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Isochorismate synthase isoforms from elicited cell cultures of *Rubia tinctorum*

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Abstract

Elicitation of *Rubia tinctorum* cell cultures with a *Pythium aphanidermatum* elicitor leads to a doubling of anthraquinone content which is preceded by a large rise in isochorismate synthase (ICS; EC 5.4.99.6) activity. Two ICS isoforms were purified from the elicited cultures. Both isoforms had an absolute requirement for Mg^{2+}. Isoform I and II had a K_m for chorismate of 365 and 466 μM, respectively. Gel filtration indicated a molecular mass of approximately 67 kD for both isoforms. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: *Rubia tinctorum* L; Rubiaceae; Cell cultures; Isochorismate synthase; Elicitation

1. Introduction

The shikimate pathway is an essential part of the metabolism of plants and bacteria. It not only provides precursors for the synthesis of the aromatic amino acids, but also for large variety of secondary metabolites (Herrmann, 1995). Many secondary compounds are biosynthesized directly from the aromatic amino acids but metabolites such as phylloquinones, menaquinones and anthraquinones are synthesized via pathways branching off at chorismate (Fig. 1) (Poulsen & Verpoorte, 1991). The first, common step in these biosynthetic pathways is the conversion of chorismate into isochorismate catalyzed by isochorismate synthase (ICS; EC 5.4.99.6) (Poulsen & Verpoorte, 1991). As all green plants need phylloquinones, ICS is an important enzyme in plant metabolism.

ICS uses the same substrate as anthranilate synthase (AS) and chorismate mutase (CM) which makes regulated partitioning of chorismate vitally important (Poulsen & Verpoorte, 1991). The regulation of the chorismate flow into the different pathways may be accomplished by modulation of the relative amounts of the competing enzymes. Alternatively, biochemical properties of the enzymes such as affinity for chorismate and reaction velocity may dictate precursor flows. These properties may be influenced allosterically by certain key metabolites. So information on characteristics of the enzymes involved is of vital importance to understand how precursor flow is regulated under different circumstances.

Such information is already available for AS (Romero, Roberts, & Phillipson, 1995) and CM (Poulsen & Verpoorte, 1991) from a number of plant species. Genes encoding AS and CM have been cloned, which allowed investigation of gene expression patterns under different circumstances of precursor demand (Bohlmann, Deluca, Eilert, & Martin, 1995; Eberhard, Bischoff, Raesecke, Amrhein, & Schmid, 1996). In contrast, only limited information is available on isochorismate synthases from plants. In *Catharanthus roseus* cell cultures, ICS is involved in the elicitation-induced synthesis of 2,3-dihydroxy-

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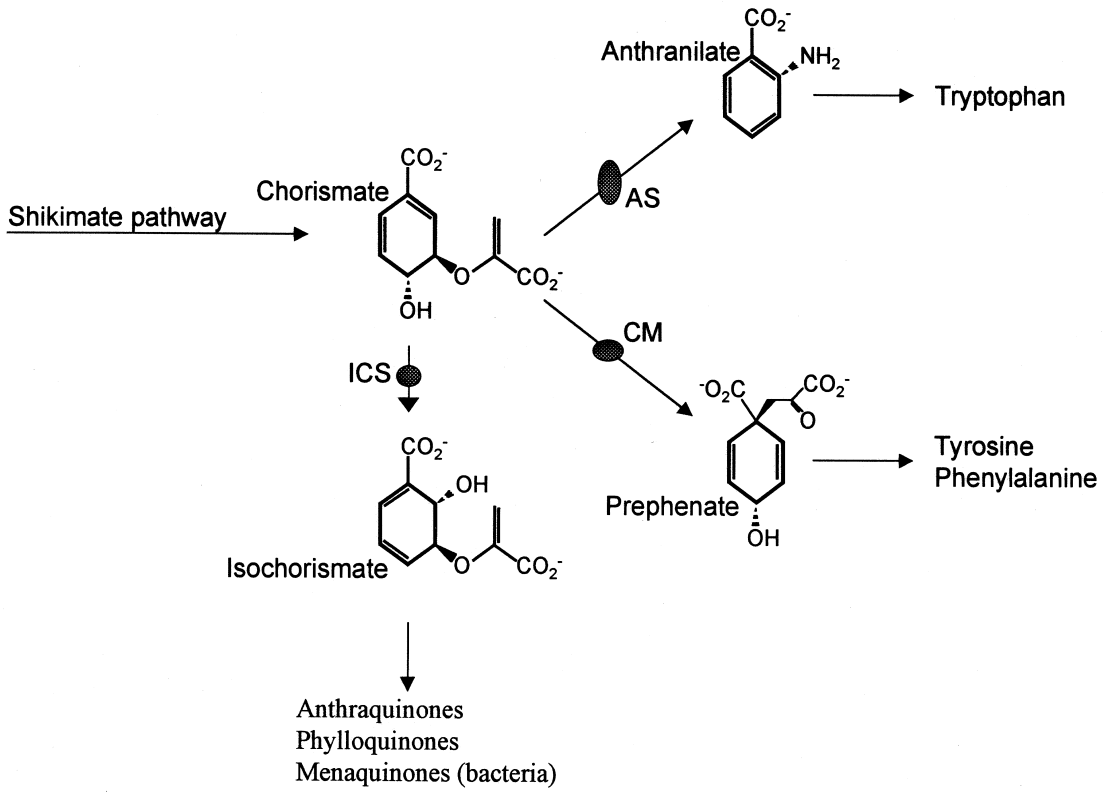

Fig. 1. Role and position of isochorismate synthase (ICS), anthranilate synthase (AS) and chorismate mutase (CM) in the metabolism of plants and microorganisms.

benzoate (Moreno, Van der Heijden, & Verpoorte, 1994). A plant ICS was purified for the first time to homogeneity from these cultures (Van Tegelen, Moreno, Croes, Venpoorte, & Wullems, 1999). Sequence information from the purified protein led to the cloning of an ICS cDNA (Accession number AJ006065) encoding a protein with a predicted molecular mass of 64 kD. The purification method used revealed the presence of two 64 kD ICS isoforms, which seem to be encoded by the same gene. ICS has been partially purified from anthraquinone-synthesizing cell cultures of *Galium mollugo*, but in this system only one ICS protein has been identified (Leduec, Birgel, Mueller, & Leistner, 1997). Recently a cDNA sequence encoding a putative ICS from *Arabidopsis thaliani* was deposited in the EMBL database (Accession number AF 078080) by H. Meng, G. Peter, and G. Pullman.

Much more information is available on ICSs in bacteria. ICS genes have been cloned from at least five different species (Ozenberger, Brickman, & McIntosh, 1989; Serino, Reimmann, Baur, Beyeler, & Haas, 1995; Rowland, Grossman, Osburne, & Taber, 1996; Daruwala, Kwon, Meganathan, & Hudspeth, 1996). The ICS genes fall into two groups, one group involved in the biosynthesis of siderophores such as enterobactin, 2,3-dihydroxybenzoate and amonabactin, the other group involved in the biosynthesis of menaquinones. In *Escherichia coli* the two isochorismate synthases involved in the two pathways have quite dissimilar biochemical properties (Daruwala, Bhattacharyya, Kwon, & Meganathan, 1997).

Because of its involvement in the biosynthesis of phylloquinones, ICS needs to be active in all plants. Therefore it is worthwhile to purify and characterize ICS proteins from a number of different plant species. The discrepancy in the number of isoforms in *C. roseus* and *G. mollugo* (Leduec et al., 1997), indicates that differences may exist between plants. Purification of ICS is hampered by the low ICS activity in leaves. This probably mirrors the very low rate of isochorismate formation needed to sustain phylloquinone levels (Lichtenthaler, 1968). A suitable system would produce a large amount of isochorismate-derived metabolites, and therefore possess a high level of ICS activity. An important group of metabolites formed from isochorismate in plants are the anthraquinones (Simantaris & Leistner, 1989). The large amount of anthraquinones produced by cell cultures of *Rubia tinctorum* L. makes these cultures an excellent source from which to purify ICS. Here we report on the purification and biochemical characterization of ICS from these cultures.

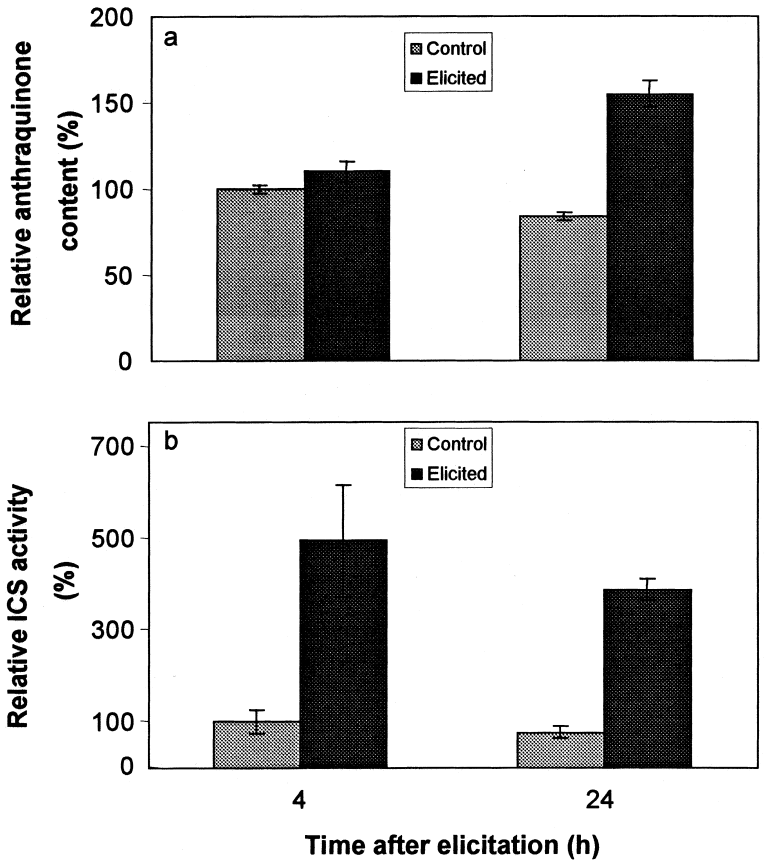

Fig. 2. Effect of elicitation with a *Pythium aphanidermatum* elicitor on anthraquinone content (a) and ICS activity (b) of *R. tinctorum* cell cultures. Results are given as means ± S.E. ($n = 3$).

2. Results

2.1. Elicitation and stability of ICS

To optimize the starting material for the purification of ICS from *R. tinctorum* cells we tested various media for their effects on ICS activity. There were only marginal differences (results not shown), but anthraquinone biosynthesis could be elicited by a culture filtrate of *Pythium aphanidermatum* (Fig. 2). Within 24 h anthraquinone content of the cells had doubled. The rise in anthraquinone biosynthesis is accompanied by a fivefold increase in ICS activity within 4 h of elicitation. The elevated ICS activity is sustained for at least 24 h. Cells harvested 6 h after application of the elicitor were used for the purification of ICS.

Preliminary experiments with partially purified ICS preparations showed that the enzyme was rather unstable. All ICS activity in the sample was lost within 48 h when the enzyme was stored at 4°. Storage at 30° decreased the stability of the enzyme even more. Stability of the enzyme was increased by adding 10% glycerol, 1 mM EDTA, 1 mM DTT, 200 mM KCl or a combination of these supplements (Fig. 3). A low pH caused irreversible inactivation of the enzyme. Although the effects of the protease inhibitors PMSF and leupeptin were variable, negative effects were never found.

2.2. Purification of ICS

The data from the stability experiments were used to design the purification scheme for ICS from elicited cells. Because of the stabilizing effect of potassium chloride, it was substituted for ammonium sulfate in hydrophobic interaction chromatography, and for sodium chloride in dye affinity and anion exchange chromatography. Glycerol, DTT and EDTA were added to all buffers. Strategy and results from a typical experiment are shown in Table 1. All ICS activity in a crude extract was precipitated with ammonium sulfate (40–60% cut), which resulted in a fourfold increase in specific activity. A large portion of the colored anthraquinones co-precipitated with the ICS activity. Subsequent chromatography on PhenylSepharose CL-4B not only separated ICS activity from 85% of the protein but also resulted in the loss of the contaminating anthraquinones, which bound strongly to the column (Fig. 4a). Pooled desalted fractions of the

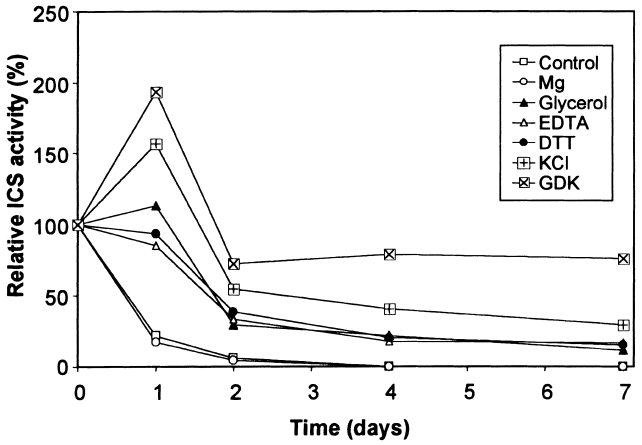

Fig. 3. Stability of ICS in various buffers. Details are given in the insert. GDK = glycerol, DTT and KCl.

PhenylSepharose column were further purified on a BlueA column (Fig. 4b).

Anion exchange chromatography on MonoQ (Fig. 4c) revealed the presence of two ICS activity peaks in the eluate. The ratio between the peaks was approximately 1–8, a number found in several independent purifications. Re-injection of fractions of either peak did not result in the appearance of more than one peak. Although the purification method employed yielded two significantly purified isoforms of ICS (purification factors 298 and 340 respectively for isoform I and II), analysis on native and SDS–PAGE revealed that neither isoform was purified to homogeneity (results not shown). Attempts to purify ICS further on other columns did not result in improved purification or even resulted in complete loss of enzyme activity (MonoS and hydroxy-appatite).

2.3. Characterization of ICS isoforms

For biochemical characterization MonoQ samples were used that were completely free of AS and CM activities. Maximal ICS activity was at pH 7.5–9, with 50% activity present at pH 7 and 10. Incubation of the isoforms at low or high pH led to irreversible loss of enzyme activity.

The activity of ICS was dependent on Mg^{2+}. The K_m for Mg^{2+} of the purified isoforms was 1.2 mM for isoform I and 1.45 mM for isoform II. No divalent metal ion could substitute for Mg^{2+} in the ICS reaction.

With respect to the substrate chorismate the two isoforms showed typical saturation curves and followed Michaelis–Menten kinetics. The K_m values for chorismate were 365 and 466 μM respectively for isoform I and II. To test possible inhibitory or stimulatory effects of aromatic amino acids on the activity of ICS, enzyme assays were performed in the presence of tyrosine, phenylalanine or tryptophan. None of the aromatic amino acids had any effect on ICS activity. Addition of several potential cofactors did not affect the activity of the enzyme.

Gel filtration chromatography on a Superose 12 column was used to estimate the molecular weight of the ICS isoforms. In both cases ICS activity eluted at the same retention time as BSA, indicating a molecular weight of approximately 67 kD.

3. Discussion

Because information on isochorismate synthase is limited to enzymes from bacteria and a very small number of plant species we purified the enzyme from anthraquinone producing cell cultures of *R. tinctorum*. Partial purification of the rather unstable enzyme was achieved when elicited cell cultures were used for extraction and the procedure was carried out in the continuous presence of DTT, glycerol and KCl. The stabilization by dithiothreitol is probably caused by protection of sulfhydryl groups. Similar results were found for ICS proteins from *E. coli* (Daruwala et al., 1997) and *G. mollugo* (Leduec et al., 1997).

The purification method yielded two isoforms of ICS after the final MonoQ step. The two peaks represent two isoforms rather than artifacts caused by the purification method because several independent purifications yielded a similar ratio of the two isoforms (1–8), and re-chromatography of either ICS isoform on

Table 1
Purification of ICS from 700 grams of elicited cells of *Rubia tinctorum*

Fraction	Activity (pkat)	Protein (mg)	Specific activity (pkat/mg)	Yield (%)	Purification factor
Crude extract	37,884	1043	36.3	100	1
$(NH_4)_2SO_4$ pellet	44,669	332	134	118	3.7
Pool PhenylSepharose	12,686	35.4	975	34	26.9
Pool BlueA	1838	1.2	1540	4.9	42.4
MonoQ 36 (ICS I)	51.5	0.005	10,842	0.14	299
MonoQ 48 (ICS II)	449	0.056	8090	1.19	223

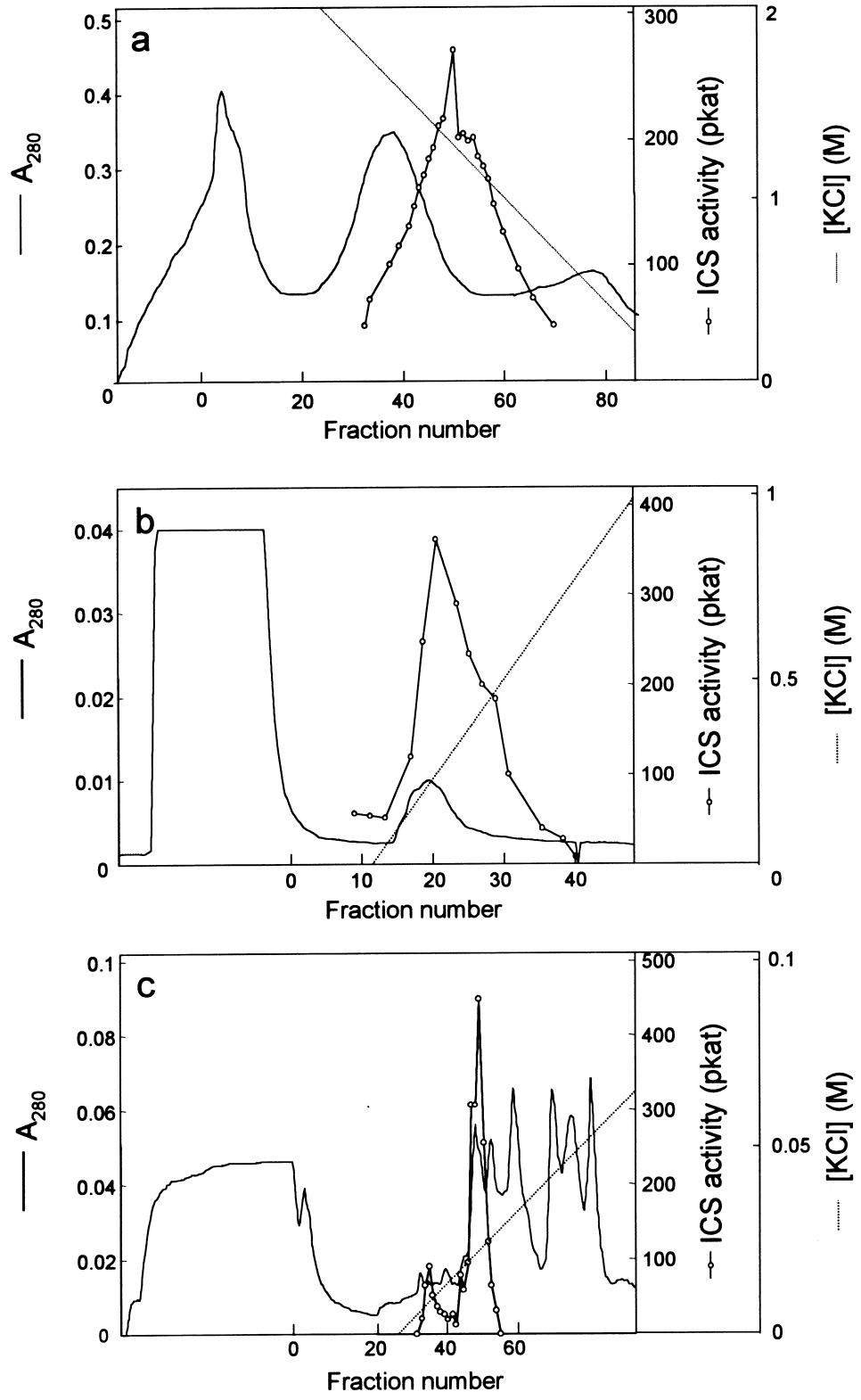

Fig. 4. (a) Elution profile for *R. tinctorum* ICS from a PhenylSepharose CL-4B column. (b) Purification of ICS on a BlueA column. (c) Separation of two ICS isoforms on a Pharmacia MonoQ column.

MonoQ resulted in the appearance of only that isoform in the chromatogram. Like all ICS proteins purified so far, both isoforms have a strict requirement for magnesium. This is consistent with the reaction mechanism which has been proposed for the conversion of chorismate into isochorismate, involving a magnesium-

coordinated transition state (Poulsen, Bongaerts, & Verpoorte, 1993).

Also in other aspects the two *R. tinctorum* ICS isoforms are very similar as is inferred from the only minor differences in affinity for the substrate, in pH optimum, and estimated molecular weight. From elicited cell cultures of *C. roseus* also two isoforms of ICS have been isolated, though at a different ratio (1–2) (Van Tegelen, Moreno, Croes, Venpoorte, & Wullems, 1999). Their characteristics such as molecular weight, pH dependency, and substrate affinity are similar to those of their counterparts in *R. tinctorum*. The two isoforms in *C. roseus* do not much differ from each other and are probably encoded by a single gene (Van Tegelen, Moreno, Croes, Venpoorte, & Wullems, 1999). The ICS isoforms of *Rubia* and *Catharanthus* resemble each other in their biochemical properties and are also similar to the ICS protein isolated from cell cultures of *G. mollugo* (Leduec et al., 1997). This suggests that ICS proteins and genes are strongly conserved.

In contrast, the two ICS proteins in *E. coli* and *Bacillus subtilis* differ in biochemical characteristics (Daruwala et al., 1997). The two proteins have distinct functions in bacterial metabolism and are encoded by separate genes (Daruwala et al., 1996; Rowland & Taber, 1996). One enzyme is involved in the biosynthesis of menaquinones and the other protein catalyzes a step in the biosynthesis of siderophores.

In plants, most enzymes of the shikimate pathway exist in at least two isoforms encoded by differentially regulated genes (Schmid & Amrhein, 1995). It is unknown yet how many genes code for ICS in *Rubia*. Cloning of (an) ICS gene(s) may answer this question and give insight in the regulation and function of the two isoforms.

The activities of the two ICS isoforms are not influenced by aromatic amino acids, in contrast to some isoforms of the other chorismate-utilizing enzymes (Walsh, Liu, Rusnak, & Sakaitaini, 1990; Poulsen & Verpoorte, 1991). This means that the first committed step in anthraquinone biosynthesis is not under allosteric control of the aromatic amino acids. Through a different mechanism, more chorismate may become available for conversion by ICS, when high concentrations of the aromatic amino acids inhibit AS and CM activities.

4. Experimental

4.1. Plant material

Cell cultures of *R. tinctorum* were grown in Gamborg's B5 medium (Gamborg, 1970) containing 2% sucrose, 2 mg l^{-1} 2,4-D, 0.5 mg l^{-1} NAA, 0.5 mg l^{-1} IAA, and 0.2 mg l^{-1} kinetin in the dark at 25° on a gyratory shaker at 120 rpm. Subculturing was done every seven days by a threefold dilution into fresh medium.

4.2. Elicitation

Cells cultures were elicited with an autoclaved *P. aphanidermatum* culture filtrate as described in (Moreno et al., 1994) 4 d after subculturing.

4.3. ICS assay

ICS activity was determined according to (Poulsen, Van der Heijden, & Verpoorte, 1991) with slight modifications. The incubation mixture (250 μl) contained 0.1 M Tris–HCl, pH 7.5, 2 mM Ba-chorismate, 10 mM $MgCl_2$, and enzyme extract (crude extracts 125 μl, column fractions 10–100 μl). After incubation for 60 min at 30° the reaction was stopped with 62.5 μl MeOH: *sec*-BuOH (1:1 v/v). The samples were centrifuged and analyzed by HPLC. Assay mixtures (250 μl) for determination of pH optima contained 190 μl of 0.2 M stock solutions of the various buffers (citrate, pH 4–6; bis-Tris, pH 6–7; Tris–HCl, pH 7–9 and glycine, pH 9–10). All other assay components were dissolved in distilled water.

4.4. Anthraquinone determination

0.3 g of cells were extracted with 2 ml 80% ethanol at 80°C for one hour. The colored fluid was separated from the nearly white pellet, which was washed twice. Samples from the combined extracts were measured using a spectrophotometer at 434 nm. Alizarin was used as a standard.

4.5. Protein determination

Protein concentrations were determined in microtiter plates using the Bradford microassay method (Bradford, 1976) with BSA as a standard. SDS- and native PAGE were carried out on a PhastSystem using precast 8–25% gradient gels. Proteins were visualized by silver staining (Davis, Dibner, & Battey, 1986).

4.6. Enzyme extraction

R. tinctorum cells were harvested by suction after 4 h of elicitation, washed once with water, immediately frozen in liquid nitrogen and stored at −80°. 700 g of frozen cells were homogenized in a Waring Blender equipped with a stainless-steel bucket. One ml of extraction buffer (0.1 M Tris–HCl pH 7.5, 10% glycerol (v/v), 1 mM DTT, 0.2 mM PMSF, 10 mM leupeptin and 1 mM EDTA) and 50 mg polyvinylpyrrolidone were added per g fr. wt. After

thawing, the homogenate was centrifuged at 10,000*g* for 30 min to remove cell debris. The supernatant is referred to as crude extract.

4.7. Purification of ICS

To the crude extract solid $(NH_4)_2SO_4$ was added to 40% saturation. After stirring for 20 min the ppt. was removed by centrifugation at 10,000*g* for 30 min. More ammonium sulfate was added to the supernatant to 60% saturation. The ppt. was collected by centrifugation at 10,000*g* for 30 min. The pellet was dissolved in 50 ml buffer A (20 mM triethanolamine–HCl, pH 7.5, 10% (v/v) glycerol, 1 mM DTT, 1 mM EDTA and 0.2 mM PMSF), and solid KCl was added to a final concentration of 2 M. After centrifugation at 13,000*g* for 15 min, the supernatant was applied to a PhenylSepharose CL-4B column (72 ml, 2.6×13.5 cm) equilibrated in buffer B (buffer A + 2 M KCl). After washing the column with 300 ml buffer B, ICS was eluted with a 700 ml linear gradient from buffer B to A, followed by 150 ml buffer A, at a flow of 1 ml min^{-1}. Fractions of 10 ml were collected. Fractions containing ICS activity were pooled and concentrated using an Amicon unit equipped with a 50 kD cut-off membrane. The concentrate was desalted by gel filtration over Sephadex G-25, equilibrated in buffer A and applied to a 20 ml BlueA column. After application the flow was stopped for one half-hour to allow binding. The column was washed by reverse flow (0.25 ml min^{-1}) with 40 ml buffer A. ICS was eluted in the same way with a 160 ml gradient from buffer A to 50% buffer B and fractions of 4 ml were collected. Fractions containing ICS activity were pooled, concentrated and desalted on Sephadex G-25 columns equilibrated with buffer C (20 mM triethanolamine–HCl pH 8.0, 5% (v/v) glycerol and 1 mM DTT). The desalted sample was applied to a MonoQ HR 5/5 column equilibrated in buffer C. The column was washed with 16 ml buffer C and ICS was eluted with a 80 ml linear gradient from buffer C to D (buffer C + 0.5 M KCl). The flow was 0.5 ml min^{-1} and fractions of 0.5 ml were collected.

4.8. Determination of molecular mass

50 μl of MonoQ fractions containing either isoform I or isoform II were separated on a Superose 12 HR (3.2 mm × 300 mm) column on a Smart system. The column was equilibrated and eluted with a buffer containing 0.1 M triethanolamine (pH 7.5), 5% glycerol, 1 mM DTT, 200 mM KCl. The column was calibrated with aldolase (158,000), BSA (67,000), albumin (43,000), catalase (23,200), and ribonuclease (13,700).

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