



INTERACTION OF 5-HYDROXYMETHYL-FURFURAL WITH HYDROXYMETHYLBILANE SYNTHASE

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Key Word Index—*Lepidium sativum*; Brassicaceae; cress; chlorophyll biosynthesis; hydroxymethylbilane synthase; 5-hydroxymethylfurfural; porphobilinogen.

Abstract—The inhibition of chlorophyll biosynthesis in greening cress seedlings (*Lepidium sativum* L.) by 5-hydroxymethylfurfural (5-HMF), a natural compound isolated from the bulbs of *Gladiolus* spp. was investigated *in vitro* and *in vivo*. A direct reaction between 5-HMF and the chlorophyll precursor porphobilinogen was observed at pH 1.0 but not at pH 8.0. The enzymatic conversion of porphobilinogen to hydroxymethylbilane was inhibited by 5-HMF with a K_i of about 30 mM. Enzyme kinetics showed that the inhibition was non-competitive. The activity of hydroxymethylbilane synthase increased during 72 hr development of cress seedlings in darkness. In the light, there was a further increase of enzyme activity. The light dependent increase in activity was completely abolished when the seedlings were incubated with 5-HMF. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Tetrapyrroles, the so-called pigments of life [1–3], are nearly ubiquitous in all organisms. The most abundant tetrapyrroles are heme, the prosthetic group of hemoglobin and several cytochromes, and chlorophyll, the prosthetic group of chlorophyll-binding proteins. Their biosynthesis can differ at the beginning, namely formation of 5-aminolevulinate, and at the end, namely insertion of the central metal into protoporphyrin and subsequent steps. However, most of the intermediate steps from 5-aminolevulinate to protoporphyrin are identical for heme and chlorophyll biosynthesis. The corresponding enzymes have been found in animals, plants and bacteria [3].

Inhibitors of chlorophyll biosynthesis are of interest as potential herbicides. Preference is given as a rule to those compounds that inhibit either one of the first or one of the last steps of chlorophyll biosynthesis that is not part of mammalian heme biosynthesis. The expected lack of interference with mammalian heme biosynthesis should result in low toxicity to animals and man. However, interesting inhibitors of intermediate steps of chlorophyll biosynthesis have also been described, e.g. diphenylether herbicides that inhibit protoporphyrinogen oxidase, an enzyme common to heme and chlorophyll biosynthesis [4, 5].

We isolated and identified recently two natural inhibitors of chlorophyll biosynthesis from bulbs of *Gladiolus* spp. [6]. The first compound, 3-hydroxybenzoic acid methylester, turned out to inhibit

magnesium chelatase activity [7]. The second compound, 5-hydroxymethylfurfural (5-HMF), did not interfere with the step of magnesium insertion [8]. The chemical structure of the compound prompted us to investigate two possible points of interference. (1) 5-HMF is discussed as reactive intermediate in the reaction of sugars with amino-compounds ('Maillard reaction') that can take place not only *in vitro* but also *in vivo* [9]. The process may be significant in the context of increased cross-linking of proteins with increased age [10]. Since the chlorophyll precursors 5-aminolevulinate and porphobilinogen carry amino groups, we wanted to know whether the reactive aldehyde group of 5-HMF could react with these intermediates and remove them from the biosynthetic chain *in vivo*. The preferred compound for this investigation is porphobilinogen, because it does not contain an oxo group and cannot undergo self-condensation, unlike 5-aminolevulinate. (2) 5-HMF and porphobilinogen have a substituted five-membered heterocyclic ring in common. We wanted to know whether the enzyme hydroxymethylbilane synthase (synonym: porphobilinogen deaminase, EC 4.3.1.8) could bind 5-HMF instead of porphobilinogen. Competitive inhibition was expected in this case. The enzyme catalyses the condensation of four molecules of porphobilinogen to the tetrapyrrole hydroxymethylbilane. Hydroxymethylbilane synthase is the first enzyme of chlorophyll biosynthesis with an elucidated crystal structure [11]. The enzyme produces its own dipyrromethane cofactor [12, 13], to which the

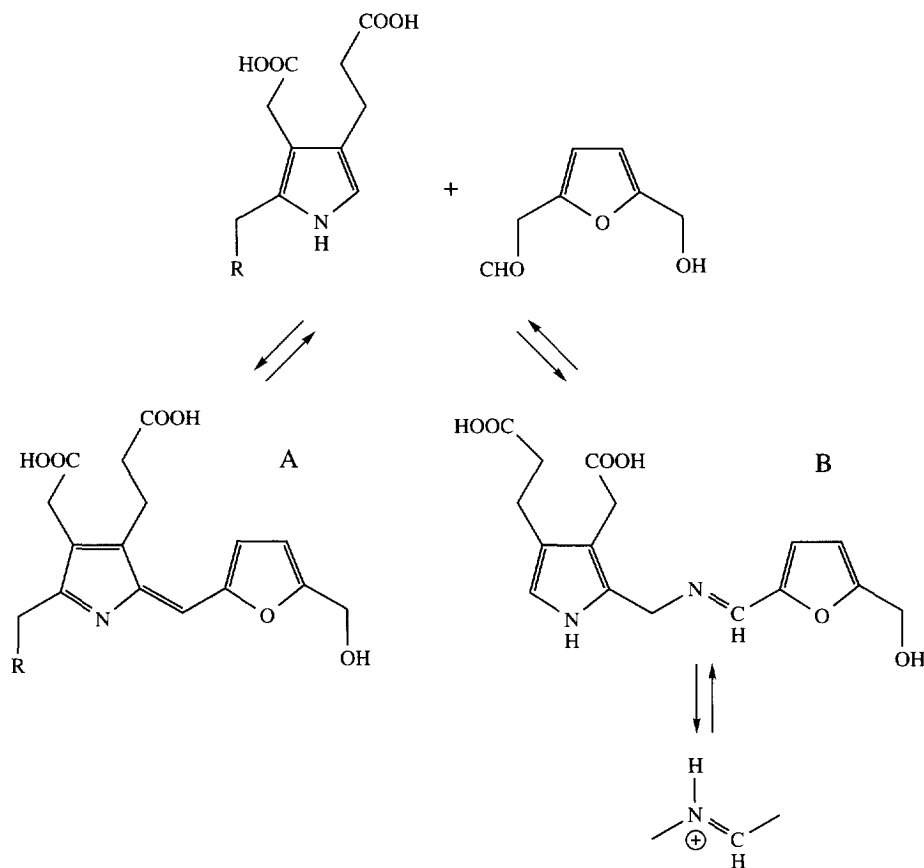


Fig. 1. Possible reactions of porphobilinogen and 5-hydroxymethylfurfural (5-HMF). (A) Addition of 5-HMF at the free α -position of porphobilinogen. (B) Formation of the Schiff base and its reversible protonation. R, $-\text{NH}_2$ for free porphobilinogen or holoenzyme-oligopyrrole for enzyme-bound porphobilinogen.

additional porphobilinogen molecules are condensed. Some properties of the enzyme have recently been reviewed [3, 14, 15]. The investigation described here gave two unexpected effects of 5-HMF: non-competitive inhibition of hydroxymethylbilane synthase activity and inhibition of light-dependent accumulation of hydroxymethylbilane synthase protein.

RESULTS AND DISCUSSION

Chemical reaction between 5-hydroxymethylfurfural and porphobilinogen

Precondition for investigation of the inhibitory action of 5-HMF upon the activity of hydroxymethylbilane synthase was the investigation of possible non-enzymatic interactions with the substrate, porphobilinogen. Two possible reactions can be envisaged (Fig. 1), the addition of the aldehyde group of 5-HMF to the α -pyrrolic position of porphobilinogen (reaction A, Fig. 1) and the formation of a Schiff base (reaction B, Fig. 1).

Incubation of porphobilinogen with 5-HMF was performed at pH 1 and 8. The absorption spectra recorded during the incubation (data not shown) indi-

cate an immediate reaction at pH 1. The new absorption maximum at 472 nm starts to appear within 30 sec and stays nearly constant between 5 min and 4 hr periods of incubation. The absorption maximum points to the product of reaction A (Fig. 1) which is similar to the reaction product of pyrrolic compounds with *N,N*-dimethylaminobenzaldehyde ('Ehrlich reaction') absorbing at around 500 nm. In contrast, no absorption peak appears between 350 and 700 nm within 4 hr at pH 8. Subsequent acidification yields the compound absorbing at 472 nm with the same kinetics as direct incubation at pH 1 but no absorption peak at 380 nm, the expected maximum of the protonated Schiff base (reaction B, Fig. 1). This means that no direct interaction of 5-HMF with the substrate occurs during the enzyme reaction at pH 8. Porphobilinogen and 5-HMF are still present unchanged after a 4 hr-period of incubation at pH 8 and react then immediately after acidification.

Interference of 5-hydroxymethylfurfural with hydroxymethylbilane synthase in vitro

Before the enzyme was incubated with the inhibitor, the pH optimum of the enzyme reaction was deter-

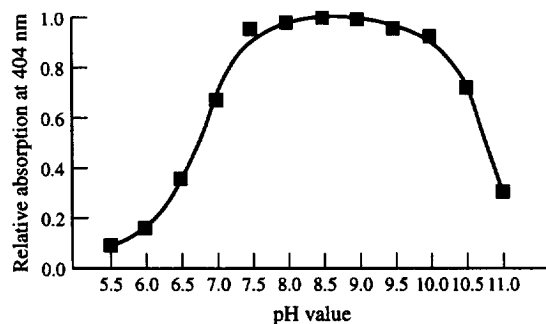


Fig. 2. Activity of hydroxymethylbilane synthase from cress seedlings at various pH values. The activity is expressed as the amount of uroporphyrin I produced under standard conditions, determined at the absorption maximum (404 nm).

mined (Fig. 2). We obtained a nearly symmetrical curve with a broad maximum of activity between pH 7.5 and 10.0. The activity dropped sharply at both the acid and alkaline side of these limits. The optimum is broader than that described for the enzyme from *Arabidopsis thaliana* [16]. The subsequent investigations were performed at pH 8.0. This value is within the optimum of activity and not too far away from the physiological pH.

In a series of experiments, the reaction was carried with constant enzyme concentration, varying substrate concentration, and with various (constant) concentrations of 5-HMF. The results were plotted in the form of a Lineweaver–Burk diagram (Fig. 3).

Three aspects of the results are worth mentioning:

(1) Since the enzymic reaction requires 4 identical substrate molecules for formation of one product molecule, a linear dependency of $1/v$ on the reciprocal of the fourth power ($1/c^4$) could have been expected. This was not the case. A linear relationship in this diagram was only obtained when the substrate concentration

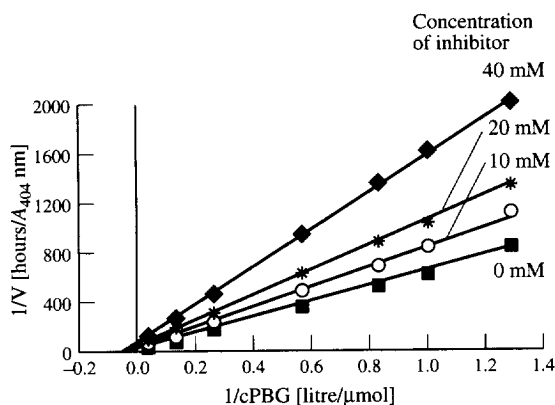


Fig. 3. Lineweaver–Burk diagram of the conversion of porphobilinogen to hydroxymethylbilane, catalysed by hydroxymethylbilane synthase, in the presence of various concentrations of the inhibitor 5-HMF.

was plotted as the reciprocal of the first power ($1/c$). A similar finding was reported by Juknat *et al.* [17] for hydroxymethylbilane synthase from *Scenedesmus obliquus*. The authors explained this finding with the assumption of a sequential displacement mechanism, the release of ammonia being the kinetically irreversible step.

(2) The Lineweaver–Burk plot shows clearly that the inhibition of the hydroxymethylbilane synthase reaction by 5-HMF is non-competitive. The presence of 5-HMF leads to a decrease of the reaction velocity but does not lead to any change in the K_M value. One could have speculated that the structures of porphobilinogen and 5-HMF (see Fig. 1) are similar enough to compete for the same binding site. However, the experimental results lead to the conclusion that the enzyme must contain a binding site for the inhibitor that is different from the active site of the enzyme. The kinetics exclude also the possibility of a direct reaction between 5-HMF and enzyme-bound porphobilinogen or any enzyme-bound intermediate. Thus, the kinetic measurements confirm the previous conclusion from spectroscopic measurements on lack of reaction between porphobilinogen and 5-HMF.

The K_M value of hydroxymethylbilane synthase from cress seedlings was determined from the plot of Fig. 3 to be $15 \mu\text{M}$. This value is similar to that of the enzyme from *Arabidopsis thaliana* ($17 \pm 4 \mu\text{M}$, [16]) or *Escherichia coli* [18] but smaller than that of algae (e.g. *Scenedesmus obliquus*, $89 \mu\text{M}$, [17] or *Chlorella vulgaris*, $85\text{--}90 \mu\text{M}$, [19]).

(3) The K_i value for 50% inhibition of the enzyme reaction can be evaluated from Fig. 3 to be about 30 mM 5-HMF. This is at variance with the K_i value of 6.7 mM 5-HMF determined previously for 50% inhibition of chlorophyll accumulation in greening cress seedlings [6]. This discrepancy prompted us to investigate the enzyme activity after treatment of young cress seedlings with 5-HMF. We used the same conditions that were used for chlorophyll accumulation and its inhibition by 5-HMF.

Interference of 5-hydroxymethylfurfural with hydroxymethylbilane synthase in vivo

The investigation included determination of hydroxymethylbilane synthase activity during the early development of cress seedlings. At first, a micro-method for measuring the enzyme activity had to be developed (see Experimental). The enzyme activity was then determined in samples of 10 seedlings that were 8–96 hr old (Fig. 4). In darkness (closed squares in Fig. 4), the enzyme activity was very low at the beginning, then increased until the age of 72 hr and declined thereafter. The reserve compounds of the seeds were probably exhausted after growth for 72 hr. The seedlings that were irradiated for the last 8 hr before harvest (open squares in Fig. 4), contained more enzyme activity than the dark controls. This

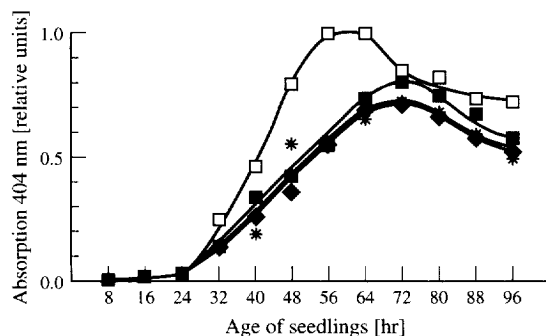


Fig. 4. Enzyme activity of hydroxymethylbilane synthase depending on the age of cress seedlings. Closed squares, H₂O control dark-grown; open squares, H₂O control after irradiation for 8 hr; closed rhombi, incubated with 5-HMF in darkness; stars, incubated with 5-HMF after irradiation for 8 hr. The values are total enzyme activity per 10 cress seedlings after the respective treatment.

increase became significant when the cotyledons had left the seed coat (40 hr after imbibition and later). The maximum of enzyme activity was reached at 56 hr after imbibition under these conditions. The activity then declined so that the same value was obtained for irradiated plants and dark controls at 72 hr.

When the seedlings were incubated with 10 mM 5-HMF instead of H₂O, no light-dependent increase in enzyme activity was obtained over the values in darkness (stars and rhombi in Fig. 4). The maximum of enzyme activity was reached 72 hr after imbibition like in the H₂O dark-control. The enzyme activity of the plants incubated with 5-HMF was only slightly below the values for the dark control. This agrees with the *in vitro* inhibition (see Fig. 3) if one considers that some 5-HMF could have been metabolized during incubation of the plants and a certain dilution occurred by the enzyme preparation.

The most remarkable effect was the lack of increase of enzyme activity after irradiation when the plants were incubated with 5-HMF. The increase in activity after irradiation could mean either light-activation of pre-existing enzyme or light-dependent accumulation of enzyme protein. Both processes are well-known for various plant enzymes. In the first case, the amount of enzyme protein does not change. In the second case, there is an increase in enzyme protein. To distinguish between these possibilities, we investigated the proteins of non-irradiated and irradiated plants of various age by Western blotting (Fig. 5). In the dark controls [Fig. 5(A)] the signal for the hydroxymethylbilane synthase protein can be detected in 56–96 hr-old seedlings. The signal of irradiated plants [Fig. 5(B)] can be detected in 40 hr-old seedlings. It is stronger in the extract from irradiated plants than in the dark controls at every age. This means that the light-dependent increase in enzyme activity is based on increased enzyme protein accumulation. It is most probably

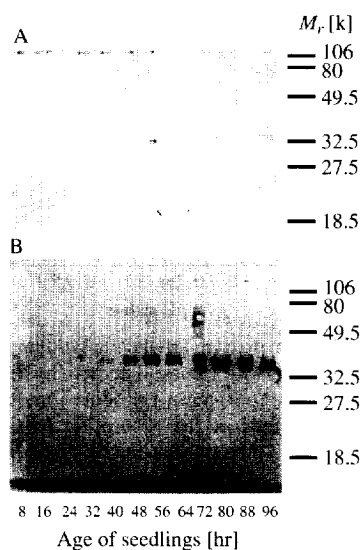


Fig. 5. Western blot for detection of hydroxymethylbilane synthase protein in dark controls (A) and irradiated plants (B). Dark-grown plants of the indicated age were either directly extracted (A) or irradiated for 8 hr and then extracted (B). The separation of soluble proteins by SDS-PAGE was followed by transfer to nitrocellulose and staining with antibodies raised against the enzyme from *Arabidopsis thaliana*. The amount of protein in each lane corresponds to that from 0.25 seedling.

caused by enzyme synthesis. Hence the effect of 5-HMF was apparently inhibition of light-dependent synthesis of this enzyme. The effect was significant at a concentration of 5-HMF (< 10 mM) at which only minor inhibition of enzyme activity *in vitro* was observed.

The previously observed inhibition of chlorophyll accumulation after incubation with 5-HMF [6] can be caused by interaction with hydroxymethylbilane synthase in two ways, inhibition of enzyme activity and inhibition of enzyme formation. The latter process dominates at low concentrations of 5-HMF. The significance of inhibition of enzyme formation rather than inhibition of activity is corroborated by the finding that the effect of 5-HMF strongly depends on the time point of incubation of cress seedlings with the inhibitor. The largest inhibition of chlorophyll accumulation was observed when incubation with 5-HMF was started 8 hr after imbibition of the seeds [6] but much less inhibition was found when the incubation was started later (unpublished results).

In higher plants, there is only one plastid-located hydroxymethylbilane synthase [20] that produces the precursor for tetrapyrroles of plastids, mitochondria and cytosol. Inhibition of light-dependent formation of this enzyme or inhibition of its activity must retard development of plants beyond inhibition of chlorophyll synthesis. However, we cannot exclude the possibility that synthesis of other enzymes was affected by the incubation with 5-HMF, as well. This would open

up the interesting possibility that 5-HMF acts as an inhibitor of light-dependent development and that chlorophyll accumulation is merely a measure for such developmental processes. The occurrence of 5-HMF in non-green tissue of *Gladiolus* bulbs is in agreement with such a view.

EXPERIMENTAL

Plant material. Seeds (7.5 g dry wt, about 4000 seeds) of garden cress (*Lepidium sativum*) L. cv. Armada, J. Wagner, Heidelberg/Germany were imbibed with deionized H₂O for 10 min and then evenly distributed over a filter paper (35 × 20 cm) moistened with 40 ml H₂O in a plastic container (35 × 20 × 5 cm) with cover. Germination occurred in darkness at 25 ± 1° and 80% relative humidity. After 48 hr, the plastic containers with the seedlings were transferred from darkness to a field of white light (5 W m⁻², produced by four fluorescent lamps L18W/25 Osram) and irradiated for 8 hr. The seedlings (fr. wt including seed coats 70 g) were then stored at -20°.

Enzyme preparation. The seedlings from eight containers (560 g fr. wt) were thawed and extracted with 400 ml 50 mM Na-Pi buffer, pH 8, in a Waring blender. Cell debris, seed coats etc. were removed by filtration through a sieve and by subsequent centrifugation at 4400 g for 10 min. The crude extract (400 ml) was diluted with the same vol. of Me₂CO. The resulting ppt (mainly nucleic acids) was removed by filtration. The clear filtrate was diluted with 1200 ml Me₂CO (final Me₂CO concn 80%) and kept overnight at -20°. The protein ppt was collected by centrifugation (3500 g for 10 min) and dissolved in 40 ml H₂O. The soln was then quickly (within 5 min) heated to 55° with stirring, kept at this temp. for 1 min and then quickly cooled to 4°. The ppt was removed by centrifugation (4400 g for 10 min). The clear supernatant was used as enzyme soln.

Preparation on the micro scale. Batches of 100 seeds were germinated in the dark. After 8 hr imbibition, H₂O was exchanged by 10 mM 5-HMF except for the controls. The seedlings were either kept in darkness for the indicated time (see Fig. 5) or transferred to the light field for the last 8 hr, harvested and kept frozen at -20° until workup. Extraction with Na-Pi buffer (0.5 ml per batch), stepwise precipitation with Me₂CO and heat denaturation of contaminants was performed as for the large scale preparation.

Enzyme test. A stock soln of 0.7 mg/ml porphobilinogen in 10 mM Na-Pi buffer, pH 8, was prepd. The concn was controlled with Ehrlich's reagent, 2% (w/v) *N,N*-dimethyl-*p*-aminobenzaldehyde in 98% HCOOH-2 M HCl (7:3, v/v). For the calculation of the porphobilinogen content, the molar extinction at 555 nm ($\epsilon = 57\,700\text{ M}^{-1}\text{ cm}^{-1}$) of the reaction product was used. The stock soln was sorted in small portions at -20°. Immediately before use, it was diluted with H₂O to a concn of 70 mg porphobilinogen/ml.

One hundred microlitres of the enzyme soln (see above) were mixed with 50 µl porphobilinogen soln (70 µg/ml), inhibitor as indicated, and 50 µl 50 mM Na-Pi buffer, pH 8, and incubated in darkness at 25° for 4 hr. The reaction was then stopped with 500 µl 0.5 M HCl. The mixt. was then irradiated (daylight) for 30 min and cleared by centrifugation. The absorption of the clear supernatant at $\lambda_{\text{max}} = 404\text{ nm}$ gave the content of uroporphyrin. A straight line connecting the absorption values at 388 and 420 nm was used as background line.

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