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PURIFICATION AND PROPERTIES OF EMODIN DEOXYGENASE FROM *PYRENOCHAETA TERRESTRIS*

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Key Word Index—*Pyrenochaeta terrestris*; Sphaeropsidaceae; emodin; chrysophanol; deoxygenase.

Abstract—Emodin deoxygenase, which catalyses the reduction of emodin to chrysophanol, was purified 17-fold from crude extracts of *Pyrenochaeta terrestris*. The M_r of the enzyme was 103 000. Upward curvature was exhibited by the plot of rate vs concentration of the crude extract. The protein fraction obtained from gel filtration with Sephadex G-75 was activated by ATP plus a low M_r fraction; ATP alone or low M_r fraction alone did not increase its activity. The K_m for NADPH of the crude extract was 3 μM, that after NADPH gel filtration of the crude extract was 1.5 mM. It is proposed that ATP plus an unidentified factor increase emodin deoxygenase activity by lowering the K_m for NADPH. Iron II, which increased activity of the crude extract, inhibited activity of the partially purified enzyme by 95%. Sulphydryl reagents inhibited activity by 90%. Partially purified emodin deoxygenase activity was low in the absence of mercaptans and was increased eight-fold by the addition of dithiothreitol. It is proposed that a pair of thiol groups is required for activity and that they occur in the disulphide form in the absence of mercaptans.

INTRODUCTION

Radioactive incorporation studies in *Penicillium oxalicum* supported the step emodin to chrysophanol in the pathway of biosynthesis of the secalonic acids [1]. This conversion was demonstrated in a cell-free system from *Pyrenochaeta terrestris* [2], which produces secalonic acids [3]. Deuterium incorporation studies demonstrated the direct reduction of emodin by NADPH and supported a mechanism involving keto–phenol isomerization [4]. Emodin deoxygenase activity of the crude extract was increased by removing air and by the addition of NADPH, iron II, ATP and mercaptoethanol [2].

In the present work emodin deoxygenase was partially purified and the effects of the above additions after partial purification were compared with the effects on the activity of the crude extract. It was found that the effects of NADPH, iron II and ATP were different in partially purified extracts from their effects in the crude extracts. The results suggest regulatory mechanisms of the enzyme.

RESULTS AND DISCUSSION

Emodin deoxygenase was purified 17-fold from a crude extract from *P. terrestris*; the yield was 11% (Table 1). A major band and several smaller bands were observed after SDS PAGE of the purified enzyme. The position of the major band corresponded to the position of ovalbumin with M_r 45 000. The M_r of emodin deoxygenase, determined by gel filtration, was 103 000. If the major band in the SDS gel is emodin deoxygenase, the enzyme may be a dimer with a subunit M_r of 45 000–50 000.

The crude extract was stable for 4 months at −20° [1] but the partially purified enzyme was less stable. The enzyme after ammonium sulphate fractionation and Sephadex G-75 column chromatography lost 40% of its activity in 24 hr at 5° and 88% of its activity in 24 hr at −20°. The loss of activity after 45 min at 25° was 67%. With 1.7 mM NADPH, the loss of activity under the latter conditions was 15%.

The optimum pH of the enzyme purified by the Bio-Gel A-0.5 m gel filtration step had a pH optimum at 6.5. The pH values at one-half maximum activity were 6.0 and 7.7; there was a shoulder in the pH activity curve at pH 7.0–7.5. A marked upward curvature was observed in the plot of rate of reaction vs concentration of crude extract (Fig. 1). The plot of rate vs concentration after fractionation with ammonium sulphate and Bio-Gel A-0.5 m (Bio-Gel enzyme) was linear.

Hyperbolic plots of rate vs NADPH concentration were obtained with crude extracts and with the enzyme fractionated by gel filtration with Sephadex G-75 (G-75 enzyme). The K_m of NADPH was 0.0029 ± 0.0007 mM in the crude extract and 1.5 ± 0.1 mM with G-75 enzyme suggesting a markedly greater affinity of the enzyme in the crude extract for NADPH.

ATP increased emodin deoxygenase activity of the crude extract over a broad concentration range (Fig. 2). Maximum activity was observed from 30 to 70 mM ATP; there was little inhibition at the highest ATP concentrations. The concentration for half maximum activity was *ca* 8 mM ATP. Addition of 0.83 mM ATP alone or addition of the low M_r fraction from the G-75 gel filtration

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Table 1. Partial purification of emodin deoxygenase from *Pyrenochaeta terrestris*

	Protein (mg)	Activity (nKat)	Yield %	Sp. activity (pKat mg^{-1})	Purification (-fold)
Crude extract	189	1.51	100	8.0	1.0
$(NH_4)_2 SO_4$	71	1.32	86	18.5	2.3
Sephadex G-75	50	1.01	67	20.5	2.6
DE-52	31	0.82	54	26	3.3
Sephadex S-200	7.8	0.47	31	60	7.5
Matrex Green A	1.2	0.17	11	138	17.3

See Experimental for purification procedures.

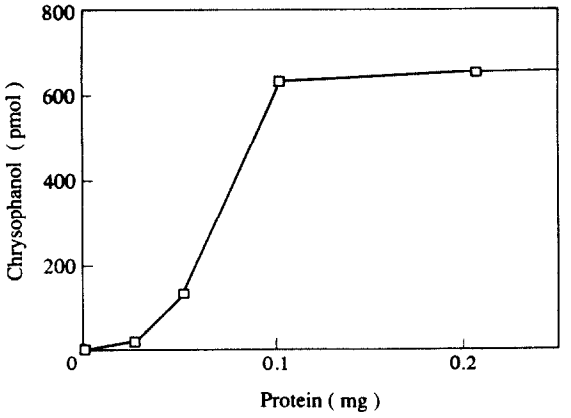

Fig. 1. Emodin deoxygenase activity as a function of concentration of crude extract. The concentration of NADPH was 0.75 mM, ATP 6.3 mM. The total volume was 0.6 ml. Incubation time 15 min.

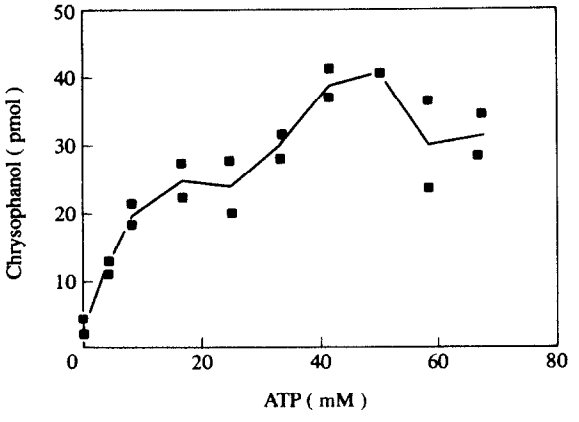

Fig. 2. Effect of concentration of ATP on emodin deoxygenase activity of crude extract. The assay mixture contained 0.24 mM NADPH, 42 mM Mg^{2+} and 0.4 mg protein in a final volume of 0.6 ml. Incubation time 30 min.

alone to G-75 enzyme did not affect emodin deoxygenase activity (Table 2). However, addition of fraction 2 and 0.83 mM ATP together gave a 220% increase in activity. ATP at 4.2 and 8.3 mM inhibited activity of the G-75 enzyme (Fig. 3) whereas emodin deoxygenase was activated in the crude extract in this concentration range (Fig. 2). In the experiment shown in Fig. 3, stimulation of activity by the addition of fraction 2 with 0.83 mM ATP was 35% compared to 220% in the experiment in Table 2, with substantially greater stimulation at the inhibitory concentrations of ATP (Fig. 3).

Table 2. Effect of ATP, fraction 2 and ADP on emodin deoxygenase activity of G-75 enzyme

Additions*	Chrysophanol (pmol)
G-75 enzyme	31
G-75 enzyme + ATP	31
G-75 enzyme + fraction 2	29
G-75 enzyme + fraction 2 + ATP	100
G-75 enzyme + fraction 2 + ADP	38

See Experimental for assay procedure and preparation of G-75 enzyme and fraction 2.

*The concentration of NADPH was 3.3 mM and the ATP or ADP concentration was 0.83 mM. The volume of fraction 2 added was 75 μl. Protein added was 0.19 mg. Total volume was 0.3 ml. Incubation time 90 min.

A low M_r component present in fraction 2 and ATP coactivate emodin deoxygenase. At higher concentrations of ATP the low M_r component also prevents inhibition of emodin deoxygenase activity by ATP. The increase in activity resulting from the interaction of low M_r compound with ATP may reflect a decrease in the K_m for NADPH, since the crude extract which contains the low M_r component has a 500-fold lower K_m for NADPH (with added ATP) than G-75 enzyme. The interaction between the low M_r component and ATP may contribute to the upward curvature in the rate vs concentration of crude extract plot (Fig. 1). The low activity at low crude extract concentration can be attributed to inhibition by added ATP (6.3 mM). The concentration of the low M_r component in the incubation mixture would increase as the concentration of crude extract increased resulting in decreased inhibition and initiation of activation by ATP. The identity of the low M_r component is at present unknown.

The addition of iron II, which increased the activity of the crude extract [2], inhibited the Bio-Gel enzyme (Table 3). The activation of emodin deoxygenase by iron II apparently involves interaction with a substance in the crude extract removed during the partial purification of the enzyme.

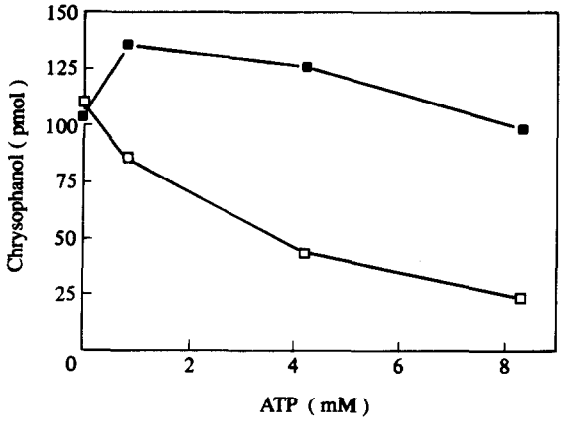

Fig. 3. Effect of concentration of ATP on emodin deoxygenase activity of G-75 enzyme with (solid squares) and without (open squares) addition of fraction 2. The assay mixture contained 1.7 mM NADPH, 100 μl fraction 2 and 0.15 mg protein in a final volume of 0.3 ml. Incubation time 90 min.

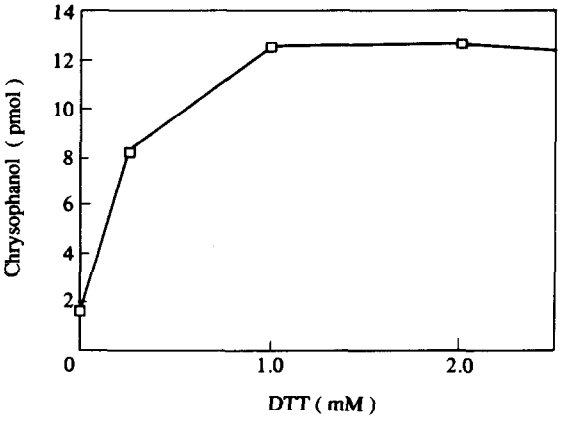

Fig. 4. Effect of DTT on emodin deoxygenase activity of Bio-Gel enzyme prepared without DTT or 2-mercaptoethanol. The Bio-Gel enzyme was preincubated at various concentrations of DTT at 25° for 25 min and emodin deoxygenase activity measured. The assay mixture contained the preincubation mixture (0.097 mg protein), 1.7 mM NADPH and 5 μM ^{3}H-emodin in a total volume of 0.3 ml. Incubation time 90 min.

Table 3. Effect of iron II and ATP on emodin deoxygenase activity of crude extract and Bio-Gel enzyme

Additions*	Chrysophanol (pmol) Crude extract	Bio-Gel enzyme
$-Fe^{2+}$, − ATP	46	42
$-Fe^{2+}$, + ATP	217	39
$+Fe^{2+}$, − ATP	214	2
$+Fe^{2+}$, + ATP	303	2

*The assay mixture contained 1.7 mM NADPH and 4.2 mM ATP. Protein content was 0.44 mg. Total volume was 0.3 ml. Incubation time 60 min.

Table 4. Effect of thiol reagents on emodin deoxygenase activity of Bio-Gel enzyme*

Thiol reagent (mM)	Chrysophanol (pmol) − DTT	+ DTT
—	23	30
p-HOMB	2	2
Diamide	2	6
N-Ethylmaleimide	2	2
Cd^{2+}	2	3

See Experimental for assay procedure and preparation of Bio-Gel enzyme,

*Bio-Gel enzyme containing 50 mM Tris–HCl pH 7.6, 1 mM EDTA, 0.50 mM dithiothreitol (DTT) and 0.075 mg protein was preincubated with 3 mM diamide, *N*-ethylmaleimide, cadmium II chloride, or 1.0 mM *p*-hydroxymercuribenzoate (*p*-HOMB). NADPH (1.7 mM) and 5 μM ^{3}H-emodin were added with or without 2.0 mM DTT (*p*-HOMB) or 10 mM DTT (diamide, NEM, $CdCl_2$) and the mixture incubated at 25° for 120 min.

It was previously reported [2] that the activity of the crude extract was low when prepared without mercaptoethanol, and that the activity was increased by the addition of mercaptoethanol to the assay mixture. A similar effect of mercaptans on activity was observed with partially purified enzyme. The enzyme fractionated on Bio-Gel A-0.5 m without added mercaptoethanol or dithiothreitol had minimal activity. Addition of dithiothreitol increased the activity (Fig. 4). The increased activity with mercaptoethanol, or dithiothreitol, suggests that a sulphydryl group is essential for activity and that this group is reversibly oxidized, possibly to a disulphide, in the absence of added mercaptan.

p-Hydroxymercuribenzoate and *N*-ethylmaleimide inhibited emodin deoxygenase activity of Bio-Gel enzyme (Table 4) further supporting the presence of an essential sulphydryl group. Treatment with diamide, which is proposed to oxidize a pair of sulphydryl groups to a disulphide group [5], and cadmium II, which is thought to inhibit enzymes that contain dithiols [6], also inhibited activity (Table 4). These results support the involvement of a pair of thiols that are essential for activity and which are readily oxidized in air to the disulphide. Addition of mercaptoethanol, or dithiothreitol, is necessary to maintain the enzyme in the active dithiol form. In the cell, thioredoxin, or a related compound, may replace DTT or 2-mercaptoethanol in forming the active dithiol [7].

Emodin deoxygenase catalyses the conversion of emodin to chrysophanol. Chrysophanol is the precursor of the secalonic acids [1]. Comparing the structures of chrysophanol and the secalonic acids, it is evident that several enzymes are involved in the conversion of chrysophanol to the secalonic acids. Emodin deoxygenase catalyses an early step and thus may be subject to regulation. The enzyme appears to be regulated by both the energy charge (ATP) and conditions that would affect the equilibrium between sulphydryl groups and disulphide. An unidentified metabolite is a coactivator with ATP.

EXPERIMENTAL

General. Sephadex G-75, Sephacryl-200, dithiothreitol, *p*-hydroxymercuribenzoate, diamide and *N*-ethylmaleimide were from Sigma. DE-52 was obtained from Whatman Biosystems. Bio-Gel A-0.5 m was purchased from Bio-Rad.

Culture conditions and assay. Culture conditions of *P. terrestris* and prepn of crude extract were as described previously [2, 3]. Two incubation mixts were used in the assay of emodin deoxygenase activity. For expts with crude extract and G-75 enzyme the assay components were those used previously [2] with minor changes, viz. 15 mM $FeCl_2$, 12 mM 2-mercaptoethanol, 6 mM ^{3}H-emodin, 20% (v/v) glycerol, NADPH and ATP at the indicated concns, and 50 mM K-Pi buffer pH 7.4. When enzyme prepns after $(NH_4)_2SO_4$ fractionation were assayed, the incubation mixt contained enzyme, 1.7 mM NADPH, 6 mM ^{3}H-emodin, 12 mM 2-mercaptoethanol, 1 mM EDTA in 50 mM Tris–HCl pH 7.6. The final vol. was 0.3 or 0.6 ml. The mixt was flushed with N_2 and then incubated for various times at 25°. After incubation, samples were extracted and assayed as described previously [2]. The pmol chrysophanol formed was determined from the sp. ract. of ^{3}H-emodin of 104 Ci/mol; the efficiency of counting was 0.47 cpm dpm^{-1}.

Sephadex G-75 column chromatography. The crude extract was applied to a Sephadex G-75 column equilibrated with buffer A (50 mM K-Pi buffer pH 7.4, 20% (v/v) glycerol, 1 mM EDTA, 12 mM 2-mercaptoethanol). The column was eluted with buffer A; two peaks at 280 nm were observed in the eluted frs. Frs corresponding to the first peak, which contained protein and emodin deoxygenase activity, were combined (G-75 enzyme). Frs corresponding to the second peak, which was devoid of protein were also combined (fr. 2).

Partial purification of emodin deoxygenase. The crude extract was brought to 65% satn by the addition of solid $(NH_4)_2SO_4$. After stirring for 15 min the mixt was centrifuged and the pellet mixed with buffer B containing 1% (w/v) Triton X-100, 1 mM EDTA, 12 mM 2-mercaptoethanol, 50 mM Tris–HCl pH 7.6). The mixt was centrifuged at $250\,000 \times g$ for 90 min and the supernatant applied to a Sephadex G-75 column equilibrated with buffer B containing 25 mM KCl. The enzyme activity eluted in the first peak of A_{280}.

Active frs were combined and this enzyme was applied to a DE-52(DEAE cellulose) column equilibrated with buffer B containing 25 mM KCl. Proteins were eluted with a linear gradient from 25 to 300 mM KCl. The activity eluted from the column at 100 mM KCl. Active fractions were again combined and the enzyme concd on an Amicon concentrator with a PM-5 membrane to a small vol. The sample was fractionated on a Sephacryl S-200 column with buffer B as eluant. Active fractions were pooled and the sample loaded on a Matrex Green A column equilibrated with buffer B. After washing with buffer B, the activity was eluted with buffer B containing 2 mM NADP.

Determination of M_r. Sephacryl S-200 gel filtration of the sample from DE-52 chromatography was carried out on a 2×99 cm column with buffer B containing 0.20 mM KCl. Elution vols of emodin deoxygenase activity, cytochrome *c*, trypsinogen, egg albumin, bovine albumin, alcohol dehydrogenase and α-amylase were all measured. M_r was determined from a curve of log M_r vs elution vol.

Bio-Gel enzyme. The 65% $(NH_4)_2SO_4$ fr was fractionated on a Bio-gel A-0.5 m gel filtration column with buffer B and frs with emodin deoxygenase activity were combined (Bio-Gel enzyme).

Effect of pH on activity. Bio-Gel enzyme (2 ml) was subjected to ultrafiltration through a 10K membrane Centricon 10 microconcentrator until the min enzyme residue was left on the filter. 50 mM buffer, MES 5.5, 6.0; MOPS 6.5, 7.0; Tris 7.5, 8.0, 8.5, (2 ml) containing 12 mM 2-mercaptoethanol was added and mixed with the retained enzyme residue. This soln was then assayed for enzyme activity. The incubation mixt contained 0.12 mg protein, 3.3 mM NADPH, 12 mM 2-mercaptoethanol, 5 mM ^{3}H-emodin and 50 mM buffer in a final vol of 0.3 ml. Incubation time was 2 hr.

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