Two Different Chalcone Synthase Activities from Spinach

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Chalcone synthase activity was found in enzyme preparations from spinach. In homogenates of young leaves two different activities of the enzyme could be separated by DEAE-ion exchange chromatography and chromatofocusing. Both activities formed naringenin with [2-^{14}C] malonyl-CoA and 4-coumaroyl-CoA as substrates. They exhibited only slight differences in substrate specificity. For both activities 4-coumaroyl-CoA proved to be the most suitable substrate at both pH 6.8 and 8.0. Eriodictyol and homoeriodictyol formation from caffeoyl-CoA and feruloyl-CoA, respectively, only occured at pH 6.8. The formation of naringenin by the two activities was maximal at pH 7.5–8.0 and dependent upon the DTE-concentration in the assay mixture.

Introduction

Chalcone synthase, the key enzyme in flavonoid biosynthesis, catalyses the formation of 2′,4,4′,6′-tetrahydroxychalcone by condensation of three molecules of malonyl-CoA with one molecule of 4-coumaroyl-CoA. The first enzymic studies on enzyme preparations from cell suspension cultures of parsley [1, 2] followed investigations on the enzyme activity from different plant systems and different parts of plants [3–9]. Both caffeoyl-CoA and feruloyl-CoA can substitute for 4-coumaroyl-CoA in the condensation reaction [3, 4].

Recently immunological studies were used to determine the localization of chalcone synthase in tulip anthers [10]. Further immunohistological studies using anthers as an enzyme source were limited by the lack of suitable developmental stages for the enzyme isolation which were only available during a short time of the year. Spinach leaves were therefore chosen for further studies. For the first time, the existence of two different activities of chalcone synthase could be shown. Some characteristics of the two activities are reported here.

Materials and Methods

Plant material

Spinach plants were cultivated in the botanical garden of Münster.

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Chemicals

[2-^{14}C] malonyl-CoA (1.71 GBq/mmol) was purchased from N. E. N., Boston, Mass. The hydroxycinnamoyl-CoA esters were synthesized by Dr. R. Sütfeld and Mr. P. A. Bäumker as described previously [11].

Buffer solutions

A: 0.1 M potassium phosphate buffer, pH 6.8, containing 40 mM ascorbate and 14 mM glutathione,
B: 50 mM imidazole, pH 6.5,
C: 25 mM piperazine, pH 5.35,
D: Polybuffer 74 (Pharmacia), 1 : 10 dilution, pH 4.0,
E: 0.05 M potassium phosphate buffer, pH 6.8.
The buffers B–E contained 6 mM DTE.

Enzyme preparation

All steps were carried out at 0–4 °C. 20 g leaves (2–4.5 cm; Fig. 1) were homogenized with 5.0 g Polyclar AT (Serva) and 400 ml buffer A for 1 min in a Waring blendor. The homogenate was filtered through nylon cloth and centrifuged for 5 min at 10 000 × g. Solid $(NH_4)_2SO_4$ was added to the supernatant. The protein fraction which precipitated between 50 and 80% saturation was collected by centrifugation at 27 500 × g for 20 min. The pellet was dissolved in buffer B for ion exchange chromatography and buffer C for chromatofocusing.

Ion exchange chromatography

The protein solution was desalted by passing through a column of Sephadex G-25 equilibrated

with buffer B. The protein fraction was loaded onto an ion exchange column packed with DEAE-Trisacryl M (LKB; 1.6×15 cm) which had been equilibrated with buffer B. Unbound protein was removed by washing with two gel volumes of the same buffer. The elution of bound protein was performed by applying a linear salt gradient from 0–500 mM NaCl using 10 gel volumes of buffer (flow rate: 10 cm/h). Fractions of 50 drops were collected.

Chromatofocusing

The combined active fractions of the DEAE-column with respect to the first and second activity and a 50–80% $(NH_4)_2SO_4$-solution prepared as described above were desalted by passing through a column of Sephadex G-25 equilibrated with buffer C. The protein fraction was then applied to a chromatofocusing column (PBE 94, Pharmacia; 0.9× 50 cm) which had been equilibrated with buffer C. Elution was carried out with buffer D (flow rate: 24 cm/h). Fractions of 50 drops were collected.

Molecular sieving

A 50–80% $(NH_4)_2SO_4$-precipitated protein pellet was dissolved in 10 ml of buffer E and applied to a Sephadex G-150 column (2.6×92 cm) equilibrated with the same buffer. The protein separation was carried out at a constant flow rate of 8 cm/h. Fractions of 50 drops were collected.

Enzyme assay

The synthase assay was slightly modified according to Sütfeld *et al.* [11]. The reaction mixture contained in a total volume of 125 µl: 100 µl 0.1 M potassium phosphate buffer, pH 8.0, 0.5 nmol of hydroxycinnamoyl-CoA in 10 µl of the same buffer, 1.1 nmol of [2-^{14}C] malonyl-CoA in 5 µl of hydrochloric acid, pH 3.5 and 10 µl enzyme solution resulting in a final DTE-concentration of 0.5 mM. After incubation for 30 min at 30 °C the reaction was stopped by adding 20 µl methanol and 200 µl ethylacetate [12]. The reaction products were immediately extracted. An aliqout of 100 µl of the organic extract was used for scintillation counting and a portion of 80 µl for thin layer chromatography.

Determination of pH optimum

Incubations were carried out with 100 µl 0.1 M potassium phosphate buffer, pH between 6.0 and 8.5, 10 µl 4-coumaroyl-CoA, 5 µl [2-^{14}C] malonyl-CoA and 10 µl enzyme solution.

Effect of DTE-concentration on product formation

Reaction mixtures contained 10 µl 4-coumaroyl-CoA, 5 µl [2-^{14}C] malonyl-CoA, 10 µl enzyme solution and 100 µl 0.1 M potassium phosphate buffer, pH 8.0, containing 0, 1, 3 or 6 mM DTE. Consequently the final DTE-concentrations in the enzyme assay were 0.5, 1.3, 2.9 and 5.3 mM, respectively.

Identification of the reaction products

The ethylacetate-soluble reaction products were co-chromatographed in 15% EtOH on cellulose plates (Merck) with an authentic sample of the expected flavanone. The radioactive zone corresponding to the reference compound was scraped off, eluted with methanol and evaporated to dryness under nitrogen. The residue was redissolved in methanol and co-chromatographed in either chloroform : acetic acid : water = 10 : 9 : 1 (v/v/v) or 30% acetic acid. Co-chromatography on thin layer silica gel plates (Kieselgel 60, Merck) was performed in the solvent system hexane : ethylacetate : methanol = 50 : 50 : 1 (v/v/v) as described above. The chosen assay did not allow the proof of chalcones but only of the corresponding flavanones [8].

Results

Young leaves showed the highest specific chalcone synthase activity and consequently proved to be the most suitable enzyme source for the purification procedure (Fig. 1).

Two different activities of chalcone synthase were separated using DEAE-ion exchange chromatography and chromatofocusing. From the DEAE-column the first activity (A I) was eluted at a concentration of 110 mM NaCl, the second (A II) at 160 mM NaCl (Fig. 3). Subjecting A I and A II to

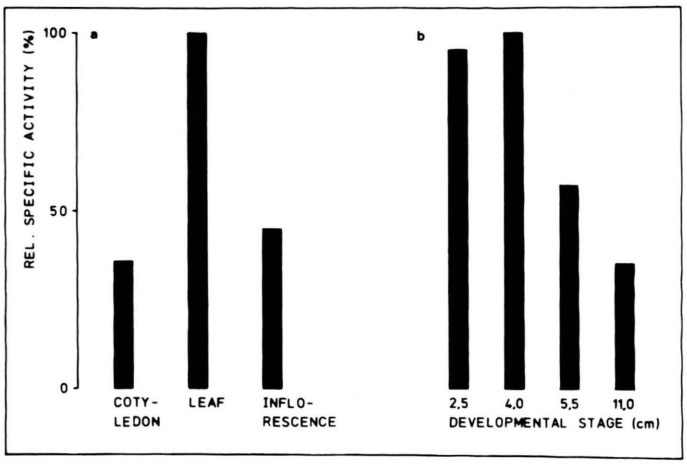

Fig. 1. Chalcone synthase activity; a) in different organs of spinach, b) as a function of the developmental stage of leaves. The highest specific activity is set as 100%.

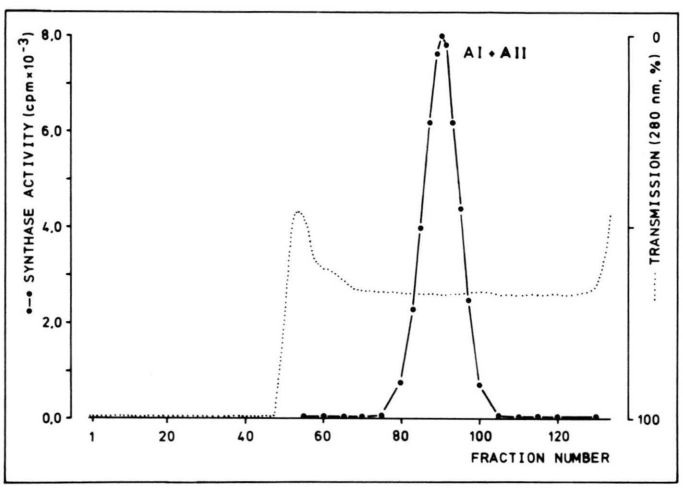

Fig. 2. Molecular sieving on Sephadex G-150. The two chalcone synthase activities (A I and A II) are not separated.

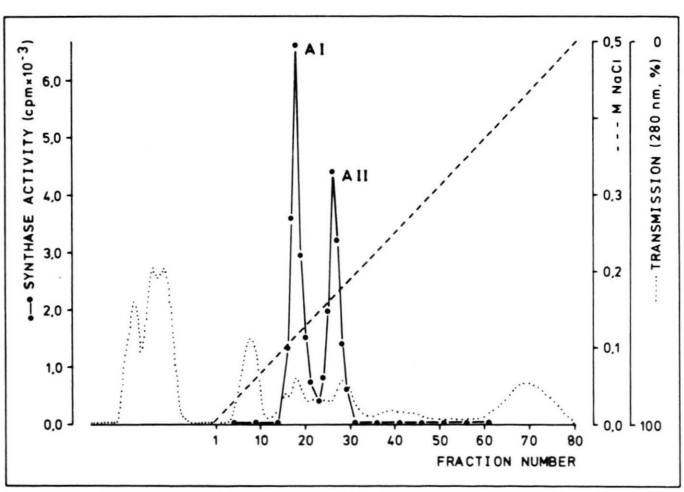

Fig. 3. Separation of two different chalcone synthase activities (A I, A II) by DEAE-ion exchange chromatography.

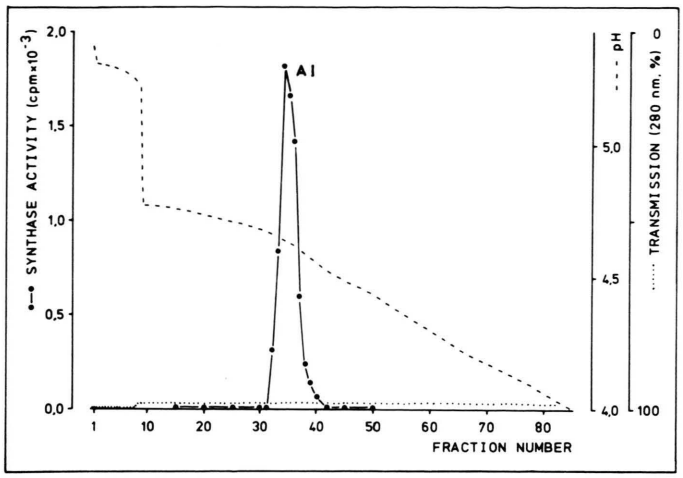

Fig. 4. Chromatofocusing of chalcone synthase activity A I.

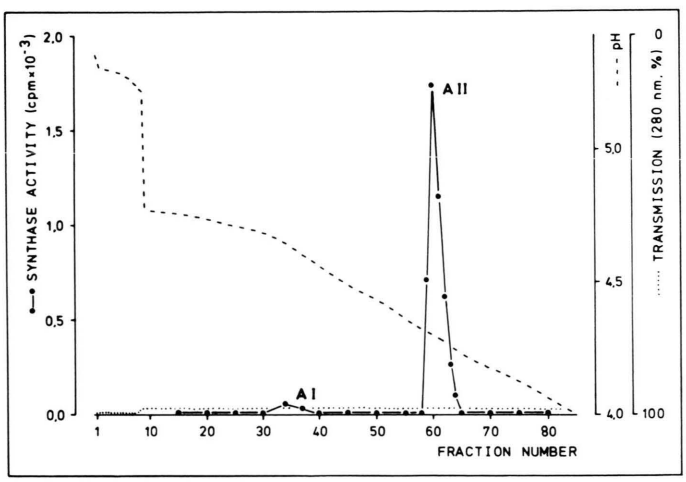

Fig. 5. Chromatofocusing of chalcone synthase activity A II. Since the separation of the two activities by ion exchange chromatography is not complete a residual activity of A I is also detected.

Table I. Substrate specificity of A I and A II at two different pH-values.

Substrate	pH 6,8				pH 8,0			
	A I		A II		A I		A II	
	Total activity [%] [a]	Appr. ratio of flavanone to other products [b]	Total activity [%] [a]	Appr. ratio of flavanone to other products [b]	Total activity [%] [a]	Appr. ratio of flavanone to other products [b]	Total activity [%] [a]	Appr. ratio of flavanone to other products [b]
4-coumaroyl-CoA	100	1:0	100	1:0	100	1:0	100	1:0
caffeoyl-CoA	10	2:1	10	2:1	3	0:1	4	0:1
feruloyl-CoA	61	1:1	39	1:1	30	0:1	21	0:1
sinapoyl-CoA	3	[c]	2	[c]	1	[c]	1	[c]

[a] Total activity of the enzymic conversion of malonyl-CoA to ethylacetate-solube products (s. Whitehead *et al.* (1983)); total activity with 4-coumaroyl-CoA as substrate is set as 100%.
[b] Only enzymatically formed products.
[c] Not determined, due to the low amount of total activity.

chromatofocusing resulted in elution of A I at pH 4.65 (Fig. 4) and elution of A II at pH 4.30 (Fig. 5). Both activities were released from the chromatofocusing column at the corresponding pH values when a protein solution, which had not been pre-separated by ion-exchange chromatography, was chromatofocused. Molecular sieving by Sephadex G-150 did not separate the two activities (Fig. 2).

Incubation of [2-^{14}C] malonyl-CoA and 4-coumaroyl-CoA with A I or A II and co-chromatography of the ethylacetate-soluble reaction products in three different solvent systems with an authentic sample of reference compound indicated that both activities form naringenin. The two activities did not exhibit differences in pH optimum. Maximum product formation with [2-^{14}C] malonyl-CoA and 4-coumaroyl-CoA as substrates was observed at pH 7.5 – 8.0. Increasing the DTE-concentration in the incubation mixture above 0.5 mM did not increase the total activity. The absence of DTE has been shown to cause a loss of activity of about 40% relative to the final DTE-concentration of 0.5 mM. Cellulose thin layer chromatograms developed in 15% EtOH indicated that, at a final DTE-concentration of 0.5 mM in the reaction mixture, the main product (P I; R_f: 0.25) was naringenin. A further product (P II; R_f: 0.80) is assumed to be formed non-enzymatically. It appeared in a comparable amount on the chromatogram of the blank. Increasing DTE-concentrations led to decreasing amounts of naringenin, enhanced production of P II and the formation of a further product (P III; R_f: 0.70), which was formed in increasing amounts. The appearance of a fourth product (P IV; R_f: 0.40) was restricted to a few chromatograms and was not clearly dependent upon the DTE-concentration.

The two activities exhibited only slight differences in substrate specificity. 4-coumaroyl-CoA was the most suitable substrate for both activities. The amounts of total product formed by A I and A II at two different pH values with caffeoyl-CoA, feruloyl-CoA or sinapoyl-CoA as substrates are presented in Table I. Co- and rechromatography of the products with an authentic sample of the corresponding flavanone showed that eriodictyol and homoeriodictyol formation by both A I and A II only occured at pH 6.8. In this case, besides the flavanone and P II the occurrence of P III and of a new product (P V; R_f: 0.85) was observed on cellulose thin layer plates in 15% EtOH. At pH 8.0 production of P II, P III, P V and in addition of another product (P VI; R_f: 0.35) took place. Formation of eriodictyol and homoeriodictyol was not observed. Due to the low amount of total product formed with sinapoyl-CoA as substrate identification of the reaction products was not definite.

Discussion

Chalcone synthase activity which has been demonstrated in various plant systems [1–9] has now been shown in spinach leaves. High specific activity was found in young leaves. Comparable results relative to petals have already been reported [13]. For the first time, in enzyme preparations from young spinach leaves two different activities of chalcone synthase were separated by means of ion exchange chromatography and chromatofocusing. The two activities were not separated by molecular sieving. Therefore both activities are assumed to have the same molecular weight. SDS gel electrophoresis of each of the two proteins revealed one major band with a molecular weight of approximately 45 000. Furthermore the two proteins exhibit similar substrate specificities and identical pH optima for naringenin formation. They do not differ in their behaviour towards varying DTE-concentration during incubation.

Compared with chalcone synthase preparations from other plants [1–9] the two activities from spinach leaves show a similar pH optimum for naringenin formation. With respect to substrate specificity the non-spinach enzymes exhibit either only naringenin formation from 4-coumaroyl-CoA [5–7, 9] or naringenin and eriodictyol production from 4-coumaroyl-CoA and caffeoyl-CoA, respectively. In the latter case, a difference in pH optimum for the production of the two flavanones was observed. Maximum naringenin formation occured at pH 8.0, maximum eriodictyol formation at pH 6.5–7.0 [3, 8]. Only small amounts of homoeriodictyol were formed from feruloyl-CoA at the lower pH values [3]. The enzyme preparation from tulip anthers formed besides naringenin both eriodictyol and homoeriodictyol at pH 7.6 – 8.0 [4]. These three flavanones are also formed by the two activities from spinach leaves. Similar to some enzyme preparations mentioned above eriodictyol and homoeriodictyol were only formed at the lower pH value. A

higher conversion of feruloyl-CoA than of caffeoyl-CoA has so far not been observed.

The effect of varying thiol concentrations in the assay mixture on the enzyme activity has been described in several reports [6, 7, 9]. An increasing rate of reaction was induced by increasing thiol concentrations in the incubation mixture. The activities of the two spinach leaf proteins were not stimulated by DTE at concentrations above 0.5 mM. In agreement with the above-mentioned reports, the formation of naringenin decreased at higher DTE-concentrations. At a final DTE-concentration of 0.5 mM naringenin and P II were the only reaction products.

Acknowledgement

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