FEBS Letters 356 (1994) 221–225

FEBS 14880

The 'pterocarpan synthase' of alfalfa: association and co-induction of vestitone reductase and 7,2′-dihydroxy-4′-methoxy-isoflavanol (DMI) dehydratase, the two final enzymes in medicarpin biosynthesis

Lining Guo, Richard A. Dixon, Nancy L. Paiva*

Plant Biology Division, The Samuel Roberts Noble Foundation, P.O. Box 2180, Ardmore, OK 73402, USA

Received 2 September 1994; revised version received 3 November 1994

Abstract Vestitone reductase and 7,2′-dihydroxy-4′-methoxy-isoflavanol (DMI) dehydratase are the two final enzymes in medicarpin biosynthesis in alfalfa (*Medicago sativa*). Although two independent enzymes, vestitone reductase and DMI dehydratase can be loosely associated in low ionic strength buffers, presumably by a weak protein–protein interaction. The activities of vestitone reductase and DMI dehydratase increased approximately 3-fold 6 hours after elicitor treatment in alfalfa suspension cell culture. The activities remained at maximal levels for 40 hours, correlating with a steady increase in the medicarpin content of the cells. Medicarpin produced in vitro from vestitone by the action of vestitone reductase and DMI dehydratase was found to be (−)-medicarpin (6a*R*,11a*R*-medicarpin), possessing the same stereochemistry as medicarpin produced in vivo.

Key words: Pterocarpan biosynthesis; Vestitone reductase; DMI dehydratase; Stereochemistry; *Medicago sativa* L.; Phytoalexin

1. Introduction

Attacks by fungal pathogens stimulate the biosynthesis of antimicrobial pterocarpan phytoalexins in leguminous plants [1]. Medicarpin is the major pterocarpan phytoalexin which accumulates in infected alfalfa (*Medicago sativa* L.) [2], a major forage crop in North America and other parts of the world. The observation that medicarpin is toxic to several fungal pathogens of alfalfa, along with other correlative evidence, suggests that medicarpin biosynthesis plays an important role in the defense response of alfalfa [2,3].

Several enzymes in the pathway leading to medicarpin and other pterocarpans are well characterized; the first six enzymes, from phenylalanine ammonia-lyase to chalcone isomerase, have been purified and/or cloned from various species, including alfalfa [3,4]. Until recently, isoflavone reductase (IFR) (see Fig. 1) was the only pterocarpan- or isoflavonoid-specific enzyme purified or cloned [5]. IFR from alfalfa has been shown to stereospecifically reduce 2′-hydroxy-formononetin, a 2′-hydroxylated isoflavone, to (3*R*)-vestitone, a 2′-hydroxylated isoflavanone. It had been reported earlier that the final step in medicarpin biosynthesis was the conversion of vestitone to medicarpin by a single enzyme, 'pterocarpan synthase' (PTS) [6,7]. Analogous results were reported for the synthesis of a similar pterocarpan in soybean [8]. Like the earlier enzymes in the pathway, PTS activity was shown to increase following elicitation of plant cell cultures, but only very preliminary purification progress was reported [6,8].

Recently, we have found that the conversion of vestitone to medicarpin is catalyzed by two independent enzymes [9]. The first enzyme, vestitone reductase, catalyzes the NADPH-dependent reduction of vestitone to 7,2′-dihydroxy-4′-methoxy-isoflavanol (DMI). The second enzyme, DMI dehydratase, catalyzes the loss of water and closure of an ether ring to form medicarpin (Fig. 1). The exact mechanism of ring closure remains uncertain; both a carbocation and a quinone methide intermediate have been proposed [10]. We have purified vestitone reductase to homogeneity. It appears to be a monomer of 38 kDa as determined by SDS-PAGE, with strict substrate stereo-specificity for (3*R*)-vestitone with a K_m value of 45 μM. The second enzyme, DMI dehydratase, was partially purified. It has a native molecular mass of 38 kDa and a K_m value of 5 μM for DMI [9], but its product stereo-specificity was not determined. Only (−) or (6a*R*,11a*R*)-medicarpin accumulates in alfalfa cells.

We report here that vestitone reductase and DMI dehydratase can form a weak association, probably via protein–protein interactions, although they co-elute around the monomer molecular weights under normal conditions of gel-filtration (explaining the previous conclusions that 'pterocarpan synthase' was one enzyme). Both activities are rapidly induced, with similar kinetics, in elicitor-treated alfalfa cell suspension cultures. DMI dehydratase produces only (6a*R*,11a*R*)medicarpin from 7,2′-dihydroxy-4-methoxy-isoflavanol, consistent with the stereochemistry of the medicarpin accumulated in alfalfa and the stereochemical outcomes of the preceeding isoflavone reductase and vestitone reductase reactions.

2. Materials and methods

2.1. Plant material

Callus and suspension cultures of alfalfa were initiated and maintained as described [11]. Alfalfa suspension cells were treated with yeast elicitor and harvested at different times as described [5].

2.2. Chemicals

Vestitone was produced by reducing 2′-hydroxyformononetin using NADPH as the reducing agent and the cloned alfalfa isoflavone reductase (IFR) expressed in *Escherichia coli* as the catalyst [5]. This enzymatic reduction initially produces (3*R*)-vestitone which can gradually racemize in solution. Since vestitone reductase can only use (3*R*)-vestitone as substrate and is not inhibited by (3*S*)-vestitone [9], the crude vestitone was racemized under basic conditions to stabilize the percentage of useable isomer [12]. Racemic vestitone was purified by semi-preparative HPLC [9,12] for use in all assays.

7,2′-Dihydroxy-4′-methoxy-isoflavanol (DMI) was synthesized from large scale vestitone reductase reactions (see below) using purified vestitone reductase from alfalfa [9]. DMI was purified by semi-preparative

*Corresponding author. Fax: (1) (405) 221 7380.

SSDI 0014-5793(94)01267-9

HPLC, taking care to avoid any contact with acidic solutions. After dissolving in methanol, the concentration of DMI was adjusted based on the relative absorptivities of DMI and medicarpin at 287 nm, as determined by HPLC monitoring of the DMI dehydratase assay (see below).

(−)-Medicarpin (6a*R*,11a*R*-medicarpin) was purified from alfalfa roots by semi-preparative HPLC as described previously [13]. (+)-Medicarpin (6a*S*,11a*S*-medicarpin) was extracted from infected peanut leaves [14] and purified by the same method used for (−)-medicarpin. The identity and stereochemistry of the two forms of medicarpin were confirmed by UV, MS, NMR, CD, and HPLC retention time (N. Paiva, unpublished results).

2.3. Extraction and assay of vestitone reductase and DMI dehydratase

Frozen cells (1 g) were homogenized with 2 ml of 0.2 M Tris-HCl, pH 7.5, containing 10% glycerol and 14 mM β-mercaptoethanol. Polyvinylpolypyrrolidone (PVPP; 10% w/w) was then added to the homogenate and mixed for 10 min. The mixture was then centrifuged at 27,500 × *g* for 40 min and the pellet discarded. The supernatant was used directly for enzyme assays.

The standard vestitone reductase assay mixture contained 0.2 M sodium phosphate buffer, pH 6.0, 1 mM NADPH, 50 μM vestitone, and enzyme in a total volume of 500 μl, as described previously [6]. Reactions were conducted at 30°C for 15 min and terminated by adding 1 ml of ethyl acetate. After partitioning, 0.75 ml of organic phase was taken to dryness under a stream of N_2 and redissolved in 150 μl of methanol; a 20 μl aliquot was used for HPLC analysis (System Gold Model 126 HPLC system equipped with a Model 168 photodiode array detector, Beckman, Fullerton, CA). Compounds were separated on a C18 column (250 × 4.6 mm, 5 μm packing, J.T. Baker, Phillipsburg, NJ) using a non-acidic gradient system (80% H_2O/20% CH_3CN to 40% H_2O/60% CH_3CN in 20 min, 1.5 ml/min) [9]. One unit is defined as the amount of enzyme reducing 1 μmol of vestitone per hour.

The standard DMI dehydratase assay mixture contained 0.2 M sodium phosphate buffer, pH 6.0, 50 μM DMI, and enzyme in a total volume of 500 μl [9]. Reactions were conducted at 30°C for 5 min, and then terminated and assayed by HPLC as described above for the vestitone reductase assay, using the non-acidic gradient system. One unit is defined as the amount of enzyme synthesizing 1 μmol of medicarpin per hour.

2.4. Measurement of medicarpin

Medicarpin and its conjugated form, medicarpin-3-*O*-glucoside-6″-*O*-malonate (MGM) were extracted from frozen cells using cold acetone and quantified by HPLC as described previously [15]. 'Total' medicarpin is the sum of the values obtained for these two forms of medicarpin.

2.5. Identification of the stereochemistry of medicarpin

The medicarpin generated by DMI dehydratase or by mild acid treatment of DMI was separated by HPLC as described for the enzyme assay. The medicarpin peak was collected, extracted three times with an equal volume of ethyl acetate, and the organic phases pooled and dried. The stereochemistry of medicarpin was then determined by chromatography using an analytical-scale chiral HPLC column (Chiralpak OT(+), 250 × 4.6 mm, 5 μm packing, with a 50 mm precolumn; Daicel Chemical Industries, Ltd., Japan) using isocratic elution (100% MeOH, 0°C, 0.75 ml/min) [16]. By comparing the elution profiles of standard (−)-medicarpin and (+)-medicarpin, it was determined that the (+)-medicarpin eluted first (8.3 min) and the (−)-medicarpin second (12.4 min), as previously reported.

2.6. Gel filtration conditions

Gel filtration was performed on a FPLC Superdex 75 HR 10/30 column (Pharmacia) with buffers indicated in the text at a flow rate of 0.5 ml/min at 4°C. Fractions (0.5 ml) were collected and assayed directly for vestitone reductase and DMI dehydratase activity.

3. Results

3.1. Interaction of vestitone reductase and DMI dehydratase

We have shown that the conversion of vestitone to medicarpin is catalyzed by two enzymes, vestitone reductase (34 kDa, native molecular weight) and DMI dehydratase (38 kDa) [9]. Using gel filtration, we tested whether or not these two enzymes can interact. Twenty units of purified vestitone reductase (0.04 mg protein) were mixed with 100 units of partially purified DMI dehydratase (0.06 mg total protein) and chromatographed over a Superdex 75 FPLC column using 20 mM potassium phosphate buffer, pH 7.5, containing 7 mM mercaptoethanol, and 100 mM KCl. Fig. 2A shows the elution profile of a mixture of molecular weight standards, 66 kDa, 44 kDa, 17 kDa, and 1.35 kDa. Fig. 2B shows the elution profile of vestitone reductase and DMI dehydratase. Vestitone reductase eluted at 20.5 min (34 ± 2 kDa) and DMI dehydratase at 21 min (38 ± 2 kDa), as previously observed [9]. However, when we omitted the 100 mM KCl from the chromatography buffer, part of the vestitone reductase activity and all of the DMI dehydratase activity were shifted to 17.5 min (70 ± 4 kDa) (Fig. 2C). Omitting or including 100 mM KCl did not change the elution times of the molecular weight standards, vestitone reductase alone, or DMI dehydratase alone.

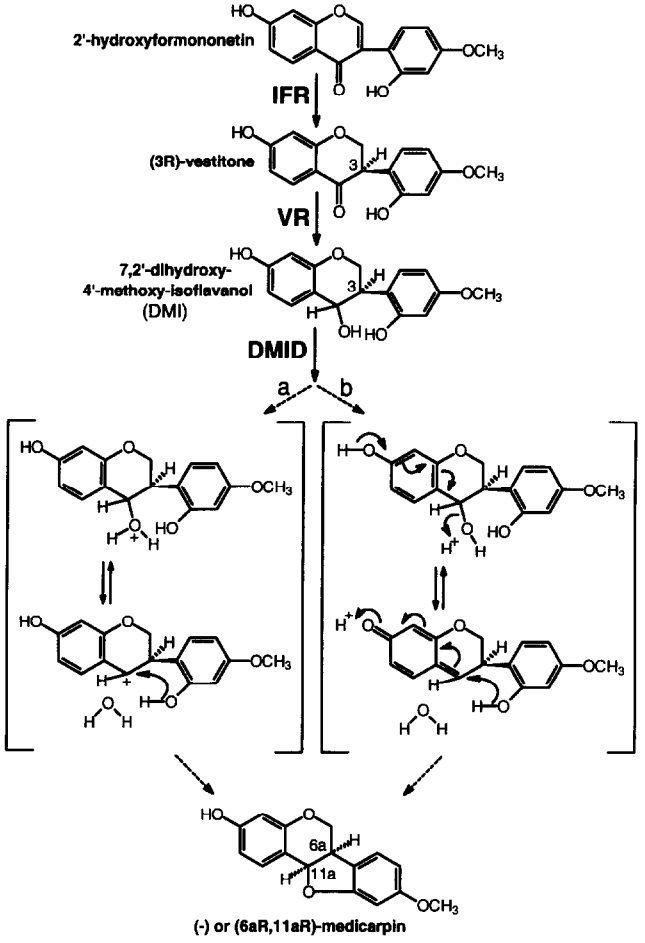

Fig. 1. The final steps of medicarpin biosynthesis in alfalfa. The isoflavanone (3*R*)-vestitone is reduced by vestitone reductase to 7,2′-dihydroxy-4′-methoxy-isoflavanol (DMI). DMI dehydratase catalyzes the loss of H_2O and ring closure to the final pterocarpan, via either a carbocation (a) or quinone methide (b) intermediate. The names of the enzymes are abbreviated as follows: IFR, isoflavone reductase; VR, vestitone reductase; DMID, DMI dehydratase.

3.2. Elicitation of vestitone reductase and DMI dehydratase in alfalfa suspension cell culture

Fig. 3 shows the changes in the activities of vestitone reductase and DMI dehydratase, as well as total medicarpin accumulation, in alfalfa suspension cell cultures following treatment with an elicitor preparation derived from yeast. The induction of vestitone reductase (Fig. 3A) and DMI dehydratase (Fig. 3B) followed the same pattern. The activities rapidly increased about 3-fold, with near maximal activities being reached within 6 to 8 h and the activities remaining high throughout the 48 h time course. Total medicarpin levels (free medicarpin and the malonylated glucoside of medicarpin (MGM)) also increased steadily after these enzyme levels increased (Fig. 3C), as reported previously [5].

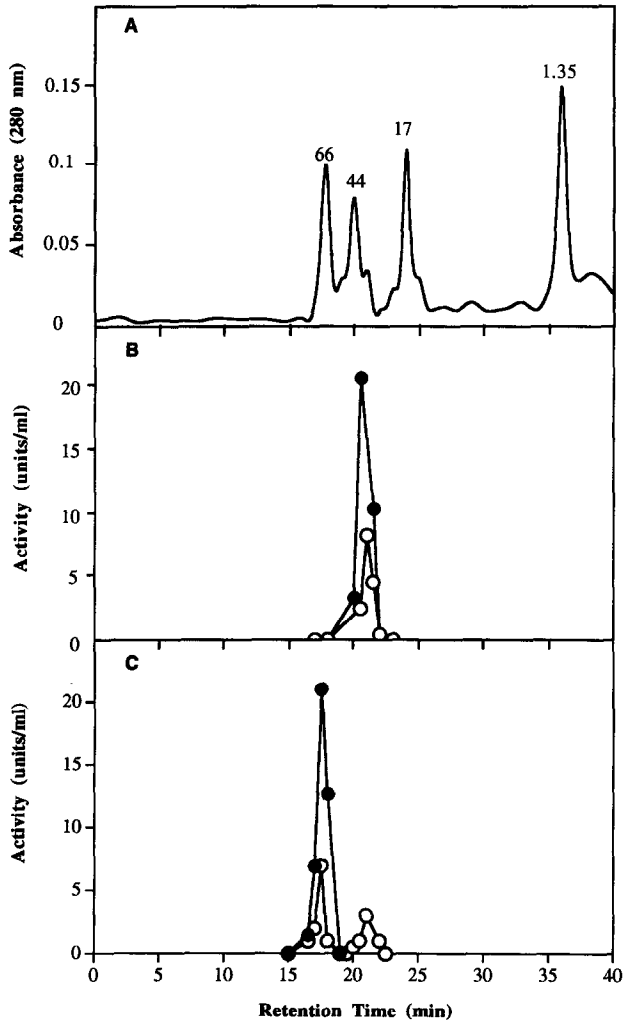

Fig. 2. Effect of salt concentration on the elution of vestitone reductase and DMI dehydratase during gel filtration on an FPLC Superdex 75 column. (A) A mixture of molecular weight standards: BSA, 66 kDa; ovalbumin (chicken), 44 kDa; myoglobin (horse), 17 kDa; and vitamin B-12, 1.35 kDa. (B) Mixture of purified vestitone reductase (20 units, 0.04 mg total protein) and partially purified DMI dehydratase (100 units, 0.06 mg total protein). The vestitone reductase activity (○) and DMI dehydratase activity (●) in collected fractions are indicated. The chromatography buffer was 20 mM potassium phosphate buffer, pH 7.5, containing 7 mM β-mercaptoethanol and 100 mM KCl. (C) Same protein mixtures and elution conditions as in (B), except that KCl was omitted from the chromatography buffer.

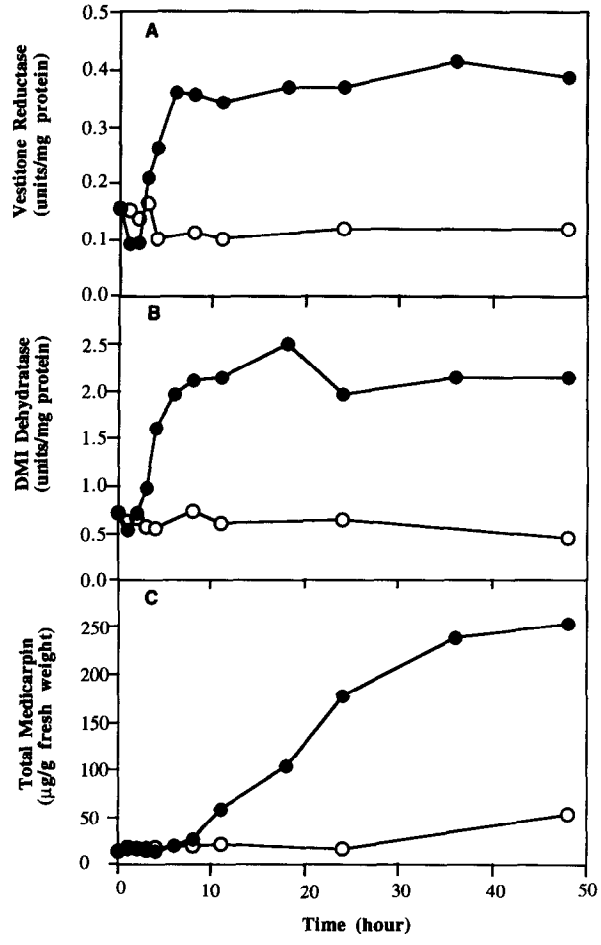

Fig. 3. Changes in activities of vestitone reductase (A) and DMI dehydratase (B) and total medicarpin levels (C) in alfalfa suspension cell cultures in response to fungal elicitor treatment (●) or in unelicited controls (○). Filter-sterilized elicitor was added to the cultures four days after subculture, and then samples were collected at the times indicated.

3.3. Stereochemistry of medicarpin produced by the action of vestitone reductase and DMI dehydratase

It has been reported that infected alfalfa leaves and elicited cell cultures only accumulate (−)- or (6a*R*,11a*R*)-medicarpin [1,13]. Isoflavone reductase creates the first chiral center by reducing an achiral substrate to (3*R*)-vestitone (the C3 of vestitone corresponds to C6a of medicarpin; see Fig. 1) [5]. We have recently shown that vestitone reductase will only accept (3*R*)-vestitone as its substrate; (3*S*)-vestitone neither serves as a substrate, nor does it inhibit the reaction [9]. The stereochemistry of the in vitro product of DMI dehydratase has not yet been investigated. It is also possible to convert DMI to medicarpin by non-enzymatic means. Heating DMI in the presence of acid will cause dehydration and ring closure [9], but the stereochemistry of the product of this reaction has not been determined previously.

DMI was synthesized by reducing vestitone with vestitone reductase and then purified by HPLC. A portion of the DMI was then reacted with DMI dehydratase and the in vitro, enzymatically produced medicarpin was purified. Similarly, another portion of the DMI was treated with HCl (0.1 M HCl, 15 min,

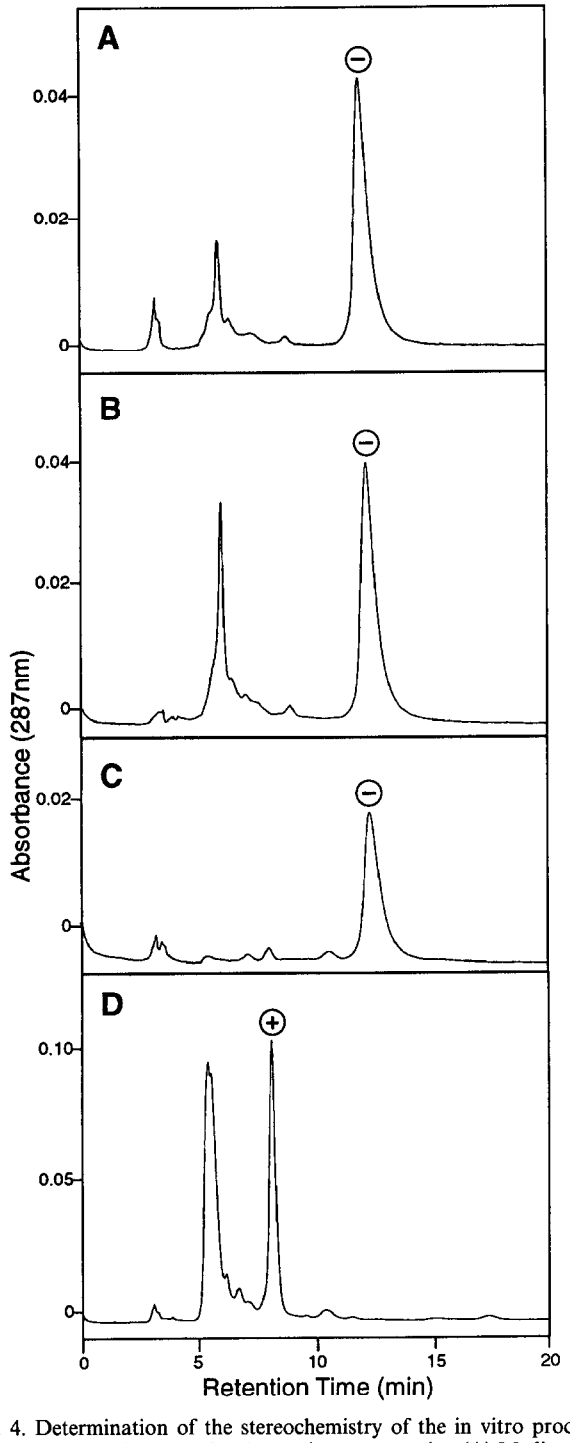

Fig. 4. Determination of the stereochemistry of the in vitro product medicarpin by chiral HPLC column chromatography. (A) Medicarpin produced from the in vitro reduction of vestitone by vestitone reductase to produce DMI, followed by ring closure catalyzed by DMI dehydratase. (B) Medicarpin produced as in (A), except ring closure was catalyzed by heating with acid. (C) (−)-Medicarpin standard, (D) (+)-Medicarpin standard. Peaks assigned to (+) or (−) medicarpin are labeled with a (+) or (−) symbol. Other peaks in the chromatograms do not have the UV spectrum of medicarpin, and are due to solvent effects or impurities.

30°C) and the resulting medicarpin was purified. The stereochemistry of each preparation of medicarpin was then determined by chromatography using a chiral HPLC column. The medicarpin produced by the in vitro DMI dehydratase reaction and by the acid-catalyzed dehydration had identical retention times on the chiral column (Fig. 4A and B). This retention time (12.4 min) matched that of (6a*R*,11a*R*)-medicarpin (Fig. 4C); no trace of (6a*S*,11a*S*)-medicarpin (Fig. 4D) was observed.

4. Discussion

The gel filtration results indicate that although vestitone reductase and DMI dehydratase are two independent enzymes, they can bind together by a weak protein–protein interaction. This weak interaction can be disrupted by 100 mM KCl. Judging by the molecular weight shift, the complex formed between vestitone reductase and DMI dehydratase probably contains one molecule of each enzyme. The incomplete shift of vestitone reductase activity was due to the presence of a molar excess of vestitone reductase. Presumably, the close association of vestitone reductase and DMI dehydratase in vivo would facilitate the synthesis of medicarpin by minimizing the need for diffusion of the DMI intermediate. A similar weak protein–protein interaction has been proposed between chalcone reductase (CHR) and chalcone synthase (CHS), two enzymes earlier in the pathway [17,18], and high salt concentrations also inhibited the interaction of CHR and CHS [17]. Also, several authors have presented both experimental evidence and theoretical arguments suggesting that many of the flavonoid biosynthetic enzymes are arranged sequentially in a large complex, loosely associated with the cytosolic side of the membrane of the endoplasmic reticulum or other organelle [19,20]. The structural basis for the protein–protein interaction between vestitone reductase and DMI dehydratase will be investigated using molecular modelling following the cloning and sequencing of these two genes.

Medicarpin is rapidly accumulated in alfalfa suspension cell cultures following addition of yeast elicitor. The activities of early pathway enzymes common to both phenylpropanoid and flavonoid metabolism, such as phenylalanine ammonia-lyase, cinnamic acid 4-hydroxylase, chalcone synthase and chalcone isomerase, are rapidly but transiently increased (2- to 30-fold within 10 to 15 h) [3,21]. In contrast, the pterocarpan-specific isoflavone reductase showed an equally rapid increase in enzyme activity (~10-fold in 12 h) but remained at high levels until more than 48 h post-elicitation, long after the activities of early enzymes had returned to control levels [5]. Here we report that the activities of vestitone reductase and DMI dehydratase, the two final enzymes in the pathway, both increased 3- to 4-fold within 8 h of elicitation. Both activities remained high throughout the remainder of the 48-hour time course, yielding a pattern much more similar to that of isoflavone reductase than to the early pathway enzymes. This level of increase is in agreement with the 2- to 3-fold increase previously observed in alfalfa cells for 'pterocarpan synthase' [5], which represents the total conversion from vestitone to medicarpin; similar results were obtained in soybean [6] and chickpea [8] cultures for analogous 'pterocarpan synthase' activities. The higher levels of late pathway enzymes correlates well with the continued increase in the medicarpin content of the elicited cultures.

Analysis by chiral HPLC chromatography indicates that only (−)- or (6a*R*,11a*R*)-medicarpin is produced by either acid-catalyzed dehydration or the action of DMI dehydratase in vitro on enzymatically produced DMI. This is consistent with

reports that alfalfa plants and cell cultures only accumulate (6a*R*,11a*R*)-medicarpin *in vivo*. Isoflavone reductase produces (3*R*)-vestitone, which is in turn the only isomer of vestitone utilized by vestitone reductase [5,9]. The stereochemistry of the 6a carbon is presumably fixed by the stereospecificity of vestitone reductase; once the keto group in vestitone is reduced, it is difficult chemically to invert the stereochemistry at the 3*R* position of DMI. Whether the subsequent dehydration and ring closure proceeds via a cationic or quinone methide transition state, the carbon corresponding to 11a becomes achiral (Fig. 1). We believe that the configuration of the second chiral center (11a*R*) is determined by the configuration of the first (6a*R*). The R configuration at the 6a carbon puts the aromatic hydroxyl above the plane of the rest of the molecule, and the new C–O bond must form on this side, to avoid undue ring strain. The stereochemistry of the pterocarpan system can be extremely important in determining the antifungal activity against certain pathogens ([22]; J. Blount, R.A. Dixon, H.D. VanEtten and N. Paiva, unpublished results). Efforts are underway to determine how legumes which synthesize (+) and (−) pterocarpans differ in the final steps of the pathway; preliminary results indicate that the vestitone reductase substrate stereo-specificities are reversed (N. Paiva, unpublished results).

Acknowledgements: We thank Allyson Wilkins for assistance in preparing the manuscript and Cuc Ly for assistance in preparing Fig. 4. We also thank Drs. Jacqueline Pallas and Gerardo Rodriguez-Alvarado for critically reviewing the manuscript.

References

[1] Ingham, J.L. (1982) in: Phytoalexins (Bailey, J.A. and Mansfield, J.W., Eds.) pp. 21–80, Wiley, New York.
[2] Higgins, V.J. (1972) Physiol. Plant Pathol. 2, 289–300.
[3] Dixon, R.A., Choudhary, A.D., Dalkin, K., Edwards, R., Fahrendorf, T., Gowri, G., Harrison, M., Lamb, C.J., Loake, G.J., Maxwell, C.A., Orr, J. and Paiva, N.L. (1992) in: Phenolic Metabolism in Plants (Stafford, H.A. and Ibrahim, R.K., Eds.) pp. 91–138, Plenum Press, New York.
[4] Fahrendorf, T. and Dixon, R.A. (1993) Arch. Biochem. Biophys. 305, 509–515.
[5] Paiva, N.L., Edwards, R., Sun, Y., Hrazdina, G. and Dixon R. A. (1991) Plant Mol. Biol. 17, 653–667.
[6] Bless, W. and Barz, W. (1988) FEBS Lett. 235, 47–50.
[7] Barz, W. and Welle, R. (1992) in: Phenolic Metabolism in Plants (Stafford, H.A. and Ibrahim, R.K., Eds.) pp. 139–164, Plenum Press, New York.
[8] Fischer, D., Ebenau-Jehle, C. and Grisebach, H. (1990) Phytochemistry 29, 2879–2882.
[9] Guo, L., Dixon, R.A. and Paiva, N.L. (1994) J. Biol. Chem. 269, 22372–22378.
[10] Stoessl, A. (1982) in: Phytoalexins (Bailey, J.A. and Mansfield, J.W., Eds.) pp. 133–180, Wiley, New York.
[11] Jorrin, J. and Dixon, R.A. (1989) Plant Physiol. 92, 447–455.
[12] Blount, J.W., Dixon, R.A. and Paiva, N.L. (1992) Physiol. Mol. Plant Pathol. 41, 333–349.
[13] Kessmann, H., Edwards, R., Geno, P.W. and Dixon, R.A. (1990) Plant Physiol. 94, 227–232.
[14] Strange, R.N., Ingham, J.L., Cole, D.L., Cavill, M.E., Edwards, C., Cooksey, C.J. and Garratt, P.J. (1985) Z. Naturforsch. 40c, 313–316.
[15] Edwards, R. and Kessmann, H. (1992) in: Molecular Plant Pathology: A Practical Approach (Gurr, S.J., McPherson, M.J. and Bowles, D.J., Eds.) vol. 2, pp. 45–62, Oxford University Press, New York.
[16] Antus, S., Bauer, R., Gottsegen, A. and Wagner, H. (1990) J. Chromatography 508, 217–219.
[17] Welle, R. and Grisebach, H. (1988) FEBS Lett. 236, 221–225.
[18] Welle, R., Schröder, G., Schiltz, E., Grisebach, H. and Schröder, J. (1991). Eur. J. Biochem. 196, 423–430.
[19] Stafford, H.A. (1990) Flavonoid Metabolism, CRC Press, Boca Raton, Florida.
[20] Hrazdina, G. and Wagner, G.J. (1985) Arch. Biochem. Biophys. 237, 88–100.
[21] Dalkin, K., Edwards, R., Edington, B. and Dixon, R.A. (1989) Plant Physiol. 92, 440–446.
[22] VanEtten, H.D., Matthews, D.E. and Matthews, P.S. (1989) Annu. Rev. Phytopathol. 27, 143–164.