 (Fig. 1). The absorption maximum was *ca.* 450 nm (Fig. 2).

2.3. Catalytic properties

The optimum pH value of the cationic peroxidase was 5.76, the pH values for half maximal activity were 4.8 and 7.3. The reciprocal of the activity of the cationic peroxidase ($1/v$) was significantly related to the reciprocal of H_2O_2 concentration ($1/(s)$): $1/v = 0.00157 + 0.00160/(s)$ ($r = 0.983$); thus, the apparent V_{max} (637 μmol/mg protein/min.) and K_m (1.02 mmol/l H_2O_2) could be obtained (data not shown).

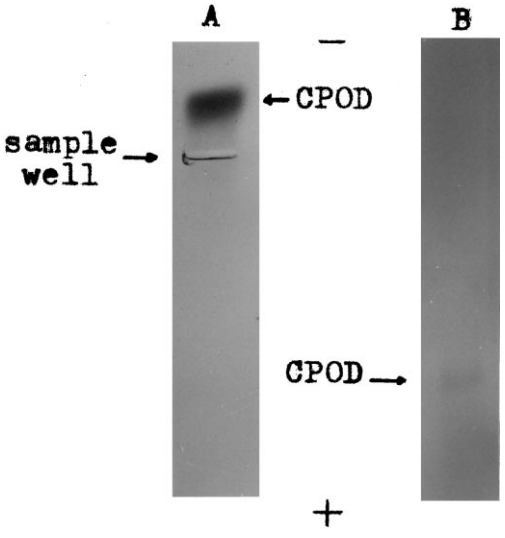

Fig. 1. Identification of cationic peroxidase (CPOD) by native- (A) and SDS–PAGE (B, the protein of standard M_r was not shown).

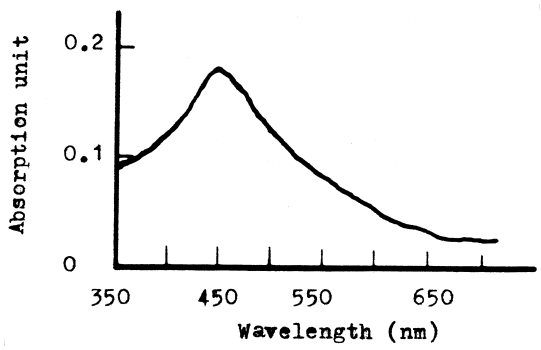

Fig. 2. Photospectrum of the purified cationic peroxidase.

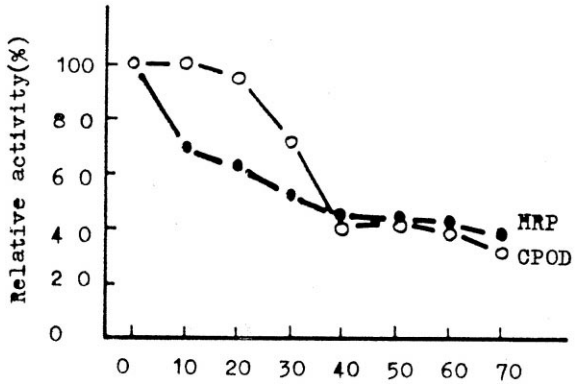

Fig. 3. Stability of cationic peroxidase and horse radish peroxidase at 60°.

Comparing the thermostability of the cationic peroxidase with that of horse radish peroxidase (HRP), we can conclude that the purified cationic peroxidase is more stable than HRP at high temperature (Fig. 3).

Table 2
Effect of inorganic salts on the activity of the purified cationic peroxidase from leaves of *Vitis pseudoreticulata*

Salt	Relative activity*
300 mM	
NaCl	133 ± 7
Na_2SO_4	124 ± 10
KNO_3	148 ± 19
KH_2PO_4	105 ± 6
KCl	129 ± 28
$CaCl_2$	119 ± 16
$Ca(NO_3)_2$	143 ± 12
$MgSO_4$	122 ± 10
150 mM	
$ZnSO_4$	152 ± 19
$MnSO_4$	143 ± 12
$(NH_4)_2SO_4$	128 ± 4

* Percentage of the activity of purified cationic peroxidase in medium without inorganic salts ($n = 8$).

The cationic peroxidase was also stimulated obviously by 150 mmol/1 $ZnSO_4$, $MnSO_4$, or $(NH_4)_2SO_4$ and 300 mmol/1 NaCI, Na_2SO_4, KNO_3, KCl, $CaCl_2$, $Ca(NO_3)_2$, or $MgSO_4$, but the effect of low concentrations of these inorganic salts or high concentration of KH_2PO_4 was not obvious (Table 2). The effect of inorganic ions for stimulating the cationic peroxidase followed the order: (1) NO_3^-, SO_4^{2-}, Cl^-, (2) Zn^{2+}, Mn^{2+}, NH_4^+, but the differences among K^+, Na^+, Ca^{2+}, Mg^{2+}, was not significant. Thus, the cationic peroxidase may be very important in maintaining a high level peroxidase, when expression and activity of other peroxidase were inhibited due to the accumulation of inorganic ions under salt stress. However, the expression of the cationic peroxidase under stress conditions remains unclear.

3. Experimental

3.1. Enzyme purification

Leaves of *Vitis pseudoreticulata* were obtained from wild grape germplasm plot of the North–Western Agricultural University. Leaf fragments (200 g) were homogenized in 300 ml 20 mM Tris–acetate buffer (pH 7.4) with a homogenizer for 2×30 s at 18000 rpm. The disrupted tissues were removed by filtration and centrifugation (12000 *g*, for 20 min). The supernatant was treated with insoluble PVP to remove the pigment followed by filtration. It was then loaded to a DEAE–cellulose column equilibrated with 20 mM Tris–acetate (pH 7.4), and enzyme was eluted with the same buffer. The elutant was adjusted to pH 8 and applied to a synthesized zeolite column (1.5 cm×15 cm, equilibrated with buffer B) (Liao 1996), washed with buffer B (20 mM) Tris–acetate, pH 8, then eluted with buffer B containing 6.5% (w/v) NaCl. The active fractions were pooled and dialyzed against 20 mmol/1 Tris–acetate, pH 7.4, then conc 10-fold and stored at 4°.

3.2. Enzyme assays

Activity was expressed in U (μmol/min) using 4 mM guaiacol and 10 mM H_2O_2 at 32.5° in 0.2 M Tris–acetate buffer at the estimated optimum pH. An absorption coefficiency of 26.6 $mM^{-1}\,cm^{-1}$ at 470 nm was used (Amako, Chen & Adada, 1994).

Thermal stability was investigated by incubating enzyme soln at 60° and compared with HRP. Spectral measurement was performed using a Shimadzu CS-930 spectrophotometer. Activity measurement and protein content were measured using a Shimadzu UV-120 spectrophotometer. Protein content was determined according to the method of (Bradford, 1976).

3.3. Electrophoresis

SDS–PAGE was performed by the method of (Laemmli, 1976) followed by Coomassie Brilliant staining. Native PAGE was performed on a LKB horizontal electrophoresis instrument (Amako, Chen & Adada, 1994). The gel was developed for peroxidase activity by the following procedures: (1) washing the gel with H_2O 2 times each for 20 s, (2) soaking the gel in 0.2 mg/ml benzidine for 10 min, (3) incubating in 8.8 mM H_2O_2, 1 M NaCl, 4 mM $CoCl_2$, 50 mM Tris–acetate, pH 6.

References

Asada, K. (1987). *Physiologia Plantarum, 85,* 235.

Greppin, H., Penel, C., and Gaspar, T., *Molecular and Physiological Aspects of Plant Peroxidase*. University of Geneva, Geneva, 1986.

Everse, J., Everse, K. E., and Grisham, M. B., *Peroxidases in Chemistry and Biology*, Vol. 2. FL, CRC Press, Boca Raton, 1991.

Bowler, C., Van Montagu, M., & Inze, D. (1992). *Annual Review of Plant Physiology and Plant Molecular Biology, 43,* 83.

Foyer, C. H., Lelandadais, M., & Kunert, K. J. (1994). *Physiologia Plantarum, 92,* 696.

Inze, D., & Van Montagu, M. (1995). *Current Opinion of Biotechnology, 6,* 153.

Liao, X. R., & Zhu, X. C. (1996). *Life Chemistry (Chinese), 16*(6), 19.

Hernandez, J. A., Corpas, F. J., Gomez, M., Del Rio, L. A., & Sevilla, F. (1993). *Physiologia Plantarum, 89,* 103.

Hernandez, J. A., Del Rio, L. A., & Sevilla, F. (1994). *The New Phytologist, 126,* 37.

Hernandez, J. A., Olmos, E., Corpas, F. J., Sevilla, F., & Del Rio, L. A. (1995). *Plant Science (Limerick), 105,* 151.

Olmos, E., Hernandez, J. A., Sevilla, F., & Hellin, E. (1994). *The Journal of Plant Physiology, 144,* 594.

Liao, X. R., *Doctorate Thesis*, North–Western Agricultural University, Yangling, Shaanxi, 1996.

Amako, K., Chen, G. X., & Adada, K. (1994). *Plant and Cell Physiology, 35,* 497.

Bradford, M. M. (1976). *Analytical Biochemistry, 72,* 248.

Laemmli, U. K. (1970). *Nature, 227,* 680.