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NADPH: BIOCHANIN A OXIDOREDUCTASE FROM THE FUNGUS *FUSARIUM JAVANICUM*

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Key Word Index—*Fusarium javanicum*; Hyphomycetes; NADPH: biochanin A oxidoreductase; isoflavone metabolism; biochanin A.

Abstract—A soluble enzyme which catalyses the NADPH-dependent reduction of the heterocyclic double bond of the isoflavone biochanin A (5,7-dihydroxy-4'-methoxy-isoflavone) yielding the corresponding isoflavanone was isolated from the fungus *Fusarium javanicum*. The NADPH: biochanin A oxidoreductase was constitutively present in the mycelium with an extractable average activity of 4 pkat/g fresh weight. The enzyme was purified *ca* 4500 fold to apparent homogeneity. The native enzyme had M_r of *ca* 87 000 and consisted of two identical subunits of M_r 43 000. The enzyme reaction showed a pH-optimum at pH 7.5 and a temperature optimum between 30 and 35°. The apparent K_m values were 43 μM for biochanin A and 190 μM for NADPH with a maximum velocity of 4 mkat/kg protein. The enzyme exhibited a remarkable substrate specificity for biochanin A.

INTRODUCTION

Among the defence mechanisms which plants have developed against the attack of micro-organisms, the isoflavonoids are of considerable interest, due to their differential fungitoxic properties [1–3]. Numerous phytopathogenic fungi were shown to be potent degraders of such isoflavonoid pre- or post-infectional inhibitors [3–9].

Biochanin A (1, Fig. 1), an isoflavone considered to be a preinfectional inhibitor [5, 10–12], is known to accumulate in high concentrations in several Leguminosae [13], mainly as the malonylglucoside conjugate. The decomposition of the malonylglucoside moiety by *Fusarium javanicum* was previously reported and explained by the subsequent action of an esterase and a glucosidase detected in the mycelium [14]. It was further demonstrated that the fungus completely degraded the aglycone to carbon dioxide and a number of transiently accumulating metabolites [9, 14]. The degradative sequence starts by the conversion of the isoflavone biochanin A to the corresponding isoflavanone (dihydrobiochanin A, 2, Fig. 1).

Other *Fusarium* species or *Ascochyta rabiei* apply alternative starting reactions for the isoflavone catabolism such as 4'-*O*-demethylation or 3'-hydroxylation [4, 15, 16]. In our studies on the fungal catabolism of fungitoxic plant defense compounds we have investigated the expression of enzyme activities for biochanin A degradation in *Fusarium javanicum*. We now report on the isolation and characterization of a NADPH: biochanin A oxidoreductase, the putative starting enzyme in the degradation of this isoflavone.

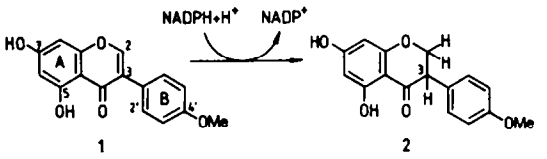

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Fig. 1. Reaction catalysed by NADPH: biochanin A oxidoreductase from *Fusarium javanicum* yielding an isoflavanone (2) from an isoflavone (1).

RESULTS AND DISCUSSION

Enzyme reaction

In crude protein preparations obtained from the fungus *Fusarium javanicum*, grown on a complex medium, enzyme activity for the conversion of the isoflavone biochanin A to the corresponding isoflavanone was detected. The reaction product was unequivocally shown to be dihydrobiochanin A (2) according to our previous chromatographic and spectroscopic data [9]. The enzyme reaction strictly depended on NADPH as cosubstrate whereas NADH showed 30% of the maximum activity only (Table 1). The enzyme reaction thus proceeds as depicted in Fig. 1.

The oxidoreductase was found to be a soluble protein. Furthermore, the enzyme activity could be totally extracted from the fungal mycelium with buffer B. This is in contrast to esterase- and β-glucosidase activities previously shown to be involved in the degradation of biochanin A 7-*O*-glucoside 6''-*O*-malonate by this fungus [14]. A portion of these enzymes was tightly bound to the insoluble fungal cell debris. Preincubation of the mycelium in biochanin A solutions (0.1 mM) for up to 10 hr did not lead to any increase in the activity of the

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oxidoreductase. We must thus conclude that this enzyme is constitutively expressed in *F. javanicum*. Data obtained from *in vivo* studies [9, 14] and from other investigations on the enzymes involved in biochanin A degradation by *Fusarium javanicum* (unpublished) support the assumption that the enzymes subsequent to dihydrobiochanin A are inducible either by biochanin A or by the corresponding substrate [9, 14].

Enzyme purification

Attempts to purify the oxidoreductase were hampered by the very low enzyme activity in the mycelium (4 pkat/g fr. wt), due to the low concentration of the enzyme (*ca* 0.02% of the soluble proteins). Despite this, a 4600-fold increase in specific activity with a 12% yield of activity and a 0.003% yield of total protein was finally reached (Table 2). Affinity chromatography on Blue Sepharose CL 6 B and subsequent elution with NADPH represented the best purification step (Table 1). When applied in a 50 mM potassium phosphate buffer at pH 7.5 the enzyme was tightly bound to the triazin dye and could not be eluted from the gel with salt solutions up to 40-fold higher in ionic strength. Though numerous compounds were tested (see Experimental) only NADPH was a successful eluant for the enzyme from the Blue Sepharose ligands. This competitive behaviour of ligand and NADPH is in agreement with the high specificity of the enzyme for NADPH and also indicates that the enzyme possesses specific binding sites for the folded dinucleotide structure of the reduced cosubstrate [17–19]. The pronounced difference in the elution potential between NADPH and NADP further supports the assumption that the enzyme may form a stable complex with the reduced form of the cosubstrate and that the oxidized form is readily released from it after hydride transfer. The enzyme finally obtained after the affinity chromatography step (Table 1) was shown to be homogeneous as judged by SDS-PAGE and was used for further characterization.

When the blue sepharose step was replaced by Fast Protein Liquid Chromatography (FPLC) on a Mono Q column, an efficient preparative purification was obtained. With this method a 700-fold increase in specific activity was achieved which resulted in a final value of 2.5 mkat/kg protein, a yield of 2.5% total activity and a 0.003% yield of protein respectively. The enzyme could be stored at −25° in the presence of 20% (v/v) glycerol without significant loss of activity. Enzyme solutions kept at 4° showed a decrease in enzyme activity with a half life of about 2 weeks.

Table 1. Relative activities of the purified oxidoreductase with various substrates and cosubstrates

Substrate (0.2 mM)	Cosubstrate (2 mM)	Rel. activity (%)
Biochanin A	NADPH	100
Biochanin A	NADH	30 ± 4
Pratensein	NADPH	78 ± 10
Genistein	NAPDH	44 ± 5

Structure of the enzyme

Gel chromatography indicated a M_r of 87000–88000 for the native enzyme as estimated from 4 independent experiments. SDS-PAGE with the purified enzyme revealed one single protein band with an M_r of *ca* 43000–44000. No other proteins could be detected, either with Coomassie Blue staining or by the silver staining method. These results suggest a dimeric structure with monomers of 43000. Sedimentation analysis by ultracentrifugation with alcohol dehydrogenase as reference protein resulted in much higher values for the M_r in the range of 240000. Though only one single, distinct band for the reductase activity could be detected in the sucrose gradients, polymerization cannot be excluded, but more likely the enzyme contains metal atoms leading to a high density, and thus an overestimated M_r could have been shown by this method.

Kinetic properties

The purified NADPH:biochanin A oxidoreductase exhibited a broad pH optimum at pH 7.5 with considerable enzyme activities also in the range of pH 5–8. The temperature optimum was between 30–35°, while a temperature above 40° rapidly inactivated the enzyme. The enzyme reaction was linear up to 50% conversion of biochanin A, both for the amount of protein in the assay and for incubation time (up to 4 hr at 30°). The values for K_m of biochanin A and NADPH were (43 ± 5) μM and (190 ± 20) μM, respectively, and V_{max} was measured as (4.2 ± 0.2) mkat/kg. Several flavones, chalcones and isoflavones (see Experimental) were tested to determine the substrate specificity of the purified oxidoreductase. In addition to biochanin A, an enzymic reduction could only be measured with the isoflavones pratensein (5,7,3'-

Table 2. Purification of the NADPH: Biochanin A oxidoreductase from *Fusarium javanicum*

Purification step	Protein (mg)	Total activity (pkat)	Specific activity (μ kat/kg)	Recovery (%)	Purification (− fold)
Crude extract	857	1105	1.3	100	1
$(NH_4)_2SO_4$ fractionation 40–60% saturation	327	803	2.5	73	1.9
DEAE—Sephacel	39.3	583	14.8	53	11.4
Ultrogel AcA 34	6.8	475	69.9	43	54
Blue Sepharose CL 6B (Elution with NADPH)	0.023	134	5920	12	4550

trihydroxy-4'-methoxy-isoflavone) and genistein (5,7,4'-trihydroxy-isoflavone) (Table 1). Flavones, chalcones and 5-deoxy-isoflavones, respectively, were not accepted as substrates by the oxidoreductase. The enzyme is thus characterized by an absolute specificity for ring A (5,7-dihydroxy pattern) and a pronounced specificity for ring B (4'-substituent) of isoflavones. These results on substrate specificity are in agreement with the observation that the fungus readily degrades the isoflavones listed in Table 1, whereas 5-deoxy-isoflavones are not catabolized [16, 20]. The NADPH: biochanin A oxidoreductase represents another example of a highly specific fungal reductase [21].

The enzymatic reaction shown in the Fig. 1 is comparable with another oxidoreductase reaction recently found in a plant system where 2'-hydroxy-5-deoxy-isoflavones are converted to the corresponding isoflavanones [22]. This enzyme is part of the pterocarpan phytoalexin biosynthesis in chickpea and offers to be absolutely specific for a 5-deoxy-2'-hydroxy substitution pattern.

EXPERIMENTAL

Chemicals. 2,5,7-Trihydroxy-4'-methoxy-isoflavone was obtained from Prof. Grisebach (University of Freiburg, F.R.G.), all other phenolic compounds were from our previous studies [5, 9, 14, 20]. DTE (dithio-erythritol), NADPH, NADH, NADP, NAD, 2'-AMP and nicotinamide were purchased from Serva (Heidelberg, F.R.G.). DEAE-Sephacel, Blue Sepharose CL 6 B and Sephadex G-25 (medium) came from Pharmacia (Freiburg, F.R.G.). Ultrogel AcA 34 was a product of LKB (Gräfelfing, F.R.G.).

Buffer systems. The buffers were: buffer A, 50 mM K-Pi, pH 7.5, containing 2 mM DTE; buffer B, 100 mM K-Pi, pH 7.5, containing 2 mM DTE. For estimating the pH-optimum of the enzyme reaction the following buffers were used: pH range 2–3, 100 mM glycine/HCl; pH 4–5, 100 mM tri-Na_3-citrate; pH 5–8, buffer B; pH 7–8.5, 100 mM Tris–HCl; pH 9–12, 100 mM glycine/NaOH; each buffer included 2 mM DTE.

Fungus. Fusarium javanicum (Koord), CBS 203.32 obtained from the Centraalbureau voor Schimmelcultures (Baarn, Netherlands), was stored, grown and harvested as previously described [14].

Enzyme purification. The mycelium harvested 4 days after inoculation was washed in buffer A and stored in liquid N_2. After thawing in buffer B (1–2 ml/g fr. wt) it was further homogenized in a chilled mortar with 20% (w/w) quartz sand. All procedures were carried out at 4°. The homogenate was centrifuged (30 000 *g*, 30 min) and the supernatant was fractionated by dropwise addition of a saturated $(NH_4)_2SO_4$ soln in two steps between 0–40% and 40–60% satn. The precipitated protein was removed by centrifugation (30 000 *g*, 20 min). Protein from the second precipitation step was dissolved in buffer A and the soln was desalted on Sephadex G-25 (PD-10) using buffer A. The protein material was then applied to a DEAE-Sephacel column (29 × 450 mm). After elution of unbound protein (500 ml buffer A) a linear gradient of NaCl in buffer A with a slope of 0–0.7 M and a total vol. of 500 ml was started. Proteins were eluted with a flowrate of 50 ml/hr and fractions of 4 ml each were collected. Fractions with pronounced oxidoreductase activity were pooled and concentrated by ultrafiltration (PM 10, Amicon, Osterhout, Netherlands) to a vol. of 10 ml. This sample was immediately subjected to Ultrogel AcA 34 chromatography (column 26 × 860 mm) using buffer A and fraction sizes of 4 ml. The fractions with oxidoreductase activity were pooled and used for the final affinity chromatography on Blue Sepharose CL 6 B (column 15 × 50 mm). Unbound protein was eluted with 600 ml buffer A. A selective elution of the oxidoreductase activity could be achieved by the use of 5 ml 10 mM NADPH in buffer A (flowrate 50 ml/hr, fraction size 1.4 ml). The bulk of enzyme activity was distributed over two fractions only. Other compounds such as 0.1 M KCl, 0–2 M NaCl, 0.01 M 5'-AMP, 0.02 M 2'-AMP, 0.1 M nicotinamide, 0.01 M NAD, 0.01 M NADP, 0.01 M NADH were unable to remove the enzyme from the dye ligands. The eluants were routinely withdrawn from the fractions by gelfiltration on PD-10 columns as mentioned above. In order to maintain the binding capacity of the gel it had to be regenerated with 8 M urea following each purification procedure.

Molecular mass. The M_r of the native enzyme was determined in the course of gel filtration steps using hexokinase, chymotrypsinogen (Serva, Heidelberg, F.R.G.), bovine serum albumine and ovalbumine (Sigma, Munich, F.R.G.) as marker proteins. The void vol. and the pore volume (Ultrogel AcA 34, 26 × 860 mm) were determined with Blue Dextran 2000 (Pharmacia, Freiburg, F.R.G.) and DNP-Alanine (Serva, Heidelberg, F.R.G.), respectively.

Analytical SDS-PAGE was performed after each purification step under denaturating conditions according to ref. [23]. The stacking gel consisted of 3% and the running gel of 12.5% acrylamide. Gels (80 × 80 mm) were stained with Serva Blue G. Some gels were also stained by the silver method [24] using Bio-Rad reagents (Munich, F.R.G.). Bovine serum albumin. ovalbumin (Sigma, Munich, F.R.G.), chymotrypsinogen and myoglobulin (Serva, Heidelberg, F.R.G.) were used as markers. The mixture contained 0.2% Bromophenol Blue as tracking dye.

Sedimentation analysis was carried out according to ref. [25] using linear, isokinetic sucrose gradients [5–20% (w/v)] in buffer A with a vol. of 12 ml and protein samples of 0.1 ml. A preparative ultracentrifuge (Damon/IEC B-60) equipped with the swinging bucket rotor 488 was employed. After centrifugation (24 hr, 4°, 40 000 rpm) the tubes were fractionated dropwise from the bottom and fractions of 0.4 ml each were collected. Alcohol dehydrogenase from horseliver (Boehringer, Mannheim, F.R.G.) was used as int. ref. protein.

Enzyme assays. Standard assays consisted of 50–200 μl enzyme preparation, 100 nmol biochanin A and 500 nmol NADPH in a total vol. of 500 μl buffer A. The phenolic compounds were added with 10 μl 2-methoxy-ethanol. The assays were started with NADPH, incubated for 1 hr at 30° and stopped by adding 500 μl MeOH. Denaturated protein was removed by centrifugation and the resulting methanolic solns were directly used for HPLC analysis. Alternatively tests were stopped by the addition of 600 μl EtOAc, and the phenols were extracted by shaking for 1 min. After removal of proteins by centrifugation the EtOAc phase was recovered and evaporated to dryness. The residue was dissolved in 100 μl MeOH and submitted to HPLC analysis. The analytical system for HPLC separation of isoflavones and other phenolic compounds has been described [13, 14, 28]. The separation of biochanin A and dihydrobiochanin A was achieved with a linear gradient of 40% B in A to 60% B in A within 15 min at a flow of 0.8 ml/min, performed on a RP 18 column (Lichrosorb, Merck, Darmstadt, F.R.G.), with 1.5% H_3PO_4 (A) and CH_3CN (B). A gradient of 20% B in A to 80% B in A of the same eluants was used for chromatography of assays including other isoflavones, flavones and the chalcone as substrates. Quantitation of substrates and products was performed at 260, 290 and 320 nm, respectively, by external standardization. Alcohol dehydrogenase was assayed according to the recommendations of the producer. The data quoted are the average values obtained by three different methods of linear transformations (Lineweaver–Burk, Eadie–Hofstee, and Hanes [26, 27]).

Product identification. The identity of the reduction product obtained from biochanin A was elucidated by co-chromatography and GC/MS analyses after preparative HPLC purification. MS data identical to our previous reports [9] were obtained.

Substrate specificity. The following compounds were not accepted as substrates by the purified oxidoreductaseunder standard incubation conditions: (a) the flavones apigenin, quercetin, (b) 2',4'-dihydroxy-4-methoxy-chalcone and (c) the isoflavones orobol (3',4',5,7-tetrahydroxy-isoflavone), 2,5,7-trihydroxy-4'-methoxy-isoflavone, 3',4',5-trihydroxy-isoflavone, texasin (6,7-dihydroxy-4'-methoxy-isoflavone), formononetin (7-hydroxy-4'-methoxy-isoflavone), calycosin (3',7-dihydroxy-4'-methoxy-isoflavone), daidzein (4',7-dihydroxy-isoflavone), and 4'-hydroxy-7-methoxy-isoflavone.

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