Partial Characterization of an Enzyme from the Fungus *Ascochyta rabiei* for the Reductive Cleavage of Pterocarpan Phytoalexins to 2′-Hydroxyisoflavans

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Crude protein extracts from the chickpea (*Cicer arietinum* L.) pathogenic fungus *Ascochyta rabiei* catalyze the NADPH dependent conversion of the pterocarpan phytoalexins medicarpin and maackiain to 1) 2′-hydroxyisoflavans, 2) 1a-hydroxypterocarp-1,4-diene-3-one, and 3) 10,2′-dihydroxyisoflav-8-ene-7-one derivatives. A pterocarpan:NADPH oxidoreductase which cleaves the benzylphenyl ether bond of the phytoalexins was purified some 100-fold and partially characterized with regard to its kinetic properties. The oxidoreductase was shown to be specific for NADPH and 3-hydroxypterocarpans. The enzyme appears to be constitutively expressed by *A. rabiei.*

Introduction

Phytopathogenic fungi are often characterized by a pronounced ability to degrade the phytoalexins of their host plants [1–3]. This potential for the inactivation of defense compounds may partly determine fungal virulence [4, 5] so that characterization of such catabolic routes and of the enzymes involved will contribute to a better understanding of plant-parasite interaction.

In the course of our studies on the fungal degradation of pterocarpan phytoalexins by fungi of the genus *Fusarium* [6, 7] and by the chickpea (*Cicer arietinum* L.) pathogen *Ascochyta rabiei* [8–10] we have demonstrated the conversion of medicarpin, **1** and maackiain, **2** (Fig. 1) to 7,2′-dihydroxy-4′-methoxyisoflavan, **3**, and 7,2′-dihydroxy-4′,5′-methylenedioxyisoflavan, **4**, respectively. The cleavage of a pterocarpan to the corresponding 2′-hydroxyisoflavan has repeatedly been shown as an initial step in the fungal degradation of pterocarpan phytoalexins [1, 6, 7–9, 11]. Alternative routes for pterocarpan catabolism by fungi are the formation of a 1a-hydroxy-pterocarp-1,4-diene-3-one such as **5** or **6** [12, 13], a 2′-hydroxyisoflavanone [12, 13] or an 0-demethylation product [1, 4, 6].

Since the reductive cleavage of the benzylphenyl ether bond of **1** or **2** leading to **3** or **4** appears to be of major importance in pterocarpan fungal dissimilation we have now investigated this reaction with protein preparations of the phytopathogenic fungus *Ascochyta rabiei.* We here report on the partial characterization of the responsible pterocarpan: NADPH oxidoreductase from this fungus.

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Materials and Methods

Fungus

Ascochyta rabiei, strain III (CBS number 534.65), was used throughout. Mycelium was grown and harvested as described [8, 10]. Incubation experiments of mycelium with pterocarpans have also been reported [8, 9].

HPLC analyses

The isolation of phenolic compounds from incubation assays and their determination by HPLC followed our earlier reports [8, 10].

Enzyme purification

Mycelium (150 g fr.w.) was homogenized in a mortar at 4 °C with 25 ml potassium phosphate buffer (0.05 M; pH 7.5) containing 1 mM dithioerythritol (DTE). After centrifugation of the homogenate (40,000 × *g*) the supernatant was fractionated by the addition of a saturated ammonium sulfate solution. The protein sediment obtained between 30–50% saturation was collected by centrifugation (40,000 × *g*; 30 min) and desalted on a Sephadex

G 25 column. The protein eluate was chromatographed with potassium phosphate buffer (0.05 M; pH 7.5) containing 1 mM DTE on a DEAE-Sephacel column (3 × 24 cm) equilibrated with the same buffer solution. Proteins were eluted with a flow rate of 50 ml/h and fractions of 7.5 ml each were collected. After 165 ml had been eluted a linear gradient of 0.1 M NaCl in the same phosphate buffer was applied. Oxidoreductase activity appeared in the fractions 14–22 whereas the oxidizing enzyme activity catalyzing the formation of compounds **5** and **6** (Fig. 1) was found in the fractions beginning with number 22. The fractions with oxidoreductase activity were pooled and concentrated. This protein sample was subjected to affinity chromatography on a Blue Sepharose CL-6B-column (gel volume 7 ml) equilibrated with potassium phosphate buffer (0.05 M; pH 7.5). Protein was applied in the same buffer in a concentration of 0.5 mg/ml. Non-binding protein was eluted with altogether 70 ml of the same buffer. The oxidoreductase was finally eluted with a 10 mM NADPH-solution and fractions of 1.4 ml each were collected. Enzyme activity was found in the fractions 14–16 which were pooled, desalted and concentrated.

Gel electrophoresis

Analytical SDS gel electrophoresis and the protein markers have been described [14].

Enzyme assays

NADPH : pterocarpan oxidoreductase was assayed with (−)-maackiain or (±)-medicarpin. The standard reaction mixture contained 370 µl potassium phosphate buffer (0.05 M; pH 7.5; 1 mM DTE), 20 µl NADPH (20 mM), 10 µl pterocarpan solution (10 mM) and 100 µl protein preparation. The reaction was started by the addition of substrate, incubated at 30 °C for 5–30 min and stopped with ethylacetate. Phenolics were assayed by HPLC as described [8, 10].

Protein concentrations were determined according to Bradford [15] with bovine serum albumine as reference. For the determination of the pH optimum the following buffers were used: potassium phosphate buffer, 0.1 M, pH 5–8; TRIS/HCl, 0.1 M, pH 7–10; glycine/NaCl/NaOH, 0.1 M, pH 9–12.

For determination of the substrate specificity the following compounds were also tested but they were not accepted as substrates by the purified oxidoreductase: 3,9-dimethoxypterocarpan, trifolirhizin, glyceollin I, II and III, 11a-methylpterocarpan, 6a-hydroxy-11a-methylpterocarpan, coumestrol, 3,9-di-0-methylcoumestrol and 3,7-dihydroxy-9-methoxycoumestan. All substrates and compounds were from the collection of the institute.

Results

Enzyme assay

Previous investigations with cell-free protein preparations from *A. rabiei,* strain III [8, 9], had shown that both **1** and **2** were converted to three products each as shown by HPLC techniques. These products (Fig. 1) had been identified as **3**, **5** and **7** from medicarpin [8] and **4**, **6** and **8** from maackiain [9]. The formation of these conversion products of **1** or **2** depended on the presence of NADPH whereas NADH as cofactor did not lead to any enzymatically formed product.

Further incubation experiments with crude protein preparations from *A. rabiei,* strain III, and maackiain, **2**, have now shown that the ratio of the enzymatic products is greatly effected by the amount of NADPH in the incubation assays. In a concentration range of 0.8–2 mM NADPH compounds **6** and **8** are formed in a 30 to 50% yield whereas **4** was formed to only 2–6% as based on **2**. A lower NADPH concentration of 0.4 M substantially decreased the accumulation of **6** and **8** (maximum 15–20%) and increased the yield of **4** up to 16% in 45 min. The reaction rate for the formation of **6** was linear with time for 45 min, whereas in case of **4** and **8** time linearity was obtained for 15–30 min only.

Exclusion of molecular oxygen from the incubation assays by flushing with nitrogen gas resulted in an almost complete elimination of **6** and **8** with sole accumulation of **4** in nearly stoichiometric amounts as based on the amount of **2** consumed (HPLC determinations). In short time incubations (5 to 10 min) with limiting amounts of NADPH the dihydrocompound **4** was the predominant product. Using partially purified protein preparations (see below) with **4** as substrate clearly demonstrated that **8** originated from this substrate and not from **6**. Incubation experiments with the latter compound did not lead to any enzymatic conversion indicating that **6** is not an intermediate in the formation of **8**.

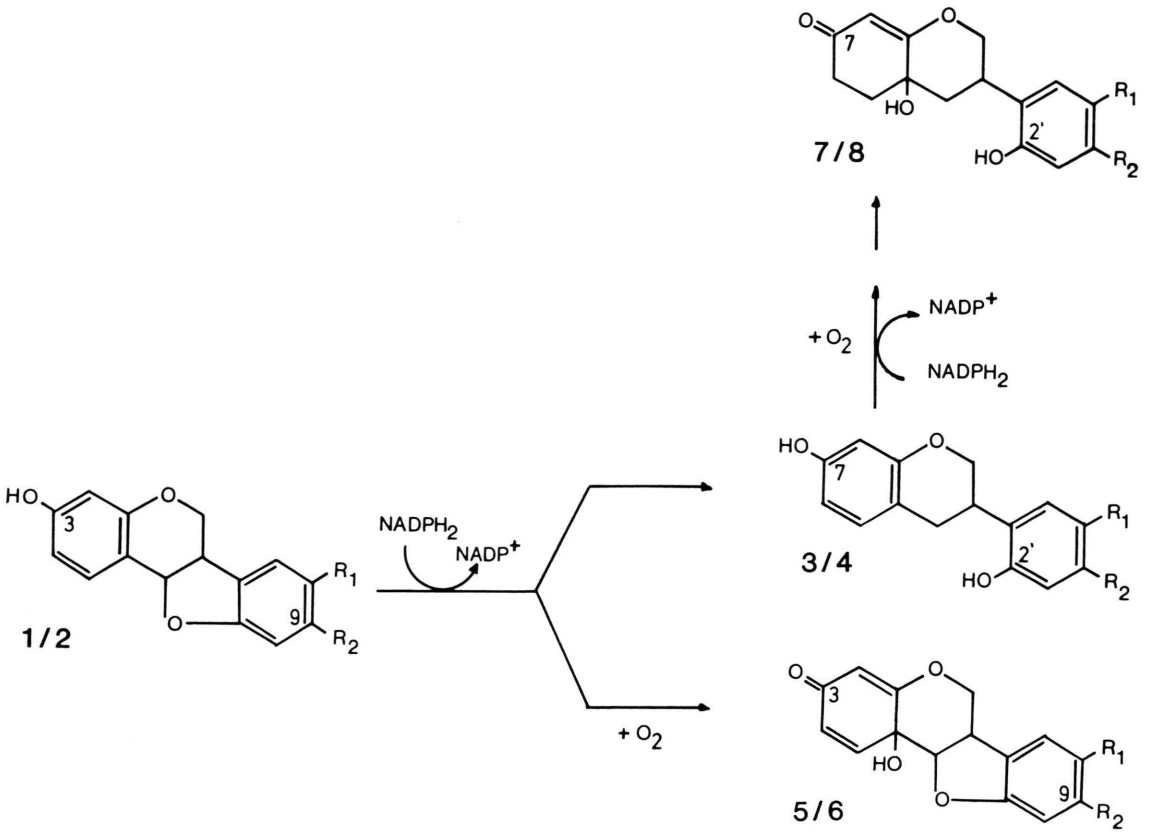

Fig. 1. The pterocarpan:NADPH oxidoreductase converts medicarpin **1** (R_1 = H; R_2 = OCH_3) to 7,2′-dihydroxy-4′-methoxyisoflavan, **3** (R_1 = H; R_2 = OCH_3) and maackiain, **2** (R_1 = R_2 = O−CH_2−O) to 7,2′-dihydroxy-4′,5′-methylenedioxyisoflavan, **4** (R_1 = R_2 = O−CH_2−O). An alternative enzyme activity from *A. rabiei* oxidizes **1** to 1a-hydroxy-9-methoxy-pterocarp-1,4-diene-3-one, **5** (R_1 = H; R_2 = OCH_3) and **2** to 1a-hydroxy-8,9-methylenedioxy-pterocarp-1,4-diene-3-one, **6** (R_1 = R_2 = O−CH_2−O). Compounds **7** (10,2′-dihydroxy-4′-methoxy-isoflav-8-ene-7-one; R_1 = H; R_2 = OCH_3) and **8** (10,2′-dihydroxy-4′,5′-methylenedioxy-isoflav-8-ene-7-one; R_1 = R_2 = O−CH_2−O) originate from **3** and **4**, respectively. Formation of compounds **5**, **6**, **7** and **8** also requires NADPH and oxygen.

Partial purification of pterocarpan:NADPH oxidoreductase

Parallel incubation assays with protein preparations from freshly harvested mycelium of *A. rabiei* and from mycelium batches preincubated with 10^{-4} M maackiain for up to 6 h showed practically identical enzyme activities for maackiain conversion to compounds **4**, **6** and **8**. This result indicates that the pterocarpan oxidoreductase and the other enzyme activities shown in Fig. 1 are constitutively expressed and that they are not induced by substrate. This expression of enzyme activities could be observed for mycelial preparations grown either on chickpea-seed-meal or potatoe homogenate media.

The pterocarpan:NADPH oxidoreductase was purified about 100-fold from 150 g fr.w. of freshly harvested mycelium of *A. rabiei* strain III using maackiain as substrate (Table). The enzyme precipitated at ammonium sulfate concentrations between 30 to 50% saturation. This step partially removed the enzyme activity leading to compound **8**. Subsequent chromatography on DEAE-Sephacel allowed a good separation of the oxidoreductase from the enzyme activity catalyzing the oxidation reaction to **6** because the oxidoreductase could be eluted from the column much earlier than the oxygenase. The most effective purification step was affinity chromatography on Blue Sepharose CL-6B. The oxidoreductase readily

Table. Purification of pterocarpan:NADPH oxidoreductase from mycelium of *Ascochyta rabiei*.

Purification step	Protein [mg]	Total act [pKat]	Spec. act [pKat/mg]	Recovery [%]	Purification (-fold)
Crude extract	571	6340	11.1	100	1.0
$(NH_4)_2SO_4$ fractionation 30–50% fraction	135	849	6.3	13.4	0.6
DEAE Sephacel	2.98	1825	613	28.8	55
Blue Sepharose CL-6B	0.43	451	1050	7.1	95

bound to the gel and could be eluted with comparatively low concentrations of NADPH. The protein thus obtained only catalyzed the formation of compounds **3** and **4** with **1** or **2** as substrates. Purity of the enzyme was checked by SDS gel electrophoresis where only one protein band could be detected by silver staining. Marker proteins applied to the slab gels showed that the molecular mass of the protein material from the oxidoreductase was in the range of 19,000–20,000.

Properties of the oxidoreductase

Stability of the enzyme activity was high in crude fractions and only partially purified samples which could be frozen and stored (−25 °C) for several days without essential loss of activity. The purified enzyme, however, could not be frozen nor stored for any periods of time without very rapid loss of enzyme activity.

The enzymatic cleavage of **1** or **2** yielding **3** or **4**, respectively, showed an optimum at pH 8.5. Below pH 6.5 and above pH 9.5 the enzyme is nearly inactive. Best enzyme activity was measured at 28–32 °C. At temperatures above 35 °C the enzyme was rapidly inactivated. The apparent K_m for NADPH was determined to be 4.5×10^{-5} M with a maximum velocity of 1.03 μkat/mg protein. NADH could not serve as a cosubstrate of the oxidoreductase. The apparent K_m value for the phytoalexins measured with maackiain was found to be 2.4×10^{-5} M with a maximum velocity of 617 nKat/mg protein.

The substrate specificity of the enzyme was measured using a considerable number of pterocarpans and analogous compounds (see Methods). Medicarpin, **1**, and maackiain, **2**, were found to be the only substrates of the enzyme. Even structurally closely related compounds such as 3,9-dimethoxypterocarpan or the pterocarpan phytoalexins trifolirhizin and the glyceollins were not converted to 2′-hydroxyisoflavans.

Discussion

These studies represent the first report on a fungal enzyme for the reductive cleavage of a pterocarpan to a 2′-hydroxyisoflavan. Furthermore, this enzyme reaction is another example of the few cases observed so far with natural products where an ether bond, an 0-glycoside or a lactone type of structure is cleaved by an enzymatic reduction and not by an oxidative or hydrolytic enzyme process [16–18].

The conversion of various natural pterocarpans to the corresponding 2′-hydroxyisoflavans has repeatedly been observed with fungi from several different genera [1, 6, 11]. The narrow substrate specificity observed for the *A. rabiei* oxidoreductase indicates that other enzymes with specificities for the various substitution patterns of the natural pterocarpans will most likely be found. The oxidoreductase described in this report will only accept as substrate pterocarpans with hydrogen in the 6a and 11a position and a hydroxyl function in position 3. This latter requirement may indicate that a carbonium ion at C-4 of the isoflavan stabilized by a protonated quinone methide intermediate formed with the hydroxyl group at C-7 might be involved in this reductive cleavage of the phenylbenzyl ether bond [1, 19]. Present investigations are devoted to an analysis of the stereochemical aspects of the oxidoreductase reaction because it is not yet known whether the enzyme will in addition to the 6aR:11aR isomer also cleave the 6aS:11aS compounds of maackiain and medicarpin [20]. Furthermore, it remains to be elucidated whether the oxidoreductase is specific for the A or B side hydrogen of NADPH [21].

The comparatively low molecular mass of the protein band observed during SDS gel electrophoresis

(appr. 19,000–20,000 dalton) leads to the assumption that a subunit of the enzyme has so far been found and that the natural enzyme possesses a larger molecular mass.

Reductive cleavage to 2′-hydroxyisoflavans (compounds **3** and **4**) and oxidative conversion to 1a-hydroxy-pterocarp-1,4-diene-3-one derivatives (compounds **5** and **6**) represent the two major initial reactions of pterocarpan disintegration by fungi [1, 4, 6]. Fig. 1 documents that both reactions are constitutively expressed by *A. rabiei* and that both reactions can be studied at the enzyme level. Investigations are presently under way to purify the oxygenase enzyme so that the quantitative ratio of the expression of both enzymes can be studied in more detail. Such knowledge will lead to a better understanding of the physiological role of these reactions in *A. rabiei* chickpea interaction.

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