Purification and characterization of α-3′,4′-anhydrovinblastine synthase (peroxidase-like) from *Catharanthus roseus* (L.) G. Don

M. Sottomayor[a], M. López-Serrano[b], F. DiCosmo[c], A. Ros Barceló[b,*]

[a] *Department of Botany and Institute for Molecular and Cell Biology, University of Porto, R. Campo Alegre, 823, 4150 Porto, Portugal*
[b] *Department of Plant Biology, University of Murcia, E-30100 Murcia, Spain*
[c] *Department of Botany, Institute of Biomedical Engineering and Department of Surgery, University of Toronto, 25 Willcocks St., Toronto, Ont. M5S 3B2, Canada*

Received 6 April 1998; revised version received 25 April 1998

Abstract An H_2O_2-dependent enzyme capable of coupling catharanthine and vindoline into α-3′,4′-anhydrovinblastine (AVLB) was purified to apparent homogeneity from *Catharanthus roseus* leaves. The enzyme shows a specific AVLB synthase activity of 1.8 nkat/mg, and a molecular weight of 45.40 kDa (SDS-PAGE). In addition to AVLB synthase activity, the purified enzyme shows peroxidase activity, and the VIS spectrum of the protein presents maxima at 404, 501 and 633 nm, indicating that it is a high spin ferric heme protein, belonging to the plant peroxidase superfamily. Kinetic studies revealed that both catharanthine and vindoline were substrates of the enzyme, AVLB being the major coupling product.

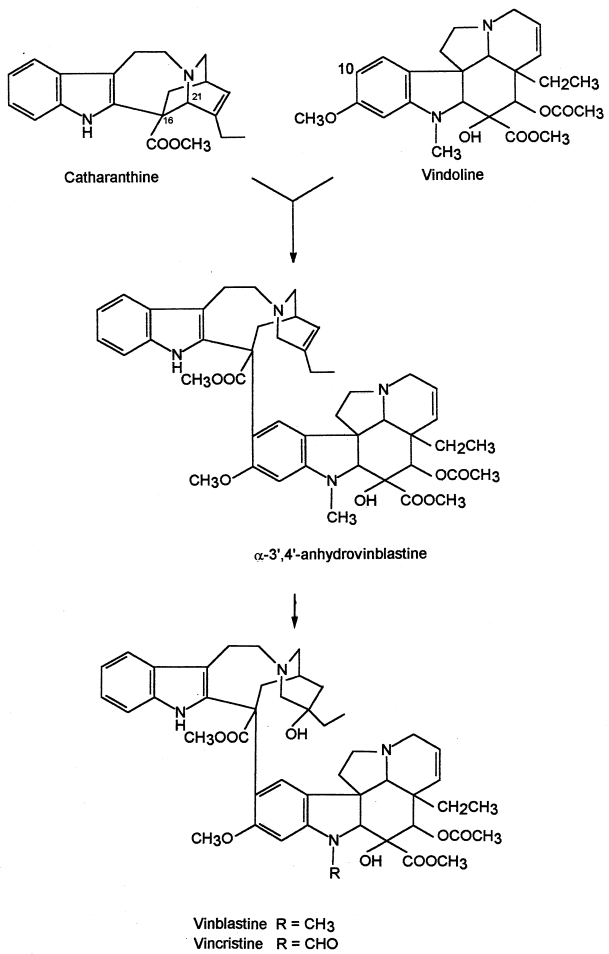
Key words: α-3′,4′-Anhydrovinblastine; α-3′,4′-Anhydrovinblastine synthase; Catharanthine; Vindoline

1. Introduction

α-3′,4′-Anhydrovinblastine (AVLB) is a dimeric monoterpenoid indole alkaloid present in *Catharanthus roseus* (L.) G. Don leaves [1–4], where it is believed to be the metabolic precursor of the anticancer drugs vinblastine (VLB) and vincristine (VCR) (Scheme 1). These compounds were the first natural anticancer agents to be clinically used [5], and are still an indispensable part of most curative regimens used in cancer chemotherapy [6].

Despite the great pharmaceutical importance of and demand for VLB and VCR, the characterization of AVLB, VLB and VCR biosynthesis, and of the enzymes involved, together with their regulation, remains incomplete in *C. roseus* leaves. In fact, although a number of enzymes of the indole alkaloid biosynthetic pathway have been isolated and extensively studied in this plant [7,8], the dimerizing step, which is catalyzed by a putative AVLB synthase, and which is particularly important from a regulatory point of view [9], has not been characterized. Feeding experiments have shown that AVLB is the direct product of the coupling of catharanthine and vindoline in planta [1] (Scheme 1), and that it may be further converted to VLB and VCR by cell-free extracts of *C. roseus* [10–12].

Preliminary studies on the coupling reaction have shown that peroxidase-like activities from *C. roseus* cell suspension cultures are capable of synthesizing AVLB [13,14], although direct proof of this assumption has only recently been possible with *C. roseus* plants [15,16]. In a preceding report [15] we demonstrated that a basic peroxidase isoenzyme located in the vacuoles of mesophyll cells of *C. roseus* leaves, where both vindoline and catharanthine, as well as AVLB, are accumulated [17–19], has AVLB synthase activity, and that this enzyme could be responsible for AVLB synthesis in vivo. The present paper reports the purification of this AVLB synthase (peroxidase-like) activity. Some properties of the enzyme are also described.



Scheme 1.

*Corresponding author. Fax: (34) (968) 363963.

Abbreviations: AVLB, α-3′,4′-anhydrovinblastine

PII S0014-5793(98)00551-1

2. Materials and methods

2.1. Plant material and chemicals

Plants of *C. roseus* (L.) G. Don (cv. Little Bright Eyes) were grown at 25°C in a growth chamber under a 16/8 h white fluorescent light photoperiod. Catharanthine sulfate and vindoline were obtained from Richter Gedeon (Budapest, Hungary). AVLB was obtained by enzymatic synthesis as reported [14], and characterized by optical rotation, circular dichroism and ^{1}H-NMR. All other chemicals used in this work were obtained from Sigma (Madrid, Spain) and were of the highest purity available.

2.2. Enzyme purification and protein determination

All extraction and purification procedures were carried out at 4°C. 418 g of leaves was homogenized in 75% (v/v) acetone at −20°C with a mortar and pestle and the residue filtered under vacuum through filter paper. The protein precipitate was washed with 75% (v/v) acetone at −20°C until all the chlorophylls were washed out. The precipitate was resuspended in 1 M KCl in 50 mM Tris-HCl, pH 7.5, gently stirred for 2 h and centrifuged at 15 000×*g* for 20 min. The supernatant (crude extract) was fractionated with solid $(NH_4)_2SO_4$, and the protein precipitated with 35% (w/v) salt saturation was discarded by centrifugation at 10 000×*g* for 30 min. The 35% (w/v) $(NH_4)_2SO_4$ soluble protein fraction was directly applied to a phenyl-Sepharose CL-4B column (35×1.5 cm) at a flow rate of 2 ml/min. After washing with two bed volumes of 1.5 M $(NH_4)_2$ SO_4 in 50 mM Tris-HCl, pH 7.5, bound proteins were eluted with a linear decreasing gradient of $(NH_4)_2SO_4$ (1.5–0.0 M $(NH_4)_2SO_4$ at a concentration change rate of 25 mmol/min with a flow rate of 2 ml/min) in 50 mM Tris-HCl, pH 7.5. Fractions of 6 ml were collected. The eight fractions showing the highest enzymatic activity were pooled and dialyzed overnight against 50 mM Tris-HCl, pH 7.5. The sample was then applied to a concanavalin A (Con A)-Sepharose 4B column (26×1 cm) equilibrated with 50 mM Tris-HCl, pH 7.5. After washing with two bed volumes of buffer, bound proteins were eluted with a linear gradient of 0–2.5 M NaCl, 0.5 M methyl α-D-mannopyranoside with a flow rate of 2 ml/min. Fractions of 2 ml were collected. The six fractions with the highest activity were pooled and directly loaded on a Sephacryl S-200 HR column (45×2.6 cm) equilibrated with 50 mM Tris-HCl, pH 7.5, which was also used for elution at a flow rate of 0.6 ml/min. Fractions of 3 ml were collected and the six fractions with the highest activity were pooled and dialyzed overnight against 20 mM MES-Tris, pH 6.0. For molecular mass determination, the column was calibrated and void volume determined with the Sigma kit for molecular weights of 12 000–200 000. The sample obtained from the above chromatography was applied to a Bio-Rad cation exchange Econopac-S column (5 cm length) equilibrated with 20 mM MES-Tris, pH 6.0. After washing with two bed volumes, basic proteins were eluted with a linear gradient of 0.0–0.35 M KCl with a flow rate of 1 ml/min. Fractions of 2 ml were collected. The three fractions showing the highest activity were pooled, and constituted the purified enzyme preparation. Protein was estimated according to Bradforf [20] using the Bio-Rad protein assay kit following the manufacturer's instructions and using bovine serum albumin as standard protein.

2.3. Assay of AVLB synthase activity by HPLC and determination of peroxidase activity

The assay of AVLB synthase activity was performed according to Sottomayor et al. [15]. The reaction assay contained 1.12 nmol of *C. roseus* protein, 300 μM vindoline, 540 μM catharanthine sulfate, and 330 μM H_2O_2 in 0.1 M MES pH 6.8, in a final volume of 1.5 ml. The reaction proceeded for 45 min at 30°C and was stopped by the addition of sodium borohydride to a final concentration of 21.1 mM. The mixture was basified to pH 9 with 0.1 ml of 28% (v/v) NH_3 aq., and the alkaloids extracted twice with ethylacetate. The combined extracts were evaporated to dryness, and the residue was dissolved in HPLC grade methanol. HPLC analyses were performed on a RP-C18 column (125×4 mm) using methanol/water containing 0.1% (v/v) triethylamine, at a flow rate of 1 ml/min, with the following multiple step gradient: 55% (v/v) methanol at 0 min, 65% (v/v) at 4 min, 67% (v/v) at 7 min, 68.5% (v/v) at 8 min, 70% (v/v) at 9 min, 71.5% (v/v) at 14 min, 90% (v/v) at 18 min and 55% (v/v) at 23 min. Due to their complexity, alkaloid leaf extracts were analyzed by HPLC in a longer RP-C18 column (250×4.6 mm) using the same methanol/water mixture containing 0.1% (v/v) triethylamine, at a flow rate of 1 ml/min, with the following multiple step gradient: 55% (v/v) methanol at 0 min, 65% (v/v) at 5 min, 70% (v/v) at 15 min, 80% (v/v) at 18 min, 90% (v/v) at 35 min and 55% (v/v) at 40 min. Alkaloids were detected at 254 nm, 270 nm and 280 nm.

Identification and quantitation of AVLB in HPLC was made by co-injection, and by comparing retention time, UV spectra and peak areas with those of an external standard. The peak corresponding to AVLB had previously been identified by ^{1}H-NMR [14]. The detection limit of the assay was of 1 pmol AVLB/s. Peroxidase activity was estimated at 30°C with 4-methoxy-α-naphthol as substrate according to Ferrer et al. [21].

2.4. Determination of the k_3 constants for AVLB synthase

The kinetic constants for AVLB synthase were determined by spectrophotometric assays of reaction media containing 0.5 μM *C. roseus* AVLB synthase, 1 μM vindoline, 1 μM catharanthine sulfate and 1 μM H_2O_2 in 20 mM Tris-MES (pH 6.0). AVLB synthase concentration was calculated using a $\varepsilon_{403} = 102.0$ mM^{-1} cm^{-1}. For kinetic measurements, the concentration of the ferric form of the enzyme ($Fe^{3+}{}_p$) and compound II (CoII) were determined using ε_{403} (CoII) = 61.85 mM^{-1} cm^{-1}, ε_{418} (CoII) = 96.51 mM^{-1} cm^{-1} and ε_{418} ($Fe^{3+}{}_p$) = 53.26 mM^{-1} cm^{-1}, according to: $A_{403} = [Fe^{3+}{}_p]\times\varepsilon_{403}$ ($Fe^{3+}{}_p$) + [CoII]×ε_{403} (CoII) and $A_{418} = [CoII]\times\varepsilon_{418}$ (CoII) + [$Fe^{3+}{}_p$]×ε_{418} ($Fe^{3+}{}_p$).

It was assumed that compound I (CoI) concentration was not significant compared with the concentrations of $Fe^{3+}{}_p$ and CoII. CoII reduction rates for vindoline and catharanthine were estimated from the progress curves of CoII. CoII reduction constants (k_3) were estimated from the CoII reduction rates, assuming that $v/([CoII][R_3N]) = k_3$.

2.5. Gel electrophoresis and staining

All types of gel electrophoresis were performed on a Pharmacia (Sevilla, Spain) PhastSystem. SDS-PAGE was carried out in PhastGel gradient medium 8-25 with PhastGel SDS buffer strips. Isoelectric focusing was performed with PhastGel Dry IEF using Pharmacia Ampholine pH 3.5–10.0 to generate the pH gradient. Protein staining was carried out with the PhastGel Silver Kit. All the procedures were performed according to Pharmacia PhastSystem protocols. Peroxidase activity was stained with 4-methoxy-α-naphthol [21].

3. Results

3.1. Purification and structural properties of AVLB synthase

AVLB synthase activity in *C. roseus* leaves is masked in

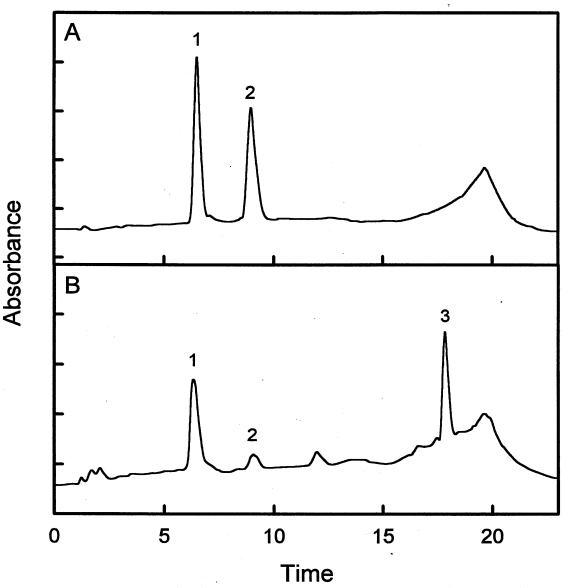


Fig. 1. HPLC profiles of the products of the activity of AVLB synthase over vindoline (1) and catharanthine (2) in the absence (A) and in the presence (B) of H_2O_2, showing the major presence of AVLB (3).

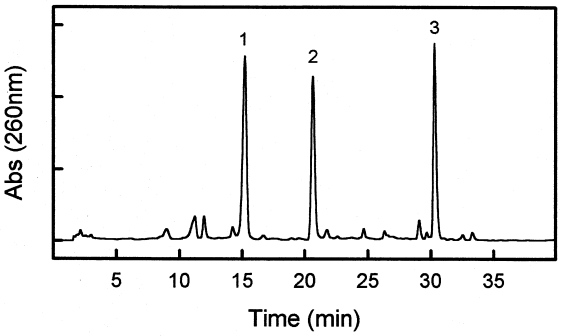


Fig. 2. HPLC profile of an alkaloid extract of *C. roseus* leaves showing the major presence of vindoline (1), catharanthine (2) and AVLB (3).

cell-free crude protein extracts, and this could explain why this enzymatic activity had not been detected until recently in *C. roseus* leaves [15]. However, this activity can be unmasked by homogenization of frozen leaves in acetone at −20°C and further chromatography on phenyl-Sepharose. For this reason, purification of AVLB synthase activity from *C. roseus* leaves was initiated with these two preliminary steps. Further purification of AVLB synthase was performed by using a protocol directed at purifying mannose-rich vacuolar glycoproteins using Con A. After Con A chromatography, gel filtration on Sephacryl S-200 and cation exchange chromatography, a highly purified AVLB synthesizing protein with a specific activity of 1.8 nkat/mg was obtained. The specificity of the reaction catalyzed by this enzyme for the coupling product is shown in Fig. 1. According to Fig. 1, the protein performs the coupling reaction of catharanthine and vindoline to yield AVLB as the major detectable coupling product, in a reaction strictly dependent on H_2O_2. In HPLC, the peak corresponding to AVLB had been previously identified by ^{1}H-NMR, optical rotation and circular dichroism [14]. The specificity of the in vitro reaction for AVLB as the major coupling product is in accordance with the alkaloid shading pattern shown by *C. roseus* leaves, in which catharanthine, vindoline, and its coupling product, AVLB, are the principal alkaloids (Fig. 2).

Protein purity was checked by SDS-PAGE and silver staining, which indicated the apparent homogeneity of the AVLB synthesizing protein (Fig. 3). The molecular weight was estimated to be 45.40 kDa by SDS-PAGE (Fig. 3) and 41.04 kDa by gel filtration in Sephacryl S-200. Thin-layer IEF confirmed the apparent homogeneity of the protein and enabled us to estimate the p*I* of the protein to be around 10.7 (Fig. 4).

The main structural properties of AVLB synthase are as follows. The VIS spectrum of the native form of AVLB synthase shows maxima at both 404 nm (Soret band), and 501 and 633 nm (α and β absorption bands), indicating that this enzyme is a high spin ferric heme protein, belonging to the plant peroxidase superfamily, the prosthetic group therefore being ferric protoporphyrin IX. In fact, this purified AVLB synthesizing protein showed peroxidase activity (1.9 μkat/mg when assayed with the non-natural substrate 4-methoxy-α-naphthol) and an *Rz* (A_{403nm}/A_{280nm}) value of 3.1 (Table 1). Because the AVLB synthase activity of this protein was masked until the phenyl-Sepharose chromatography step, it was not possible to calculate the final degree of purification starting from the acetone powder. However, since the peroxidase-like activity of this protein was not inhibited by the endogenous inhibitors of AVLB synthase activity, it was possible to express the purification degree on the basis of this enzymatic activity. When this was done, the purification degree for the protein was calculated to be 192-fold (Table 1).

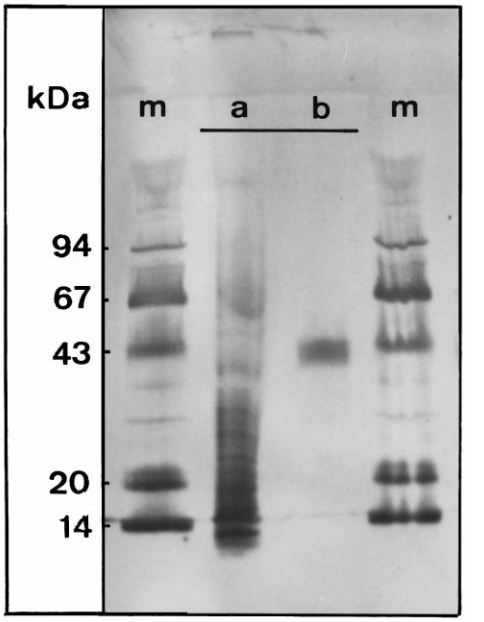


Fig. 3. SDS-PAGE of the purified AVLB synthase in Phastgel 8-25 gradient medium. Lane a: crude extract. Lane b: purified enzyme (3.2 pmol). Lanes m: molecular mass markers ranging from 94.0 (top) to 14.4 (bottom) kDa. Protein staining was performed with the PhastSystem silver kit.

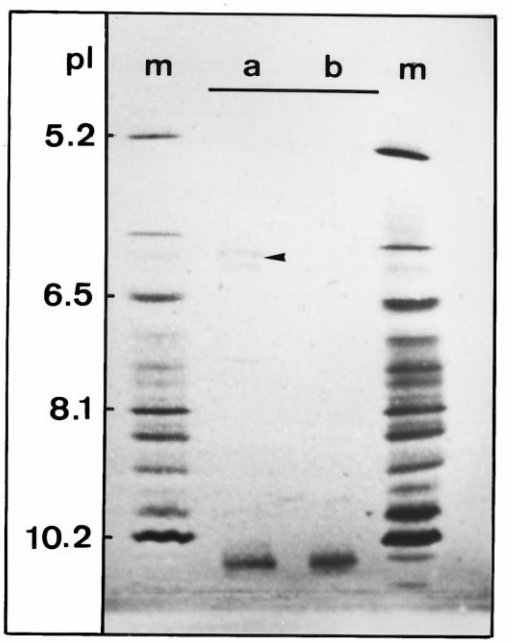


Fig. 4. IEF of the purified AVLB synthase in Phastgel dry IEF. Lane a: purified enzyme (3.2 pmol). Lane b: purified enzyme diluted 1:2. Lanes m: p*I* markers ranging from 5.20 (top) to 10.25 (bottom). Arrowhead, origin. Protein staining was performed with the PhastSystem silver kit.

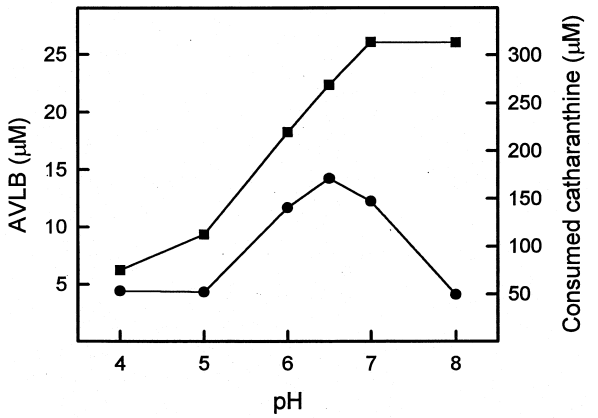


Fig. 5. Influence of pH on the AVLB synthase activity measured either as AVLB production (●) or catharanthine consumption (■).

3.2. Catalytic properties

The catalytic properties of this protein in AVLB formation were studied. The AVLB synthase activity of this protein has an optimal pH of around 6.5, although it also shows substantial activity in the 4–5 pH range (Fig. 5), which is the pH range commonly found in plant vacuoles [19].

The capacity of catharanthine and vindoline to act as reducers (substrates) of the oxidized ferric states of AVLB synthase was also studied. CoI of the enzyme was obtained upon addition of H_2O_2 to the native ferric form of AVLB synthase. In the presence of catharanthine and vindoline, the transition from CoI to CoII is so fast that it cannot be detected. That is, CoII appeared as the major intermediary species to be accumulated during the coupling reaction. Since the formation of the porphyrin π-cation radical, CoI, of AVLB synthase, and its further reduction to CoII is too fast to be detected, the effect of these alkaloids on the CoI reduction rate could not be estimated. However, the participation of CoI in the catalytic cycle of the enzyme may be inferred from the weak changes in absorbance at 411 nm (Fig. 6A), the putative isosbestic point between the ferric form of AVLB synthase and CoII.

These results indicate that CoII reduction of AVLB synthase is the rate limiting step during the coupling reaction between catharanthine and vindoline to yield AVLB. The decay of CoII to the native ferric form of the enzyme was clearly accelerated in the presence of vindoline and catharanthine (Fig. 6B). When the capacity of catharanthine alone or vindoline alone to reduce CoII of AVLB synthase was studied, it was found that both were substrates for CoII reduction. The values found for the compound II reduction constant, k_3, were 0.66 mM^{-1} s^{-1} for catharanthine and 1.17 mM^{-1} s^{-1} for vindoline.

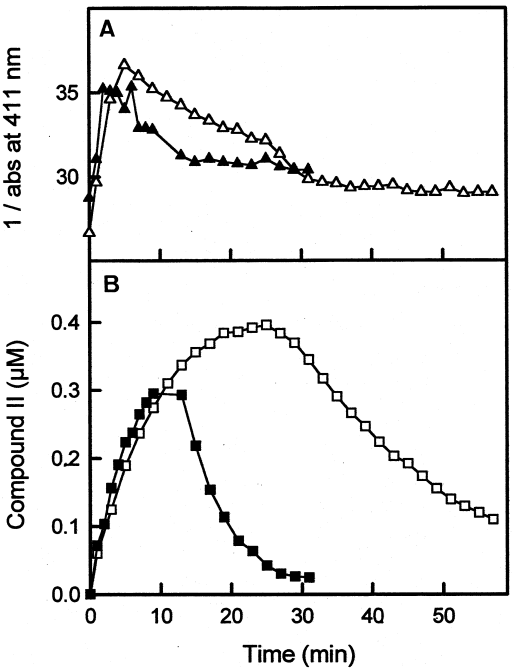


Fig. 6. A: Time course of absorbance at 411 nm, indicative of the presence of CoI, of a reaction medium containing AVLB synthase, H_2O_2, catharanthine and vindoline (closed symbols) and control in the absence of catharanthine and vindoline (open symbols). B: Time course of the evolution of CoII in the presence (closed symbols) and in the absence (open symbols) of catharanthine and vindoline.

4. Discussion

In this work, an AVLB synthase (peroxidase-like) protein capable of performing the coupling of catharanthine and vindoline to give AVLB as the main coupling product (Fig. 1) was purified from *C. roseus* leaves, and its molecular and kinetic properties are reported. This enzyme was purified until homogeneity (Figs. 3 and 4) using a five-step protocol designed to purify mannose-rich vacuolar glycoproteins. The enzyme showed a molecular weight of 45.40 kDa by SDS-PAGE (Fig. 3) and 41.04 kDa by gel filtration in Sephacryl S-200, which suggests that the enzyme is a monomeric protein, with a p*I* of around 10.7. However, p*I* determination of this extremely basic protein was hindered by the fact that there are no commercially available ampholines to create pH gradients above 10, so it is highly probable that the p*I* of AVLB synthase is actually higher than 10.7.

The VIS spectrum of the protein presents maxima at 404, 501 and 633 nm, indicating that this enzyme is a high spin ferric heme protein, belonging to the plant peroxidase superfamily, as was also demonstrated by its ability to oxidize a

Table 1
Purification of AVLB synthase (peroxidase-like) from *C. roseus* leaves

Purification step	Peroxidase activity (nkat)	Yield (%)	Specific activity (nkat/mg)	Purification (fold)	*Rz*
Crude extract	27 246	100.0	26.19	–	–
> 35% $(NH_4)_2$ SO_4	10 354	38.0	10.03	1.0	–
Phenyl-Sepharose	6 438	23.6	44.35	4.4	–
Concanavalin A	2 971	10.9	220.26	22.0	0.28
Sephacryl S-200	2 101	7.7	1 245.78	124.2	1.16
CE Econopac S	1 320	4.8	1 929.56	192.4	3.1

common phenolic substrate at the expense of H_2O_2. This protein was located in mesophyll vacuoles [15] at the level of the internal face of the tonoplast [16], and it is in direct contact with catharanthine and vindoline, two alkaloids also located in vacuoles [17–19], and which are the two substrates used by the enzyme for the synthesis of AVLB (Fig. 1). It is worth noting that vindoline and catharanthine (the substrates of the enzyme) and AVLB (the product of the enzymatic reaction) constitute the three main indole alkaloids found in *C. roseus* leaves (Fig. 2), supporting a further physiological meaning for the specificity of the in vitro reaction (Fig. 1).

Support for this statement was obtained from the specific activity of the enzyme during AVLB synthesis. Thus, in optimal conditions, the specific activity of this protein to mediate the coupling reaction between catharanthine and vindoline to yield AVLB is 1.8 nkat/mg. This specific activity towards AVLB production is of the same order as that shown by the preceding enzymes of the bis-indole alkaloid biosynthetic pathway until now purified from *C. roseus* leaves: tryptophan decarboxylase (2.7 nkat/mg) [22], the 2-oxoglutarate-dependent dioxygenase involved in vindoline biosynthesis (0.09 nkat/mg) [8] and geraniol 10-hydroxylase (0.52 nkat/mg) [23]. All these reasons appear to be sufficient evidence for considering this enzyme the true, and to date unidentified, AVLB synthase, which is ultimately responsible for AVLB synthesis in *C. roseus* leaves.

Acknowledgements: This work was partially supported by Grant AIHL HP 96-27 (AILE E-26/97) to A. Ros Barceló and M. Sottomayor, and by a grant from UNESCO (1996) and the Calouste Gulbenkian Foundation (1996) to M. Sottomayor.

References

[1] Scott, A.I., Gueritte, F. and Lee, S.L. (1978) J. Am. Chem. Soc. 100, 6253–6255.
[2] Goodbody, A.E., Watson, C.D., Chapple, C.C.S., Vukovic, J. and Misawa, M. (1988) Phytochemistry 27, 1713–1717.
[3] Balsevich, J. and Bishop, G. (1989) in: Primary and Secondary Metabolism of Plant Cell Cultures (Kurz, W.G.W., Ed.), pp. 149–153, Springer Verlag, Berlin.
[4] Naaranlahti, T., Auriola, S. and Lapinjoki, S.P. (1991) Phytochemistry 30, 1451–1453.
[5] Hamburger, M. and Hostettmann, K. (1991) Phytochemistry 30, 3864–3874.
[6] Son, J.K., Rosazza, J.P.N. and Duffel, M.W. (1990) J. Med. Chem. 33, 1845–1848.
[7] DeLuca, V. (1993) Methods Plant Biochem. 9, 345–368.
[8] Meijer, A.H., De Waal, A. and Verpoorte, R. (1993) J. Chromatogr. 635, 237–249.
[9] Sottomayor, M., DiCosmo, F. and Ros Barceló, A. (1998) Adv. Plant Physiol. (in press).
[10] Stuart, K.L., Kutney, J.P., Honda, T. and Worth, B.R. (1978) Heterocycles 9, 1391–1395.
[11] Baxter, R.L., Hasan, M., Mackenzie, N.E. and Scott, A.I. (1982) J. Chem. Soc. Chem. Commun. 791–793.
[12] Endo, T., Goodbody, A., Vukovic, J. and Misawa, M. (1987) Phytochemistry 26, 3233–3234.
[13] Endo, T., Goodbody, A., Vukovic, J. and Misawa, M. (1988) Phytochemistry 27, 2147–2149.
[14] Smith, J.I., Amouzou, E., Yamagushi, A., McLean, S. and DiCosmo, F. (1988) Biotech. Appl. Bioeng. 10, 568–575.
[15] Sottomayor, M., de Pinto, M.C., Salema, R., DiCosmo, F., Pedreño, M.A. and Ros Barceló, A. (1996) Plant Cell Environ. 19, 761–767.
[16] Sottomayor, M., Salema, R., DiCosmo, F. and Ros Barceló, A. (1996) in: Plant Peroxidases: Biochemistry and Physiology (Obinger, C., Burner, U., Penel, C. and Greppin, H., Eds.), pp. 128–133, University of Geneva, Geneva.
[17] Deus-Neumann, B. and Zenk, M.H. (1984) Planta 162, 250–260.
[18] Brisson, L., Charest, P.M., De Luca, V. and Ibrahim, R.K. (1992) Phytochemistry 31, 465–470.
[19] Wink, M. (1993) J. Exp. Bot. 44, 231–246.
[20] Bradford, M.G. (1976) Anal. Biochem. 72, 248–254.
[21] Ferrer, M.A., Calderón, A.A., Muñoz, R. and Ros Barceló, A. (1990) Phytochem. Anal. 1, 63–69.
[22] Pennings, E.J.M., Verpoorte, R., Goddijn, O.J.M. and Hoge, J.H.C. (1989) J. Chromatogr. 483, 311–318.
[23] DeCarolis, E. and DeLuca, V. (1994) Plant Cell Tiss. Org. Cult. 38, 281–287.