The monomeric polypeptide comprises the functional flavanone 3β-hydroxylase from *Petunia hybrida*

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Abstract Flavanone 3β-hydroxylase catalyzes the Fe^{II}/oxoglutarate-dependent hydroxylation of (2*S*)-flavanones to (2*R*,3*R*)-dihydroflavonols in the biosynthesis of flavonoids, catechins and anthocyanidins. The enzyme had been partially purified from *Petunia hybrida* and proposed to be active as a dimer of roughly 75 kDa in size. More recently, the *Petunia* 3β-hydroxylase was cloned and shown to be encoded in a 41 655 Da polypeptide. In order to characterize the molecular composition, the enzyme was expressed in a highly active state in *Escherichia coli* and purified to apparent homogeneity. Size exclusion chromatographies of the pure, recombinant enzyme revealed that this flavanone 3β-hydroxylase exists in functional monomeric and oligomeric forms. Protein cross-linking experiments employing a specific homobifunctional sulfhydryl group reagent or the photochemical activation of tryptophan residues confirmed the tendency of the enzyme to aggregate to oligomeric complexes in solution. Thorough equilibrium sedimentation analyses, however, revealed a molecular mass of 39.2 ± 12 kDa for the recombinant flavanone 3β-hydroxylase. The result implies that the monomeric polypeptide comprises the catalytically active flavanone 3β-hydroxylase of *P. hybrida*, which may readily associate in vivo with other proteins.

Key words: Flavonoid biosynthesis; Monomeric structure; Flavanone 3β-hydroxylase; 2-Oxoglutarate-dependent dioxygenase; *Petunia hybrida*

1. Introduction

Flavanone 3β-hydroxylase (FHT) catalyzes the hydroxylation of (2*S*)-flavanones to (2*R*/3*R*)-dihydroflavonols, and this reaction is pivotal to the formation of flavonols, anthocyanidins and catechins [1]. FHT activity requires the presence of ferrous iron and 2-oxoglutarate for the reduction of molecular oxygen to yield the hydroxylated substrate besides succinate and carbon dioxide. FHT thus classifies as a member of the intermolecular dioxygenases [2,3] which are involved in a broad spectrum of primary and secondary biosynthetic pathways, e.g. the mammalian formation of carnitine [4], the microbial conversion of thymidine to uracil [5–7] or of penicillins to cephalosporins [8] as well as the biosynthesis of plant secondary metabolites such as alkaloids [9,10] and gibberellins [11].

The FHT in crude extracts from young petals of *Petunia hybrida* had been described as a rather labile enzyme and was initially purified under semi-anaerobic conditions in the presence of ascorbate, 2-oxoglutarate and ferrous iron [2]. A molecular mass of 75 ± 3 kDa was estimated for the native *Petunia* FHT from size exclusion chromatography, whereas M_r values of 37 000 and 35 000 were determined by two-dimensional electrophoresis under non-denaturing conditions, suggesting a composition of two subunits for the active enzyme [2,3]. Later, the FHT cDNA was cloned from a *Petunia* cDNA library, and the identity of the clone was verified by the expression of highly active recombinant FHT in *Escherichia coli* [12]. Denaturing gel electrophoresis and immunoblotting of the recombinant enzyme revealed a single band corresponding to a molecular weight of 41 000 to 42 000 [12,13], while the size exclusion chromatography of the native recombinant FHT on Superdex 75 again suggested a homodimer composition [13]. This discrepancy disclosed for the first time that the FHT polypeptide is capable of forming aggregates in solution and added support to the idea that functional native FHT might be a homodimeric enzyme. The dimeric enzyme composition has been reported also for several other 2-oxoglutarate-dependent dioxygenases, which include the γ-butyrobetaine hydroxylases from *Pseudomonas*, human kidney or calf liver [14], the 2,4-dichlorophenoxyacetate/α-ketoglutarate dioxygenase encoded by the tfdA gene of plasmid pJP4 [15] as well as the α-ketoglutarate-dependent taurine dioxygenase from *E. coli* [16]. It was thus conceivable that the structural organization of *Petunia* FHT might follow the same rules.

This report presents a thorough reexamination of the molecular composition of *Petunia* FHT by size exclusion chromatography, protein cross-linking experiments and finally equilibrium sedimentation analysis.

2. Materials and methods

2.1. Recombinant Petunia FHT

Petunia FHT was expressed in *E. coli* [17], and the enzyme was purified from the clear supernatants of crude bacterial extracts (30 000 × *g*, 5°C, 10 min) through four steps of chromatography as described earlier [17] or by successive chromatographies on a Fractogel EMD BioSEC (S) column (Merck, Darmstadt, Germany) and anionic exchange on a Fractogel EMD DEAE (S) column (Merck) (Lukačin, R., Gröning, I., Schiltz, E., Britsch, L. and Matern, U., submitted for publication).

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Abbreviations: ACC oxidase, 1-aminocyclopropane-1-carboxylic acid oxidase; FHT, flavanone 3β-hydroxylase; DAOCS, deacetoxycephalosporin C synthase; DTT, dithiothreitol; IPNS, isopenicillin N synthase; SDS–PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate

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2.2. Size exclusion chromatography

The molecular weight of native FHT was estimated by size exclusion chromatography on a Fractogel EMD BioSEC (S) column (600×16 mm; Merck), equilibrated with 0.05 M Tris–HCl buffer, pH 7.5, containing 0.1 M NaCl and 2 mM DTT. The column was calibrated with Blue Dextran 2000 (void volume) and thyreoglobulin (669 kDa), ferritin (440 kDa), catalase (240 kDa), bovine serum albumin (67 kDa), egg albumin (45 kDa), carbonic anhydrase (29 kDa) and equine myoglobin (17.8 kDa) as the reference proteins. The partially purified FHT (585 μg protein) or either one of the reference samples was applied in 200 μl buffer, and individual runs were recorded as described earlier [18].

2.3. Protein cross-linking

2.3.1. Chemical cross-linking. Cross-linking of homogeneous FHT (5.8 μg in 500 μl total of the incubation mixture) with bis(maleidohexane) was performed according to a published procedure [19,20]. The reaction was started by the addition of 25 μl of 10 mM bis(maleidohexane) dissolved in dimethyl sulfoxide (final concentration 0.5 mM). A blank, containing the same amount of FHT but lacking bis(maleidohexane), was incubated under the same conditions. Subsequently, the protein was precipitated by the addition of trichloroacetic acid (20% final concentration) and collected by centrifugation. The protein pellets were washed several times with cold acetone (−20°C), each pellet was dissolved in 30 μl of sample buffer and subjected to SDS–PAGE followed by immunoblotting.

2.3.2. Irradiation cross-linking. The solution of pure FHT (approximately 1.5 μg/60 μl for each experiment in 70 mM potassium phosphate buffer, pH 6.8) was placed in droplets on a glass plate, covered with a thin quartz platelet and exposed for 30 min at 4°C to ultraviolet B radiation. A Philips TL 40-W/12 fluorescent tube (λ_{max} = 310 nm, fluorescence rate = 7 W m^{-2}) served as the ultraviolet B source, and the spectrum of the tube was measured with a double monochromator spectroradiometer Optronic model 754 (Orlando, USA). Triton X-100 (final concentration 1%) was added to one of the samples in order to assay the detergent effect on the cross-linking process. The analysis of the irradiated FHT samples was carried out as described for chemical cross-linking.

2.4. Analytical ultracentrifugation

Ultracentrifugation was performed in a Beckman XL-A analytical ultracentrifuge equipped with absorption scanner optics using the An-60 4-place rotor with double sector charcoal filled epon centerpieces.

For equilibrium measurements, 150 μl of the respective sample was centrifuged at 18 000 rpm. Scans were taken in 60 min intervals for a total of 30 h. The measured concentration profile remained unchanged for the last 12 h indicating equilibrium to be attained. All scans from these 12 h were averaged to yield the final equilibrium concentration profile. A theoretical concentration distribution can be calculated according to

$$c(x) = c(x_m)\cdot e^{\frac{M(1-\bar{\nu}\rho)}{2RT}\omega^2(x^2-x_m^2)}$$

where $c(x)$ and $c(x_m)$ are concentrations at x or x_m (meniscus position) respectively, M is the molar mass ν and ρ are the partial specific volume of the solute and the density of the solution, respectively. ω is the angular velocity of the rotor, R is the gas constant and T the absolute temperature.

Sedimentation velocity was measured at 45 000 rpm using 400 μl of the respective sample. Scans were taken at approximately 9 min time intervals and the sedimentation constant was determined by simulating the position and shape of the moving boundary using numeric integration of Lamm's differential equation [21,22] which takes into account sedimentational movement and diffusional broadening of the sedimenting boundary simultaneously. The sedimentation coefficient of a sedimenting molecule is determined by mass and by the friction with the surrounding solvent:

$$s = \frac{m(1-\bar{\nu}\rho)}{f}$$

s, sedimentation coefficient; m, mass of the molecule; ν and ρ, the partial specific volume of the solute and the density of the solution, respectively; f, frictional coefficient.

2.5. General assays

The FHT activity was determined with (2*S*)-[4a,6,8-^{14}C]naringenin (2.18 GBq/mmol) as reported previously [2], and SDS–PAGE separation of proteins was carried out according to Laemmli [23]. The detection of FHT polypeptides was accomplished by immunoblotting [24]. The purified IgG fraction from the immune serum was used, which provided strong inhibition of FHT activity [3]. These antibodies were highly specific and did not cross-react with *E. coli* proteins [12]. Protein amounts were quantified after precipitation with trichloroacetic acid and employing the Lowry procedure [25,26].

3. Results

3.1. Size exclusion chromatography of recombinant FHT

Various 2-oxoglutarate-dependent dioxygenases are known to oligomerize easily, and monomers [27] as well as aggregates of dimers [27], trimers as in case of DAOCS [28,29] or tetramers [27] have been reported. It is difficult, under these circumstances, to distinguish the functional enzyme composition. The spatial structure of *Petunia* FHT appears to closely resemble those of IPNS and DAOCS [27] (Lukačin, R., Gröning, I., Pieper, U. and Matern, U., submitted for publication), and the data supposedly suggest that the FHT polypeptide of 41.5 kDa is also prone to aggregate in solution. Partially purified recombinant FHT was therefore subjected to size exclusion chromatography on a calibrated Fractogel EMD BioSEC (S) column. This material is suitable to fractionate polypeptides over the range of 5 to 1000 kDa, and the column performance was carefully monitored by plotting the log of molecular weight standards vs. the elution ratios $K_{av} = (V_e - V_o)/(V_t - V_o)$. The elution profile of FHT which was monitored by enzyme assays (Fig. 1, top) and Western blotting (Fig. 1, bottom) covered the range of 30 to 400 kDa, suggesting that a significant part of the enzyme had formed aggregates of higher order. Nevertheless, the maximal enzyme activity was always recovered in those fractions corresponding to an M_r of 44 000 ± 3000 (Fig. 1). The results remained virtually the same on variation of the DTT concentration, and the concentration was routinely kept high enough (2 mM) to suppress the oxidation of protein sulfhydryl and covalent ligation of polypeptides.

3.2. Protein cross-linking

Chemical and irradiation cross-linking experiments were conducted in order to visualize a reversible interaction of FHT polypeptides. The incubation of pure FHT in the presence of bis(maleidohexane), a homobifunctional sulfhydryl group cross-linking reagent, generated several new protein bands in the range of 120 kDa and 180 kDa as observed on SDS–PAGE separation and Western blotting (Fig. 2A). The results supported the conclusions drawn from size exclusion chromatographies and indicated that a significant proportion of the FHT polypeptides had associated close enough to become cross-linked via their surface-exposed cysteine residues. The apparent sizes of the cross-linked aggregates were much larger than expected for dimers or tetramers of the 41.5 kDa polypeptide, possibly due to anomalous migration of the cross-linked proteins in denaturing gels, which has often been described in the literature [20,30,31]. The band hybridizing in Western blots at about 120 kDa (Fig. 2A), however, might be composed of an FHT trimer, which would be compatible with the reported trimeric composition of crystallized 2-oxoglutarate-dependent DAOCS [28]. The FHT polypeptide

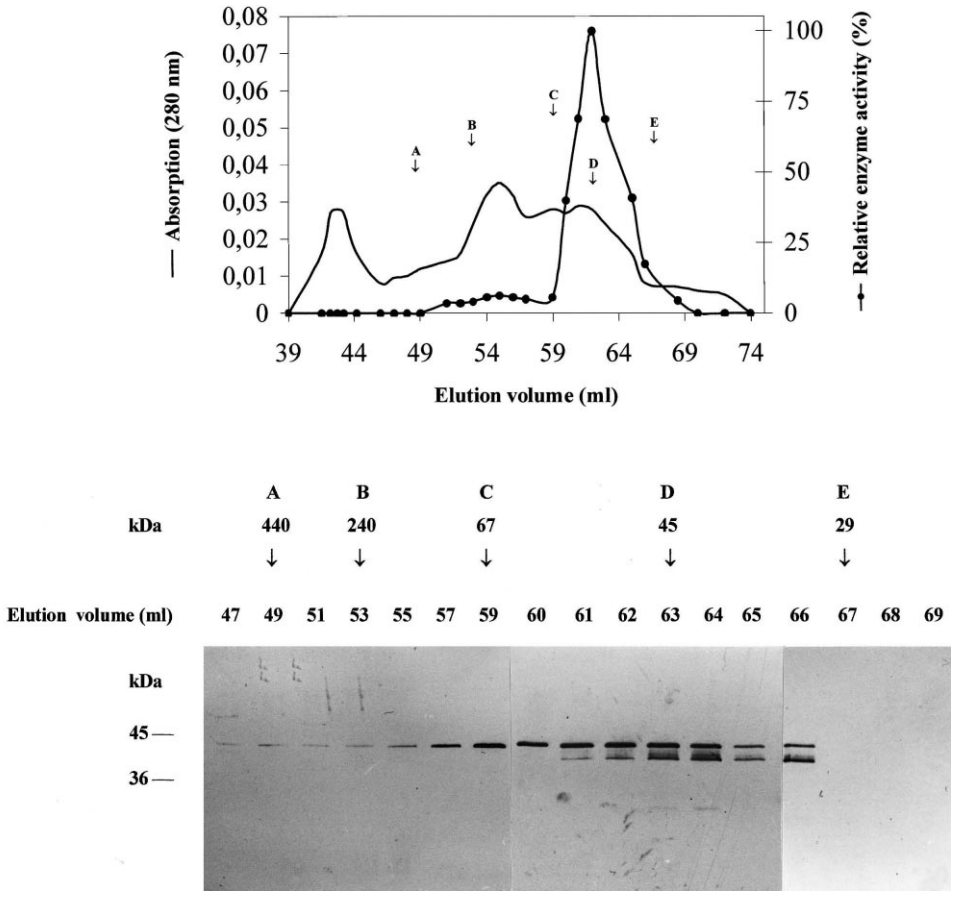

Fig. 1. Size exclusion chromatography of recombinant *Petunia* FHT. The enzyme expressed in *E. coli* had been partially purified through hydrophobic interaction chromatography on Fractogel EMD Butyl (S) (Lukačin, R., Gröning, I., Schiltz, E., Britsch, L. and Matern, U., submitted for publication), and size exclusion chromatography was performed on a Fractogel EMD BioSEC (S) column (119 ml). The enzyme was applied to the column (585 μg protein/200 μl buffer) and fractionated at a flow rate of 1 ml/min with 50 mM Tris–HCl buffer, pH 7.5, containing 0.1 M NaCl and 2 mM DTT. The absorbance was recorded at 280 nm, and the elution of FHT was monitored by enzyme assays (top) as well as by immunoblotting (bottom). Western blots (4 μg protein/lane) of the eluate fractions (bottom) revealed the elution of FHT (about 44 kDa) over a relatively broad size range, and the elution volumes of reference proteins (A to D) used for calibration of the column are marked by arrows for comparison (top and bottom). A, ferritin; B, catalase; C, bovine serum albumin; D, egg albumin; E, carbonic anhydrase. The FHT antibodies [3,12] cross-react also with the catalytically inactive, primary proteolytic FHT fragment of 38 kDa, which is generated rapidly in crude extracts and lacks a short C-terminal portion.

contains five cysteine residues all of which could account for the observed cross-linking. These residues are clustered with Cys-60 and Cys-67 in one region and Cys-172, Cys-193 and Cys-208 as part of a conserved amphipathic α-helix spanning the amino acids 165 to 210 [32].

The association of enzyme polypeptides might also be favored by other forces, and hydrophobic interactions had been assumed for the *Petunia* FHT [32] as well as for ACC oxidase [34]. In a number of 2-oxoglutarate-dependent dioxygenases, a putative leucine zipper forms part of the conserved amphipathic α-helix (α_6 in IPNS) that was shown to be exposed to the surface in case of the DAOCS and also IPNS polypeptides [28,33]. However, these leucine residues face the core of the enzymes and are not available for cross-linking [33]. As an alternative, it appeared possible that Trp residues in the neighborhood of Cys-60 and Cys-67 and/or of the α-helix participate in the interaction, and Trp-70 as well as Trp-156 out of five tryptophan residues total in FHT seemed to be a promising choice. Accordingly, the cross-linking of FHT polypeptides was examined by activation of tryptophan residues under ultraviolet B irradiation. The irradiation of pure FHT for 30 min caused the formation of two larger protein bands which were detected by immunoblotting (Fig. 2B). The addition of Triton X-100 (final concentration 1%) to the pure FHT prior to the irradiation did not affect the result, indicating that this mild detergent does not interfere with the interaction of FHT polypeptides (Fig. 2B). Tryptophan residues of the individual FHT polypeptides in solution are thus likely to associate in close proximity and may function as the primary sites of interaction under favorable conditions. This again raises the question on the true state of aggregation of functional FHT.

3.3. Sedimentation analysis

The equilibrium sedimentation of highly active, pure FHT (Fig. 3) revealed a molar mass of 39 ± 4 kg/mole showing the protein to be a monomer. Nevertheless, the non-random distribution of the residuals near the bottom of the cell indicated the presence of some material with a larger molar mass.

FHT protein sedimented with a rate of $S_{20,W} = 3.2 \pm 0.1$ S which corresponds to a frictional ratio of 1.3 assuming the protein to be a monomer. If the protein were a dimer, a much

larger frictional ratio (2.0) improbable for a folded protein would result. Addition of 4 mM Fe^{II} and 4 mM 2-oxoglutarate, respectively, did not change the sedimentation rate. For a single sedimenting species the shape of the boundary is determined by the mass of the sedimenting species with a lower mass leading to a broadening of the boundary. If additional species with different sedimentation coefficients are present, the sedimentation boundary will be broadened too, regardless whether the additional species have a larger or smaller sedimentation coefficient than the main species. Analysis of the shape of the sedimenting boundary resulted in an apparent molar mass much lower than the mass of the monomeric unit (Fig. 4). Since low molar mass impurities can be excluded (Fig. 5), this indicates the presence of some aggregated protein as observed in the equilibrium measurements.

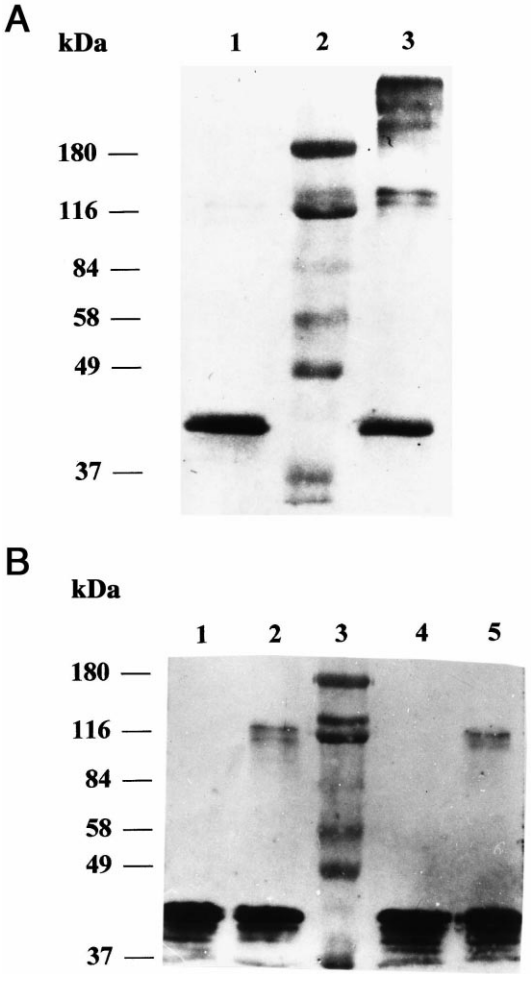

Fig. 2. Western blots of pure *Petunia* FHT after chemical or irradiation cross-linking. Homogeneous recombinant FHT was cross-linked by chemical (A) or irradiation treatment (B), and the proteins were separated by SDS–PAGE on 8.5% separation gels and blotted on nitrocellulose filters for the cross-reaction with *anti*-FHT antiserum [3]. The relative mobilities of protein bands were compared to those of reference proteins (lane 2 in A, lane 3 in B) ranging from 180 to 37 kDa (SDS-7B, Sigma, Deisenhofen). For chemical cross-linking (A), the enzyme (5.8 μg protein in 0.5 ml total volume) was incubated for 1 h at 20°C with DMSO (lane 1, control) or in the additional presence of 0.5 mM bis(maleidohexane) (lane 3). Irradiation cross-linking (B) was accomplished by exposing the enzyme (1.5 μg protein/60 μl in 70 mM potassium phosphate buffer, pH 6.8) for 30 min at 4°C to ultraviolet B radiation (lane 2); the control incubation (lane 1) was carried out in the dark. The same incubations were conducted in parallel in the presence of 1% Triton X-100 (lanes 5 and 4).

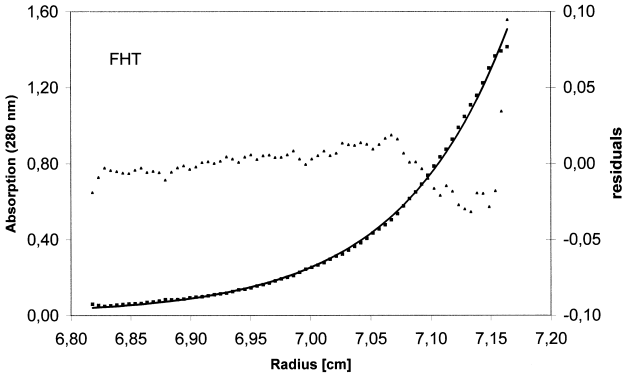

Fig. 3. Analytical equilibrium centrifugation of FHT. The ultracentrifugation of FHT (50 μg/ml) was carried out in 20 mM Tris–HCl buffer, pH 7.0, at 18 000 rpm. The solid line represents a concentration profile calculated for a molar mass of 39.2 ± 4 kg and a partial specific volume of $7.401 \cdot 10^{-4}$ m^3 kg^{-1} (calculated from the amino acid composition of FHT). Deviations of the calculated from the measured curve are given as residuals (note the difference in scale).

4. Discussion

The data collected from size exclusion chromatographies as well as from sedimentation analysis of highly active, recombinant *Petunia* FHT unequivocally demonstrated that the monomeric FHT polypeptide comprises the functional enzyme. Nevertheless, the pronounced tendency of the enzyme polypeptide to oligomerize has become obvious. This tendency had already been observed during purification of the FHT from *Petunia* tissues [2,3] as well as on purification of the recombinant enzyme; it is noteworthy that a high proportion of the recombinant enzyme associated also with the particulate fraction of the crude bacterial extracts [12]. Taken together, these observations appear to support a model, which predicts the association in planta of FHT in either soluble protein complexes of higher order or in context with other peripheral membrane-associated proteins as proposed, for ex-

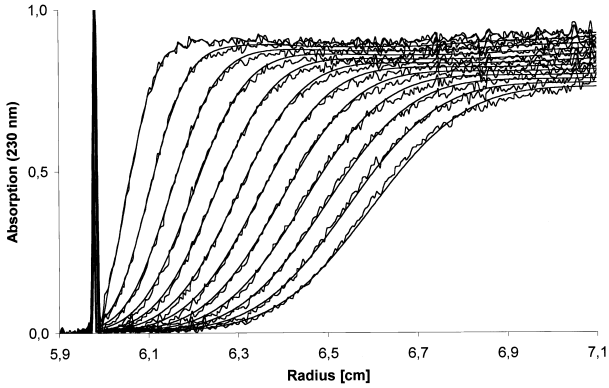

Fig. 4. Analysis of sedimentation boundaries of FHT. The sedimentation of 100 μg/ml FHT in 20 mM Tris–HCl, pH 7.0, was monitored in the analytical ultracentrifuge at 45 000 rpm. Scans were taken at 550 s intervals. The smooth line represents the theoretical boundaries for a single sedimenting species of a molar mass of 39 kg/mole and a sedimentation coefficient of 3.2 S.

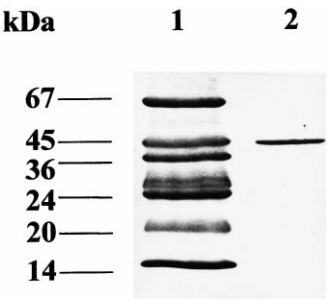

Fig. 5. Purity of *Petunia* FHT. The recombinant enzyme had been purified in a highly active state (32 mkat/kg) (Lukačin, R., Gröning, I., Schiltz, E., Britsch, L. and Matern, U., submitted for publication), and the homogeneity was examined by SDS–PAGE analysis. Coomassie staining of the protein (2 μg) ruled out any contaminating protein that might have interfered with the analytical ultracentrifugation analysis.

ample, for the efficient organization of phenylpropanoid biosynthetic pathways [35]. The assembly of several flavonoid enzymes in a macromolecular complex has recently been verified by two-hybrid assays for *Arabidopsis* [36]. The self-aggregation of FHT polypeptides observed in vitro is likely a consequence of the isolation which disrupts any kind of weak interactions that may exist in vivo with other proteins or membrane components. The significance of such interactions for the localization of the enzyme in situ remains to be established. The concept is in accordance with the proposed membrane association of ACC oxidase, which had been characterized as another closely related, soluble non-heme iron(II) enzyme from plants [34].

The oligomerization in vitro was also reported recently for microbial DAOCS, a 2-oxoglutarate-dependent dioxygenase which converts the penicillin N thiazolidine ring to the six-membered cephem ring of deacetoxycepalosporin C [28,29]. The X-ray diffraction analysis of this enzyme revealed the trimeric structure of crystals, in which the C-terminal residues of one DAOCS polypeptide protrude towards the active site of the adjacent polypeptide forming a cyclic triple. Size exclusion experiments had indicated further that DAOCS forms an equilibrium of monomeric and trimeric forms in solution, which can be shifted in the presence of cofactors. While the addition of 2-oxoglutarate to DAOCS solutions had no effect, ferrous iron shifted the equilibrium in favor of the monomeric DAOCS, and the combination of 2-oxoglutarate with ferrous iron caused nearly the complete dissociation of the complex to the monomeric DAOCS [29]. The addition of ferrous iron or 2-oxoglutarate (up to 4 mM) to FHT solutions, in comparison, did not affect the ratio of monomeric vs. oligomeric polypeptides as revealed by sedimentation analysis (Fig. 4). Considering the close structural similarity of *Petunia* FHT with DAOCS (Lukačin, R., Gröning, I., Pieper, U. and Matern, U., submitted for publication), these findings are fully complementary. Both enzymes are functional as monomers but are capable of associating to higher aggregates depending on the microenvironment. Both enzymes catalyze a single pivotal step in chains of consecutive reaction leading to either flavonols and related compounds or cephalosporins, and their aggregation in situ with enzymes providing the substrates or converting the products might be required for high efficiency.

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