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ENZYMES FROM *CATHARANTHUS ROSEUS* CELL SUSPENSION CULTURES THAT COUPLE VINDOLINE AND CATHARANTHINE TO FORM 3′,4′-ANHYDROVINBLASTINE

TSUYOSHI ENDO*, ANNE GOODBODY, JOHN VUKOVIC and MASANARU MISAWA

Allelix Inc., 6850 Goreway Drive, Mississauga, Ontario, L4V 1P1, Canada

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Key Word Index–*Catharanthus roseus*; Apocynaceae; tissue culture; enzyme purification; biotransformation; peroxidase; indole alkaloids; anticancer agent; vinblastine; 3′,4′-anhydrovinblastine.

Abstract—The enzymes catalysing the coupling of vindoline and catharanthine to 3′,4′-anhydrovinblastine were partially purified from cell suspension cultures of *Catharanthus roseus*. At least five isoenzymes were involved in the coupling reaction, and the peroxidase nature of these enzymes was demonstrated.

INTRODUCTION

Vinblastine and vincristine, proven anticancer agents from *Catharanthus roseus*, are among the most expensive drugs in pharmaceutical markets because of their relatively low abundance in intact plants. These so called dimeric alkaloids are products of the coupling of vindoline and catharanthine, two of the major alkaloids found in leaves of *C. roseus*.

In a previous paper [1], we demonstrated that an enzyme system involved in the coupling of vindoline and catharanthine to form dimeric alkaloids was present in cell suspension cultures of *Catharanthus roseus*. A substantial yield of 3′,4′-anhydrovinblastine (AVLB), an immediate precursor of vinblastine, was obtained with the optimized conditions. This report describes the partial purification and further characterization of the coupling enzyme.

RESULTS AND DISCUSSION

A procedure of enzyme purification from cell suspension cultures of *Catharanthus roseus* is summarized in Table 1. From fraction L of the Sephacryl S-200 gel filtration (Fig. 1), 4 isoenzymes with different pI values were isolated with isoelectric focusing (Fig. 2). These enzymes showed apparently identical M_rs with HPLC gel filtration. From fraction S, one cationic enzyme was isolated. Molecular weights and pI values of these enzymes are listed in Table 2.

The presence of isoenzymes coupling vindoline and catharanthine to form AVLB, suggests the involvement of non-specific oxidative enzymes such as peroxidases in this system. This assumption led us to examine peroxidase activity as well as coupling activity in fractions from Sephacryl S-200 gel filtration and isoelectric focusing. As seen in Figs 1 and 2, coupling activity and peroxidase activity were closely associated. This indicates the peroxidase nature of the enriched enzymes catalysing coupling. We therefore examined the effect of hydrogen peroxide on the coupling reaction by using these isolated enzymes, and the results obtained with isoenzyme L-I showed that hydrogen peroxide could replace FMN, and gave a 22% increase in yield. Manganous ion improved the yield by a further 6%.

The specific activities of enriched enzymes for the coupling systems are compared with the specific peroxidase activity toward *o*-phenylenediamine (*o*PD) and hydrogen peroxide (typical substrates for measurement of peroxidase activity) in Table 3. The specific activity for hydrogen peroxide-dependent coupling is proportional to the specific peroxidase activity (towards *o*PD) among

Table 1. Purification procedure

	Total protein (mg)	Specific activity (μmol AVLB/hr/mg)	Relative purity
Crude	2550		
70% $(NH_4)_2SO_4$	2340	0.098	1.00
DEAE Fractogel	810	0.260	2.65
(Fraction L)			
S 200	191	0.512	5.22
Isoelectric focusing			
L-I	0.679	21.0	214
L-II	0.758	15.1	154
L-III	0.833	16.9	172
L-IV	1.04	18.8	192
(Fraction S)			
S 200	19.5	1.23	12.5
Isoelectric focusing	0.36	13.1	134

See Experimental for details of enzyme purification procedure and coupling assay.

*Present Address: Research Center for Cell and Tissue Culture, Faculty of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606, Japan.

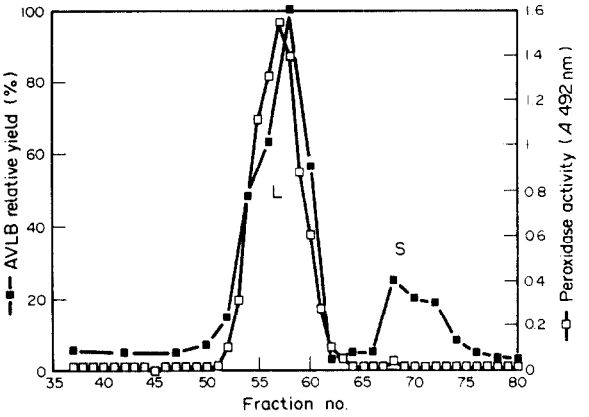

Fig. 1. Sephacryl S-200 gel filtration.

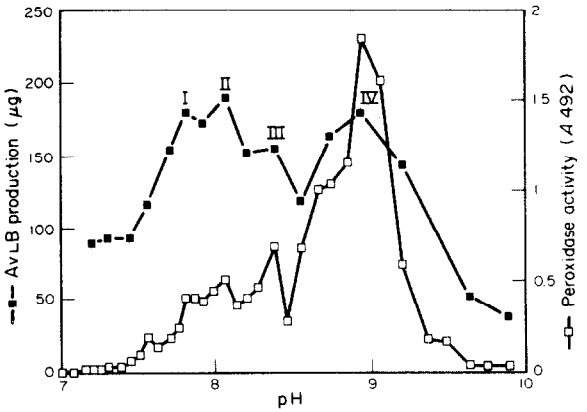

Fig. 2. Isoelectric focusing of fraction L from Sephacryl S-200 gel filtration.

Table 2. Purification of coupling enzymes from C. roseus cells

Enzyme	M_r	pI
L-I	37 000	7.8
L-II	37 000	8.0
L-III	37 000	8.3
L-IV	37 000	9.0
S	15 000	10.5

Molecular weights were estimated from calibration with known standards on HPLC gel filtration. pI values were obtained from isoelectric focusing.

the four isoenzymes, while the specific activity for FMN-dependent coupling does not show a correlation with either. For example, isoenzyme L-I had the highest specific activity for FMN-dependent coupling among the four enzymes, but had the lowest specific activities for both hydrogen peroxide-dependent coupling and oxidation of *o*PD.

Table 3. Specific activities

Enzyme	(A) Coupling FMN (μmol/hr/mg)	(B) Coupling H_2O_2 (μmol/hr/mg)	(C) Peroxidase *o*PD (OD_{492} 10 min/mg)	(A)/(C)	(B)/(C)
L-I	21.0	8.4	0.88	23.8	9.5
L-II	15.1	13.5	1.01	15.0	13.4
L-III	16.9	14.7	1.24	13.6	11.9
L-IV	18.8	28.3	2.66	7.1	10.6

Reaction conditions are described in the Experimental.

Table 4. Maximum yields of AVLB obtained with different enzymes

Enzyme	AVLB yield (μg)
L-I	438
L-II	436
L-III	447
L-IV	495
S	431
HRP	365

Incubation: 75 min at 30°. Reaction mixture contained 0.15 mM H_2O_2, 1 mM $MnCl_2$, 0.5 mg catharanthine HCl, 0.5 mg vindoline, and 0.1 mg enzyme

The role of FMN in this system is not clear. In ethylene formation from methional, Ku *et al.* [2,3] demonstrated that light mediated action of FMN could replace the enzymatic reaction with peroxidase and hydrogen peroxide. We also found that coupling in the presence of FMN is reduced in the dark (results not shown).

The maximum yields of AVLB obtained with different isoenzymes are shown in Table 4. In spite of the different specific activities, each isoenzyme gave similar conversion yields ranging from 43 to 50%. This indicates that the factor limiting AVLB yield in the system is not the enzyme activity. Instability of substrates (especially catharanthine) and product in the reaction mixture may be the limiting factors. Also, commercially available horseradish peroxidase (Sigma) was found to be capable of yielding a significant amount of AVLB, and this system is described in more detail elsewhere [4].

Identification of the AVLB produced in these studies has been supported by data from TLC, HPLC and MS. It is known that the biologically active dimers must have the α-configuration at the C-18′ position between the aspidosperma and iboga moieties [5]. CD analysis of the product derived from horseradish peroxidase-mediated coupling confirmed that the correct stereochemistry exists [4]. ^{1}H NMR analysis of this product has recently been performed and the data have further confirmed its identity as α-coupled AVLB. The coupling product obtained with enzymes extracted from *C. roseus* suspension cultures has also been analysed with ^{1}H NMR, and was found to have the same spectrum as an authentic stan-

dard (Drs F. DiCosmo, J. Smith and E. Amouzou, personal communication).

In the chemical synthesis of AVLB, catharanthine *N*-oxide was employed to couple with vindoline [6]. Although in the biological pathway, any activated form of catharanthine has not yet been isolated, it is plausible to assume that oxidative activation of catharanthine would take place before condensative coupling with vindoline occurs. The peroxidases are possibly involved in the activation of catharanthine and the actual coupling of activated catharanthine and vindoline could be a non-enzymatic reaction. However, it is difficult to clarify the mechanism of the reaction because accumulation of the activated catharanthine has not been investigated in this system.

Throughout this study, it has been demonstrated that at least five enzymes possessing peroxidase activity were involved in the coupling of vindoline and catharanthine in cell-free extracts of suspension cultures from *C. roseus*. Some of these peroxidases, if not all, may be responsible for the biosynthesis of dimeric alkaloids in intact plants of *C. roseus*. Peroxidases are known to be involved in phytohormone metabolism (degradation of IAA [7] and ethylene synthesis [2]) and in secondary metabolism such as lignin synthesis [8]. Stuart *et al.* [9] have shown that horseradish peroxidase was capable of oxidizing AVLB to leurosine, one of the major dimeric alkaloids in *C. roseus* plants. We have also observed that significant amounts of leurosine were formed as a by-product in our coupling systems with purified enzymes (both FMN-dependent and hydrogen peroxide-dependent systems), which suggests the involvement of endogenous peroxidases in this oxidation step *in vivo*. To our knowledge, the biosynthesis of dimeric indole alkaloids is the sole system among all types of alkaloid biosyntheses so far reported, in which participation of peroxidases has been demonstrated.

EXPERIMENTAL

Cell suspension cultures. A cell line of *C. roseus*, JWM* [1], was used as the enzyme source. Two-week old suspension cultures were harvested and stored at −20°.

Enzyme purification. Methods of extraction of enzyme from the frozen cells and $(NH_4)_2SO_4$ pptn were described in a previous report [1]. A typical purification procedure follows.

1. *DEAE Fractogel chromatography.* Protein pellet (2.4 g) was dissolved in 20 ml Tris–HCl buffer (20 mM, pH 8), then desalted with a Biogel P-6 column (Bio-Rad). The desalted soln (30 ml) was put on the top of a DEAE Fractogel column (2.5 × 2 cm, bed vol. 10 ml, TSK DEAE-650, EM Science) and eluted with the same buffer. Active enzyme was eluted in the first 45 ml fraction while more than 65% of the protein was adsorbed on the ion exchange gel (see Table 1).

2. *Sephacryl S-200 gel filtration.* The active fraction was applied to a Sephacryl S-200 column (2.2 × 200 cm, bed vol. 980 ml) equilibrated with the Tris–HCl buffer. The protein was eluted, at a flow rate of 20 ml/hr, and 10 ml fractions were collected. The activity appeared as two peaks (fractions L and S in Fig. 1). Each of these fractions (70 ml) was concentrated by ultrafiltration (M_r 5000 cutoff) to 20 ml and desalted with a Biogel P-6 column equilibrated with H_2O.

3. *Isoelectric focusing.* Concd fractions L and S were separately applied to a density gradient isoelectric focusing column (110 ml) containing 1% carrier ampholyte (pH 3–10:pH 6–8 = 1:4), and run at 800 V for 36 hr. The separation of fraction L by this method is shown in Fig. 2.

Assay of coupling activity. The reaction mixture contained enzyme, 0.5 mg catharanthine HCl, 0.5 mg vindoline, 1 mM FMN (or 0.15 mM H_2O_2), and 1 mM $MnCl_2$ in 6 ml Tris–HCl buffer (100 mM, pH 6.8). The incubation was performed at 30° for 1 hr. The reaction was stopped by adding 28% NH_4OH (to pH 9.5). Before the alkaloids were extracted with EtOAc by phase partitioning, an excess amount of $NaBH_4$ was added to the reaction mixture to recover AVLB [1]. AVLB was quantified with TLC (solvent system, Et_2O–$CHCl_3$–MeOH = 5:3:1) and scanned at 280 nm.

Assay of peroxidase activity. The enzyme was incubated with *o*-phenylenediamine (*o*PD, 0.4 mg/ml) and 3% H_2O_2 (10 μl/ml) in Tris–HCl buffer (100 mM, pH 6.8) at room temp. for 10 min in darkness. The reaction was stopped by adding 5 N H_2SO_4 (0.1 ml /ml), and then the *A* at 492 nm was measured.

Protein concentration was determined after ref. [9]. Horseradish peroxidase (Sigma) was used as a standard.

Estimation of M_r. The M_r of purified enzymes were estimated by HPLC on gel filtration columns of Protein Pak 125 and Protein Pak 60 (Waters) in series. Tris–HCl (100 mM, pH 7) was used as the eluting buffer with a flow rate of 1 ml/min. Protein was monitored at 280 nm and 417 nm.

REFERENCES

1. Misawa, M., Endo, T., Goodbody, A., Vukovic, J. Choi, L. S. L. and Kutney, J. P. (1988) *Phytochemistry* **27**, (in press).
2. Ku, H. S., Yang, S. F. and Pratt, H. K. (1967) *Arch. Biochem. Biophys.* 118, 756.
3. Ku, H. S., Yang, S. F. and Pratt, H. K. (1969). *Phytochemistry* **8**, 567.
4. Goodbody, A., Endo, T., Vukovic, J., Kutney, J. P. Choi, L. S. L. and Misawa, M. (1988) *Planta Med.* (in press).
5. Cordell, G. A. (1978) in Encyclopedia of Chemical Technology Vol. 1 (Kirk, R. E., Othmer, D. F. eds), pp. 883–943. Wiley, New York.
6. Kutney, J. P., Hibino, T., Jahngen, E., Okutani, T., Ratcliffe, A. H., Treasurywala, A. M. and Wunderly, S. (1976) *Helv. Chim. Acta.* **59**, 2858.
7. Kenten, R. H. (1955) *Biochem. J.* **59**, 110.
8. Freundenberg, K., Reznik, H., Bosenberg, H. and Rasenach, D. (1952) *Chem. Ber.* **85**, 641.
9. Stuart, K. L., Kutney, J. P., Honda, T. and Worth, B. R. (1978) *Heterocycles* **9**, 1391.
10. Bradford, M. (1976) *Anal. Biochem.* **72**, 248.