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Stereoselective coupling of hemigossypol to form (+)-gossypol in moco cotton is mediated by a dirigent protein

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A B S T R A C T

The terpenoid gossypol, a secondary metabolite found in the cotton plant, is synthesized by a free radical dimerization of hemigossypol. Gossypol exists as an atropisomeric mixture because of restricted rotation around the central binaphthyl bond. The dimerization of hemigossypol is regiospecific in cotton. In the case of some moco cotton, the dimerization also exhibits a high level of stereoselectivity. The mechanism that controls this stereoselective dimerization is poorly understood. In this paper, we demonstrate that a dirigent protein controls this stereoselective dimerization process. A partially purified protein preparation from cotton flower petals, which by itself is unable to convert hemigossypol to gossypol, converts hemigossypol with a 30% atropisomeric excess into (+)-gossypol when combined with an exogenous laccase, which by itself produces racemic gossypol.

1. Introduction

Cottonseed is composed of ~22.5% of a high quality protein. The estimate of US cottonseed production for 2007 is over 6.5 million metric tons (National Agricultural Statistics Service, USDA, 2007). This is sufficient protein to provide the total protein needs for over 71 million people for 1 year based on the USDA recommendation of 56.6 g of protein per day per person. However, cottonseed is under utilized as a food/feed source because of the presence of a toxic compound called gossypol (**2**). Gossypol (**2**) occurs in the seed, foliage and roots of cotton; it imparts resistance against herbivorous insects and pathogens, and is biosynthesized by the free radical coupling of two molecules of hemigossypol (**1**) that yield both atropisomers (**2a**/**2b**) (Fig. 1) (Benedict et al., 2006). The (+)-gossypol (**2a**) has an absolute configuration of *S* and its stereo configuration is shown in Fig. 1 (Jaroszewski et al., 1992). In most commercial cottons (*Gossypium hirsutum*) grown in the U.S., the ratio of (+)- to (−)-gossypol (**2a**/**2b**) is approximately 3:2 (Zhou and Lin, 1988; Cass et al., 1991). However, this ratio can be as high as 98:2 in some moco cotton (*G. hirsutum* var. *marie-galante*) grown in Brazil (Cass et al., 1991; Stipanovic et al., 2005). Significantly, only (−)-gossypol (**2b**) is toxic to animals. Toxicity toward insects and pathogens is not affected by the (+)- to (−)-gossypol (**2a**/**2b**) ratio (Stipanovic et al., 2006; Puckhaber et al., 2002). Therefore, if the ratio could be shifted predominately to (+)-gossypol (**2a**), cottonseed could be used as a feed for both ruminant and non-ruminant animals.

Stereoselective free radical coupling reactions are a poorly understood biochemical process involved in plant secondary metabolite biosynthesis, as is the case for (+)-gossypol (**2a**) formation. Perhaps the best studied biological bimolecular free radical coupling reaction is the regio- and stereoselective dimerization of *E*-coniferyl alcohol to either (+)- or (−)-pinoresinol. Lewis' group (Davin et al., 1997) elegantly demonstrated the intervention of what they call a dirigent protein. The dirigent protein, isolated from *Forsythia intermedia*, by itself has no catalytic activity to convert *E*-coniferyl alcohol to pinoresinols. However, when the dirigent protein is combined with a laccase from *F. intermedia*, only (+)-pinoresinol is formed. If the dirigent protein is missing from this reaction, the result is formation of a racemic mixture of products. Boiling deactivates the protein (Hall and Lewis, 2002). Davin and Lewis (2000) proposed that the dirigent protein captures the *E*-coniferyl alcohol-derived free radical and directs the stereoselective coupling. Homologous dirigent DNA sequences have been detected in both gymnosperms and dicotyledonous angiosperms

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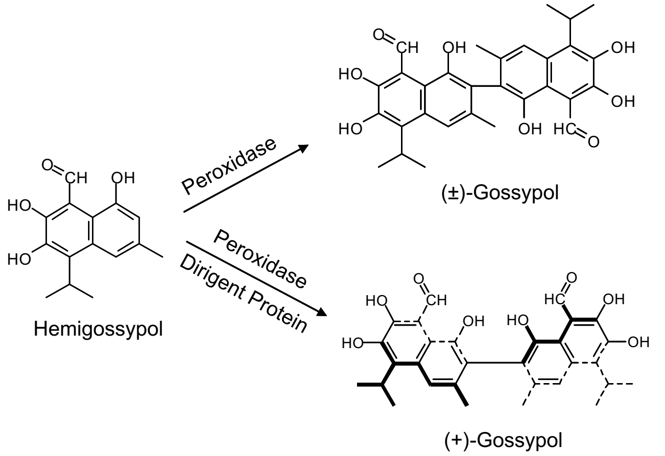

Fig. 1. Biosynthesis of gossypol by free radical coupling of hemigossypol generated by peroxidase in the presence or absence of a cotton dirigent protein.

(Gang et al., 1999). In 2000, Davin and Lewis showed that a protein in flaxseed produces the corresponding (−)-antipode. Thus, non-catalytic proteins can control the regio- and stereoselective dimerization of free-radical species. Recently, Zhu et al. (2007) obtained two dirigent-like cDNA clones, *Gbd 1* and *Gbd 2* from cotton (*Gossypium barbadense*). The two genes shared identities of 82% and were induced after infection by a fungal pathogen *Verticillium dahliae*. Phylogenetic analysis of dirigent and dirigent-like proteins (Ralph et al., 2007) indicated that the two *G. barbadense* genes belonged to subfamily DIR-b/d, one of the five subfamilies whose biochemical function are yet not established. Genes encoding dirigent proteins for the regio- and stereoselective coupling of *E*-coniferyl alcohol belonged to subfamily DIR-a.

Only one such dirigent protein has been characterized functionally to show that the protein guided the regio- and stereoselective coupling of the lignan and lignin pathway substrate *E*-coniferyl alcohol. Dirigent proteins that guided regio- and stereoselective coupling of other substrates have not been demonstrated, even though *in vivo* formation of regio- and stereoselectively coupled products of phenolic substrates are ubiquitous in nature. Herein, we show that a (+)-gossypol-forming dirigent protein mediates a peroxidase catalyzed reaction to steer the stereoselective coupling of a terpenoid pathway substrate, hemigossypol (**1**), to (+)-gossypol (**2a**).

2. Results and discussion

*2.1. Enzymatic and non-enzymatic formation of (+)- and (-)-gossypol (**2a**) and (**2b**) from hemigossypol (**1**)*

We have previously demonstrated (Benedict et al., 2006) that embryo extracts of the *G. hirsutum* var. *marie-galante* (moco cotton) preferentially converted hemigossypol (**1**) to (+)-gossypol (**2a**) [72% (+)-gossypol (**2a**) vs. 28% (−)-gossypol (**2b**)]. In this study, we used flower petal tissue of moco cotton as the source material because they contained a higher % of (+)-gossypol (**2a**) than seeds, and are relatively free of tannins and other interfering factors that can rapidly degrade proteins.

In the present investigation, three assays to measure the formation of (+)- and (−)-gossypol (**2a**) and (**2b**) from hemigossypol (**1**) were investigated using various co-substrates. The protein preparation with oxygen as co-substrate is referred to below as the "oxidase assay", the preparation with hydrogen peroxide as co-substrate is referred to below as the "peroxidase assay", and the preparation with oxygen as co-substrate and supplemented with an excess of exogenous laccase is referred to below as the "dirigent assay". In each case, controls were also studied that includes: (1) a no protein preparation; (2) a boiled protein preparation; (3) a no hemigossypol (**1**) preparation; and (4) a 0 reaction time preparation. After 10–60 min, each reaction was extracted with EtOAc/hexane (1:1, v/v) and the organic phases concentrated to dryness. The residues were derivatized with D-alaninol and the derivatives of (+)- and (−)-gossypol (**2a**) and (**2b**) were qualitatively and quantitatively analyzed using HPLC (Kim et al., 1996). A series of experiments were designed to determine if the active agent was a stereoselective enzyme or a dirigent type protein.

Dimerization of hemigossypol (**1**) with exogenous peroxidase, laccase, or ammonium persulfate gave a racemic mixture of gossypol [49.7 ± 0.3%, 49.4 ± 0.5%, and 51.1 ± 1.6% of (+)-gossypol (**2a**), respectively]. In contrast, when hemigossypol (**1**) was added to a crude enzyme preparation of moco flower petals using oxygen as co-substrate (oxidase assay), (+)-gossypol (**2a**) was preferentially formed [74% (+)-gossypol (**2a**), 2.2 nmol total gossypol/h/g tissue (Table 1)]. When H_2O_2 was added to the crude flower petal preparation (peroxidase assay), the rate of total gossypol (**2a**) formation by the naturally occurring peroxidase in the crude protein preparation [11.4 nmol total gossypol/h/g tissue] was about five-fold higher than in the oxidase assay. Here again, (+)-gossypol (**2a**) was preferentially formed (79%) (Table 1). When exogenous laccase was added to the same protein preparation followed by hemigossypol (**1**) (Table 1, dirigent assay) using oxygen as co-substrate, the % of (+)-gossypol (**2a**) (78.0%) was slightly higher compared to that formed in the oxidase assay and the total nmol of gossypol/h/g tissue was ca. 10 times that observed in the oxidase assay (Table 1, oxidase assay). When the crude preparation was boiled, and then exogenous laccase was added, only racemic gossypol (**2a**/**2b**) was formed (Table 1, control assay).

In the dirigent assay, a large excess of laccase [more than 10-fold excess of oxidase activity compared to that in the crude enzyme preparation (oxidase assay)] is present; as shown above, exogenous laccase alone produced racemic gossypol (**2a**/**2b**). If a stereoselective oxidase (laccase or peroxidase) was responsible for controlling the (+)- to (−)-gossypol ratio (**2a**/**2b**), one would expect the (+)- to (−)-ratio to decrease when the exogenous laccase was added, this was because the added laccase would be competing for the same substrate, and relatively more racemic gossypol (**2a**/**2b**) would be produced. Furthermore, if a stereoselective oxidase were involved, based on the total nmol of gossypol (**2**) produced per hour per gram fresh tissue in the oxidase assay and dirigent assay and the (+)-gossypol (**2a**) percentage in the oxidase assay as shown in Table 1, only 52.3% of (+)-gossypol (**2a**) would be

Table 1
Total and (+)-gossypol formation from hemigossypol by the crude protein preparation's oxidase activity, peroxidase activity, and dirigent activity

Assay type	Oxidase/co-substrate		Substrate		Enzyme		% (+)-Gossypol	Total nmol gossypol/h/g tissue
Oxidase	O_2	+	HG	+	Crude enzyme	→	74.2 ± 0.4	2.2 ± 0.3
Peroxidase	H_2O_2	+	HG	+	Crude enzyme	→	79.4 ± 1.1	11.4 ± 0.9
Dirigent	Laccase O_2	+	HG	+	Crude enzyme	→	77.9 ± 0.3	22.3 ± 2.4
Control	Laccase O_2	+	HG	+	Boiled crude enzyme	→	50.7 ± 0.8	19.6 ± 2.5

Aliquots of 240 μL crude enzyme preparation were used in each assay. Assays were done in triplicate.

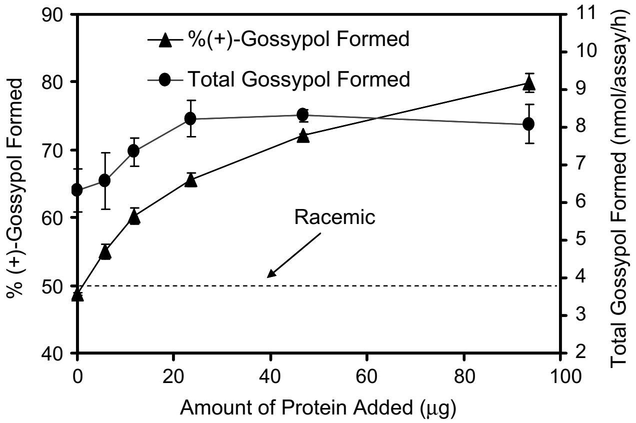

Fig. 2. Dependence of % (+)-gossypol formation and total gossypol formation on the amount of crude dirigent protein preparation added to the dirigent assay mixture containing hemigossypol and exogenous laccase. Experiments were done in triplicate.

formed in the dirigent assay. As seen, the opposite was observed and the percent (+)-gossypol (**2a**) increased slightly. These results argue against the involvement of a stereoselective oxidase, and they are consistent with the operation of a dirigent type protein.

The (+)-gossypol (**2a**) percentage increased steadily from racemic (ca. 50%) to 80% in the dirigent assay, when the amount of protein preparation of the flower petal added to the assay increased from 0 to 93 µg (Fig. 2), consistent with a mechanism involving a dirigent protein. Again, all assays contained at least a 10-fold excess of oxidase activity from exogenous laccase than that contained in the crude enzyme preparation. The rate of total gossypol (**2a**) formation increased ca. 30% when the amount of protein added in the dirigent assay was increased to ca. 23 µg. The rate remained almost constant when the added amount of protein in the assay increased further. A slight increase (ca. 10%) in the rate of total gossypol formation is expected due to the presence of endogenous oxidases in the crude protein preparation.

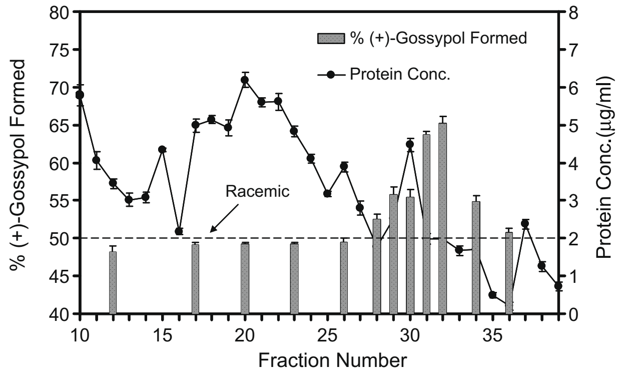

Fig. 3. Protein concentration and % (+)-gossypol formation in fractions purified by a CM-Sepharose FF column with hemigossypol and laccase in the dirigent assay. Aliquots of 240 µL column fractions were used in each assay and assays were done in triplicate.

2.2. Preferential formation of (+)-gossypol (*2a*) from hemigossypol (*1*) using a partially purified enzyme preparation

To further explore the biochemical mechanism for the (+)-gossypol (**2a**) formation, we subjected the crude protein preparation to chromatography on a CM-Sepharose FF column. The protein concentration of the individual fractions collected from the column was determined (Fig. 3). Selected fractions were assayed using the dirigent assay (i.e., enzyme preparation and exogenous laccase) and the percent (+)-gossypol (**2a**) formed in the assays were determined. Excess (+)-gossypol (**2a**) was produced by fractions 28–34. Fraction 32 was selected for further study because it showed the highest activity. An aliquot of the original crude enzyme preparation that also gave approximately 65% (+)-gossypol (**2a**) in a dirigent assay (Table 2, assay #1) was also selected. In Table 2, we compared the ability of these two preparations to form (+)-gossypol (**2a**) in the dirigent assay, peroxidase assay, and oxidase assay. In the peroxidase and oxidase assays (Table 2, assays #7 and 8, respectively), the no enzyme control experiments formed about 30 and 40 pmol of gossypol (**2**), respectively. Thus, values close to these numbers indicate that no or very little gossypol (**2**) was produced. In the peroxidase assay (Table 2, assay #2), which included the crude enzyme, hemigossypol (**1**) and H_2O_2, over 65 times more total gossypol (**2**) was produced as compared to the purified fraction (Table 2, assay #5). The purified fraction produced less gossypol (**2**) in the peroxidase assay (Table 2, assay #5) than in the assay with no enzyme added (Table 2, assay #7). This demonstrates that the column successfully separated the protein that controls the (+)- to (−)-gossypol (**2a**/**2b**) ratio from the endogenous peroxidase that is present in the crude preparation. Similarly, when hemigossypol (**1**) was added alone to the crude enzyme preparation with oxygen as co-substrate (Table 2, assay #3), 646 pmol gossypol/assay/h was produced; yet no gossypol (**2**) was produced in the oxidase assay with Fraction 32 (Table 2, assay #6). However, when Fraction 32 was mixed with laccase (Table 2, assay #4), 9743 pmol of gossypol (**2**) were produced of which 65% was (+)-gossypol (**2a**). Thus, the protein in Fraction 32 was incapable of converting hemigossypol (**1**) into gossypol (**2a**) by itself, but required the addition of an oxidative enzyme such as laccase. When laccase was added, gossypol (**2a**) was readily produced with a (+)- to (−)-gossypol (**2a**/**2b**) ratio of 13:7. These results demonstrate that the stereoselective formation of (+)-gossypol (**2a**) in moco cotton was mediated by a dirigent protein.

Table 2
Comparison of CM-Sepharose FF column Fraction 32 and the crude enzyme preparation for ability to form (+)-gossypol as determined by their respective dirigent protein activity, peroxidase activity, and oxidase activity

Assay # and type	Oxidase/co-substrate		Substrate		Enzyme	% (+)-Gossypol	Total pmol gossypol/assay/h
1. Dirigent	Laccase O_2	+	HG	+	Crude enzyme	65.4 ± 0.6	6812 ± 1374
2. Peroxidase	H_2O_2	+	HG	+	Crude enzyme	67.1 ± 1.4	1696 ± 246
3. Oxidase	O_2	+	HG	+	Crude enzyme	64.0 ± 1.1	646 ± 35
4. Dirigent	Laccase O_2	+	HG	+	Fraction #32	65.3 ± 0.9	9743 ± 1829
5. Peroxidase	H_2O_2	+	HG	+	Fraction #32	68.4 ± 5.8	26 ± 12
6. Oxidase	O_2	+	HG	+	Fraction #32	59.7 ± 6.3	34 ± 17
7. Peroxidase control	H_2O_2	+	HG			57.3 ± 4.5	30 ± 9
8. Oxidase control	O_2	+	HG			54.9 ± 2.3	40 ± 13

Aliquots of 23 µL of crude enzyme preparation were used in assays #1, 2, and 3. Aliquots of 240 µL of Fraction 32 were used in assays #4, 5, and 6. Assays were done in triplicate.

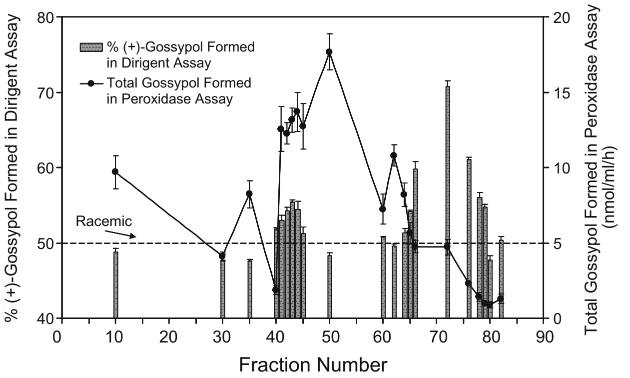

Fig. 4. Dirigent activity and peroxidase activity in fractions from Q-Sepharose FF column. Dirigent activity is expressed as % (+)-gossypol formed in dirigent assay and peroxidase activity is expressed as total gossypol formed in peroxidase assay. Aliquots of 240 μL column fractions were used in each assay and assays were done in triplicate.

2.3. Native molecular mass and substrate specificity of the (+)-gossypol-forming dirigent protein

We also used a Q-Sepharose FF column to fractionate the dirigent protein. The separation between the peroxidase activity and dirigent activity peaks was much less than that achieved by CM-Sepharose FF column (Fig. 4), but the overall recovery of dirigent activity was at least 20-fold higher. That is, twelve 5 mL fractions (Fractions #67–78, Fig. 4) from the Q-Sepharose FF column provided >60% (+)-gossypol (**2a**) dirigent activity, while only two to three fractions from the CM-Sepharose FF column gave >60% (+)-gossypol (**2a**) dirigent activity. Pooled dirigent active fractions from the Q-Sepharose FF column was used to determine the native molecular mass of the dirigent protein on a calibrated gel filtration column. A major peak and a minor peak corresponding to native molecular mass of 77.2 and 122.5 kDa were observed, reflecting the tendency of dirigent proteins to form aggregates. The (+)-gossypol-forming dirigent protein obtained from pooled active fractions of Q-Sepharose FF column converted a structurally related substrate present in some cotton species, hemigossypol 6-methyl ether, to (+)-gossypol 6,6′-dimethyl ether, but it did so less efficiently [81.7 ± 0.5% vs. 62.7 ± 0.8% (+)-gossypol (**2a**) from hemigossypol (**1**) and hemigossypol 6-methyl ether, respectively]. Very low levels of gossypol 6-methyl ether are present in the seed of some cotton cultivars, but are not observed in moco cottonseed (Stipanovic et al., 2005).

2.4. Concluding remarks

These experiments established that a dirigent protein controls the stereoselective coupling of hemigossypol (**1**) free radicals generated by cotton peroxidases for the preferential formation of (+)-gossypol (**2a**) in moco cotton. We have also partially purified and characterized the dirigent protein. Our current knowledge concerning the properties of the dirigent protein will enable us to design new approaches to purify this protein to homogeneity, obtain partial protein sequence information, and clone the encoding gene.

3. Experimental

3.1. Chemicals and general experimental procedures

Hemigossypol (**1**) and hemigossypol 6-methyl ether were obtained from cotton stele extracts by the procedure of Bell (1967). SDS–PAGE of the protein preparations was carried out according to the procedures of Laemmli (1970). Protein concentration was determined by the method of Bradford (1977).

3.2. Plant material

Plants of *G. hirsutum* var. *marie-galante* (moco cotton) were grown and maintained in a greenhouse in 2 gal pots. Unopened flower petal tissues were collected on the day of blooming and frozen in liquid N_2 and stored at −80° C.

3.2.1. Dirigent assay

This assay is designed to detect the presence of dirigent protein even with the enzyme preparations containing no free radical generating ability. Thus, crude enzyme preparations and fractions collected during purification were assayed in a total volume of 300 μL 0.1 M KPi buffer, pH 5.5 containing 104 μM hemigossypol (**1**) (20 μL, 1.56 mM HG dissolved in ethanol), *Coriolus versicolor* laccase (8.3 mU), and the enzyme preparations (up to 100 μg proteins). The assay mixtures were incubated for 10 min at 30° C. Controls were run either without *C. versicolor* laccase, without HG, without enzyme preparation, or with boiled (10 min) enzyme preparation. The reaction products and substrate were extracted three times with 160 μL EtOAc/hexane (1:1, v/v) and the organic phases were concentrated to dryness. The residues were derivatized with D-alaninol and the resulting diastereomeric adducts formed from (+) and (−)-gossypol (**2a**) and (**2b**) were qualitatively and quantitatively analyzed on a HPLC by the method of Kim et al. (1996).

3.2.2. Oxidase assay

The oxidative coupling of hemigossypol (**1**) for the formation of gossypol (**2**) by the enzyme preparation's endogenous laccase type oxidase activity was assayed in a total volume of 300 μL 0.1 M KPi buffer, pH 5.5 containing 104 μM hemigossypol (**1**), the enzyme preparations (up to 100 μg proteins), and oxygen from the air. The assay mixtures were incubated for 30 min at 30° C. Controls were run either without HG, without enzyme preparation, or with a boiled (10 min) enzyme preparation. The reaction products and substrate were analyzed as described in the above paragraph.

3.2.3. Peroxidase assay

The oxidative coupling of hemigossypol (**1**) for the formation of gossypol (**2**) by the enzyme preparation's endogenous peroxidase activity were assayed in a total volume of 300 μL 0.1 M KPi buffer, pH 5.5 containing 104 μM hemigossypol (**1**), 5 mM hydrogen peroxide and the enzyme preparations (up to 100 μg proteins). The assay mixture was incubated for 30 min at 30° C. Controls were run either without hydrogen peroxide, without HG, without enzyme preparation, or with boiled (10 min) enzyme preparation. The reaction products and substrate were analyzed as described in the above paragraph.

3.2.4. Gossypol formation from hemigossypol by horseradish peroxidase, C. versicolor laccase, and ammonium persulfate

Dimerization of hemigossypol (**1**) with exogenous peroxidase, laccase or ammonium persulfate were carried out with horseradish peroxidase (0.13 U, 100 ng) as in the "peroxidase assay" without enzyme preparations, with *C. versicolor* laccase in the "dirigent assay" without enzyme preparations, and with ammonium persulfate (100 mM) in the "oxidase assay" without enzyme preparations, respectively.

3.3. Protein extraction and purification

All extractions and purifications were conducted at 4° C. Storage of protein preparations was at −20° C with 10% glycerol added.

Buffer exchange and concentration were carried out either with dialysis tubes (10 kDa cut-off) or Centricon-10 concentrators (Amicon).

3.3.1. Protein extraction

The frozen tissue was ground to a fine powder in liquid N_2 in a mortar. Cold acetone (−20° C) was added to the ground powder (5 mL/g tissue) and the mixture was stirred for 10 min. After centrifugation at 40,000*g* for 10 min, the supernatant was decanted and discarded. The pellet was suspended in the cold acetone and the above procedure was repeated three more times. The resulting pale pellet was suspended in 0.1 M KPi buffer, pH 5.5 containing 1.5 M NaCl (5 mL/g tissue) and stirred for 1 h. The mixture was centrifuged at 12,000*g* for 10 min. The supernatant was buffer exchanged into 10 mM KPi buffer, pH 5.5, stored at −20° C after addition of 10% glycerol, and used as a crude enzyme preparation. The crude enzyme preparation was very stable under these storage conditions and no appreciable loss of dirigent activity and peroxidase activity was observed up to 1 year of storage.

3.3.2. Purification on CM-Sepharose FF column

The crude enzyme preparation (from 40 g flower tissue) was buffer exchanged into 40 mM Mes buffer, pH 5.0 and loaded on to a 1.5 × 18.0-cm CM-Sepharose FF column which had been equilibrated with 40 mM Mes buffer, pH 5.0. The column was eluted first with 25 mL 40 mM Mes buffer, pH 5.0, followed by a linear gradient of 75 mL 40 mM Mes buffer, pH 5.0 and 75 mL 40 mM Mes buffer, pH 5.0 containing 1 M NaCl at a flow rate of 1 mL/min. Five-milliliter fractions were collected and assayed for “dirigent”, “peroxidase”, “oxidase” activities, and protein content.

3.3.3. Purification on Q-Sepharose FF column

The crude enzyme preparation (from 40 g flower tissue) was also fractionated on a 1.5 × 18.0-cm Q-Sepharose FF column after buffer exchanging into 10 mM KPi, pH 8.0 buffer. The fractionation was identical as with CM-Sepharose FF column purification except the 40 mM Mes, pH 5.0 component was replaced by 10 mM KPi, pH 8.0.

3.4. Molecular mass determination of the native dirigent protein

The dirigent active fractions from the Q-Sepharose FF column were pooled, buffer exchanged, and concentrated to 0.5 mL in 10 mM KPi, pH 5.5 containing 0.2 M NaCl. This sample was loaded onto a 190 mL Ultrogel AcA34 gel filtration column previously calibrated with six known molecular mass standards and eluted with the same buffer at a flow rate of 1 mL/min. Fractions of 1 mL were collected and assayed for dirigent activity. A major dirigent activity eluted at a retention time of 116 min and a minor dirigent activity eluted at 108 min; these retention times were used to determine the molecular mass of the dirigent protein.

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