Purification and Properties of Chalcone Synthase from Cell Suspension Cultures of Soybean

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Incubation of soybean cell suspension cultures in B-5 medium containing 0.4 M sucrose caused induction of chalcone synthase (CHS) up to a specific activity of 8 μkat/kg. CHS from these cultures was purified to apparent homogeneity by a 5-step procedure. Isoelectric focussing of pure CHS gave a major band at pH 5.45 and two weaker bands at pH 5.35 and 5.5. At least the bands at pH 5.35 and 5.45 had CHS activity. Analysis of pure CHS by two-dimensional electrophoresis gave a set of six proteins with a M_r of 40 kDa and pIs between 6.0 and 6.6. One-dimensional PAGE of CHS under non-denaturing conditions gave three closely spaced protein bands. A specific antibody was raised against soybean CHS which cross-reacted with parsley CHS.

Attempts to find synthesis of deoxychalcone or of the corresponding 7,4′-dihydroxyflavanone with CHS of different purification stages and with various cofactors failed.

Introduction

Synthesis of 2′,4,4′,6′-tetrahydroxychalcone from 4-coumaroyl-CoA and three molecules of malonyl-CoA is catalyzed by the enzyme chalcone synthase [1]. This key enzyme of flavonoid biosynthesis has been purified from a number of plants and plant cell cultures [1, 2].

We were interested in studying chalcone synthase from soybean (*Glycine max*. L. Merrill) in more detail for the following reasons: 1. The biosynthesis and induction of glyceollin, the phytoalexin of soybean, has been studied extensively [3, 4]. Since glyceollin originates from the deoxychalcone, 2′,4,4′-trihydroxychalcone, but soybean also synthesizes flavonoids derived from tetrahydroxychalcone, it was of interest to find out whether more than one chalcone synthase is present in soybean and if synthesis of deoxychalcone could be detected *in vitro*.

2. We wanted to obtain an antibody against the soybean CHS which could be used in studies on induction of glyceollin synthesis in soybean by infection by *Phytophthora megasperma* f. sp. *glycinea*.

Abbreviations: CHS, chalcone synthase; PAGE, polyacrylamide gel electrophoresis; TLC, thin layer chromatography.

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Materials and Methods

Chemicals

[2-^{14}C]Malonyl-CoA (2.18 GBq/mmol) was from Amersham Buchler (Braunschweig, FRG). Cinnamoyl-CoA esters and flavonoids were from our laboratory collection. The CHS-antiserum from parsley was a gift from K. Hahlbrock, Cologne.

Buffers

All buffers were degassed and saturated with nitrogen before addition of ascorbate.

Buffer A, 0.25 M Tris-HCl, pH 7.4; buffer B, 0.1 M imidazole, pH 6.8, containing 10% sucrose and 20 mM ascorbate; buffer C, 0.05 M imidazole, pH 6.7 containing 10% glycerol and 20 mM ascorbate; buffer D, 0.04 M sodium/potassium phosphate buffer, pH 7.8, containing 10% glycerol and 20 mM ascorbate; buffer E, as buffer D but with 0.08 M phosphate; buffer F, as buffer D but with 0.24 M phosphate.

Cell cultures and induction

Cell suspension cultures of soybean (*Glycine max* cv. Harosoy 63) were propagated as described [5] and were transferred every 7th day to fresh medium. For induction of chalcone synthase 6-day-old cells were incubated for 25 h in fresh B5 medium containing 0.6 M sucrose. Harvested cells were frozen in liquid nitrogen.

Assay for chalcone synthase

The assay was carried out as described previously [6, 7].

Purification of chalcone synthase

All the following operations were carried out between 4 °C and 8 °C. Column chromatography was carried out with an FPLC system (Pharmacia). In buffers containing ascorbate, protein was monitored at 350 nm.

Step 1. Frozen soybean cells (3.7 kg) were thawed in 4.4 l buffer A containing 48 g ascorbate and 350 g Dowex 1×2 (Cl^- form equilibrated with buffer A) and ground in two portions in a mortar for 1 h with 400 g quartz sand each. The slurry was filtered through glass-wool and centrifuged for 20 min at $17000\times g$. The supernatant was again filtered through glass-wool.

Step 2. A solution of $MgCl_2$ (1 M) was slowly added to the crude extract up to a final concentration of 30 mM $MgCl_2$. After stirring for 20 min the slurry was centrifuged for 20 min at $42000\times g$. A 10% solution of polyethyleneimine (adjusted with HCl to pH 7.5) was then slowly added to the supernatant with stirring to produce a final concentration of 0.3%. After 20 min stirring the precipitate was spun down at $17000\times g$ for 20 min and the supernatant liquid was brought to 40% saturation with solid $(NH_4)_2SO_4$. After 15 min the suspension was centrifuged at $17000\times g$ for 20 min and the precipitate was discarded. The supernatant was brought to 80% saturation with $(NH_4)_2SO_4$ and the protein collected as described. The supernatant was discarded. The precipitate was dissolved in 187 ml buffer B and the solution cleared by centrifugation.

Step 3. The above solution (145 ml) was applied to a column (5×43 cm) of DGP6 (Bio Rad) which had been equilibrated with buffer C. Protein eluted with buffer C was directly applied to a column (5×10.5 cm) of Q-Sepharose (Pharmacia). After absorption of the protein the column was washed with 400 ml buffer C. Chalcone synthase was eluted with a linear gradient of 0 to 300 mM NaCl in buffer C at a flow rate of 4 ml/min. As shown in Fig. 2 two pools with CHS activity were collected.

Step 4. The two pools of chalcone synthase from step 3 were each concentrated by ultrafiltration and were then each applied to a column (5×87 cm) of Sephacryl S 200 which had been equilibrated with buffer D. CHS was eluted with buffer. D. Maximal enzyme activity appeared at 870 ml. The exclusion volume was 530 ml.

Step 5. The CHS-containing fractions from step 4 were concentrated by ultrafiltration and were then each applied to a hydroxyapatite column (2.6×6.4 cm) which had been equilibrated with buffer D. After washing with buffers D and E the enzyme was eluted with a linear gradient of 80 mM–240 mM phosphate buffer (buffers E and F). CHS was eluted between 134 mM –184 mM.

Analytical methods

Thin layer chromatography was performed on cellulose plates (E. Merck, Darmstadt, FRG) with the following solvent systems (ratios by volume): 1) 15% acetic acid; 2) 5% acetic acid; 3) chloroform/acetic acid/water (10:9:1). For two-dimensional separations solvents 1 and 3 were used. Flavonoids were detected under ultraviolet light or by spraying the plates with a 0.2% solution of Fast Blue salt (Merck) and fuming with ammonia. Radioactive substances were detected with a TLC-analyzer (Berthold, Wildbad). SDS-gel electrophoresis was carried out with flat-bed gels [8] in the buffer system of Laemmli [9]. Isoelectric focussing was performed on PAGplates with ampholine pH 5–6.5 (LKB, Gräfelfing) according to instructions of the producer.

Translation system

The *in vitro* translation of total RNA was carried out as described previously [10].

Western Blots

The Western blots were carried out as described [11, 12]. To prevent proteolysis in crude cell extracts the cells were ground in a mortar in liquid nitrogen and the fine powder was then boiled for 10 min in SDS buffer (250 mM Tris-HCl (pH 7.5), 20 mM EDTA, 5% SDS).

Results

Induction of chalcone synthase in soybean cell suspension cultures with sucrose

The induction of CHS in soybean cell cultures by osmotic stress [13] was tested in the following way. Six-day-old cells were transferred to fresh B-5 medium and incubated for 10 h. To one part of the

cells was then added sucrose to a final concentration of 0.4 M; the other part remained in the B-5 medium. The CHS activity of the cells after various times of induction was determined and the results are shown in Fig. 1. Control cells which had not been transferred to fresh medium contained only very low enzyme activity. Transfer to fresh medium caused an increase to about 2 μkat/kg protein after 16 h; with sucrose almost 8 μkat/kg were reached after 22 h (12 h after sucrose addition).

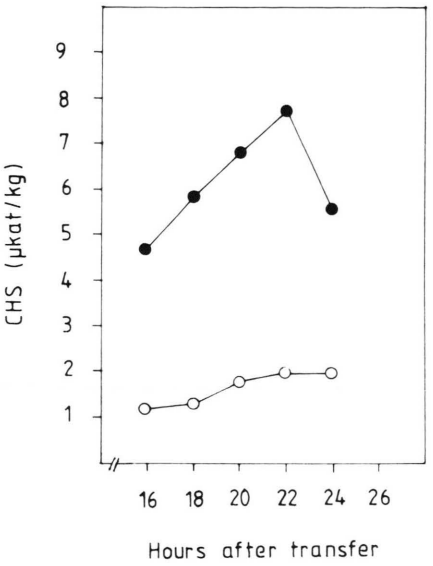

Fig. 1. Induction of CHS in soybean cell cultures with sucrose. Cells transferred to fresh B-5 medium (○); cells treated with 0.4 M sucrose 10 h after transfer to fresh medium (●).

Purification of chalcone synthase

CHS from sucrose induced soybean cell cultures was purified to apparent homogeneity by a 5-step procedure which is summarized in Table I. Ascorbate was added to the nitrogen-saturated buffers on the basis of experience gained in the isolation of CHS from parsley cell cultures [7]. The elution profile of CHS from Q-sepharose (step 3) consistently gave two partially separated peaks of activity which were pooled separately (Fig. 2). However, further purification of the two separate fractions with CHS activity on Sephacryl S200 and hydroxyapatite gave proteins with identical position on SDS gels (Fig. 3). Pool 2 contained an impurity at 22 kDa in addition to the CHS subunit at 40 kDa. In another purification protocol hydrophobic chromatography on phenyl Sepharose was included. Such CHS preparations showed two bands on SDS gels, an upper band with M_r 40 kDa and a lower band with M_r 38 kDa.

Apparent molecular weight

The apparent M_r of CHS was determined on a calibrated column of Superose 12. CHS from soybean gave a M_r of 75 kDa ± 5 kDa. The enzyme is therefore composed of two subunits with identical M_r (compare Fig. 3), but with different isoelectric points (see below).

Isoelectric focussing

Isoelectric focussing of pure CHS in a pH gradient of 5.0 to 6.5 gave a major protein band at pH 5.45 and two weaker bands at pH 5.35 and pH 5.5 (Fig. 4).

Table I. Purification procedure for chalcone synthase from soybean cell suspension culture (3.7 kg net weight).

Purification step	Volume [ml]	Protein [mg]	Specific activity [μkat/kg protein]	Purification [-fold]	Recovery [%]
1. Crude extract	6300	15150	0.68	1	100
2. Poly(ethyleneimine), ammonium sulfate (40–80%)	145	3697	1.95	2.9	69
3a. Q-Sepharose Pool 1	50	215	8.0	11.8	16.6
4a. Sephacryl S200	90	41	26.1	38.4	10.3
5a. Hydroxyapatite	34	2.6	153	225	3.7
3b. Q-Sepharose Pool 2	70	350	7.2	10.6	24.4
4b. Sephacryl S200	88	61	17.9	26.4	10.5
5b. Hydroxyapatite	63	3.8	85	125	3.1

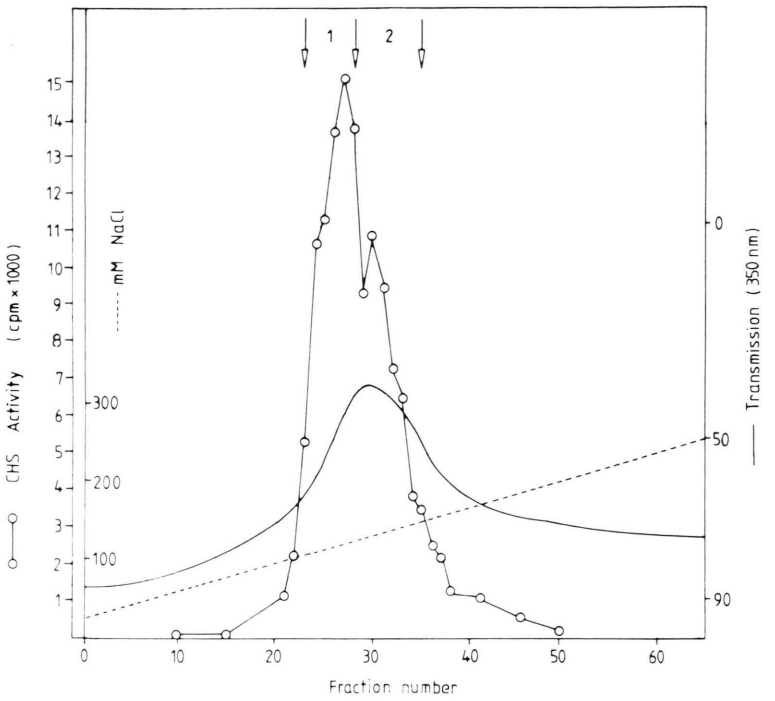

Fig. 2. Elution profile of CHS on Q-Sepharose Fast Flow.

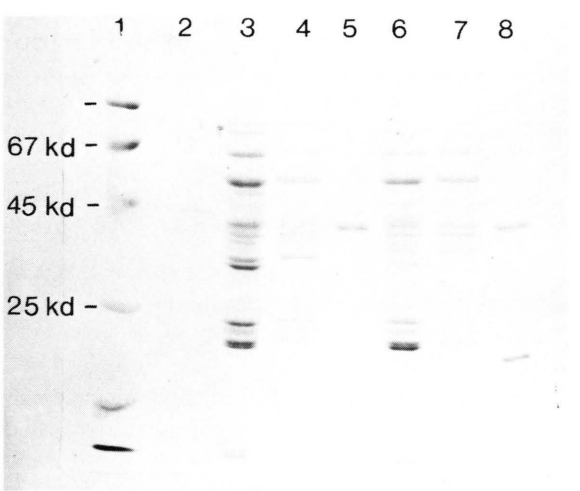

Fig. 3. SDS-gel of different purification steps for CHS (Table I). Lane 1, marker proteins; lane 2, CHS from parsley; lane 3, Q-Sepharose pool 1; lane 4, Sephacryl S200 pool 1; lane 5, hydroxyapatite pool 1; lane 6, Q-Sepharose pool 2; lane 7, S200 pool 2; lane 8, hydroxyapatite pool 2.

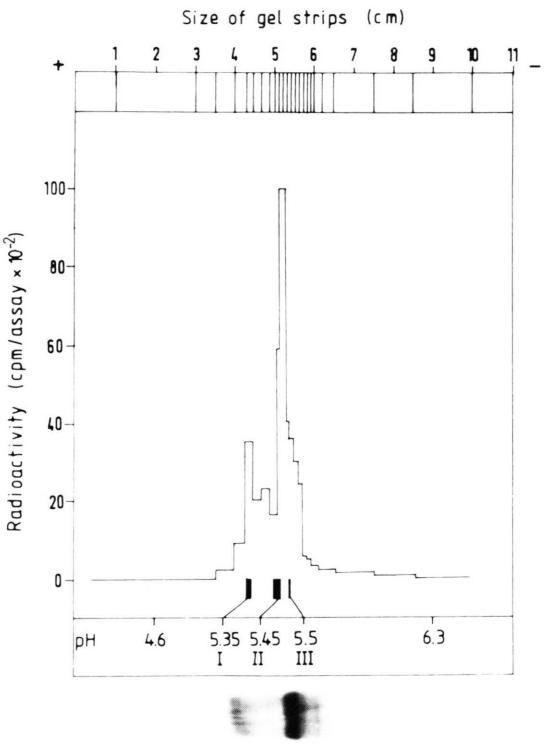

Fig. 4. Activity profile of CHS from Sephacryl S200 pool 1 after chromatofocussing on PAGplates. The lower part of the figure shows the protein bands with silver stain from a parallel run.

To test whether the proteins with different pIs are enzymatically active the gel of a parallel run was cut into strips and CHS was assayed in the individual fractions. The activity profile shown in Fig. 4 demonstrates that the proteins at pH 5.35 and 5.45 have CHS activity. Whether the weak band at pH 5.5 is also enzymatically active could not be decided. A similar CHS-activity profile was obtained with the ammonium sulfate pellet and with hydroxyapatite pool 1.

Electrophoresis

Analysis of pure CHS by two-dimensional electrophoresis [14] gave a set of 6 proteins with equal M_r of 40 kDa and isoelectric points between pH 6.0 and 6.6 (Fig. 5). This protein pattern is in agreement with results of an analysis of soybean CHS subunits synthesized either *in vivo* or *in vitro* where the same subunit pattern was observed [15]. One-dimensional PAGE of pure CHS under non-denaturating conditions gave three closely spaced but clearly separated protein bands.

Properties of chalcone synthase from soybean

The enzyme assay was linear with protein up to 30 µg and with time up to 30 min at 5.5 µg protein. The temperature optimum of the reaction was 45 °C.

Substrate specificity was tested with the coenzyme A esters of cinnamic, 4-coumaric, caffeic, ferulic and sinapic acids.

The pH optimum with 4-coumaroyl-CoA was 7.5. At this pH the only reaction product was (2*S*)-naringenin. The reaction product with caffeoyl-CoA was identified by thin layer chromatography as eriodictyol. As has been observed previously with

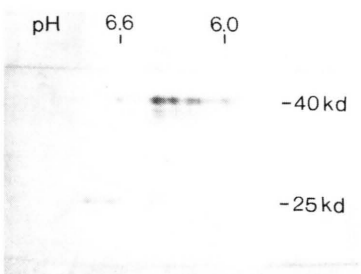

Fig. 5. Two-dimensional electropherogram of pure CHS. Silver stain.

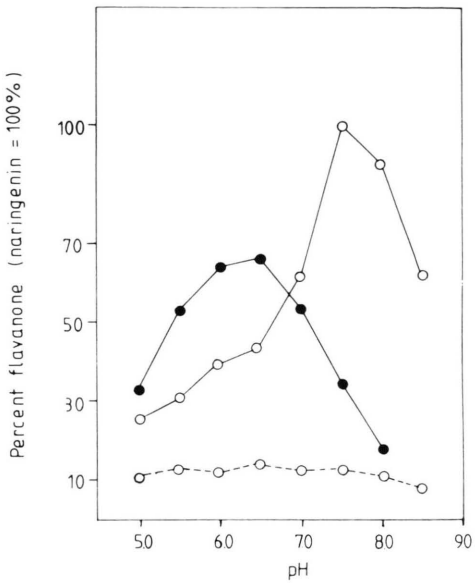

Fig. 6. Dependence of CHS activity on pH with different substrates. Naringenin (substrate 4-coumaroyl-CoA) (○); eriodictyol (substrate 4-caffeoyl-CoA) (●); pinocembrin (substrate cinnamoyl-CoA) (○---○).

CHS preparations from other plants [16–19], the pH optimum for synthesis of eriodictyol (around pH 6) was different from that for naringenin synthesis (pH 7.5) (Fig. 6). With cinnamoyl-CoA a low conversion (maximal 15%) to pinocembrin (5,7-dihydroxyflavanone) was observed which showed no pH optimum. With feruloyl-CoA one major and 3 minor reaction products were formed. None of these products was identical with homoeriodictyol (5,7,4′-trihydroxy-3′-methoxyflavanone). No reaction products were found with sinapoyl-CoA.

Attempts to find synthesis of deoxychalcone

Extracts of assays were analyzed for synthesis of liquiritigenin (7,4′-dihydroxyflavanone) or the corresponding chalcone by TLC with solvent systems 1 and 2 and in some cases by two-dimensional TLC. Solvent 1 (15% acetic acid) gave a good separation of naringenin ($R_f = 0.33$) and liquiritigenin ($R_f = 0.45$). When mercapto reagents were used in the enzyme assay instead of ascorbate a new radioactive product with $R_f = 0.73$ (solvent system 1) was formed in considerable amounts. Synthesis of this product did depend on presence of malonyl-CoA in the assay but did not require 4-coumaroyl-CoA. The reaction was

of enzymatic nature and was probably a malonylation of SH-groups in dithioerythritol, dithiothreitol and 2-mercaptoethanol.

The following cofactors (single or in combinations) were added to the CHS assay under anaerobic conditions at pH 7.5 or 6.5 and with enzyme from different purification stages: NADH, NADPH, FMN, FAD, thioredoxin, and dithionite. A number of new radioactive products were detected which in no case corresponded with deoxychalcone or deoxyflavanone.

Another series of experiments was carried out with soybean protoplasts [13] and permeabilized soybean cells. Protoplasts were lysed by osmotic shock to give a solution with 1.8 mg/ml protein and this was used for the CHS assay. No formation of deoxychalcone/flavanone could be detected. Protoplasts used for these experiments did accumulate glyceollin [13].

Furthermore, cells which had been challenged with elicitor from *Phytophthora megasperma* f.sp. *glycinea* [15] were permeabilized with DMSO [20, 21] and incubated with 4-coumaroyl-CoA and [^{14}C]malonyl-CoA for various lengths of time. Naringenin and apigenin were found in varying proportions on the radioscans of the cell extracts, but no deoxy compounds could be detected.

Antibody against CHS from soybean

An antiserum against soybean CHS was raised in rabbits with 1.24 mg pure protein. The antiserum was purified by FPLC on protein-A-Sepharose. A SDS gel of the column eluate showed only bands of the heavy and light chain IgG. In double immunodiffusion only one precipitine line was visible with crude extract from soybean cells and with CHS from Q-Sepharose pool 1. The soybean CHS antiserum showed cross-reactivity with parsley CHS. In immunotitration experiments 50% of CHS activity was precipitated with about 4 µg purified antibody. On Western blots of PAGE from cell extracts with CHS antibody three bands with 22, 38 and 40 kDa were detected. The 38-kDa band was absent when cells were ground in liquid nitrogen and immediately boiled in SDS-buffer. The cross-reactivity of the antiserum with the 22-kDa protein could be due to an impurity in the CHS preparation used for immunization. When an *in vitro* translation product of soybean mRNA was precipitated with the antibody from soybean, only one protein species with M_r 40 kDa on SDS-gel was seen. With the antibody against parsley CHS two proteins with 40 kDa and 38 kDa were visible (Fig. 7).

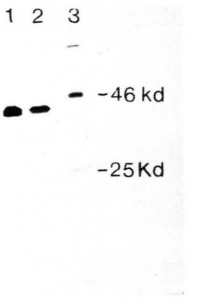

Fig. 7. *In vitro* translation of soybean RNA. Lane 1, CHS antibody from parsley; lane 2, CHS antibody from soybean; lane 3, marker proteins.

Discussion

Stimulation of soybean cell cultures with sucrose is a convenient method of obtaining cells with high CHS activity. The specific CHS activity found here is similar than the activity which has been reported for soybean cells challenged with glucan elicitor from *Pmg* [10].

By the 5-step purification procedure we obtained pure CHS which showed one protein band at M_r 40 kDa on SDS-gels. From the present and previous [15] results it can be concluded that there are no CHS species with different molecular weights present in soybean but that there exist a number of charge isomers. Analysis of CHS synthesized *in vivo* and *in vitro* by two-dimensional gel electrophoresis had shown six proteins between pH 6.1 and 7.1 [15]. The same protein pattern was found in the present work with pure CHS. Taking into account the results of IEF under non-denaturing conditions (Fig. 4) it is evident that the six proteins on O'Farrel gels are the subunits of three charge isomers of CHS. At least two of these isomers are enzymatically active (Fig. 4). The finding that there are several CHS charge isomers might be explained by the number of CHS gene copies in the soybean genome. Since CHS forms a multi-gene family of about 8–10 members (R. Wingender-Drissen and J. Schell, unpublished) the different CHS proteins most likely represent different gene products. Evidence for this can be deduced from sequence data of two CHS genes which imply some differences in the amino acid composition of the products (R. Wingender-Drissen and J. Schell,

unpublished). Two charge isomers have also been found for CHS from spinach [16].

Various attempts to detect synthesis of 5-deoxychalcone (flavanone numbering) failed. This is surprising because stimulated soybean cell cultures produce as major products glyceollin and other isoflavonoids derived from deoxychalcone. But synthesis of 5-deoxyflavonoids could not be detected *in vitro* even in experiments using a lysate of soybean protoplasts, in which cellular components are present in high concentration, or in studies in which precursors were applied to permeabilized cells.

Acknowledgements

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