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CUCURBITACIN B Δ^{23}-REDUCTASE FROM *CUCURBITA MAXIMA*

II. COFACTOR REQUIREMENTS, ENZYME KINETICS, SUBSTRATE SPECIFICITY AND OTHER CHARACTERISTICS*

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SUMMARY

1. Co-factor requirements of the cucurbitacin B Δ^{23}-reductase enzyme *viz.* electron donors (NADPH and NADH) and metal co-factors (Mn^{2+}, Fe^{2+} and Zn^{2+}) were determined.

2. Michaelis constants for cucurbitacin B and NADPH were determined. The K_m values for cucurbitacin B and NADPH are $8.01 \cdot 10^{-4}$ M and $6.04 \cdot 10^{-5}$ M, respectively, at pH 6.65.

3. The first-order velocity constant of the enzyme, as determined by both the differential method of LETORT and the first-order rate equation, was found to be in the order of $2.5 \cdot 10^{-8}$ sec^{-1}.

4. The energy of activation for the reduction of cucurbitacin B is 7615 cal·$mole^{-1}$ and a Q_{10} value of 1.52 was found.

5. The substrate specificity of cucurbitacin B Δ^{23}-reductase was investigated. Relative specificity with respect to the cucurbitacins with Δ^{23}-bonds in their side chains was found. Desmosterol and lanosterol showed no activity.

6. The reversibility of the reaction was investigated and the standard free energy change for the reduction reaction was found to be -2196 cal·$mole^{-1}$.

INTRODUCTION

The important role of cucurbitacin B Δ^{23}-reductase (NAD(P)H:cucurbitacin B Δ^{23}-oxidoreductase) in the breakdown of toxic[1] bitter principles in plants has been pointed out[2]. Reliable and accurate assay methods for this enzyme as well as its isolation and purification have been described[2]. In order to obtain more information

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about the properties of the enzyme, kinetic studies and studies concerning other enzyme characteristics were performed.

MATERIALS AND METHODS

Materials

Pure cucurbitacin A, B, C, D, E, F, and an impure β-D-glucoside of cucurbitacin E were supplied by Dr. S. REHM of the Horticultural Research Institute, Roodeplaat, Pretoria. Green Hubbard fruits were obtained from the local market and from Mr. R. STARKE of Malelane, Transvaal. NADPH and $NADP^+$ were obtained from C. F. Boehringer, Mannheim, Germany. All other chemicals were of analytical grade quality.

Reductase enzyme

Cucurbitacin B Δ^{23}-reductase was purified from immature fruits of the Green Hubbard variety of *Cucurbita maxima* as described elsewhere[2].

Assay of reductase activity

The spectrophotometric assay method at 340 mμ was employed for these studies[2]. One unit of reductase enzyme was defined as that amount which would catalyse the reduction of 1 μmole of cucurbitacin B or the oxidation of 1 μmole of NADPH per min at 25° under optimum reaction conditions[2] *viz.* pH 6.65 (pH optimum) and with $MnCl_2$ as metal cofactor (final concn. of 1 mM). All determinations were done at zero-order kinetics as described previously[2]. Protein concentration was estimated from determinations of nitrogen by the micro-Kjeldahl method.

During enzyme kinetic studies, assays with varying cucurbitacin B concentrations were performed at a constant enzyme concentration (0.03 mg protein/ml) and a NADPH concentration of 0.56 μmole/3 ml (0.19 mM). Assays with varying NADPH concentrations were performed at a cucurbitacin B concentration of 4.2 μmoles/3 ml (1.4 mM) and a constant enzyme concentration of 0.03 mg protein/ml. Under these conditions the reactions were of the first order with respect to the substrate of which the concentration was varied, and of zero order with respect to the other substrate which was present at a high and constant concentration.

RESULTS

Pyridine nucleotide requirement

NADPH and NADH were compared as co-factors for the reductase enzyme. Determinations at constant enzyme concentration and at equal molar concentrations of NADPH and NADH, employing the spectrophotometric assay method at 340 mμ, indicated that NADPH was the better electron donor by a factor of 2.58. This ratio was obtained from reaction velocities of $1.02 \cdot 10^{-2}$ $m\mu M \cdot sec^{-1}$ for NADH and $2.6 \cdot 10^{-2}$ $m\mu M \cdot sec^{-1}$ for NADPH.

Metal co-factor requirement

Crude enzyme preparations showed signs of reductase activity when assayed with the spectrophotometric method at 340 mμ, but dialysed preparations and preparations containing EDTA showed no activity.

TABLE I

EFFECT OF METAL CO-FACTORS ON CUCURBITACIN B Δ^{23}-REDUCTASE ACTIVITY

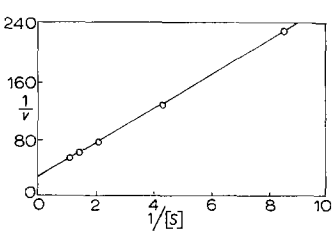
Metal co-factor	*Metal ion concn. (mM)*	*Reductase activity (units/mg enzyme × 10^5)*	*Reaction velocity ($m\mu M \cdot sec^{-1} \times 10^2$)*
Mn^{2+}	1	7.64	42.46
Mn^{2+}	3	7.46	41.44
Mn^{2+}	0.25	7.52	41.80
Fe^{2+}	1	1.52	8.43
Zn^{2+}	1	0.70	3.86
Zn^{2+}	3	0.64	3.57
Zn^{2+}	0.25	0.57	3.14
Co^{2+}	1	0.52	2.86
Mg^{2+}	1	0.22	1.22
Ca^{2+}	1	0.13	0.71
K^+	1	0.12	0.64
Na^+	1	0.09	0.50
Na^+	10	0.58	3.22
Cu^{2+}	1	0.08	0.43
No metal ion	Absent	0.00	0.00

The effect of the various metal co-factors on the activity of purified reductase enzyme was determined at the pH optimum (6.65) and at 25° in 0.05 M maleic acid–NaOH buffer. The results are summarized in Table I.

The concentration of metal ion in the reaction mixture had a marked effect on the activity. The optimum metal-ion concentration for Mn^{2+} and Zn^{2+} was found to be 1 mM. It is obvious that Mn^{2+} is the best metal co-factor for reductase activity with Fe^{2+} and Zn^{2+} the second and third best, respectively. High concentrations of a monovalent ion such as Na^+ caused some activity. Compared to Zn^{2+}, changes in pH had a much greater influence on the effectiveness of Mn^{2+} as co-factor. Studies performed in 0.05 M Tris–HCl buffer at pH 7.5, showed that Mn^{2+} and Zn^{2+} were more or less equally effective while in 0.05 M maleic acid –NaOH buffer at pH 6.65, Mn^{2+} was a much better co-factor than Zn^{2+}.

Enzyme kinetics

The results of enzyme kinetic studies were analysed according to the graphical



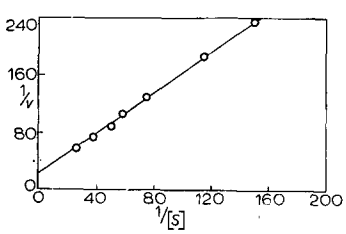


Fig. 1. Lineweaver–Burk plot of the effect of cucurbitacin B concentration (S) on cucurbitacin B Δ^{23}-reductase activity.

Fig. 2. Lineweaver–Burk plot of the effect of NADPH concentration (S) on cucurbitacin B Δ^{23}-reductase activity.

method of Lineweaver–Burk[3] and are illustrated in Figs. 1 and 2. Michaelis constants determined from EADIE plots[4] were similar to those obtained from Lineweaver–Burk plots. In all the determinations v was expressed as $m\mu M \cdot sec^{-1}$ and [S] as mM. The maximum reaction velocity (V) and K_m values obtained from the different graphical methods are summarized in Table II.

The average K_m values for cucurbitacin B and NADPH were $8.01 \cdot 10^{-4}$ M and $6.04 \cdot 10^{-5}$ M, respectively.

TABLE II

V AND K_m VALUES FOR CUCURBITACIN B AND NADPH OBTAINED FROM EADIE AND LINEWEAVER–BURK PLOTS

V is expressed in $m\mu M \cdot sec^{-1}$ and K_m in M.

Substrate	*Eadie plots*		*Lineweaver–Burk plots*		*Modified Lineweaver–Burk plots*	
	V	K_m	V	K_m	V	K_m
Cucurbitacin B	$3.28 \cdot 10^{-2}$	$7.79 \cdot 10^{-4}$	$3.57 \cdot 10^{-2}$	$8.40 \cdot 10^{-4}$	$3.32 \cdot 10^{-2}$	$7.84 \cdot 10^{-4}$
NADPH	$4.24 \cdot 10^{-2}$	$6.00 \cdot 10^{-5}$	$4.20 \cdot 10^{-2}$	$5.95 \cdot 10^{-5}$	$4.39 \cdot 10^{-2}$	$6.18 \cdot 10^{-5}$

First-order velocity constant

The first-order velocity constant (k) was determined according to the method of Letort[5] in which the straight line relationship: $\log_{10}(-dc/dt) = \log_{10}k + n \cdot \log_{10}c$, is employed, where $-dc/dt$ is the velocity in $M \cdot sec^{-1}$, k is the n^{th} order rate constant and c is the cucurbitacin B concentration. In Fig. 3, $\log_{10}(-dc/dt)$ is plotted against $\log_{10} c$.

The first-order rate constant (k) was also determined by employing the first-order rate equation[5]. The first-order velocity constant determined by the differential method is $2.61 \cdot 10^{-8}$ sec^{-1} while the first-order rate equation gives values of $2.45 \cdot 10^{-8}$ sec^{-1} and $2.53 \cdot 10^{-8}$ sec^{-1} at 5 and 10 min respectively. The average first-order velocity constant is $2.53 \cdot 10^{-8}$ sec^{-1}. The slope of the LETORT plot is 1.004. This fact

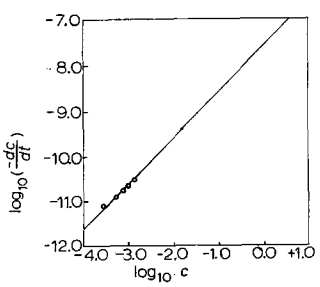


Fig. 3. The differential method for the determination of the first-order velocity constant of cucurbitacin B Δ^{23}-reductase. The velocity of the reaction ($-dc/dt$) was measured at different cucurbitacin B concentrations (varying between 0 and 1.17 mM) and a NADPH concentration of 0.187 mM at the beginning of each reaction by employing the spectrophotometric assay method at 340 mμ, under optimum reaction conditions. An enzyme concentration of 0.038 mg/ml was used. The intercept on the vertical axis where $\log_{10}c$ is equal to zero gives the velocity constant (in this case it is the first-order velocity constant). The slope of the line gives the order "n" of the reaction.

clearly demonstrated that, under the specific conditions for determining the K_m value for cucurbitacin B, the reaction was of the first-order with respect to cucurbitacin B, as was found before.

Reversibility of the reaction and the standard free energy change

The composition of the reaction mixture employed for the determination of the reversibility of the reduction of cucurbitacin B to 23,24-dihydrocucurbitacin B is given in Table III. For the purpose of comparison the forward reaction was performed under identical conditions.

TABLE III

COMPOSITION OF THE REACTION MIXTURE FOR THE CONVERSION OF 23, 24-DIHYDROCUCURBITACIN B TO CUCURBITACIN B

The composition of the reaction mixtures used in the blank determination was similar to those given in this table except that the enzyme solution was replaced by 0.05 M maleic acid–NaOH buffer (pH 6.6) containing 0.10 M NaCl. The pH of the reaction mixtures was 6.65 and the temperature 25°. Under the specific conditions of Table III, the reaction was zero order with respect to 23,24-dihydrocucurbitacin B and NADPH.

Components	*Sample cell (ml)*	*Reference cell (ml)*
0.05 M maleic acid–NaOH buffer (pH 6.55)	1.55	1.55
$MnCl_2$, final concn., 1 mM	0.20	0.20
0.057 mg enzyme in 0.05 M maleic acid–NaOH buffer (pH 6.6) containing 0.10 M NaCl	0.50	0.50
$NADP^+$, 0.97 μmoles	0.50	0.50
23,24-Dihydrocucurbitacin B in methanol, 4.4 μmoles	0.25	—
Abs. methanol	—	0.25
Total volume	3.00	3.00

The reaction velocity for the dehydrogenation of 23,24-dihydrocucurbitacin B was $0.32 \cdot 10^{-2}$ mμM$\cdot$sec^{-1}, while at the same enzyme concentration the forward reaction had a velocity of $13.07 \cdot 10^{-2}$ mμM$\cdot$sec^{-1}. The calculated ratio of reduction/dehydrogenation was 40.8:1. Thus, the reduction of curcubitacin B to 23,24-dihydrocucurbitacin B is fairly irreversible.

The standard free energy change (ΔF_1^0) for the reduction of cucurbitacin B by the reductase enzyme was determined by using the ratio mentioned above[6] which actually is a ratio of the zero-order velocity constants for the forward and reverse reactions. A value of -2196 cal$\cdot$mole^{-1} was found.

Energy of activation

The effect of temperature on reductase activity was determined by employing the spectrophotometric assay method at 340 mμ. The energy of activation for this reaction, as catalysed by the reductase enzyme, was determined from the slope of the straight line in Fig. 4, where $\log_{10}$ (kx10^3) was plotted against $1/T$ (Arrhenius plot)[5] and was found to be 7615 cal$\cdot$mole^{-1}. A Q_{10} value of 1.52 was found .

Substrate specificity

The relative specificity of the reductase enzyme with respect to the different

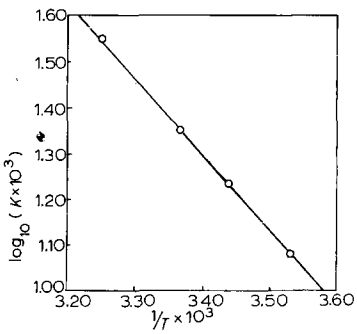


Fig. 4. Determination of the energy of activation for the reduction of cucurbitacin B by cucurbitacin B Δ^{23}-reductase. In all the determinations of reductase activity at the different temperatures (11.5, 18.1, 25.6 and 35.1°) the reaction was zero-order with respect to both cucurbitacin B and NADPH. The slope of the line is equal to $-E/2.303R$, as derived from the Arrhenius equation[6]. (E = activation energy, cal·mole^{-1}; k = zero-order velocity constant, mμM·sec^{-1}; T = absolute temperature, °K).

cucurbitacins with Δ^{23}-bonds in their side chains is given in Table IV. Unfortunately, bitter principles with unsaturated side chains, other than cucurbitacin A, B, C, D, E, F and glycosides of bitter principles, other than the β-D-glucosides of cucurbitacin E, were not available for these studies. Desmosterol and lanosterol were also tested as substrates in the form of albumin dispersions[7] but no activity was found in either case.

DISCUSSION

It is evident that the equilibrium of the reduction of cucurbitacin B to 23,24-dihydrocucurbitacin B, at pH 6.65, lies far to the right. This is supported by the

TABLE IV

THE SPECIFICITY OF THE REDUCTASE ENZYME WITH RESPECT TO THE CUCURBITACINS

The spectrophotometric assay method at 340 mμ was employed under the optimum reaction conditions for the reduction of cucurbitacin B *viz.* pH 6.65 and 1 mM $MnCl_2$ (metal co-factor). The reactions were zero-order with respect to the cucurbitacins and NADPH which were present at concentrations of 1.4 mM and 0.19 mM, respectively.

Substrate	*Reductase activity (units/ml purified enzyme × 10^5)*
β-D-Glucoside of cucurbitacin E*	101.1
Cucurbitacin B	77.1
Cucurbitacin E**	9–15
Cucurbitacin D	8.9
Cucurbitacin F	2.6
Cucurbitacin A	2.3
Cucurbitacin C	1.2

* Impure β-D-glucoside of cucurbitacin E was used.

** Cucurbitacin E at unknown concentration due to low solubility in reaction mixtures. The activity value is a rough estimation.

fairly high negative value reported for the standard free energy change (ΔF^0_1) for the reduction of cucurbitacin B.

The relative low value for the energy of activation indicated that the energy barrier for the reduction of cucurbitacin B was probably effectively lowered by the reductase enzyme.

The Michaelis constants for cucurbitacin B and NADPH were relatively low and are comparable to K_m values reported for many other oxidoreductases from plant and animal tissues.

The primary bitter principles *viz.* cucurbitacin B and E were found to be the best substrates for the reductase enzyme. The fact that the β-D-glucoside of cucurbitacin E showed a very high activity, indicated that glycosides of bitter principles which were found to be much more soluble in water than their aglycons, might be the most important substrates in the plant. However, due to the high β-glucosidase (elaterase) activity of the fruits of the Green and Golden Hubbard varieties of *C. maxima*, only aglycons of the bitter principles, which occur at very low concentrations, were found in these plants in the early stages of development (S. Rehm, personal communication). In later stages of development no bitter principles could be detected in the fruits[8], possibly due to a very effective catabolic process[2] in these plants in comparison with many other species of the Curbitaceae-family which have no breakdown pathway for bitter principles[8]. In most cases the bitter principles occurring in these species are glycosides of the various cucurbitacins[8].

Since all the bitter principles that were tested showed some activity as substrates for cucurbitacin B Δ^{23}-reductase, it is quite possible that only one reductase enzyme is responsible for the reduction of the Δ^{23}-bonds in the bitter principles with unsaturated side chains.

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