Volume 236, number 1, 221–225 FEB 06207 August 1988

Isolation of a novel NADPH-dependent reductase which coacts with chalcone synthase in the biosynthesis of 6′-deoxychalcone

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Received 13 June 1988

Enzyme synthesis of 6′-deoxychalcone from 4-coumaroyl-CoA and malonyl-CoA has been achieved, using purified soybean chalcone synthase (CHS), NADPH and a further protein (reductase). This reductase was purified to apparent homogeneity by a procedure including affinity chromatography on Blue Sepharose and elution with $NADP^+$. This enzyme has a molecular mass of about 34 kDa and consists of a single polypeptide. Synthesis of deoxychalcone also occurred with parsley CHS, NADPH and the soybean reductase. The reductase catalyzed transfer of the pro-*R* hydrogen of [4-^{3}H]NADPH to the substrate.

Deoxychalcone synthesis; Reductase; Cell culture; (Soybean)

1. INTRODUCTION

The 5-deoxy series of flavonoids and isoflavonoids, including several phytoalexins, are biosynthetically derived from 4,2′,4′-trihydroxychalcone (6′-deoxychalcone) [1]. While the synthesis of 4,2′,4′,6′-tetrahydroxychalcone (6′-hydroxychalcone) from 4-coumaroyl-CoA and 3 molecules of malonyl-CoA by the enzyme chalcone synthase (CHS) is well established [2], the enzymatic reaction for synthesis of 6′-deoxychalcone has remained unknown. Recently, Furuya et al. [3] demonstrated the NADPH-dependent synthesis of 6′-[^{14}C]deoxychalcone and of the corresponding flavanone from 4-coumaroyl-CoA and [2-^{14}C]-malonyl-CoA with a crude extract of *Glycyrrhiza echinata* cells treated with yeast extract [3]. Synthesis of 5-deoxyflavanone in the presence of NADPH was also demonstrated with extracts from *G. echinata* protoplasts [4] and from soybean cell cultures (Furuya, personal communication). Removal of the hydroxy group from 6′-hydroxychalcone in the synthesis of 6′-deoxychalcone is unlikely, since previous ^{13}C NMR studies on incorporation of [1,2-^{13}C]acetate into 5-deoxyflavonoids indicated that this reaction does not involve a chalcone intermediate with a symmetrically oxygenated A-ring (e.g. 6′-hydroxychalcone) [5–7]. Elimination of the oxygen function is therefore assumed to take place at a polyketide intermediate (fig.1). However, it remains unknown whether synthesis of 6′-deoxychalcone is catalyzed by a separate deoxychalcone synthase, by the known chalcone synthase in the presence of NADPH, or by a combination of CHS, NADPH and a reductase.

We demonstrate here that pure CHS from soybean or parsley cell cultures in the presence of NADPH does not catalyze 6′-deoxychalcone synthesis but that this reaction requires an additional protein of 34 kDa. We have purified this reductase to apparent homogeneity and report on some properties of the deoxychalcone synthase system.

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

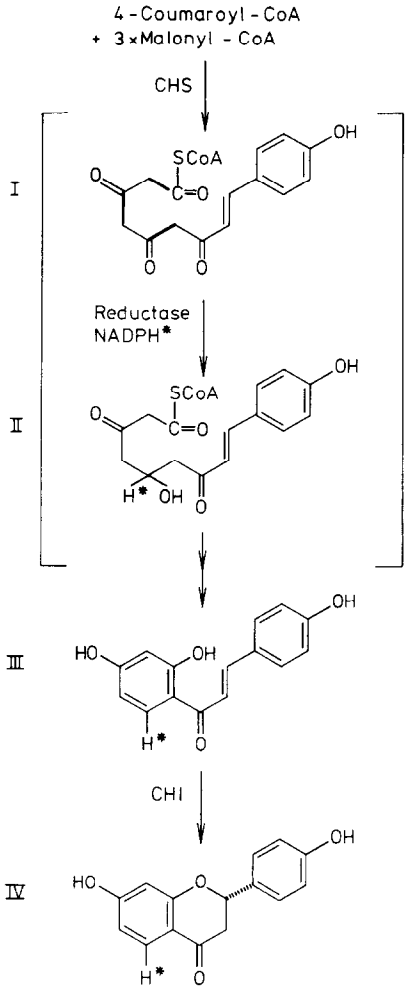

Fig.1. Postulated reaction mechanism for synthesis of 6′-deoxychalcone (III) by the co-action of chalcone synthase (CHS) and reductase. Chalcone isomerase (CHI) leads to 5-deoxyflavanone (IV).

2. MATERIALS AND METHODS

2.1. *Materials*

[2-^{14}C]Malonyl-CoA (2.18 GBq/mmol) and [4-^{3}H]NAD^{+} (81 GBq/mmol) were obtained from Amersham-Buchler (Braunschweig). FPLC columns were from Pharmacia (Freiburg). Flavonoids were from our laboratory collection.

2.2. *Cell cultures*

Cell suspension cultures of soybean (*Glycine max* cv. Harosoy 63) were propagated [8] and elicited with yeast extract as in [9].

2.3. *Buffers*

Buffer solutions were degassed under vacuum, equilibrated with nitrogen, and again degassed. The following buffers were used: (A) 0.1 M potassium phosphate (pH 6.0), (B) 50 mM imidazole-HCl (pH 6.8) with 10% glycerol and 2.8 mM mercaptoethanol and (C) 50 mM Tris-HCl (pH 8.5) with 10% glycerol and 2.8 mM mercaptoethanol.

2.4. *Assay for reductase*

The standard assay contained, in a final volume of 100 μl: 3.4 μg soybean CHS [10] (hydroxyapatite fraction), 0.09–0.27 μg reductase, 1 nmol 4-coumaroyl-CoA, 1.5 nmol [2-^{14}C]malonyl-CoA, 0.1 μmol NADPH and buffer A. Reactions were run for 30 min at 30°C and terminated by the addition of 200 μl ethyl acetate. The organic phase was separated by centrifugation, concentrated in vacuo and chromatographed on a cellulose plate with solvent 1 (in the presence of chalcone isomerase) or solvent 2 (in the absence of chalcone isomerase). The plates were scanned for radioactivity with a TLC analyzer (Berthold, Wildbad).

2.5. *Purification of reductase*

All column chromatographic steps were carried out at 4–8°C with an FPLC system. The Mono Q column was run at 20°C.

Breakage of the cells, ammonium sulfate fractionation and chromatography on Q-Sepharose were performed as described for purification of CHS [10]. Reductase activity eluted from Q-Sepharose between 45 and 75 mM NaCl and CHS between 75 and 135 mM NaCl. The reductase pool was concentrated by ultrafiltration and desalted.

This fraction (34 ml) was applied to a Blue Sepharose CL6B column (35 ml). The column was washed with buffer B and reductase was eluted with the same buffer containing 4 mM NADP^{+}. Further reductase activity was eluted with buffer B containing 500 mM NaCl. The reductase fraction eluted with NADP^{+} was concentrated by ultrafiltration and applied to a Sephacryl 200 HR column (385 ml). Reductase was eluted with buffer C. This fraction was applied to a Mono Q column (1 ml) and reductase eluted with a linear gradient of NaCl (0–200 mM) in buffer C. The main reductase activity appeared at 86–98 mM NaCl.

2.6. *Analytical methods*

TLC was performed on cellulose plates (Merck, Darmstadt) with the solvent systems (by vol.): 1, 15% acetic acid; 2, $CHCl_3$/acetic acid/water (10:9:1).

2.7. *Synthesis of [^{3}H]NADPH*

Synthesis of (4*R*)- and (4*S*)-[^{3}H]NADPH was performed as described in [11].

3. RESULTS

Soybean cell suspension cultures were treated with yeast extract to elicit phytoalexin (glyceollin) production [9]. Crude extracts from such cells incubated in the CHS assay at pH 6 in the presence of 1 mM NADPH [3] gave two new radioactive products on thin-layer chromatograms with solvents 1 and 2 which coincided with the positions of 6′-deoxychalcone and 5-deoxyflavanone,

Table 1

Purification procedure for reductase involved in 6′-deoxychalcone synthesis

Purification step	Protein (mg)	Spec. act. (μkat/kg)	Purification (-fold)	Recovery (%)
1. Q-Sepharose	330	0.39	1	100
2. Blue Sepharose	4.05	9.95	25.5	31
3. Sephacryl S200 HR	1.1	33.5	85.9	29
4. Mono-Q				
Fraction 13	0.11	109	279	9
Fraction 14	0.25	80	205	16

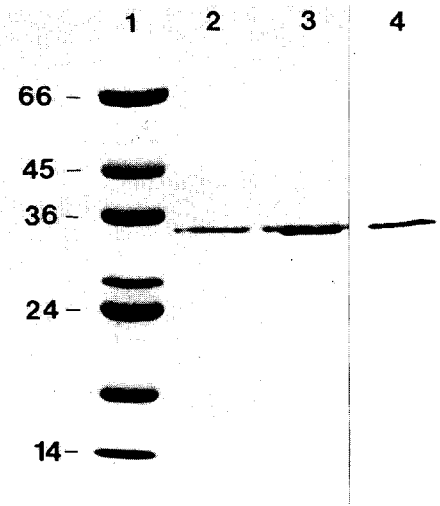

Fig.2. SDS-polyacrylamide gel of purified reductase. Lanes: 1, marker proteins; 2 (fraction 13) and 3 (fraction 14), enzyme from Mono Q, Coomassie staining; 4, enzyme from Mono Q, silver staining.

respectively. For investigating this enzyme system, advantage was taken of our purification procedure for CHS from soybean [10]. Absorption of the ammonium sulfate fraction on Q-Sepharose and elution with an NaCl gradient resulted in a peak of CHS activity which gave only very low yields of deoxychalcone in the presence of NADPH alone. However, when a fraction eluting before CHS (45–75 mM NaCl) was added, about 28% conversion to deoxychalcone/deoxyflavanone was observed. This result indicated that deoxychalcone synthesis requires in addition to CHS another protein which functions as a reductase.

Reductase activity was absorbed on Blue Sepharose and could be eluted with buffer B containing 4 mM $NADP^+$. Further purification of reductase was achieved by chromatography on Sephacryl S200 HR and Mono Q. The purification procedure is summarized in table 1. Fractions 13 and 14 from Mono Q gave a single protein band after SDS-polyacrylamide gel electrophoresis (fig.2). Incubation of 1.7 μg pure reductase in the enzyme assay yielded about 55% deoxychalcone as well as tetrahydroxychalcone (7%) and naringenin (37%). The identity of 6′-deoxychalcone and the corresponding 5-deoxyflavanone was established by cochromatography with a reference sample in several chromatographic systems including two-dimensional chromatography on cellulose HPTLC plates with solvents 1 and 2 and autoradiography. Furthermore, 6′-[^{14}C]deoxychalcone was converted with isoflavone synthase containing chalcone isomerase to [^{14}C]daidzein [12]. The latter was unequivocally identified by two-dimensional chromatography on cellulose and silica gel plates (not shown).

The following results proved that, in addition to CHS, reductase and NADPH are necessary for deoxychalcone formation. (i) Pure CHS from either soybean [10] or parsley [13] cell cultures gave, in the presence or absence of NADPH, only tetrahydroxychalcone and naringenin. (ii) Addition of pure reductase to these assay mixtures with NADPH and CHS yielded as major product deoxychalcone. (iii) When CHS was omitted no products were formed. Some of the radioscans of these experiments are shown in fig.3.

Replacement of NADPH by NADH in the enzyme assay decreased the yield of deoxychalcone by 92%. The Michaelis constant for NADPH was determined to be 17 μM. A mixture of [^{14}C]tetrahydroxychalcone/[^{14}C]naringenin, reductase and NADPH gave no reaction products.

Parallel incubations of (*R*)- and (*S*)-[4-^{3}H]-NADPH in the enzyme assay gave deoxychalcone with 166100 and 5200 cpm, respectively. This proves the pro-*R*(A) specificity of the reductase with respect to NADPH and transfer of H^- to the product as predicted from the postulated reaction mechanism (fig.1).

The molecular mass of the reductase was estimated from its elution volume using a Superose

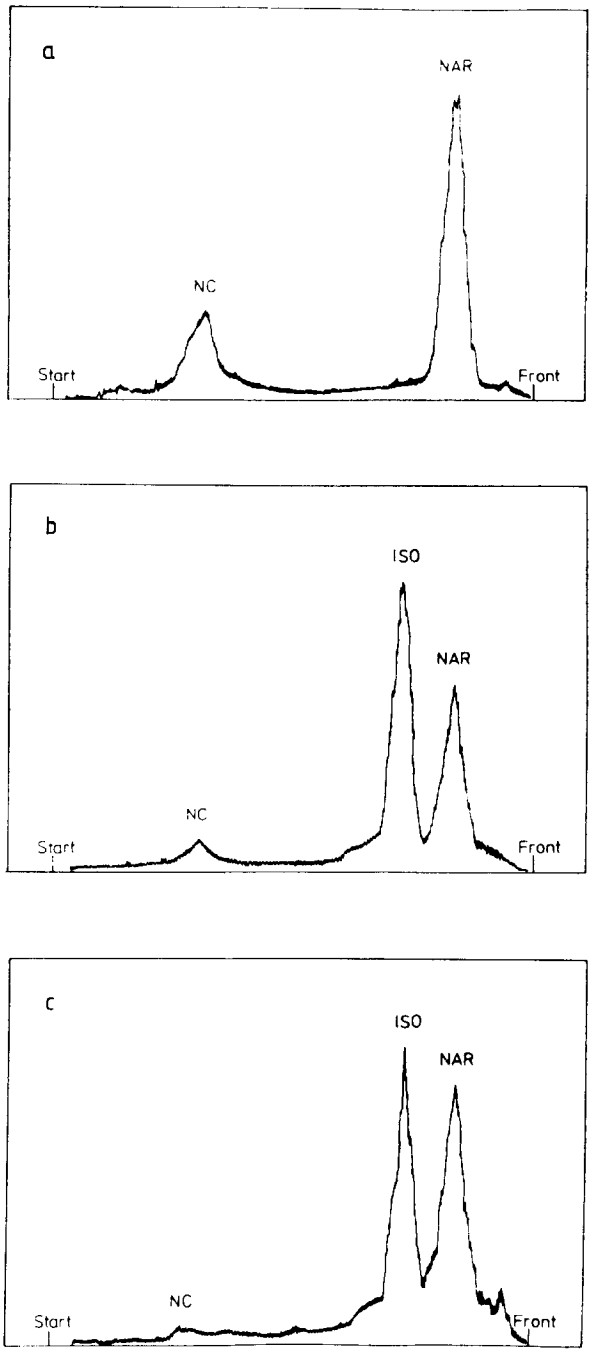

Fig.3. Radioscans of thin-layer chromatograms of enzyme incubations. Chromatograms were first developed with solvent 1, dried and developed with solvent 2. (a) Pure CHS from soybean with NADPH; (b) as (a) but with addition of pure reductase; (c) pure CHS from parsley with soybean reductase and NADPH. NC, naringenin chalcone; NAR, naringenin; ISO, 6'-deoxychalcone (isoliquiritigenin).

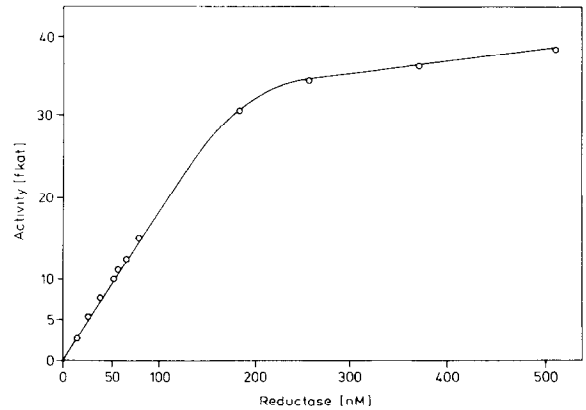

Fig.4. Dependence of 6'-deoxychalcone synthesis on reductase.

12 column as 33 ± 2.5 kDa. Since the reductase showed only one band of 34 kDa on SDS gels (fig.2), the enzyme contains one polypeptide.

Maximal deoxychalcone synthesis was observed at pH 6.0 with 81% of maximal activity at pH 5.0 and 34% at pH 8.0. With 3.4 μg CHS in the enzyme assay deoxychalcone formation was proportional to the amount of reductase added up to 0.65 μg reductase. This corresponds to an about 2:1 molar ratio of CHS reductase. A further slight increase in yield of deoxychalcone took place up to a 1:1 molar ratio of CHS:reductase (fig.4). From a Lineweaver-Burk plot, the apparent Michaelis constant of reductase with respect to CHS was found to be 0.75 μM.

4. DISCUSSION

From our results, the synthesis of 6'-deoxychalcone can be formulated as shown in fig.1. Reduction of the carbonyl group in the enzyme-bound polyketide intermediate is catalyzed by the reductase with transfer of the pro-*R* hydrogen of NADPH. Loss of water is assumed to take place after subsequent cyclization. Whether dehydration is spontaneous due to formation of the aromatic system or is also catalyzed by the reductase is unknown.

The interaction of chalcone synthase and reductase seems to be ionic, since higher salt concentrations inhibited the reaction (not shown).

The fact that combination of CHS from parsley, a plant which does not contain deoxyflavonoids,

and the reductase also gave deoxychalcone is remarkable.

Deoxychalcone synthesis in plants therefore seems to depend only on the presence of the reductase rather than on the nature of CHS. Expression of the reductase could be an important regulatory factor in the biosynthesis of soybean phytoalexins as this enzyme is obligatory for their formation. We plan to study induction of the reductase in soybean by infection with *P. megasperma* or by elicitor. For this purpose antibody against the reductase is being prepared.

Acknowledgements: The work was supported by the Deutsche Forschungsgemeinschaft (SFB 206) and Fonds der Chemischen Industrie. We thank Professor T. Furuya, Tokyo, for a copy of their communications prior to publication. We thank R. Biggs for valuable suggestions.

REFERENCES

[1] Harborne, J.B., Mabry, T.J. and Mabry, H. (1975) The Flavonoids, Chapman and Hall, London.
[2] Ebel, J. and Hahlbrock, K. (1982) in: The Flavonoids, Advances in Research (Harborne, J.B. and Mabry, T.J. eds) pp.641–679, Chapman and Hall, London.
[3] Ayabe, S.-I., Udagawa, A. and Furuya, T. (1988) Arch. Biochem. Biophys. 261, 458–462.
[4] Ayabe, S., Udagawa, A. and Furuya, T. (1988) Plant Cell Rep. 7, 35–38.
[5] Stoessl, A. and Stothers, J.B. (1979) Z. Naturforsch. C34, 87–89.
[6] Dewick, P.M., Steele, M.J., Dixon, R.A. and Whitehead, I.M. (1982) Z. Naturforsch. C37, 363–368.
[7] Ayabe, S. and Furuya, T. (1982) J. Chem. Soc. Perkin Trans. 1, 2725–2734.
[8] Ebel, J., Ayers, A.R. and Albersheim, P. (1976) Plant Physiol. 57, 775–779.
[9] Welle, R. and Grisebach, H. (1988) Arch. Biochem. Biophys. 263, 191–198.
[10] Welle, R. and Grisebach, H. (1987) Z. Naturforsch. 42c, 1200–1206.
[11] Fischer, D., Stich, K., Britsch, L. and Grisebach, H. (1988) Arch. Biochem. Biophys., in press.
[12] Kochs, G. and Grisebach, H. (1986) Eur. J. Biochem. 155, 311–318.
[13] Heller, W. and Hahlbrock, K. (1980) Arch. Biochem. Biophys. 200, 617–619.