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Purification and Antigenicity of Flavone Synthase I from Irradiated Parsley Cells

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Flavone synthase I, a soluble 2-oxoglutarate-dependent dioxygenase catalyzing the oxidation of flavanones to flavones in several *Apiaceae* species, was induced in parsley cell cultures by continuous irradiation with ultraviolet/blue light for 20 h. The enzyme was extracted from these cells and purified by a revised purification protocol including the fractionation on hydroxyapatite, Fractogel EMD DEAE, and Mono Q anion exchangers, which resulted in an apparently homogeneous flavone synthase at approximately 10-fold higher yield as compared to the previous report. The homogeneous enzyme was employed to raise an antiserum in rabbit for partial immunological characterization. The specificity of the polyclonal antibodies was demonstrated by immunotitration and Western blotting of the crude ammonium sulfate-fractionated enzyme as well as of the enzyme at various stages of the purification. High titer cross-reactivity was observed toward flavone synthase I, showing two bands in the crude extract corresponding to molecular weights of 44 and 41 kDa, respectively, while only the 41 kDa was detected on further purification. The polyclonal antiserum did not cross-react with recombinantly expressed flavanone 3β-hydroxylase from *Petunia hybrida* or flavonol synthase from *Citrus unshiu*, two related 2-oxoglutarate-dependent dioxygenases involved in the flavonoid pathway. © 2001 Academic Press

***Key Words:* *Petroselinum crispum* syn. *P. hortense*; flavonoid biosynthesis; flavone synthase I; 2-oxoglutarate-dependent dioxygenase; flavone synthase antibodies.**

The flavonoids form a very large group of abundant and well-characterized plant secondary metabolites, which has attracted increasing attention in recent years partially due to their medicinal value (1). Flavonoids show various bioactivities including the inhibition of the cyclooxygenase and/or 5-lipooxygenase activities in the arachidonate pathway. They may act in a number of ways on blood components such as platelets, monocytes, or low-density lipoprotein and have been described as potential anticancer agents in humans (1). In addition, they function as antioxidants *in vivo* by scavenging of reactive oxygen species, e.g., superoxide anion, hydroxyl radicals, or peroxy radicals (2), and the scavenging potential appears to depend on three structural requirements (Fig. 1): the *o*-dihydroxy substitution of ring B, the C_2–C_3 double bond in concert with a 4-oxo functionality in ring C, and additional 3- and 5-hydroxyl groups in rings C and A, respectively (3). These data suggest that flavones and flavonols are more potent antioxidants than flavanones.

Flavones and their glycosides form a subgroup within the more than 6400 flavonoids which have been reported so far (1). Their number exceeds 1000, and the individual compounds differ only in the substitution pattern of the flavone (2-phenyl-γ-benzopyrone) skeleton. Flavones originate from flavanones by 2,3-desaturation (Fig. 1), and this oxidation may be considered as a "branchpoint reaction" in the flavonoid pathway which paves the road to the final flavone and flavonol products. The 2,3-desaturation reaction is catalyzed by two classes of enzymes. An NADPH-dependent type of flavone synthase (FNS II)[2] was identified in a wide

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[2] Abbreviations used: FNS I, flavone synthase I; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetate; PMSF, phenylmethylsul-

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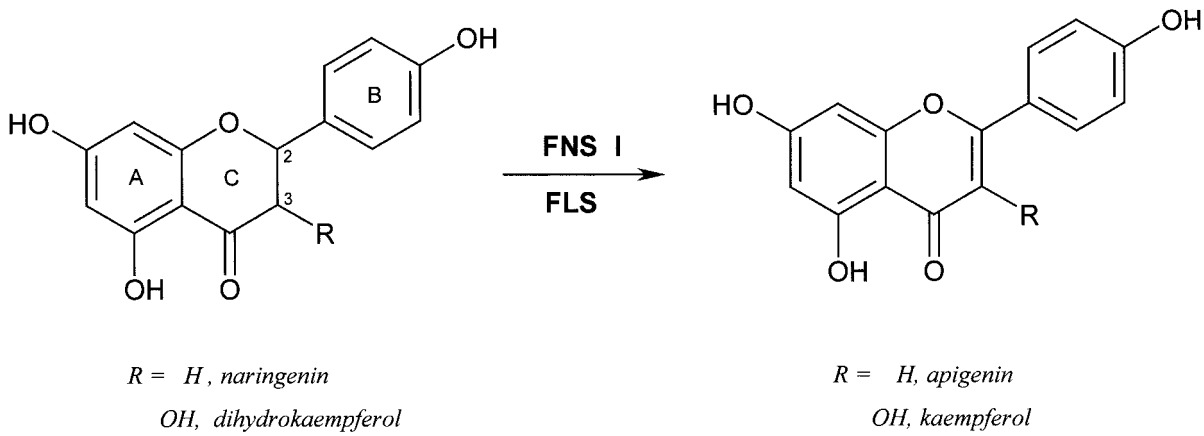

FIG. 1. Reactions catalyzed by flavone synthase (FNS), converting (2*S*)-naringenin to apigenin, or by flavonol synthase (FLS), converting dihydrokaempferol to kaempferol. The hydroxylation of (2*S*)-naringenin to dihydrokaempferol is catalyzed by flavanone 3β-hydroxylase.

range of plant species including the Fabaceae (4). This enzyme activity was initially reported from microsomal fractions of *Anthirrhinum majus* sepals (5) and shown to be inhibited by common cytochrome P450-dependent monooxygenase inhibitors (6). Based on the conserved heme-binding site in cytochrome P450 enzymes an upstream primer was designed and employed recently to isolate a full-size cDNA encoding FNS II from *Gerbera hybris* by a differential display technique. Microsomes from yeast cells expressing this FNS II cDNA catalyzed the one-step formation of labeled flavones from the corresponding ^{14}C-labeled flavanones (7). Alternatively, a soluble flavone synthase activity had been reported in 1975 from cell-free extracts of immature parsley leaves (8), which depended upon a soluble cofactor identified later as 2-oxoglutarate. This soluble enzyme was partially purified and characterized in 1981 from parsley cell suspension cultures and designated FNS I (9). FNS I required molecular oxygen, ferrous iron, 2-oxoglutarate, and ascorbate for full catalytic activity and, accordingly, was classified as a 2-oxoglutarate-dependent dioxygenase (9). The fact that different plant families use distinctly different classes of enzymes to form the same kind of metabolite (10, 11) is rather unusual and may be of evolutionary significance beyond the biosynthesis of flavonoids (12). Over the past 25 years about sixty 2-oxoglutarate-dependent dioxygenases have been reported from bacteria, fungi, plants, or vertebrates, which catalyze diverse reactions including aliphatic hydroxylation, epoxidation, desaturation, or desaturating cyclization. Most of these enzymes are involved in biosynthetic pathways other than the formation of flavonoids, e.g., leading to alkaloids, ethylene, gibberellins, penicillins, or cephalosporins (13–15), and a common structure was proposed for the entire class of dioxygenases based on conserved sequences particularly in the C-terminal portion (15).

Parsley FNS I was described as a rather unstable enzyme in crude extracts from irradiated cell cultures. Nevertheless, a partial purification was accomplished through six steps of fractionation, including chromatofocusing and hydrophobic interaction chromatography on phenyl-Superose (10), which formally enriched the enzyme activity 10-fold and yielded an inhomogeneous preparation at very low recovery (40 μg protein from 3.0 kg of irradiated parsley cells). This preparation was employed to investigate the molecular mechanism of the desaturation reaction (10), considering the option that the reaction might proceed by successive 2-hydroxylation of the flavanone substrate and 2,3-dehydratation. Detailed substrate interaction kinetics suggested, however, that 2-hydroxyflavanones do not dissociate from the FNS I during catalysis and are ineffective *in vitro* to compete with the flavanone substrate. It was postulated, therefore, that FNS I catalyzes the oxidative desaturation of the substrate by direct abstraction of the vicinal hydrogen atoms from positions 2 and 3 of the C-ring.

The present study describes a considerably improved purification of FNS I from irradiated parsley cell cultures yielding several hundred micrograms of the homogeneous and catalytically active enzyme. This enzyme was used for the generation of an antiserum as a prerequisite for characterizing the FNS I by immunological means and to distinguish the FNS I from related 2-oxoglutarate-dependent dioxygenases.

MATERIALS AND METHODS

Plants and plant cell cultures. Seedlings and plants of the Apiaceae were grown in the greenhouse of the local Botanical Gardens. Cell suspension cultures were propagated in the dark and irradiated after 7 days of subculturing for up to 50 h with ultraviolet A/blue

fonyl fluoride; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; FPLC, fast protein liquid chromatography.

light (Osram L40 W/73 and Phillips TL40W/18 light tubes at 18 W × m^{-2}). Suspension cultures of parsley (*Petroselinum crispum* syn *P. hortense*) were routinely irradiated 20 h for flavone synthase I induction and harvested as described previously (10).

Chemicals and materials. Fine biochemicals of analytical grade were purchased from Biomol (Hamburg, Germany), Roche Diagnostics (Mannheim, Germany), Merck (Darmstadt, Germany), Amersham-Pharmacia (Freiburg, Germany), Roth (Karlsruhe, Germany), Serva (Heidelberg, Germany), or Sigma (Deisenhofen, Germany). [2-^{14}C]Malonyl-CoA was from Amersham Pharmacia. The substrate, (2*S*)-[4*a*,6,8-^{14}C]naringenin (234–425 MBq/mmol), was prepared enzymatically from 4-coumaroyl-CoA and [2-^{14}C]malonyl-CoA as described previously (16) and stored at −20°C in ethyl acetate until use. Mono Q, PD-10, and protein A-Sepharose columns were purchased from Amersham-Pharmacia, hydroxyapatite HTP Bio-Gel was from Bio-Rad (München, Germany), Fractogel EMD DEAE (S) anion exchanger was from Merck, and Centriprep-10 cells were bought from Amicon (Witten, Germany). The SDS-7 markers and IEFPAG plates were purchased from Sigma and Amersham-Pharmacia, respectively.

Buffers. All buffers were degassed under vacuum, equilibrated subsequently with nitrogen, and degassed again prior to use. The following buffers were used: (A) 0.1 M imidazole-HCl, pH 6.8, containing 20 mM sodium ascorbate, 200 μM 2-oxoglutarate, 100 μM ferrous sulfate, 1 mM PMSF, 1 mM DTT, 1 mM histidine, and 5% glycerol (v/v); (B) as in A, additionally containing 0.5 M sodium chloride; (C) 50 mM imidazole-HCl, pH 6.8, containing 5 mM potassium phosphate, 1 mM DTT, 10 mM sodium ascorbate, 1 mM PMSF, and 5% glycerol (v/v); (D) 0.2 M potassium phosphate, pH 6.8, containing 10 mM sodium ascorbate, 1 mM PMSF, and 5% glycerol (v/v); (E) 50 mM imidazole-HCl, pH 7.2, containing 1 mM histidine, 1 mM DTT, 10 mM sodium ascorbate, 1 mM PMSF, and 5% glycerol (v/v); (F) as in E, containing additionally 0.5 M sodium chloride.

Enzyme purification. Irradiated parsley cells (2.3 kg) were extracted as described previously (10), and 10% polyethyleneimine that had been adjusted to pH 7.5 with HCl was added to the crude extract. The slurry was stirred for 10 min and filtered through glass wool, and the filtrate was cleared by centrifugation at 13,500*g* for 25 min. Crude FNS I was precipitated from the supernatant by the addition of solid ammonium sulfate (40 to 80% saturation). The precipitate was dissolved under a nitrogen atmosphere in a minimal volume of 0.1 M imidazole-HCl buffer, pH 6.8, containing 1 mM histidine, 50 mM sodium ascorbate, 1 mM DTT, and 1 mM PMSF. Glycerol (10%, v/v) was added, and the solution was immediately frozen in liquid nitrogen and stored at −70°C.

The FNS I was purified by FPLC at 4°C. The elution of protein was monitored at 350 nm, because sodium ascorbate had been added to all the buffers. Ammonium sulfate was removed by filtration through Sephadex G-25 (38 × 7-cm column) in buffer A at a flow rate of 8 ml/min, the enzyme fraction was applied to a Q-Sepharose fast-flow column (12 × 5 cm), and the protein was eluted in a linear gradient (650 ml) of 0–100% buffer B in buffer A. The fractions (20 ml) containing FNS I activity were pooled, reduced to 40 ml by centrifugal ultracentrifugation in Centriprep-10 cells, and subjected to Sephacryl S200 HR (80 × 5-cm column) size-exclusion chromatography in buffer A at 5 ml/min. The fractions (20 ml) containing FNS I were pooled, concentrated to 4 ml, and applied to a Bio-Rad HTP Bio-Gel hydroxyapatite column (15.5 × 2.5 cm) in buffer C. FNS I was eluted in a linear gradient (425 ml) of 0–100% buffer D in buffer C. The fractions (6 ml) containing FNS I were pooled, concentrated by ultrafiltration in Centriprep-10 cells, desalted by filtration through a PD-10 column in buffer E, and applied subsequently to a Fractogel EMD DEAE (S) anion exchanger (1-ml column) in buffer E. The proteins were eluted in a linear gradient (20 ml) of 0–100% buffer F in buffer E at a flow rate of 0.5 ml/min. Part of the FNS I was purified finally by Mono Q anion-exchange chromatography (1-ml column) under the conditions used for Fractogel chromatography, adding to an overall yield of 450 μg of the apparently homogeneous enzyme.

Enzyme assay. The FNS I activty was assayed as described previously (10) in a total volume of 100 μl, using 0.5–1 μM (2*S*)-[4*a*,6,8-^{14}C]naringenin as a substrate at pH 8.5. The assay was terminated by the addition of a concentrated aqueous EDTA solution (10 μl), the incubation mixture was subsequently extracted twice (50 and 40 μl) with ethyl acetate, and the organic phase was subjected to thin-layer chromatography and autoradiography (10). Protein was determined by the Lowry assay in the presence of deoxycholate (17, 18) using bovine serum albumin as a standard.

Electrophoresis. SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (19) utilizing the SDS-7 markers for mass calibration. Proteins in the gel were stained with Coomassie brilliant blue. Isoelectric focusing was accomplished on commercial ready-to-use IEFPAG plates, pH 6.5–4.0, and stained with Coomassie brilliant blue according to the manufacturer's instructions.

Antibodies. The homogeneous FNS I (300 μg in 400 μl of 20 mM imidazole-HCl buffer, pH 7.2, containing 5% glycerol) was supplemented with complete Freund's adjuvant and injected intraperitoneally into an 11-week-old rabbit. The rabbit received one booster injection of 100 μg FNS I/400 μl 6 weeks later, and the first serum was drawn after an additional 2 weeks followed by bleeding at 5-day intervals for three times. The preimmune serum had been taken from the same animal. The IgG fraction was isolated from the polyclonal antiserum following a published procedure (20). Briefly, an aliquot of the antiserum (25 ml) was loaded on protein A-Sepharose (30 ml column) at a flow rate of 0.5 ml/min, which had been equilibrated with 4 bed volumes of 0.1 M Tris-HCl buffer, pH 7.2. The column was washed with the same buffer until the eluate absorbance at 280 nm returned to background levels. The IgGs were eluted subsequently with 0.58% acetic acid, pH 3.0, containing 0.15 M sodium chloride (35 ml) at a flow rate of 1 ml/min, and the eluate was immediately adjusted to pH 8.5 by the dropwise addition of 1 M Tris-HCl, pH 9.0. The antibody fraction was frozen in liquid nitrogen and stored at −70°C.

Immunotitration and Western blotting. Aliquots (50 μl) of the crude enzyme collected from ammonium sulfate fractionation were incubated under gentle shaking with a serial dilution of FNS I antibodies (total volume 100 μl) at room temperature for 30 min followed by 3 h at 5°C. Subsequently, protein A-Sepharose (2 mg per vial) was added, and the incubation was continued for an additional hour. The pellet was removed by centrifugation for 10 min at 5°C (12,000*g*), and the residual FNS I activity in the supernatant was determined. The preimmune serum served as a control in parallel incubations. Western blotting was carried out as described in the literature (21) and using the FNS I polyclonal antiserum. The proteins bound to the nitrocellulose filters were visualized by staining with Ponceau S solution after immunostaining.

RESULTS

Choice of Enzyme Source

Previous activity screenings revealed that FNS I is confined to some members of the Apiaceae (10) and that irradiation with ultraviolet/blue light was required to induce the activity in dark-cultured cells (9, 10). Accordingly, cell suspension cultures of *Daucus carota, P. crispum, Ammi majus, Ammi visnaga,* and *Conium maculatum* were irradiated continuously for up to 50 h and examined for their FNS I activity. In addition, the assay was extended to different tissues (leaf, cotyledon, and root) of seedlings and mature plants of *P. crispum, D. carota, Apium graveolens, Coriandrum sativum, Anethum graveolens, Anthriscus*

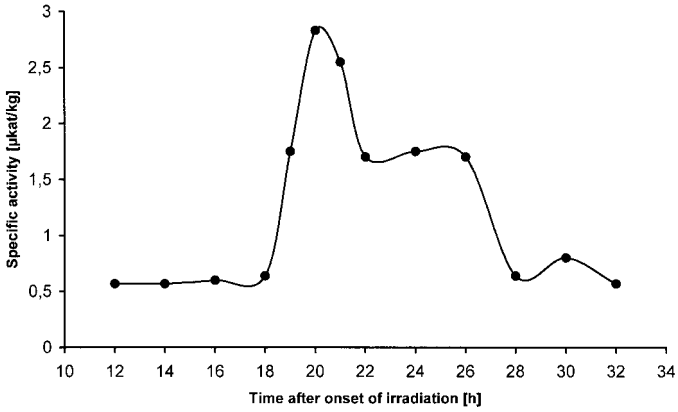

FIG. 2. Induction of flavone synthase I activity in parsley cell cultures irradiated with ultraviolet/blue light. The time course of induction was reproduced twice, and the enzyme assay was carried out as described previously (10).

sylvestris, Anthriscus cerefolium, Didiscus caeriuleus, C. maculatum, and *Orlaya grandiflora.* Considerable FNS I activity (2–3 μkat/kg) was measured in the extracts from parsley cells irradiated for 19–21 h (Fig. 2), showing a kinetic profile very similar to those of flavanone 3β-hydroxylase and chalcone synthase induction (M.-L. Heskamp and L. Britsch, unpublished), or from the cotyledons and primary leaves, respectively, of 15- and 28-day-old parsley plants irradiated for 6 h. Equivalent specific activity was determined in cotyledons or primary leaves of 13- and 23-day-old celery plants irradiated for 6 h, whereas the activity of carrot cultures remained at negligible levels, and the activity was lacking from the other plants and cell cultures tested. Due to the convenient growth and handling of suspension cultures, FNS I was isolated from irradiated parsley cells.

Enzyme Purification

The protocol developed 11 years ago (10) for the purification of FNS I from irradiated parsley cells was substantially improved by including chromatographies on hydroxyapatite as well as on Fractogel EMD DEAE tentacle type and Mono Q anion exchangers. Starting with a crude extract from 2.3 kg of frozen parsley cells, the purification through 5 steps yielded 150 μg of the homogeneous FNS I in the peak fractions from the Fractogel chromatography with an apparent purification factor of about 94 (Table I). The side fractions, which revealed minor contaminating proteins on SDS-PAGE (Fig. 3), were collected separately and subjected to an additional chromatography on a Mono Q anion-exchange column, yielding another 300 μg of homogeneous FNS I of lower specific activity (Table I). Coomassie brilliant blue stained only one protein after SDS-PAGE separation, revealing a molecular weight of 41 kDa for the FNS I polypeptide (Fig. 3). Two related

TABLE I

Purification of Flavone Synthase I from Irradiated Parsley Cells

Purification step	Protein [mg]	Specific activity [μkat/kg]	Purification [-fold]	Recovery [%]
Crude extract	11592	2.3	1.0	100
Polyethyleneimine/ ammonium sulfate (0.4–0.8)	3115	1.9	0.8	21.9
G-25/Q-Sepharose	439	4.4	1.9	7.3
Sephacryl S200 HR	99.2	11.98	5.2	4.5
Hydroxyapatite	7.8	23.40	10.2	0.7
Fractogel EMD DEAE (peak fraction)	0.15	215	93.5	0.08
Fractogel EMD DEAE (side fractions, Mono Q)[a]	0.3	67.3	29.3	0.07

[a] The side fractions from Fractogel EMD DEAE chromatography contained minor contaminating polypeptides which were removed by additional chromatography on Mono Q.

2-oxoglutarate-dependent dioxygenases, flavanone 3β-hydroxylase and flavonol synthase, are involved in the biosynthesis of flavonols in parsley (Fig. 1), and their elution characteristics were also recorded during the purification. The bulk of both of these enzymes was separated by hydroxyapatite chromatography, where the elution of FNS I required a concentration of approximately 200 mM potassium phosphate. The drop in activity during purification (Table I) demonstrated the instability of the FNS I, which appeared to be enhanced following the ammonium sulfate fractionation. As reported earlier (10), the enzyme activity was partially preserved by removing oxygen as much as possible from all buffers and utilizing high concentrations of ascorbate throughout the purification. Nevertheless,

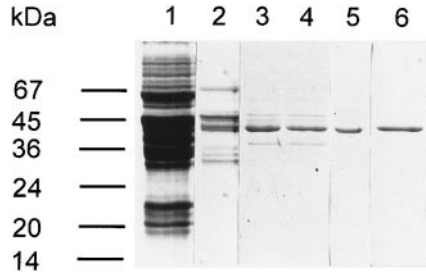

FIG. 3. SDS-PAGE separation of partially purified (lanes 1–4) and pure FNS I (lanes 5, 6) from irradiated parsley cells. The separation was accomplished in 5% stacking and 13% separation gels, and the proteins (1–2 μg/lane) were stained with Coomassie brilliant blue R-250. The purification stages correspond to the eluate from Q-Sepharose (lane 1), hydroxyapatite (lane 2), Fractogel EMD DEAE side fractions (lanes 3 and 4), Mono Q (lane 5), and Fractogel EMD DEAE peak fraction (lane 6). Molecular mass markers are indicated on the left margin.

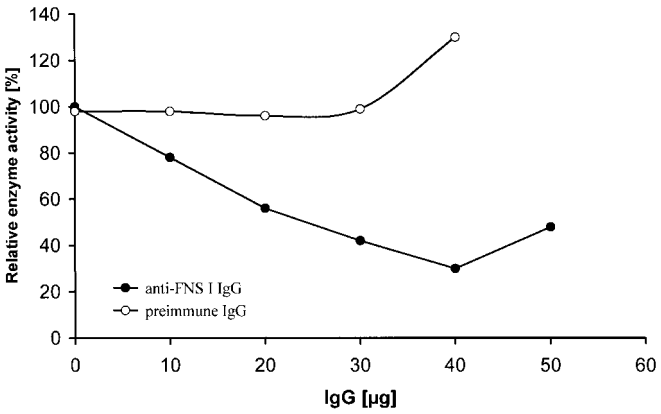

FIG. 4. Immunotitrations of FNS I in crude extract from irradiated parsley cells. The polyclonal antiserum to homogeneous FNS I had been raised in a rabbit, and various amounts of the IgG fractions of the preimmune and the immune serum were incubated for 3 h with 50 μl of the enzyme (total volume 100 μl) that had been fractionated with ammonium sulfate (40–80% saturation) from the crude parsley extract and dissolved in 0.1 M imidazole-HCl buffer, pH 6.8, containing 1 mM histidine, 50 mM sodium ascorbate, 1 mM DTT, 1 mM PMSF, and 10% glycerol (v/v). Subsequently, protein A-Sepharose (2 mg/assay) was added and the incubation was continued for an additional hour before the precipitate was removed by centrifugation. The relative FNS I activity in the supernatant was determined under standard assay conditions.

the apparent purification factor (Table I) remained rather low, and considerable losses in enzyme activity occurred even upon storage at −70°C which was necessary during the course of the purification. Overall, however, the purification protocol was sufficiently improved to yield quantities of pure and catalytically active FNS I (450 μg from 2.3 kg of cells) sufficient for immunological studies.

Immunotitration

Pure FNS I (400 μg) was employed to raise a polyclonal antiserum in an 11-week-old rabbit. The IgG factions of the preimmune and the immune sera were isolated by protein A affinity chromatography and used for immunotritrations. While the preimmune IgGs did not inhibit the FNS I activity and even showed a stimulatory effect at higher concentrations (exceeding 25 μg/100 μl assay), the IgGs from the immune serum inhibited the FNS I activity up to about 70% (Fig. 4). A relative inhibition of 50% was observed upon incubation of the crude enzyme (50 μl of the ammonium sulfate fraction) for 3 h with 27 μg of the IgG antibodies followed by protein A-Sepharose precipitation. The stimulation of activity which became apparent at high protein concentrations (Fig. 4) was likely due to protein stabilization, since FNS II was absent from the cells (9). Furthermore, neither the flavanone 3β-hydroxylase nor the favonol synthase activity of the crude parsley extract was inhibited upon incubation with the FNS I antiserum. This finding was somewhat unexpected, since these two dioxygenases were assumed to be closely related to the FNS I.

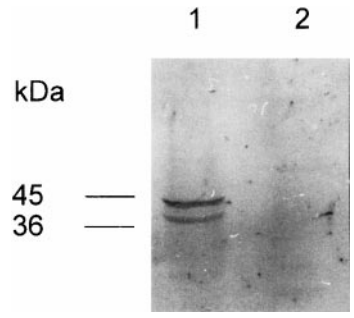

FIG. 5. Western blotting of the FNS I polypeptides in crude cell extracts, using the polyclonal rabbit antiserum. Lane 1, extract from parsley cells that had been irradiated with ultraviolet/blue light for 20 h; lane 2, extract from parsley cells continuously cultured in the dark. Molecular mass markers are indicated in the left margin.

Specificity of FNS I Antiserum

The polyclonal antiserum was used to verify the induction of FNS I upon irradiation of parsley cells and to document the process of enzyme purification. While the extracts from dark-grown parsley cells contained no cross-reacting proteins, two cross-hybridizing bands of of 44 and 41 kDa, respectively, were detected by Western blotting of the crude protein fraction that had been precipitated with ammonium sulfate shortly after extraction from the irradiated cells (Fig. 5). The pattern of two bands was always detected in fresh, crude FNS I-containing extracts (Figs. 5, 6) and was also seen in leaf extracts from garden parsley plants at different developmental stages (R. Lukačin and S. Martens, unpublished). The pattern appeared to suggest that the native enzyme of 44 kDa might be truncated by about 3 kDa in crude extracts to another form of active FNS I which was enriched on further purification (Fig. 6).

The immunological cross-reactivity of heterologous 2-oxoglutarate-dependent dioxygenases of diverse cat-

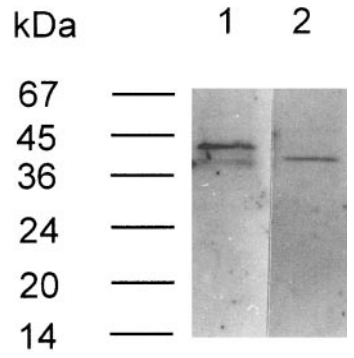

FIG. 6. Western blotting of the FNS I polypeptide in the crude extract from irradiated parsley cells (lane 1) and after final purification (0.5 μg protein, lane 2). Molecular mass markers are indicated in the left margin.

alytic function has been reported (22), and antibodies raised against a particular flavanone 3β-hydroxylase cross-reacted with the 3β-hydroxylase from many different plant sources (23, 24). Therefore, the antibodies generated to the parsley FNS I were examined in Western blots for their eventual cross-reactivities with pure, recombinantly expressed flavanone 3β-hydroxylase from *Petunia hybrida* and flavonol synthase from *Citrus unshiu* that was subcloned and expressed recently in our laboratory (F. Wellmann *et al.*, unpublished). Although these dioxygenases must be closely related to FNS I, the antibodies recognized the parsley FNS I only (data not shown).

DISCUSSION

The revised scheme of purification yielded a homogeneous FNS I of about 41 kDa from irradiated parsley cells, and the recovery (450 μg from 2.3 kg of cells) was sufficiently high for further investigations. The purified enzyme exhibited an isoelectric point of 4.85 in accordance with the p*I* of 4.8 reported earlier (10). The instability of 2-oxoglutarate-dependent dioxygenases, in general, has long been known (25), and for *Petunia* flavanone 3β-hydroxylase the proteolytic degradation in crude extracts had been reported and correlated with enzyme inactivation (23, 25, 26). The banding pattern in FNS I Western blotting might be reminiscent of the phenomenon encountered on purification of *Petunia* flavanone 3β-hydroxylase (26, 27), because in both instances a moderate rate of inactivation occurred in crude extracts which became more severe at later stages in the purification. Therefore, the relatively low recoveries of FNS I activity in the previous (10) and present studies (Table I) cannot be ascribed solely to proteolytic degradation. It remains to be established whether the parsley FNS I polypeptide is prone to proteolysis in crude extracts analogous to the *Petunia* flavanone 3β-hydroxylase (27). Alternatively, the band of 44 kDa cross-reacting with the antiserum might represent another protein which must be induced concomitantly with the FNS I in parsly cells under irradiation (Fig. 5). Recalling the fact that irradiation selectively induces the biosynthesis of ultraviolet-protecting flavonoids in parsley cells (11), such a protein is likely involved also in the flavonoid pathway and might represent another dioxygenase entity.

The homogeneous FNS I was employed to generate a polyclonal rabbit antiserum that was required for investigation of the induction and the degradation of FNS I in parsley cells and extracts, respectively. The proof of specificity is an essential prerequisite for such experiments, and the initial immunotitration of FNS I activity with the purified IgG fraction (Fig. 4) clearly demonstrated the high-affinity cross-hybridization. Surprisingly, however, the activity of flavanone 3β-hydroxylase which represents another 2-oxoglutarate-dependent dioxygenase in the parsley extracts (9) was not inhibited in these immunotitrations. This result was puzzling, since 2-oxoglutarate-dependent dioxygenases had been assumed to be closely related and to show cross-reactivity, i.e., polyclonal antibodies to desacetoxyvindoline 4-hydroxylase from *Catharanthus roseus* recognized a flavonol 6-hydroxylase described from *Chrysosplenium americanum* (22). Similarly, antibodies raised to *Petunia* flavanone 3β-hydroxylase cross-hybridized with 3β-hydroxylases from widely different plants, e.g., parsley, stock, and carnation (24), and the affinity of parsley FNS I antibodies to the endogeneous 3β-hydroxylase involved in the same secondary pathway (Fig. 1) had been anticipated. In contrast, the FNS I antibodies showed narrow specificity which was substantiated further in Western blots with *Petunia* flavanone 3β-hydroxylase (27) and yet another 2-oxoglutarate-dependent dioxygenase, flavonol synthase from *C. unshiu* expressed in *Escherichia coli* (F. Wellmann *et al.*, unpublished). FNS I and flavonol synthase catalyze the same 2,3-desaturation reaction and use flavanone substrates distinguished solely by the absence or presence of a 3-hydroxyl group (Fig. 1), a spatial difference that must impose different conformations on the enzyme polypeptides and cause the generation of divergent sets of polyclonal antibodies. Flavones are formed more commonly in plants by another type of flavone synthase, a cytochrome P450-dependent FNS II, which appears to be absent from parsley. This enzyme which had been cloned and expressed recently from *Gerbera hybrida* (7) also did not not cross-react with the parsley FNS I antibodies. The selectivity of the FNS I antibodies (Figs. 4 and 6) and the absence of FNS from dark-grown parsley cells (Fig. 5) enable the expression cloning of the FNS I as had been done with flavanone 3β-hydroxylase from *Petunia* (25).

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