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ENZYMIC SYNTHESIS OF CORYNANTHE-TYPE ALKALOIDS IN CELL CULTURES OF *CATHARANTHUS ROSEUS*: QUANTITATION BY RADIOIMMUNOASSAY*

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Key Word Index—*Catharanthus roseus*; Apocynaceae; cell cultures; ajmalicine; enzymic synthesis; radioimmunoassay.

Abstract—The soluble enzyme system from *Catharanthus roseus* cell suspension cultures which synthesizes ajmalicine, 19-epiajmalicine and tetrahydroalstonine from tryptamine and secologanin has been further characterized. The enzymic reaction was followed quantitatively by using a radioimmunoassay (RIA) with antibodies directed against ajmalicine. This RIA proved exceedingly useful in determining the enzymology of the reaction and displayed a sensitivity shown previously only by radio-tracer methods. By this method, the enzyme system was found to function optimally at pH 6.5. Sensitivity of the enzymic synthesis of ajmalicine and its stereoisomers to δ-D-gluconolactone, a β-glucosidase inhibitor, indicated the involvement of a β-glucosidase in the formation of these alkaloids. The enzyme system catalysed the formation of unnatural ajmalicine analogues from ring-substituted tryptamines.

INTRODUCTION

Several groups of workers have established by precursor feeding experiments with differentiated plants that tryptamine and the monoterpenoid secologanin serve as precursors of *Corynanthe*-type alkaloids [for review see 1, 2]. The immediate condensation product is the alkaloidal glucoside strictosidine [3, 4] which is efficiently converted into the alkaloids of the ajmalicine type. Efforts to detect significant quantities of intermediates of this conversion have so far been unsuccessful.

Scott and Lee have recently shown [5] that a crude cell-free system from *Catharanthus roseus* callus cultures can catalyse the conversion of labelled tryptamine and secologanin into ajmalicine. This experimental result was confirmed and extended by us [6] using *Catharanthus* cell suspensions. Three *Corynanthe*-type alkaloids have been isolated by large-scale incubation and chemically identified; these are ajmalicine, 19-epiajmalicine and tetrahydroalstonine. Furthermore it was shown [6] that this reaction had an absolute requirement for NADPH (NADH was less effective) and that in the absence of reduced pyridine nucleotide an intermediate accumulated which was subsequently identified as cathenamine (20, 21-didehydroajmalicine) [7].

The aim of this investigation was to characterize further the conversion of tryptamine and secologanin into ajmalicine and its two isomers. This work constitutes the first successful attempt in a phytochemical investigation to monitor quantitatively a complex enzyme-catalyzed sequence by means of radioimmunoassay [8, 9].

*Part 3 in the series 'Use of immunoassay in plant science'.

RESULTS

The assay system

The radioimmunoassay method [for review see 10] has recently been applied to the solution of phytochemical problems [11, 12]. Its specificity, sensitivity and suitability for the analysis of large numbers of samples should also make it an ideal tool for the quantitation of enzymatically catalyzed reactions. A radioimmunoassay for the determination of ajmalicine has been established [9] and used successfully for the selection of *Catharanthus* plants and cells in culture capable of producing high yields of ajmalicine [8]. The cell-free system produces ajmalicine in the range of 20 nmol per ml (*ca* 7000 ng/ml) of reaction mixture. 19-Epiajmalicine is produced in approximately twice this concentration. The assay procedure detects both alkaloids at levels of 0.1 ng per 0.1 ml of sample [9] and should therefore be highly suitable. Tetrahydroalstonine is detected with one tenth the sensitivity.

In order to demonstrate that the products formed by the cell-free system can be individually identified, 0.2 ml of a standard incubation mixture [6] was applied to a Si gel TLC-plate and chromatographed in solvent system I. In parallel with this, the products from incubation with ^{14}C-tryptamine were chromatographed in the same solvent system. Comparison of the two plates (Fig. 1a and b) showed the correspondence of labelled material with ajmalicine and its two stereoisomers (R_f 0.65). The plate containing the unlabelled material (Fig. 1b) was subsequently divided into 37 equal zones which were eluted with methanol. These fractions were diluted with H_2O and analysed by the RIA method. Fig. 1c shows the position of immunoreactive zones which formed in the presence of NADPH, while in its absence this material did not appear (Fig. 1d). Further analysis [6] proved this

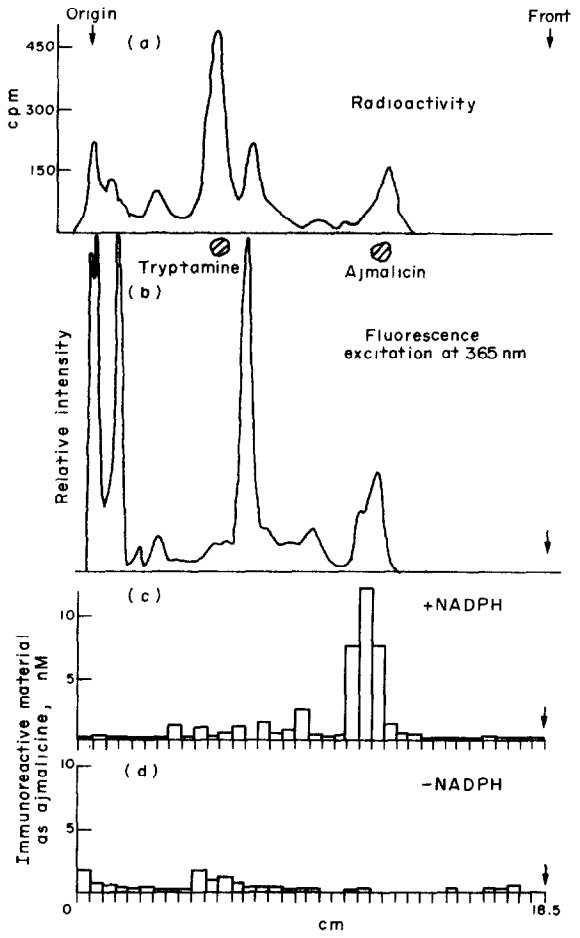


Fig. 1. TLC chromatogram of a standard incubation mixture (0.2 ml) developed in solvent system I. (a) distribution of radioactivity from tryptamine-2-^{14}C (b) fluorescence scan (c) distribution of immunoreactive material using ajmalicine antibodies (d) control experiment: standard incubation mixture, as in (c), but without NADPH

band (R_f 0.65) to contain exclusively ajmalicine and its two isomers. Incubations with either heat-denatured protein or without NADPH served as control. Recovery of ajmalicine added to the samples in six independent experiments showed an average value of 99.8% ± 3.7%. The assay was sufficiently sensitive to theoretically permit the reaction to be conducted in a total volume of less than 0.1 µl.

These results demonstrate that radioimmunoassay can be used successfully to follow enzymic reactions mediated by plant enzymes. Its sensitivity is well in the range of radiotracer experiments and greater by a factor of 500 than, for instance, the optical assay using reduced pyridine nucleotides.

Time course and protein dependence of the enzyme reaction

The kinetics of formation of the ajmalicine stereoisomers during incubation of substrate with the crude enzyme preparation is shown in Fig. 2a. The rate is linear during the first 30 min and the endpoint is reached after about 80 min (see Fig. 2a). A substantial increase of immunoreactive material could not be produced by addition of further quantities of tryptamine under conditions where secologanin or NADPH were present in excess. Furthermore the reaction is clearly enzyme-dependent (Fig. 2b); maximal product formation was achieved at a concentration of 2 mg protein/ml incubation mixture. Fig. 2 shows clearly that the RIA gives results which are comparable with conventional enzyme assay. In this case, however, a simple enzyme assay was not available and the RIA procedure proved markedly superior to the tracer assay used previously [5, 6]. Furthermore, assays (Fig. 2a) could readily be conducted in triplicate since the semi-automated RIA has a capacity of over 300 samples per worker per day.

Effect of pH value on enzyme activity

Employing the RIA procedure, the effect of different pH values was studied under standard assay condition. Over the pH range 3–9 the following buffers were used: citrate–phosphate (3–6), phosphate (6–8) and Tris-HCl (8–9). The enzyme system showed a surprisingly wide pH tolerance with an optimum around pH 6.5 (Fig. 3). Tris-buffer was clearly inhibitory for the overall reactions as compared with phosphate buffer. Assay which were run at pH 6.5 without addition of buffer showed maximal conversion to the ajmalicine isomers.

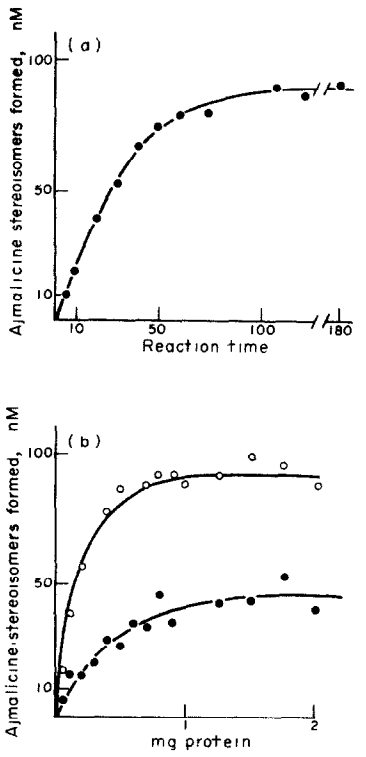


Fig. 2. (a) time course of the formation of ajmalicine stereoisomers as measured by the appearance of immunoreactive material (ajmalicine-RIA) (b) dependence of the formation of ajmalicine stereoisomers on the protein concn used. Incubation period 60 min. Two preparations of different specific activity were used (ajmalicine-RIA)

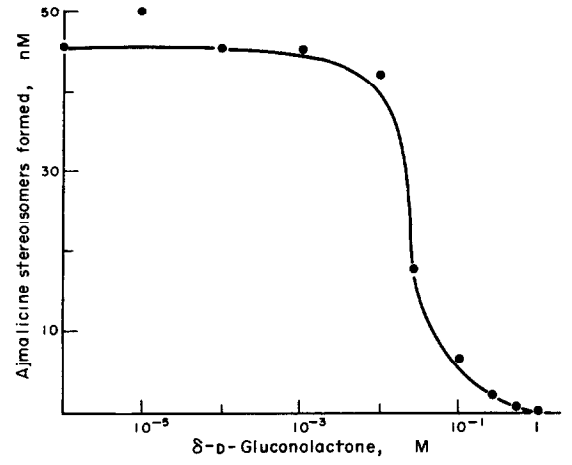


Fig. 3. pH-profile for the formation of ajmalicine stereoisomers under standard conditions. Incubation period 30 min (ajmalicine–RIA).

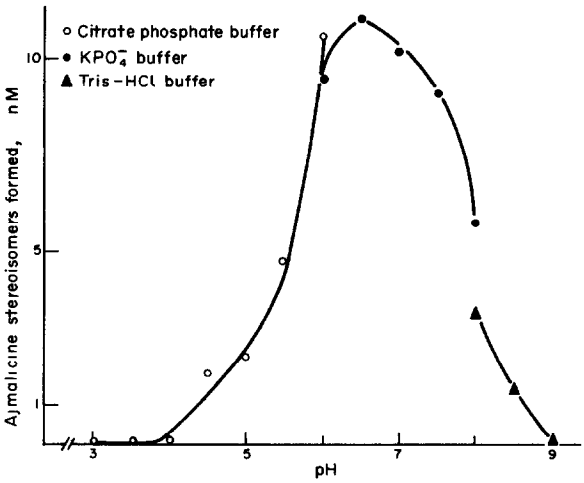


Fig. 4. Inhibition of the formation of ajmalicine stereoisomers by δ-D-gluconolactone. Incubation period 30 min (ajmalicine–RIA).

Substrate kinetics

Apparent K_m (mol/l.) values for the various substrates and reduced pyridin nucleotides were as follows: tryptamine 0.16×10^{-3}; secologanin 0.50×10^{-3}; NADPH 0.7×10^{-3}; NADH 1.5×10^{-3}. All K_m values were obtained from double reciprocal Lineweaver–Burk plots. With all the substrates and coenzymes tested the enzyme system showed normal Michaelis–Menten kinetics; no sigmoidal curves were observed. High concentrations of tryptamine in excess of 1 mM produced a slight inhibition of the overall reaction. The values were determined after an incubation period of 2 hrs. The K_m values again clearly show that NADPH is the preferred pyridine nucleotide for this reaction [6].

Substrate specificity

The enzyme system produced immunoreactive material by condensation of the following amines with secologanin: 5-fluoro- and 6-fluorotryptamine, 5-hydroxytryptamine, 5-methoxytryptamine and 7-methyltryptamine. With tryptamine but secologanic acid in place of secologanin, the reaction also yielded immuno-positive substances. Absolutely no reaction was observed with loganin or loganic acid instead of the secologanin derivatives. Since the enzyme seems to be non-specific with regard to the amine, it will be of interest to produce enzymatically ajmalicine derivatives containing substituted indole nuclei and test their pharmacological activity.

Enzyme stability

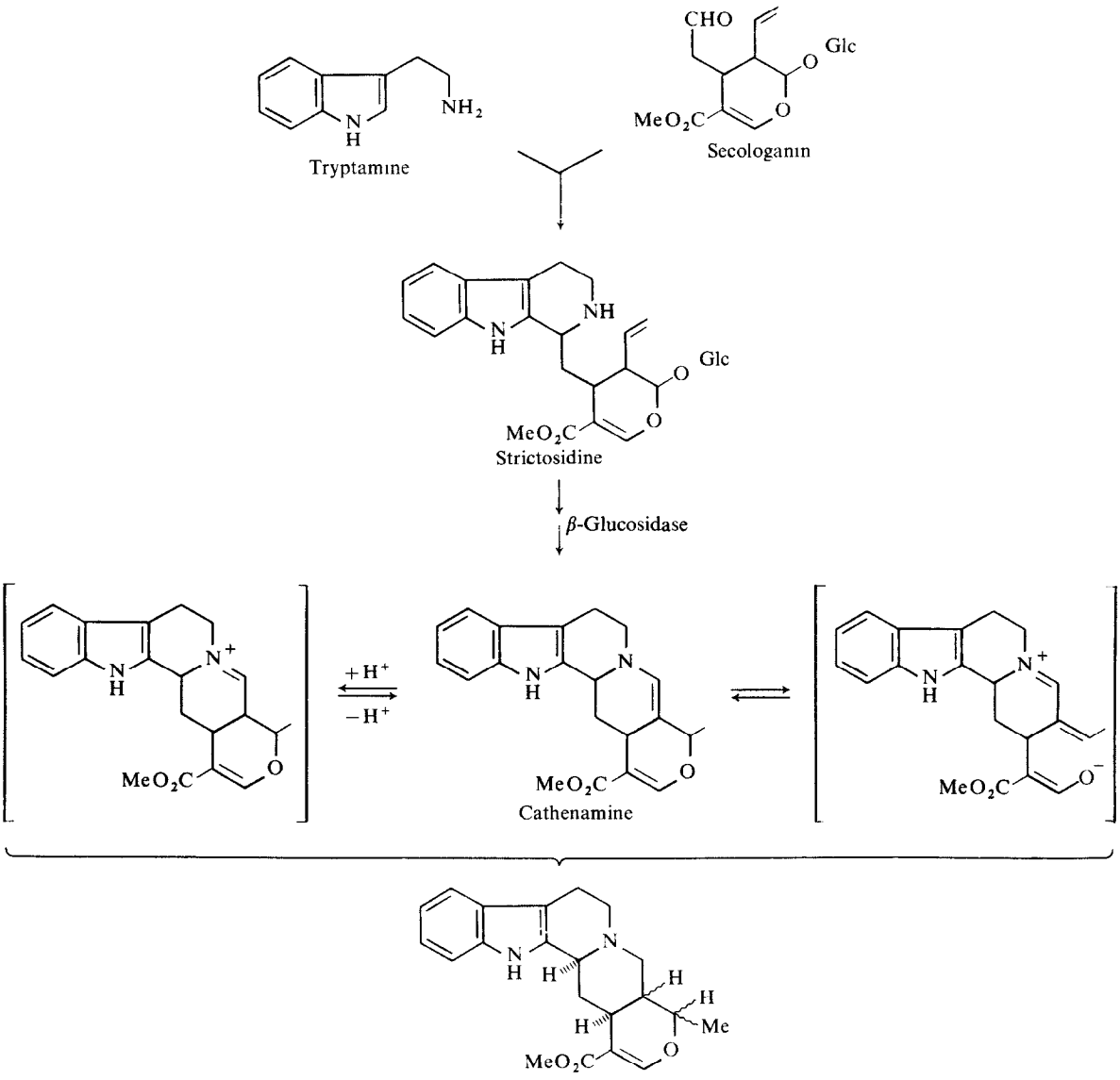
The deterioration of the enzyme system was followed over a period of 3 months at −20°. 50% loss of activity was observed after 5 weeks. The enzyme mixture was stable towards prolonged dialysis and the stability was increased by the addition of 10^{-3} M mercaptoethanol or dithiothreitol.

Inhibitors

The enzyme system is not affected by high concentrations (10^{-1} M) of Na^+, K^+, Li^+, Mg^{+2}, Ba^{+2}, but is strongly inhibited by Ca^{+2}, Fe^{+3}, Cu^{+2}, Mn^{+2}, Zn^{+2}, Co^{+2} at the same concentration. Since the conversion of the initially formed glucoside vincoside into subsequent products like cathenamine must involve a glucosidase [13] a glucosidase inhibitor was tested. δ-D-Gluconolactone is known to be a fairly specific inhibitor of plant glucosidases [14]. Fig. 4 shows the inhibition of the synthesis of ajmalicine and its isomers by the inclusion of δ-D-gluconolactone in the incubation mixture. A 70% inhibition of the reaction was achieved at 2×10^{-2} M concentration. This result supports the enzymic removal of glucose from vincoside as the next biosynthetic step, leading to an aminoaldehyde which can very plausibly be transformed into cathenamine [7], the stable product isolable in absence of NADPH.

DISCUSSION

The enzymic system [5, 6] capable of synthesizing ajmalicine from tryptamine and secologanin has been further characterized. The overall reaction must involve several enzymes. One was indirectly identified as a β-glucosidase by inhibiting the reaction with δ-D-gluconolactone, a known inhibitor of β-glucosidases [14]. This suggestion is supported by the recent independent finding [15] that in the ajmalicine-synthesizing complex a non-specific β-glucosidase might be involved. As has been shown previously [7], a second enzyme specifically reduces cathenamine (and its tautomerisation products) at the expense of NADPH to ajmalicine and its two isomers. The reaction sequence for ajmalicine biosynthesis in *Catharanthus roseus* is depicted in Scheme 1. In our opinion the importance of this investigation hinges on the demonstration that the radioimmunoassay can be used to monitor quantitatively the formation of end products by complex plant enzyme systems. Radioimmunoassay achieves a sensitivity comparable with that obtainable with radiotraces. The major advantages are the ease and precision with which quantitative figures can be obtained and the large throughput capacity of the automated procedure [9, 11, 12]. The only disadvantage, in our experience, is the fact that the setting up of an appropriate RIA system is extraordinarily timeconsuming. We



	19-H	20-H	
(a)	β	β	Ajmalicine
(b)	α	β	19-epiajmalicine
(c)	β	α	Tetrahydroalstonine

Scheme 1. Proposed biosynthetic pathway leading from tryptamine and secologanin to ajmalicine and its stereoisomers [after 3].

believe that in spite of this, RIA will prove to have wide application in studying the enzymology of secondary product formation by plant systems.

EXPERIMENTAL

Cell culture. Cell cultures of high ajmalicine- and serpentine-producing strains of *Catharanthus roseus* were used [8]. The cells were grown in a specially devised production medium [8] at 30° in 30 l. airlift fermenters with 0.3 vvm aeration After *ca* 20 days growth, the cell mass (200 g fr.wt/l.) was harvested by filtration, washed and immediately frozen with liquid N_2.

Enzyme extraction. Enzymes were extracted from the frozen cells and freed of low MW contaminants as in ref. [6].

Enzyme assay. The standard reaction mixture contained 50 μmol KPO_4^{-2} buffer (pH 7), 0.125 μmol tryptamine, 0.625 μmol secologanin 1 μmol NADPH (Boehringer, Mannheim), enzyme preparation (*ca* 1.5 mg) and H_2O in a total vol. of 0.5 ml. The reactions were conducted at 25 . A radioactive assay was conducted using tryptamine–bissuccinate-2-^{14}C (NEN) [0.2–0.6 μCi] as in ref. [6], and as solvent system (I) Me_2CO–petrol (bp 40–60°)–$NHEt_2$ (2.7.1). The plates were scanned using a Berthold (Wildbad) radioscanner II. Fluorescence was recorded using a Zeiss TLC fluorescence scanner KM 3 at an excitation wavelength at 365 nm.

Radioimmunoassay (RIA). From the enzyme incubation mixture, 0.2 ml was removed and diluted with MeOH to 5 ml. A slight ppt. which was devoid of any immuno-reactive material was allowed to settle and 0.1 ml of the MeOH soln was removed for analysis in the semi-automated ajmalicine RIA [9]. The assay was usually conducted in duplicate.

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