ALKALOID PRODUCTION IN CATHARANTHUS ROSEUS (L.) G. DON CELL CULTURES. XV.[1] SYNTHESIS OF BISINDOLE ALKALOIDS BY USE OF IMMOBILIZED ENZYME SYSTEMS

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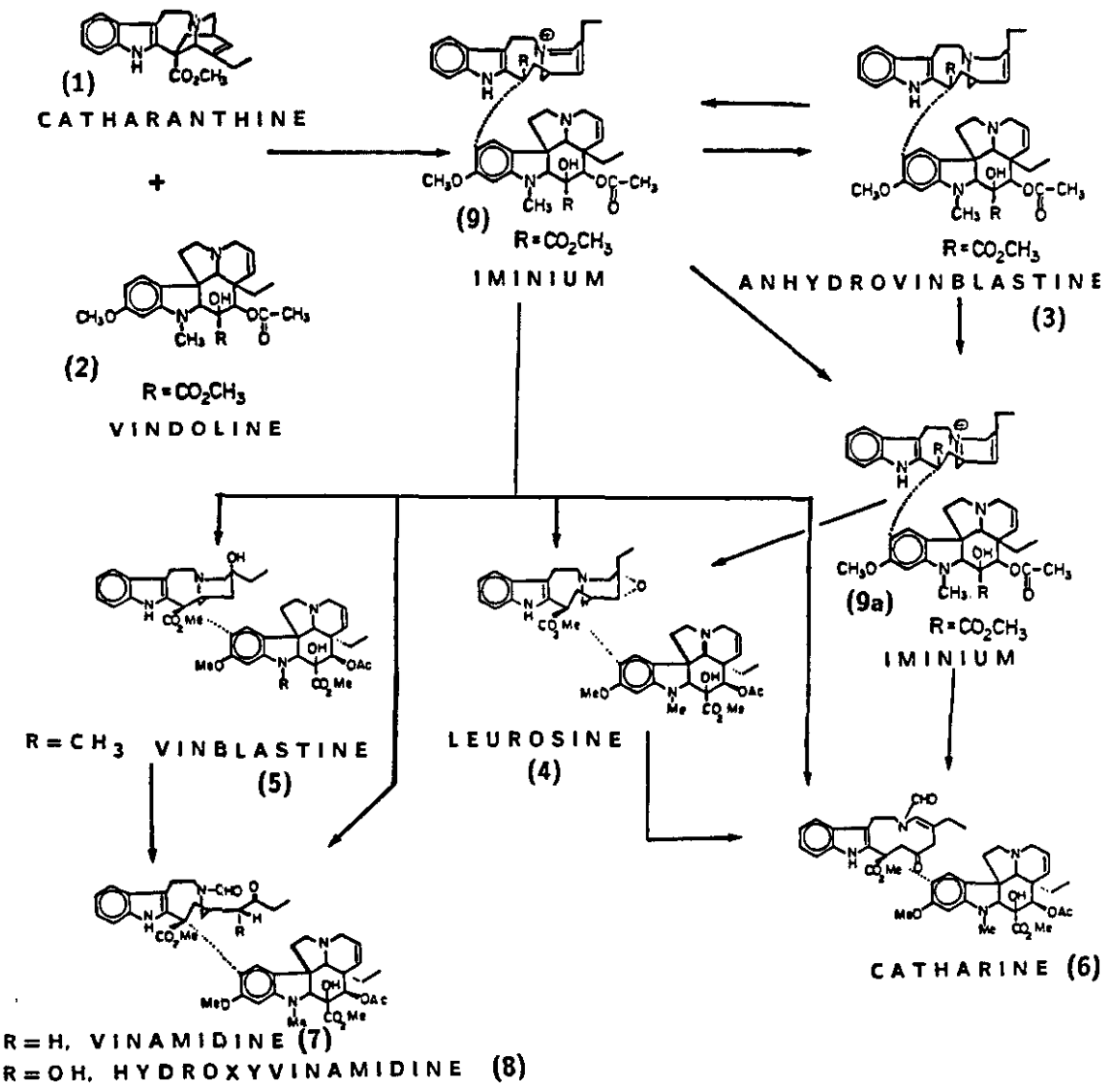
Abstract - Affinity gel bound enzyme systems obtained from Catharanthus roseus cell cultures were employed to establish a high yielding enzymatic system for the coupling of catharanthine (1) and vindoline (2) to 3',4'-anhydrovinblastine (3) and leurosine (4). Long term enzymatic activity can be preserved by this technique, an aspect which may prove important in subsequent utilization of such systems in large scale production.

In the accompanying publication[1], we described some experiments relating to the enzyme-catalyzed coupling of catharanthine (1) and vindoline (2) to an unstable dihydropyridinium intermediate (9) which is then enzymatically transformed to various bisindole alkaloids as summarized in Scheme 1. It was clear from that study that a multi-enzyme process is involved, that is, oxidative coupling of 1 and 2 to 9, followed by sequential reduction to anhydrovinblastine (3) and/or oxidation-reduction to leurosine (4), vinblastine (5), catharine (6), vinamidine (7) and hydroxyvinamidine (8). In order to optimize the yield of coupling of 1 and 2 to 9 and, in turn, to the various end products, we initiated some studies directed toward enzyme purification and/or immobilization so as to afford more optimum conditions for the desired transformations. Specifically, it was our desire to optimize, if possible, the enzyme-catalyzed coupling of 1 and 2 and obtain higher yields of appropriate end products. We wish to present our results relating to the latter coupling and illustrate that this approach can indeed allow a high yielding system portraying long term enzyme stability.

As mentioned in our accompanying publication[1], we had studied, in some detail, the influence of various cofactors, for example, β-NADP, NADPH, $MnCl_2$, FMN and FAD, in their ability to aid in the enzymatic coupling of 1 and 2. As FMN, FAD and β-NADP were shown to be important in this

Scheme 1. Overall Summary of Enzymatic Coupling of Catharanthine (1) and Vindoline (2) to Bisindole Alkaloids.



regard, we considered the application of affinity chromatography with these cofactors or their analogues as appropriate ligands in an attempt to obtain selective isolation of specific enzymes suitable for this purpose. The following commercially available affinity gels were selected: (a) FMN-Agarose, (b) β-NADP-Agarose, (c) Reactive Red 120-Agarose and (d) 2'5' ADP-Sepharose 4B. The two latter gels are known to have high affinity for β-NADP requiring enzymes[2,3].

Scheme 2. Procedure for Binding Catharanthus roseus Cell-Free Extract (C.F.E.) Proteins to Affinity Gels.

2',5'-ADP Sepharose

4 ml of 2',5'-ADP-sepharose (washed with 0.1 M Tris-HCl buffer solution, pH 7.2)
↓ 10 ml of CFE (after G25), amount of protein = 2.05 mg
↓ incubated for 16 h at 4° C

eluted with buffer solution (4 times), (1) - (4)

	Vol. of 0.1 M Tris-HCl buffer (ml)	Incubation time of each fraction (min)	Protein eluted (mg)
(1)	9.5	60	0.855
(2)	9.5	60	0.285
(3)	5.0	60	0.075
(4)	5.0	60	0.025

↓ 10 ml of CFE (after G25), amount of protein = 2.05 mg
↓ incubated for 16 h at 4° C

eluted with buffer solution (4 times), (5) - (8).

	Vol. of buffer (ml)	Incubation time of each fraction (min)	Protein eluted (mg)
(5)	9.5	60	1.235
(6)	9.0	60	0.45
(7)	10.0	60	0.20
(8)	9.5	60	0.0475

↓ 10 ml of CFE (after G25), amount of protein = 2.05 mg
↓ incubated for 16 h at 4° C

eluted with buffer solution (4 times), (9 - (12), and finally with β-NADP buffer solution (13).

	Vol. of buffer (ml)	Incubation time of each fraction (min)	Protein eluted (mg)
(9)	9.5	60	1.188
(10)	10.0	60	0.5
(11)	10.0	60	0.2
(12)	9.5	60	0.0
(13)	5 ml of 20 nM β-NADP buffer sol'n	16 h	0.028
(14)	10	60	0.00

Total amount of protein eluted (5.09 mg)

2',5'-ADP sepharose bound enzyme (enzyme conc. = 1.06 mg/4 ml of gel)
↓ stored at 4° C (27 days)
ADP-1
↓
ADP-2, ADP-3, ADP-4, ADP-5 (see Table IV)

Scheme 2 outlines the method that we have used in assessing the specific absorption/binding of proteins present in the desalted (Sephadex G25) crude cell-free extracts, as prepared according

to Scheme 2 in the accompanying publication[1], by 2',5'-ADP Sepharose 4B gels. Similar procedures were also employed with the other three affinity gels indicated above. It was of interest to note that <u>all</u> four affinity gels exhibited very strong binding of certain proteins. After the removal of non-specifically bound proteins by elution with buffer solutions, attempts to carry out specific desorption with the corresponding co-factors (FMN or β-NADP), even at high concentrations, were not successful. In fact, these proteins appeared to be immobilized by the gels. Table I illustrates the amount of protein bound to each of the four affinity gels.

Table I. Absorption of C.F.E. Protein with Various Affinity Gels.

Affinity Gel	Vol. of Affinity Gel (ml)	Total C.F.E. Protein Applied (mg)	Protein Eluted (mg)	Bound Protein (mg)	Concentration of Bound Protein (mg/ml)
FMN-Agarose	10	8.97	7.43	1.54	0.154
β-NADP-Agarose	1	2.18	1.68	0.5	0.5
Reactive Red 120-Agarose	10	5.69	2.0	3.69	0.369
2',5'-ADP-Sepharose-4B	4	6.15	5.09	1.06	0.265

These affinity gel bound enzymes were then evaluated for their ability to couple catharanthine (1) and vindoline (2), to afford the dihydropyridinium intermediate 9 (HPLC monitoring) and, in turn, the known alkaloids, 3',4'-anhydrovinblastine (3) and leurosine (4), obtained after borohydride reduction of the incubation mixture. The reaction mixtures employed are described in Table II while the incubation conditions, percent of coupling etc. are shown in Table III. Direct HPLC time-course monitoring indicated that <u>all</u> four affinity gel bound enzymes showed activity to couple catharanthine (1) and vindoline (2) (in the presence of FMN (1 eq.) and $MnCl_2$ (1 eq.)), to form initially the intermediate 9, and subsequently other metabolites.

As determined from storage and reuse of the bound enzymes (Table IV), it was shown that this technique of immobilization imparted a high degree of enzymatic stability. Maintenance of activity was demonstrated in repeated usages of these immobilized systems, as shown in Tables

Table II. Reaction Mixtures for Evaluation of Enzymatic Coupling of Catharanthine (1) and Vindoline (2) by Affinity Gel Bound Enzymes.

Reaction Mixture	Experiment No.					
	ADP-1,2,3	ADP-4	ADP-5	NADP-1 to NADP-5	R.red-1,2,3	FMN-1,2
Vindoline (mg)	3	3	6	3	3	3
Catharanthine. HCl (mg)	2.5 (1 eq.)	2.5 (+2.5*)	7.5 (1.5 eq.)	2.5 (1 eq.)	2.5 (1 eq.)	2.5 (1 eq.)
FMN (mg)	4.2	4.2	13.2	4.2	4.2	4.2
$MnCl_2$ (mg)	0.83	0.83	2.5	0.83	0.83	0.83
MeOH (ml)	0.5	0.5	1	0.5	0.5	0.5
Buffer (0.1M Tris-HCl, pH 7.2)	9	9	18	9	9	9
2',5'-ADP-Sepharose-4B bound enzyme (ml)	2	2	4	-	-	-
Reactive Red 120-Agarose bound enzyme (ml)	-	-	-	-	5	-
β-NADP-Agarose bound enzyme (ml)	-	-	-	0.5	-	-
FMN-Agarose bound enzyme (ml)	-	-	-	-	-	2.5

* Another equivalent of catharanthine.HCl (2.5 mg) was added after initial incubation of 80 min.

III and IV, the former illustrating the percent yield of coupling as well as yield of 3',4'-anhydrovinblastine (3) and leurosine (4).

Lowering the temperature of incubation from 26° to 4° C resulted in a small increase in the total yield of bisindole products (compare, for example, experiments ADP-1 and ADP-2 (38.5% versus 40.8%, Table III). Of more significance was the use of anaerobic incubation conditions for the coupling reaction. Thus under an argon atmosphere and at 4° C, total coupling yields of over 70% were attained (e.g. NADP-5 and ADP-3 in Table III). At the same time, the yield of 3',4'-anhydrovinblastine (3), obtained after a reductive work-up with $NaBH_4$, also increased.

Table III. Studies of Enzymatic Coupling of Catharanthine (1) and Vindoline (2) by Affinity Gel Bound Enzymes.

Exp.	Incubation Conditions[a]			% Yield of Coupling (Total)[b]	% Yield of (3)	% Yield of (4)
	Time (min)	Temp. (° C)	Atmos-phere			
ADP-1 (Scheme 2)	120	26	air	38.5	4.2	1.7
ADP-2 (Table IV)	60	4	air	40.8	10.8	5.2
ADP-3	160	4	Ar	74.4	20.4	5.3
ADP-4	200	4	Ar	90.3	37.7	13.4
ADP-5 (2X)	240	4	Ar	77.7	23.7	8.5
R.red-1	90	26	air	26.0	1.6	1.7
R.red-2	25	26	air	17.6	1.4	0.2
R.red-3	90	26	air	30.8	3.4	1.5
NADP-1	90	26	air	28.0	0.7	1.4
NADP-2	25	26	air	9.8	2.3	0.6
NADP-3	90	26	air	44.2	3.9	1.0
NADP-4	40	26	air	37.5	5.3	1.5
NADP-5	80	4	Ar	79.6	16.7	5.6
FMN-1	240 17 h	26 4	air air	33.3	-	1.4
FMN-2	27 h	4	air	66.4	-	1.0

a After appropriate incubation, the mixture was reduced with $NaBH_4$ prior to isolation of bisindole coupling products.

b Total coupling yield is based on vindoline (2) used and refers to total of bisindole alkaloids isolated by Sephadex LH20 chromatography. Only 3',4'-anhydrovinblastine (3) and leurosine (4) were characterized while other unknown bisindole compounds (mass spectrometric analysis) are not yet elucidated.

This is consistent with the observed formation and accummulation of the intermediate 9 obtained by direct HPLC analysis during time course monitoring of the reaction.

Table IV. Storage and Reuse of Bound Enzyme Systems for Evaluation of Coupling Activity.

Exp.	Time of Storage at 4° C in Days (unless otherwise stated)
ADP-2[1]	5
ADP-3	9
ADP-4	1
ADP-5	14
R-red-2[2]	3 h
R-red-3	4
NADP-2[2]	3 h
NADP-3	4
NADP-4	2
NADP-5	14
FMN-2[2]	7

1 To obtain ADP-2, the bound enzyme from the ADP-1 experiment (120 min incubation time, Table III) was recovered by filtration on a sintered glass funnel, washed with TRIS-HCl buffer, stored at 4° C for 5 days and then used for evaluation of coupling activity. In similar fashion, ADP-3 represents recovered enzyme from the ADP-2 experiment (Table III), stored at 4° C for 9 days and evaluated for coupling activity, etc.

2 R-red-2, NADP-2 and FMN-2 represent bound enzymes recovered from R-red 1, NADP-1, FMN-1 experiments respectively as described in Table III, and similarly evaluated for coupling activity. Preparation of R-red-1, NADP-1 and FMN-1 follows the preparation outlined for ADP-1 (Scheme 2).

It is significant that when a second equivalent of catharanthine hydrochloride was added after an initial incubation of 80 min (see experiment ADP-4 in Tables II and III), a high yield (90.3%) of coupling was observed and the best yields for the bisindole alkaloids 3 and 4 were realized. It appeared that catharanthine, by virtue of its more rapid enzymatic transformation to other products, relative to vindoline, is consumed more rapidly and therefore is not

entirely available for coupling. Indeed, we have shown, by HPLC monitoring, that catharanthine, when exposed to this enzymatic mixture, is more rapidly converted to other unidentified products than is vindoline.

It is also clear from Table III that the best immobilized system for the coupling, and particularly for the biosynthesis of 3 and 4, is the ADP-bound enzyme(s). Furthermore, the stability of intermediate 9 is highest at low temperature (4° C versus 26° C) and under an inert atmosphere (argon versus air).

In conclusion, the above studies have revealed that enzymes capable of coupling of catharanthine (1) and vindoline (2) to the unstable dihydropyridinium intermediate 9, and in turn, to the bisindole alkaloids 3',4'-anhydrovinblastine (3) and leurosine (4) can be effectively immobilized to provide a stable and high yielding system. Furthermore, immobilized enzyme systems can be stored at 4° C for long time periods and therefore their use for repeated coupling reactions so as to accumulate end products is possible. The use of this technique to achieve similar immobilized systems for the other bisindole alkaloids particularly vinblastine (5) is under consideration in our laboratory.

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REFERENCES

1. Part XIV. J. P. Kutney, C. A. Boulet, L. S. L. Choi, W. Gustowski, M. McHugh, J. Nakano, T. Nikaido, H. Tsukamoto, G. M. Hewitt, and R. Suen. 1987. Accompanying publication.
2. M. D. Scawen, J. Darbyshire, M. J. Harvey, and T. Atkinson, Biochem. J., 1982, 203, 699.
3. P. Bordelius, P. O. Larsson, and K. Mosbach, Eur. J. Biochem., 1974, 47, 81.

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