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Two-step epoxidation of hyoscyamine to scopolamine is catalyzed by bifunctional hyoscyamine 6β-hydroxylase

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In several solanaceous plants, hyoscyamine is first hydroxylated at the 6β-position, and then epoxidized to scopolamine. We expressed hyoscyamine 6β-hydroxylase (H6H) in *Escherichia coli* as a fusion protein with maltose-binding protein. The crude cell extract from the bacterium that expressed the soluble fusion protein showed a strong hydroxylase activity and a weak epoxidase activity. When 100 μM of hyoscyamine was fed to the recombinant bacterium, the alkaloid was first converted to 6β-hydroxyhyoscyamine, and then to scopolamine, which was almost the only alkaloid found in the culture after one week. Therefore, H6H catalyzes two consecutive reactions that oxidize hyoscyamine to scopolamine.

Hyoscyamine 6β-hydroxylase; Bacterial expression; Epoxidation; Scopolamine

1. INTRODUCTION

Hyoscyamine and its epoxide scopolamine are typical tropane alkaloids found in several solanaceous plants. Previous feeding experiments with alkaloid precursors [1,2] have suggested that the 6,7-epoxide bridge of scopolamine is formed by hyoscyamine by way of 6β-hydroxyhyoscyamine (Fig. 1). We discovered a 2-oxoglutarate-dependent dioxygenase that hydroxylates hyoscyamine at the 6β-position in alkaloid-producing root cultures, and named it hyoscyamine 6β-hydroxylase (H6H; EC 1.14.11.11)[3,4]. Later, we also found that a similar 2-oxoglutarate-dependent dioxygenase converts 6β-hydroxyhyoscyamine to scopolamine by dehydrogenation of the 7β-hydrogen [5]. This epoxidase activity was relatively weak and represented only 1–10% of the hydroxylase activity in partially purified enzyme preparations. Our subsequent observations indicated that H6H may be a bifunctional dioxygenase endowed with strong hydroxylase activity and comparatively weak epoxidase activity; these two activities were observed in the same fractions during partial purification [5], and transgenic tobacco and *Atropa belladonna* that expressed H6H under the cauliflower mosaic virus promoter efficiently converted hyoscyamine that was supplied either exogenously [6] or endogenously [7] to scopolamine.

Although these observations are very suggestive, we need direct evidence that H6H has both hydroxylase and epoxidase activities, and that the relatively weak epoxidase activity, together with the strong hydroxylase activity, is sufficient to oxidize hyoscyamine completely to scopolamine, without having the conversion blocked at 6β-hydroxyhyoscyamine. In this study, we first found that the monoclonal antibody mAb5 that was raised against homogeneous hydroxylase [8] inhibited both enzyme activities to the same extent. Next, we constructed a prokaryotic expression vector in which the H6H cDNA from *Hyoscyamus niger* [9] was fused to the maltose-binding protein (MBP) gene, and expressed the fusion protein in an enzymatically active form in *Escherichia coli*. Measurement of enzyme activities in the bacterial extracts and feeding of hyoscyamine to the bacterial culture demonstrated that our previous assertion was correct.

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2. MATERIALS AND METHODS

2.1. *Neutralization of enzyme activities by antibodies*

The hydroxylase was partially purified from cultured roots of *H. niger* by ammonium sulfate precipitation and butyl-column chromatography [4], and aliquots (27 pKat) were incubated with 150 μg of monoclonal antibody mAb5 [8] or preimmune IgG (Sigma) in 350 μl of 50 mM Tris-HCl (pH 7.8), 100 mM NaCl at 25°C for 1 h. Reaction mixtures were then added to the enzyme solutions to start the reactions.

2.2. *Expression vectors*

The strategy for constructing pMH1 is illustrated in Fig. 2. *H. niger* H6H cDNA, into which a *Nco*I site had been introduced at the translation initiation ATG codon, was excised from pNHH3 [9] by *Nco*I and *Nsp*I, and ligated with pTV199N (Takara Shuzo, Kyoto) that had been digested by *Nco*I and *Sph*I. H6H cDNA without the poly (A) tail was excised from the resultant plasmid pNHH312 by *Nco*I and *Sal* I, and cloned into pTV118N (Takara Shuzo) to produce pN8H. pN8H was digested with *Nco*I, filled in with dNTP/Klenow, and ligated with

an *Eco*RI linker (pCCGAATTCGG; Takara Shuzo). The *Pst*I/*Eco*RI H6H fragment from pN8HE was then cloned into pMAL-c (New England Biolabs) to give pMH1. In the expression vector, the C-terminus of MBP is fused in frame to the N-terminus of H6H via a short peptide containing a recognition site of the protease factor Xa. pMH1 was transformed into *E. coli* JM109.

2.3. *Isolation of recombinant H6H protein*

An overnight culture was diluted 100-fold with LB broth containing 60 μg/ml ampicillin, grown at 37°C until OD_{660} became 0.4, and induced with 0.4 mM isopropylthio-β-D-galactoside (IPTG) for 2 days at 10°C. Bacteria were spun down at 3,000 × *g* for 10 min, suspended in TND buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM dithiothreitol), and, after one freeze–thaw cycle, lysed by sonication. The lysed cells were centrifuged at 10,000 × *g* for 20 min, and the fusion protein in the supernatant precipitated at ammonium sulfate concentrations of between 40% and 60% saturated. The precipitate was dissolved in TND buffer and desalted by passage through NAP-5 or PD-10 columns (Pharmacia). Protein concentrations were determined according to Bradford [10].

2.4. *Cleavage of fusion protein with factor Xa*

The partially purified fusion protein (350 μg) was incubated with 3.6 μg of factor Xa (New England Biolabs) in 500 μl of TND buffer containing 20% glycerol, at 4°C for 4 days.

2.5. *SDS-PAGE and Immunoblotting*

SDS-PAGE on 12.5% separation gel and immunoblotting using a mouse anti-H6H monoclonal antibody mAb5 were performed according to Hashimoto et al. [8]. The antiserum for MBP was supplied with the Protein Fusion & Purification System kit (New England Biolabs).

2.6. *Enzyme assay*

The hydroxylase activity and the epoxidase activity were assayed by gas–liquid chromatography as described previously [5]. Hyoscyamine and 6β-hydroxyhyoscyamine were, respectively, the substrates used for the assays of hydroxylase and epoxidase.

2.7. *Hyoscyamine feeding*

The fusion protein was induced in a 200 ml culture by IPTG as described above. Concurrently with the addition of IPTG, hyoscyamine hydrobromide was added to a final concentration of 0.1 mM. At several time intervals, 5 ml of the culture was harvested and centrifuged to separate bacterial cells and medium. Some of the cells were lysed as above and, after centrifugation, the crude cell extracts and the culture medium were desalted separately through PD-10 columns for the enzyme assay. Tropane alkaloids in the cells and the medium were quantified as described previously [11].

3. RESULTS AND DISCUSSION

3.1. *Inhibition of enzyme activities by antibodies*

We first asked whether the monoclonal antibody mAb5 that was raised against homogeneous hydroxylase and is capable of inhibiting hydroxylase activity [8] could inhibit epoxidase activity as well. The hydroxylase preparation partially purified from the cultured roots of *H. niger* also contained a weak epoxidase activity (about 3% of the hydroxylase activity). Incubation with preimmune IgG did not inhibit either enzyme activities, but mAb5 reduced both activities to about 30% of the controls (Table I). The results indicate that hydroxylase and epoxidase share a common epitope to mAb5.

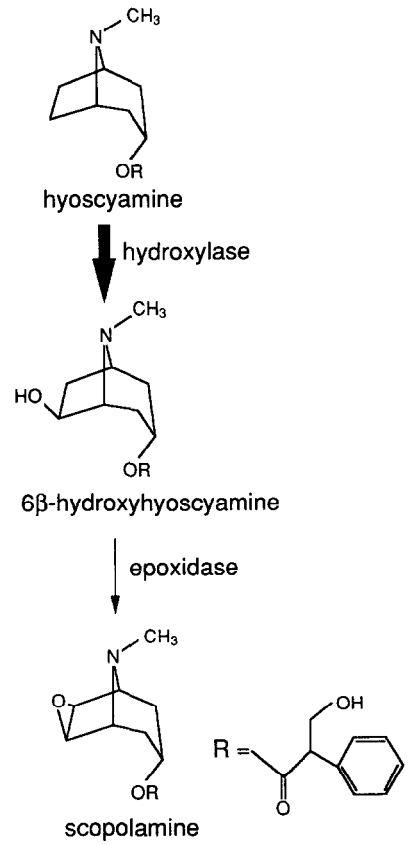

Fig. 1. Conversion of hyoscyamine to scopolamine by way of 6β-hydroxyhyoscyamine. The present study demonstrates that hyoscyamine 6β-hydroxylase catalyzes both the first hydroxylation reaction and the second epoxidation reaction.

3.2. *Production of recombinant H6H*

To gain further insight into the identity of hydroxylase and epoxidase, we expressed the H6H cDNA isolated from *H. niger* in *E. coli*. When an intact H6H protein was translated from the first ATG of the cDNA under the control of the *lacZ* promoter, we obtained only low levels of H6H enzyme activity (typically 1–2 pKat/mg soluble protein) (see [9], for example). Subsequent experiments, however, showed that expression of H6H as a fusion protein and incubation of the recombinant bacteria at a low temperature effectively increased the H6H enzymatic activity in the culture. The prokaryotic expression vector pMH1 (Fig. 2) provided an N-terminal MBP and a C-terminal H6H protein connected with a linker peptide containing a factor Xa cleavage site, under the *tac* promoter. The *E. coli* harboring pMH1 was cultured at 10°C for 2 days after the addition of 0.4 mM IPTG. The soluble fraction of the culture contained a prominent protein of 81 kDa (Fig. 3A, arrowhead), which was recognized by antibodies specific to MBP (Fig. 3B) and H6H (Fig. 3C). Cleavage of the fusion protein with factor Xa produced two predominant polypeptides of 42 kDa and 39 kDa. The 42-kDa polypeptide was recognized by the anti-MBP

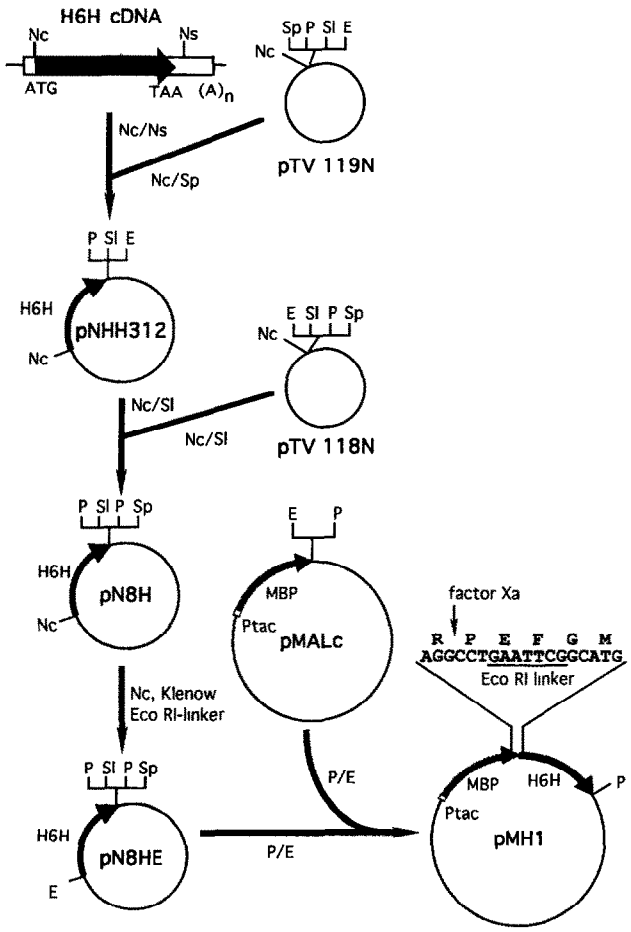

Fig. 2. Construction of the H6H expression vector pMH1. E, *Eco*RI; Nc, *Nco*I; Ns, *Nsp*I; P, *Pst*I; SI, *Sal*I; Sp, *Sph*I.

antibody, and was thus identified as MBP, while the 39-kDa polypeptide was recognized by the anti-H6H antibody, and thus was H6H. The molecular weights for these polypeptides agreed well with those for MBP and H6H.

3.3. *H6H shows both the hydroxylase and the epoxidase activities*

However, complete cleavage of 100 μg of the fusion protein required incubation with more than 1 μg of factor Xa for 4 days at 4°C, and resulted in a 68% loss of the H6H enzyme activity. Therefore, we measured the enzyme activities of the fusion protein in the crude enzyme extracts from the recombinant bacteria. The bacterium that had been transformed with pMH1 showed both hydroxylase and epoxidase activities, whereas the bacteria that had been transformed with the vector alone showed neither activity (Table II). The epoxidase activity was only 2.4% of the hydroxylase activity. This clearly demonstrated that the H6H cDNA of *H. niger* encodes a bifunctional 2-oxoglutarate-dependent dioxygenase with a strong hydroxylase activity and a relatively weak epoxidase activity.

The hydroxylation reaction inserts molecular oxygen into the C6-Hβ bond of the tropane ring, while the epoxidation reaction removes the 7β-hydrogen and the hydrogen from the 6β-hydroxyl. We have proposed a model that explains how a single enzyme might catalyze two types of reactions [5]. The model is based on a highly reactive ferryl enzyme intermediate, which we postulate homolytically cleaves a nearby C-H bond and is an enzyme species common to all of the 2-oxoglutarate-dependent dioxygenases and related enzymes. The group, which has been tentatively called the 2-oxoacid-dependent oxygenase family [12], catalyzes a wide variety of reactions, including hydroxylations, desaturations and ring expansions. Probably because of the common ferryl intermediate, enzymes of this family occasionally catalyze more than one reaction; for example, a bifunctional enzyme from *Cephalosporium acremonium* catalyzes both the ring expansion of penicillin N to deacetoxycephalosporin C and the successive hydroxylation of the initial reaction product [13], clavaminate synthase of *Streptomyces clavuligerus* catalyzes the oxidative cyclization/desaturation of proclavaminic acid to clavaminic acid by way of dihydroclavaminic acid [14], and thymine 7-hydroxylase of *Neurospora crassa* catalyzes three sequential reactions leading from thymine to uracil-5-carboxylic acid that involve a hydroxylation, conversion of an alcohol to an aldehyde, and conversion of an aldehyde to a carboxylic acid [15].

3.4. *Bioconversion of hyoscyamine to scopolamine in* E. coli

6β-Hydroxyhyoscyamine does not usually accumulate in plants that accumulate scopolamine. To see whether the low epoxidase activity (as compared to the hydroxylase activity) of H6H blocks the epoxidation process at 6β-hydroxyhyoscyamine, we fed hyoscyamine at a concentration of 0.1 mM to *E. coli* harboring pMH1 or vector pMALc. IPTG was added to the culture at the same time as hyoscyamine, and the bacterium was incubated at 10°C for 7 days. No hydroxylase activity nor any conversion of the alkaloid were observed in the bacterium harboring the vector alone (not

Table I

Monoclonal antibody mAb5 inhibits two oxygenase activities

Antibody	Enzyme activity (pKat)	
	Hydroxylase	Epoxidase
Preimmune IgG	25.8 (100)	0.88 (100)
mAb5	8.3 (32)	0.26 (30)

An enzyme preparation partially purified from cultured *H. niger* roots was assayed for the activities of hyoscyamine 6β-hydroxylase and 6β-hydroxyhyoscyamine epoxidase in the presence of antibodies. The figures in parentheses denote enzyme activities expressed as percentages of the preimmune controls. The values are the average of two measurements.

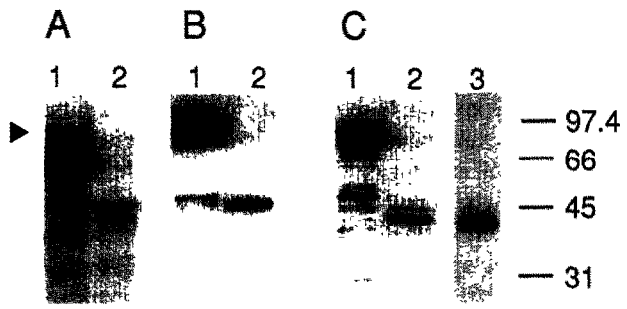

Fig. 3. SDS-PAGE/immunoblot analysis of the fusion protein. (A) Stained with Coomassie brilliant blue. (B) Immunoblot with an anti-MBP antibody. (C) Immunoblot with an anti-H6H antibody mAb5. Lanes 1 and 2, proteins before and after cleavage with factor Xa, respectively. Lane 3 of C, H6H purified from cultured roots of *H. niger*. The arrow points to the fusion protein between MBP and H6H. Molecular weights of the standard proteins are indicated on the right in kDa.

shown). The hydroxylase activity in the bacterium harboring pMH1, however, increased dramatically during the first three days, and then leveled off (Fig. 4A). The enzyme activity was not detected in the culture medium. Fig. 4B shows that hyoscyamine that had been added to the medium decreased to an undetectable level at day 4. 6β-Hydroxyhyoscyamine appeared soon after the hyoscyamine feeding, increased up to about 0.06 mM at day 3, and then decreased to a trace amount at day 7. Scopolamine first became detectable at day 2 and continued to increase until finally it dominated the alkaloid pool in the culture at day 7. These alkaloids were found almost exclusively in the medium, indicating that the bacterial cell membrane is freely permeable to these alkaloids. Similar findings have also been reported for strictosidine synthase when a recombinant bacterium expressing the synthase converted exogenously fed substrates to strictosidine [16].

When larger amounts of hyoscyamine were fed to the culture, the conversion of 6β-hydroxyhyoscyamine to scopolamine was less efficient (not shown). These results suggest that when a sufficient amount of the catalyst H6H is present, scopolamine is produced without considerable accumulation of the intermediate 6β-hydroxyhyoscyamine, but when the amount of H6H is limiting (in relation to the supply of hyoscyamine), the intermediate accumulates. When hyoscyamine was fed to the culture medium of transgenic tobacco plantlets that expressed H6H constitutively, conversion to scopolamine was efficient in the leaf where the supply of hyoscyamine through the vascular cylinder was limited [6], but 6β-hydroxyhyoscyamine accumulated in the root where a large amount of hyoscyamine was available (our unpublished results). It should also be noted that 6β-hydroxyhyoscyamine was originally isolated in considerable amounts from hybrids between plants that produced large amounts of hyoscyamine and plants with low alkaloid contents but with high ratios of scopolamine to hyoscyamine [17,18].

This study has provided direct evidence that a bifunctional enzyme catalyzes two sequential steps in the formation of the epoxide bridge in scopolamine, and has suggested that the amount of this enzyme may be rate-limiting for the accumulation of scopolamine in plants. Although the expression level of the fusion protein is not particularly high at present, further studies may achieve much higher production levels of recombinant H6H in bacteria. Such recombinant bacteria would be very useful for developing a bioconversion process to

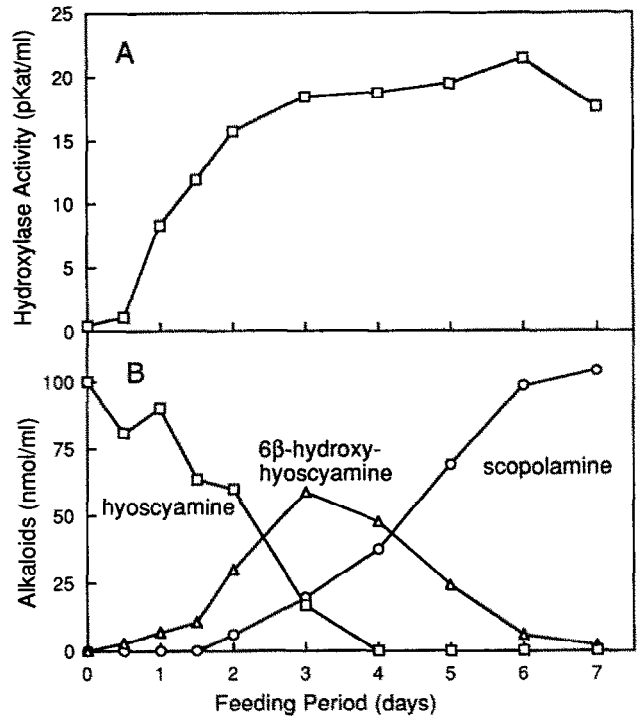

Fig. 4. Bioconversion of hyoscyamine to scopolamine in the recombinant bacterium. Hyoscyamine at 100 nmol/ml and IPTG at 0.4 mM was added at day 0 to *E. coli* expressing the fusion protein, which were then cultured at 10°C for the indicated periods. (A) The hydroxylase activity of H6H. (B) Tropane alkaloids in the culture.

Table II

H6H cDNA encodes an enzyme with two oxygenase activities

Plasmid	Enzyme activity (pKat/mg protein)	
	Hydroxylase	Epoxidase
pMH1	81.5 ± 4.8 (100)	1.9 ± 0.1 (2.4)
pMALc	0.0 ± 0.0	0.0 ± 0.0

Crude cell extracts were prepared from bacteria harboring the plasmids and assayed for the activities of hyoscyamine 6β-hydroxylase and 6β-hydroxyhyoscyamine epoxidase. pMH1 expresses an H6H protein fused to a maltose-binding protein, whereas pMALc encodes only a maltose-binding protein. The values are the average of five measurements (± S.D.).

transform hyoscyamine to scopolamine, which has a much higher commercial demand than hyoscyamine. Excretion of alkaloids in the culture medium may simplify the downstream recovery process in bioreactors.

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