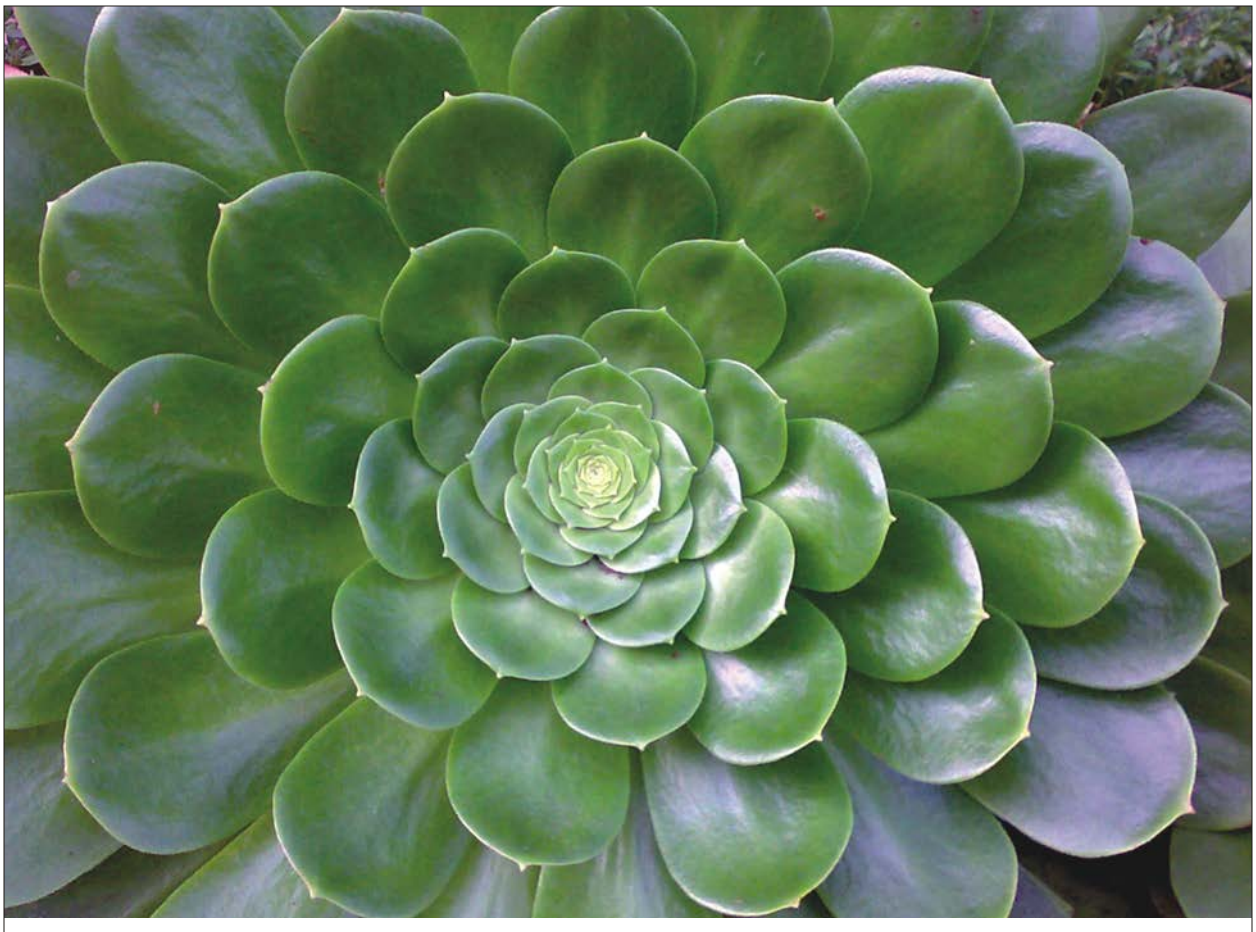

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Bioconversion of Ajmalicine to Serpentine in *Catharanthus roseus* Roots

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Abstract: Ajmalicine and serpentine are indole alkaloids of considerable medicinal importance. In *Cathranthus roseus* roots, the amount of serpentine is considerably higher as compared to that of ajmalicine. The crude alkaloid, ajmalicine and serpentine, per plant root biomass, increased from one leaf pair seedling stage to five stages. The increase was more pronounced with respect to serpentine. There is conformity between the time course of serpentine to ajmalicine ratio and that of the activity of peroxidase, the enzyme involved in the conversion of ajmalicine to serpentine. Ajmalicine is converted *in vitro* into serpentine by horseradish basic peroxidases (1.11.1.7). In *C. roseus* root extracts, since a high quantity of serpentine is associated with protein, the bioconversion results using HPLC are not conclusive. A radioactive procedure, involving the use of radio labeled ^{3}H-ajmalicine, has been standardized for bioconversion studies. The ^{3}H-ajmalicine was synthesized by sodium borotritide reduction of serpentine. Using labeled ajmalicine, it has been conclusively demonstrated that ajmalicine is converted efficiently into serpentine by peroxidase in *C. roseus* roots.

Key words: Bioconversion, *Catharanthus roseus*, indole alkaloid, radioactive

Introduction

Catharanthus roseus (L. Don, Sadabahar, family *Apocyanceae*) is the most significant alkaloid bearing plant, the roots of which contain biologically active alkaloids such as ajmalicine and its tetradehydroderivative serpentine. Ajmalicine is used in the treatment of circulatory diseases, especially in the relief of obstruction of normal cerebral blood flow and hypersensitivity (Misra *et al*., 1999). Serpentine is not used as such, but converted to ajmalicine by hydrogenation. The percentage of ajmalicine and serpentine in roots of 50 days old plant has been reported to be 0.5 and 1.2% respectively (Arens *et al*., 1999). In *C. roseus* roots, a major portion of ajmalicine is converted into serpentine. It has been proposed that ajmalicine gets trapped inside the vacuoles and is converted into serpentine (Renudin, 1989; Blom *et al*., 1991).

Enzymology of biosynthesis of ajmalicine and their compartmentation has been worked out (Misra *et al*., 1996). Activity of tryptophan decarboxylase and anthranilate synthase, two enzymes in the pathway leading to ajmalicine, increased after induction with glucose (Schlatmann *et al*., 1995). Peroxidases have been implicated in the oxidation of ajmalicine into serpentine. In cultured cells, there was a striking conformity between the time course of serpentine content and the activity of

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peroxidase (Blom *et al*., 1991). Light grown cell cultures had a twenty-fold higher vacuolar peroxidase activity than in dark grown cell cultures and chloroplasts might play a fundamental role in serpentine biosynthesis (Misra *et al*., 1996). Exogenous ajmalicine get catabolized by *C. roseus* suspension culture (Schlatmann *et al*., 1995). The production and the degradation rates of ajmalicine were relatively high at the beginning of the growth phase while the degradation rate was also high in the stationery phase. Sometimes serpentine gets accumulated during the later stage of the stationary phase of the growth cycle.

Ajmalicine can be converted efficiently into serpentine by peroxidase extracted from vacuoles and by intact isolated vacuoles (Blom et al., 1991). The biotransformation results using the whole root crude extracts were, however, not conclusive because of the association of large amount of serpentine with the proteins. In the present investigation, radio labeled ^{3}H-ajmalicine has been synthesized in order to establish *in vitro* conversion of ^{3}H-ajmalicine into ^{3}H-serpentine by *C. roseus* root extracts.

Materials and Methods

Plants, Substrates and Standards

One-month-old *Catharanthus roseus* (L.) Don roots were used (Misra and Gupta, 2005) as an experimental material for biotransformation studies, whereas for developmental studies, the root samples were taken from plants at one leaf pair to five leaf pair stage. ^{3}H-Sodium borotritide (total activity 100 mCi; specific activity 8.5 Ci/mmole) was purchased from Amersham International for synthesis of radio labeled ajmalicine. Ajmalicine and serpentine were obtained from Dr. Gopal Rao of CIMAP field station, Bangalore, India and Dr. Falak of Central Drug Research Institute, Lucknow, India, respectively.

Extraction and HPLC Analysis of Alkaloids

A known amount of dried root powder was homogenized in ethyl alcohol (90%) and the alkaloids were extracted (Loyola-Vargas *et al*., 1992; Dos Santos *et al*., 1994; Endo *et al*., 1987). The ethanolic extract was evaporated to dryness. The residue was dissolved in distilled water and mixed with conc. HCl (final concentration 3%). An equal volume of ethyl acetate was added. The aqueous phase was collected, adjusted to pH 8.5 with ammonia and extracted with chloroform. The chloroformic phase containing the alkaloids was collected and evaporated to dryness to get crude alkaloids. An aliquot of the alkaloid extract was loaded on a C_{18} reverse phase analytical cartridge and eluted at a flow rate of 1.0 mL min^{-1} with the elution buffer (methanol; acetonitrile: 0.01 M ammonium acetate+0.1% triethyl amine in 1:1:2.25 ratio) at pH 8.2. The alkaloids were detected at 254 nm and identified by their retention time (ajmalicine 14.69 min, serpentine 5 .03 min) (Fig. 1a). The experiment repeated five times.

Preparation of Enzyme Extracts

A known amount of the root tissue was homogenized in 0.1 M Tris HCl buffer, pH 7.5 containing 5 mM DTT in the presence of 50% PVPP (w/w) at 4°C. The homogenate was strained through muslin cloth and centrifuged at 18,000 xg for 30 min. The supernatant was collected and used as the crude enzyme preparation.

$(NH_4)_2SO_4$ Fractionation of the Crude Enzyme Extract

The peroxidase enzyme activity was concentrated from crude enzyme by precipitation with 40-90% ammonium sulphate (w/v). The precipitated protein were dissolved in extraction buffer and dialyzed against the extraction buffer. The clear solution thus obtained was used for biotransformation.

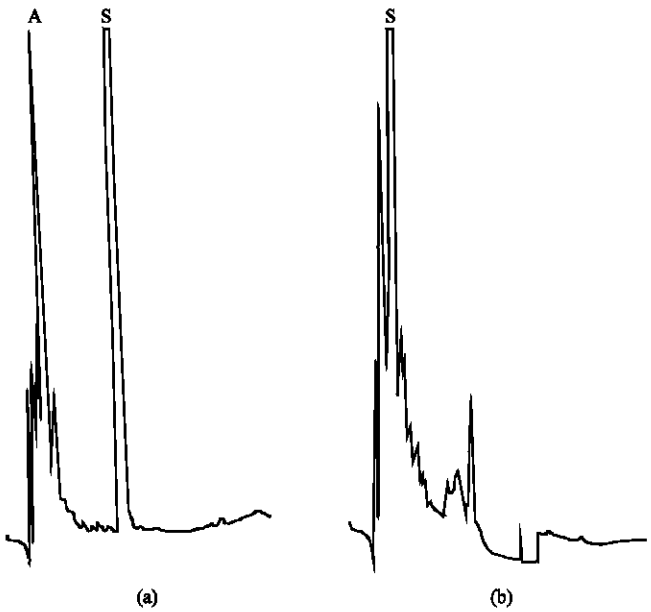


Fig. 1: a. HPLC chromatogram of the standard Ajmalicine (A), Serpentine (S). b.HPLC chromatogram of the alkaloids extracted from 40-90% Ammonium sulphate precipitated protein fraction of *C. roseus* root extract.

The peroxidase was assayed as described by Misra *et al.* (1995). The assay mixture consisted of guaiacol (20 mM) and H_2O_2 (0.3%) in sodium phosphate buffer (0.1 M, pH 6.1) at 30°C. The reaction was monitored at 470 nm. Enzyme activity was expressed as change in absorbance at 470 nm min mg of protein. One enzyme unit was defined as a change of 1 O.D. min mg protein. Protein was determined by Bradford method (1976). Each value represents mean of five replicates and SD determined.

Oxidation of Ajmalicine to Serpentine

A slight modification of the method described by Fillippelli (1986) was used for the oxidation of ajmalicine to serpentine by horseradish peroxidase. The reaction mixture consisted of 50 μg ajmalicine, 20 mM H_2O_2, 0.1 M phosphate buffer, pH 6.1 and horseradish peroxidase (10.0 units mg^{-1} of the substrate ajmalicine). The reaction mixture was incubated for 16 h at 30°C. After incubation, the alkaloids were extracted and analyzed by HPLC as described earlier. To determine the conversion of ajmalicine to serpentine in *C. roseus* roots, the reaction conditions were the same as described for the horseradish peroxidase mediated oxidation of ajmalicine, except that the horseradish peroxidase was replaced by the root extract or the 40-90% ammonium sulphate fractionated protein. Each value represents mean of five replicates and SD determined.

Preparation of Radioactive ^{3}H-ajmalicine

A methanolic solution of serpentine (8.18 mg; 11.76 mole) was added to sodium borotritide (0.54 mg; 11.76 mole) at room temperature. After 10 min, when the yellow colour of the solution decolorized, an additional amount of 20 mg of sodium borohydride (cold) was added and the reaction mixture was left for 5 h. After completion of the reaction, the mixture was diluted with methanol. TLC of the reaction mixture showed complete conversion of serpentine into radioactive ajmalicine (Rf = 0.58 on basic alumna plate, chloroform: methanol, 99.5: 0.5 v/v). The specific activity of the labeled ^{3}H-ajmalicine synthesized was 39.3 Ci/mole. Experiments repeated at least five times.

Oxidation of ^{3}H-ajmalicine to Serpentine

Oxidation of ^{3}H-ajmalicine to serpentine was performed as described in case of horseradish peroxidase. The reaction mixture consisted of 50 µg ajmalicine [10 µg ^{3}H-ajmalicine+40 µg cold (unlabeled) ajmalicine], 20 mM H_2O_2, 0.1 M phosphate buffer, pH 6.1 and root protein (25-500 µg). The reaction mixture was incubated for 4,8,16,20,24 h at 30°C. After incubation, the alkaloids were extracted and separated by TLC. The ajmalicine and serpentine spots were scraped and counted using PPO-POPOP-Toluene cocktail and LKB Rack Beta scintillation counter. The counting efficiency of scintillation counter for ^{3}H was 53%. Each value represents mean of five replicates and SD determined.

Autoradiography of the Labeled Product

The TLC plate having radiolabeled ajmalicine and serpentine spots were exposed to the X-ray film in a cassette and kept at 4°C in a deep freezer. The X-ray film was removed after 45 days and developed.

Results and Discussion

Developmental Profile of Ajmalicine, Serpentine and Peroxidase Activity in Roots of Catharanthus Roseus

The crude alkaloids, ajmalicine, serpentine and peroxidase activity per plant root biomass increased from one leaf pair seedling stage to five-leaf pair seedling stage (Table 1). Serpentine is the main alkaloid in seedlings with one leaf pair and ajmalicine is found in very low amounts. The amount of serpentine increased markedly with the plant age as compared to that of ajmalicine. Peroxidase has been implicated in the oxidation of ajmalicine to serpentine in *C. roseus* (Blom *et al*., 1991). Present results suggest conformity in the time course of serpentine and ajmalicine content and that of the activity of peroxidases, the enzyme involved in the conversion of ajmalicine to serpentine.

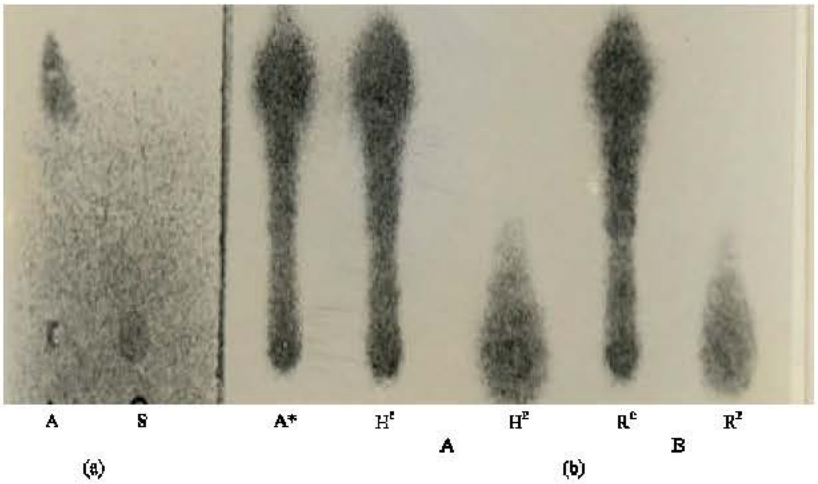


Fig. 2:a TLC of Authentic samples Ajmalicine (A) and Serpentine (S) [solvent system; chloroform: methanol, 99.5:0.5%]. b. Autoradiogram of the TLC plate depicting bioconversion of ajmalicine to serpentine by Horseradish peroxidases (A); HC (Control), HE (Experimental) and 40-90% ammonium sulphate precipitated protein fraction of *C. roseus* roots extract (B); CC (Control), CE (Experimental). The symbol A* denotes standard labeled ajmalicine.

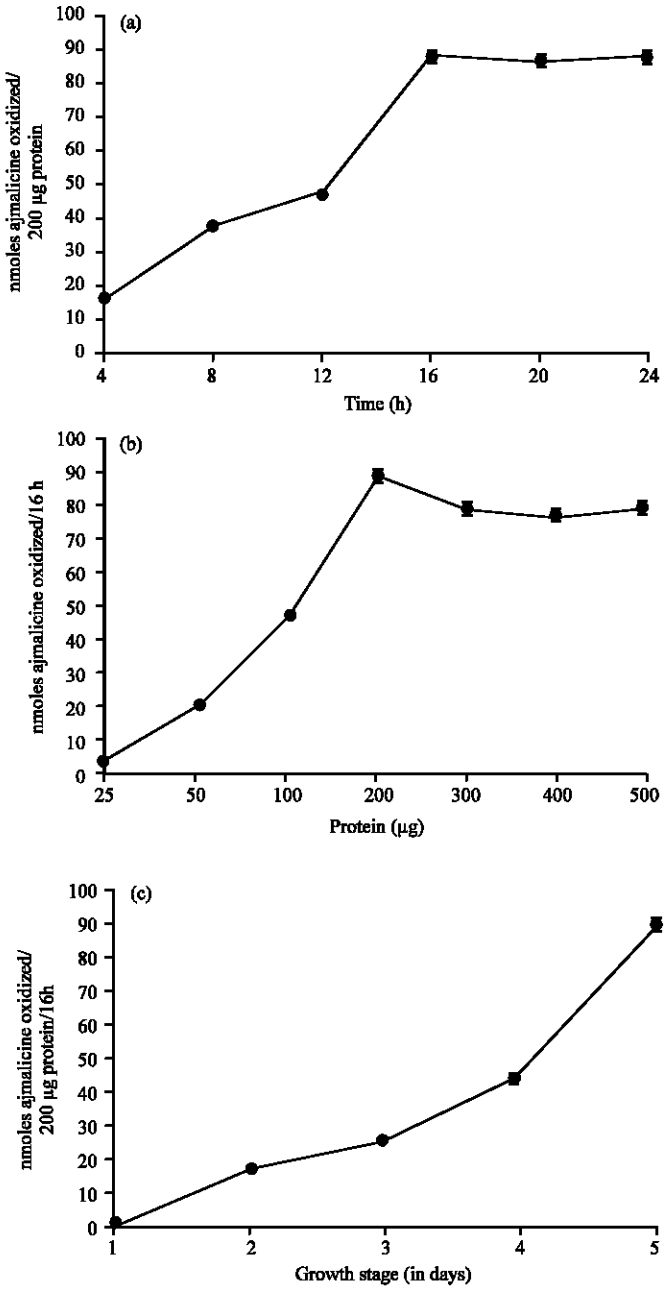
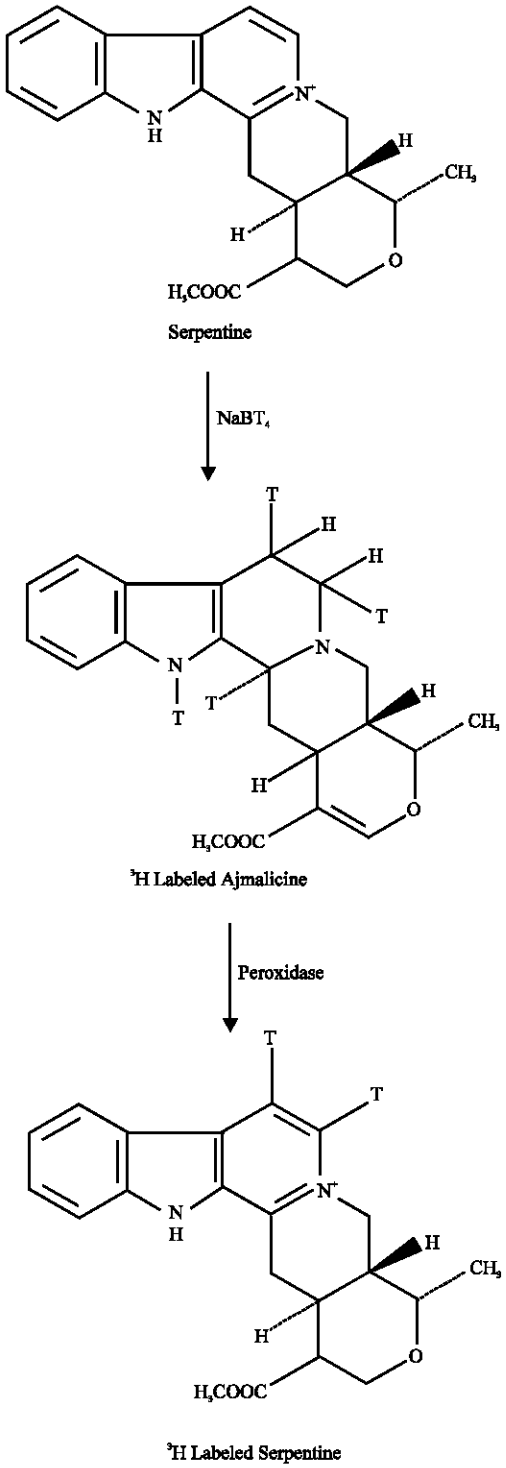


Fig. 3: a.Time course of labeled ajmalicine oxidized. 200 µg of protein was used in each assay. Each value represents mean of five replicate and SD determined. b. The conversion of labeled ajmalicine into serpentine by different protein concentrations. Each value represents mean of five replicate and SD determined. c. The bioconversion of ajmalicine into serpentine at different growth stages. The number 1 to 5 represents gradient increase in seedling growth in terms of leaf pairs. Each value represents mean of five replicate and SD determined


Serpentine
NaBT4
3H Labeled Ajmalicine
Peroxidase
3H Labeled Serpentine

Scheme 1

Table 1: Developmental profile of indole alkaloids and peroxidase in *C. roseus* Roots. Each value represents mean of five replicates and SD determined.

	Growth stages (no. of leaf pairs)				
Parameters	1	2	3	4	5
Crude alkaloids/seedling root biomass (µg)	38±0.26	62±0.25	113.6±1.1	272.2±1.4	646.7±2.3
Ajmalicine/seedling root biomass (µg)	0.14±0.06	0.09±0.001	0.2±0.05	1.0±0.12	2.57±0.25
Serpentine/seedling root biomass (µg)	1.12±0.16	3.0±0.18	7.09±0.23	45.6±1.4	116.5±1.8
Serpentine/ajmalicine ratio	8.0±0.12	33.3±1.2	35.5±0.56	45.6±1.0	45.3±1.2
Peroxidase activity					
∆ O.D./min/seedling root biomass$x10^{-1}$	0.011±0.005	0.015±0.002	0.037±0.004	0.171±0.05	0.35±0.3
∆ O.D. min mg protein	-	0.1±0.02	0.13±0.04	0.17±0.05	0.196±0.08

Biotransformation of Ajmalicine to Serpentine in Catharanthus Roseus Roots

In *C. roseus* roots, the level of serpentine is considerably higher as compared to that of ajmalicine (Table 1). HPLC results showed that although the horseradish basic peroxidase is able to bring about the conversion of ajmalicine to serpentine (Blom *et al*., 1991) but due to association of high quantities of serpentine even in peroxidase rich ammonium sulphate precipitated protein fraction of the root extract (Fig 1b), the results about bioconversion using root extract are not conclusive. Serpentine has a positive charge on its nitrogen atom; therefore, it gets precipitated with protein molecules. Because it is an anhydronium, the strong base serpentine, however, will always be in charged form and, therefore, its precipitation along with proteins cannot be avoided. A radioactive procedure involving the use of radiolabeled (^{3}H)-ajmalicine for biotransformation studies have, therefore, been standardized.

The reduction of serpentine with sodium borotritide ($NaBT_4$) afforded radio labeled ajmalicine (Scheme I). It has been observed that hydroboration of alkenes is mostly stereospecific cis addition. The radio labeled ajmalicine so obtained was utilized for *in vitro*/*in vivo* experiments. Using labeled ajmalicine, it has been found that ajmalicine gets converted efficiently into serpentine by horseradish peroxidase (Fig. 2, Lane II and III) and 40-90% $(NH_4)_2SO_4$ precipitated protein (Fig. 2, Lane IV and V). The autoradiography of the reaction products indicated the formation of radiolabeled serpentine from ajmalicine (Fig. 2). The radioactive serpentine had the same Rf value as authentic serpentine. A possible explanation for the formation of radio labeled serpentine is that the dehydrogenation of ajmalicine with peroxidase is probably a stereospecific cis elimination reaction that results in the formation of radiolabeled serpentine (Scheme I).

The 40-90% $(NH_4)_2SO_4$ precipitation protein fraction was incubated with labeled ajmalicine for different time periods to determine the linearity of the bioconversion of ajmalicine to serpentine with time (Fig. 3a). The bioconversion of ajmalicine was also performed with different concentration of the protein (25-500 µg) to get maximal oxidation of ajmalicine under the existing conditions. The substrate ajmalicine was used at saturating levels. The maximum oxidation of ajmalicine could be achieved by incubating saturating levels of the substrate with 200 µg of protein for 16 h (Fig. 3b). Using this procedure it has been observed that the oxidation of ajmalicine in roots increased with plant age from one leaf pair seedling stage to five-leaf pair stage (Fig. 3c) concomitant to the peroxidase activity and relative levels of ajmalicine and serpentine (Table 1).

Conclusions

By using radiolabeled ajmalicine, it has been conclusively demonstrated that in *C. roseus* roots, ajmalicine gets oxidized efficiently into serpentine by peroxidase. This is the first report of *in vitro* demonstration of the bioconversion of ajmalicine to serpentine by *C. roseus* root extract. Study is in progress to purify and characterize the peroxidase enzyme in *C. roseus* roots.

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