

Molecular and Biochemical Characterization of an Enzyme Responsible for the Formation of Hypericin in St. John's Wort (*Hypericum perforatum* L.)*

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Harsh Pal Bais‡§, Ramarao Vepachedu‡§, Christopher B. Lawrence¶, Frank R. Stermitz||, and Jorge M. Vivanco‡**‡‡

From the ‡Department of Horticulture and Landscape Architecture, the **Cell and Molecular Biology Program, the ¶Department of Bioagricultural Sciences and Pest Management, and the ||Department of Chemistry, Colorado State University, Fort Collins, Colorado 80523-1173

A major gene termed *Hyp-1* encoding for hypericin (HyH) biosynthesis was cloned and characterized from *Hypericum perforatum* (St. John's wort) cell cultures. *H. perforatum* leaves are widely used as an herbal remedy in the treatment of mild to moderate depression. Hypericin, a photosensitive and red-colored naphthodianthrone, has been reported as the bioactive compound responsible for reversing the depression symptoms. In this study a novel red-color-based colony screening method for examining a cDNA library (λ -Triplex2) derived from *H. perforatum* cell cultures revealed the gene responsible for hypericin biosynthesis after the administration of emodin, a precursor of hypericin. The selected clones were expressed in *Escherichia coli* (BM 25.8 line) and were further screened for biosynthesis of emodin to hypericin, which resulted in an 84.6% conversion. The full-length cDNA sequence of *Hyp-1* is 782 nucleotides in length with an open reading frame of 477 nucleotides coding for a protein of 159 amino acids, with a 45.1% homology to Bet.v.1 class allergens. Reverse transcriptase-PCR analysis showed high levels of *Hyp-1* transcripts in dark-grown cell cultures compared with the levels in light-grown cell cultures and leaves. Southern blot analysis showed the presence of a single *Hyp-1* gene in *H. perforatum*. Furthermore, *Hyp-1* was expressed with a His₆ affinity tag linked to its N terminal region using the expression vector pET-28a, and the recombinant *Hyp-1* protein was able to convert HyH from emodin under *in vitro* conditions. HyH product inhibition was observed with emodin analogues, rhein, rhein methyl ester, and DNA3-55-1. Our results demonstrate a direct and complex conversion of emodin to HyH that is solely catalyzed by *Hyp-1*, a Bet.v.1 class allergen from *H. perforatum*.

Plants have been estimated to collectively synthesize more than 100,000 different secondary metabolites, many of which have applications as medicinal compounds (1). One such compound is hypericin (HyH),¹ a naphthodianthrone produced in the dorsal leaf glands of *Hypericum perforatum* L., commonly known as St. John's wort (2). Standardized extracts of St. John's wort containing 3% HyH have been successfully tested in numerous studies for the treatment of mild to moderate depression (2, 3). HyH, a red naphthodianthrone, has been reported as the active compound found in St. John's wort and has been shown to act as a monoamine oxidase inhibitor (4). As is true for most anthraquinones, there are no published descriptions of the biosynthetic pathway that leads to HyH production.

The majority of commercially available St. John's wort formulations are crude extracts containing minor proportions of HyH combined with flavonoids, phenols, tannins, and volatile oils, which may account for some of the reported side effects of the extracts (5, 6), such as nausea and vomiting. These side effects are more severe than those encountered with synthetic antidepressants (4). Studies have shown that St. John's wort formulations may activate the cytochrome P450 (*CYP3A*) pathway, accelerating the breakdown of other drugs (5, 7). For instance, the blood concentrations of the anti-clotting drug warfarin were dramatically reduced when taken together with St. John's wort extracts (5, 8). Similar drug dilution cases were observed with birth control pills and coronary transplant anti-rejection medications (5). Recent reports indicate some metabolites other than HyH as the cause of these unwanted drug interactions in St. John's wort extracts (5, 9). Thus, the purification and bioprocessing of HyH, the antidepressant compound in St. John's wort, may be of medical importance in the treatment of depression, and in avoiding the side effects associated with this herbal remedy.

Little is known about the biosynthesis of hypericin other than that it lies on the polyketide pathway, presumably through emodin (Fig. 1) (10), and with protohypericin as the penultimate precursor (11). Synthetically, it is readily prepared by treating emodin dianthrone with ferrous sulfate and pyridine-*N*-oxide in pyridine, followed by light irradiation of the formed protohypericin (10, 11). This synthesis could be in part a biomimetic one. However, nothing is known about the sequence of steps in *H. perforatum* or other plants that lead to protohypericin and finally to HyH, and no enzymes or genes in this pathway have been purified or cloned.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AAN65449.

§ Both authors contributed equally to this work.

‡‡ To whom correspondence should be addressed: Dept. of Horticulture and Landscape Architecture, 217 Shepardson, Colorado State University, Fort Collins, CO 80523-1173. Tel.: 970-491-7170; Fax: 970-491-7745; E-mail: jvivanco@amar.colostate.edu.

¹ The abbreviations used are: HyH, hypericin; HPLC, high-performance liquid chromatography; RT, reverse transcriptase; LC-MS, liquid chromatography-mass spectrometry; PR, pathogenesis-related.

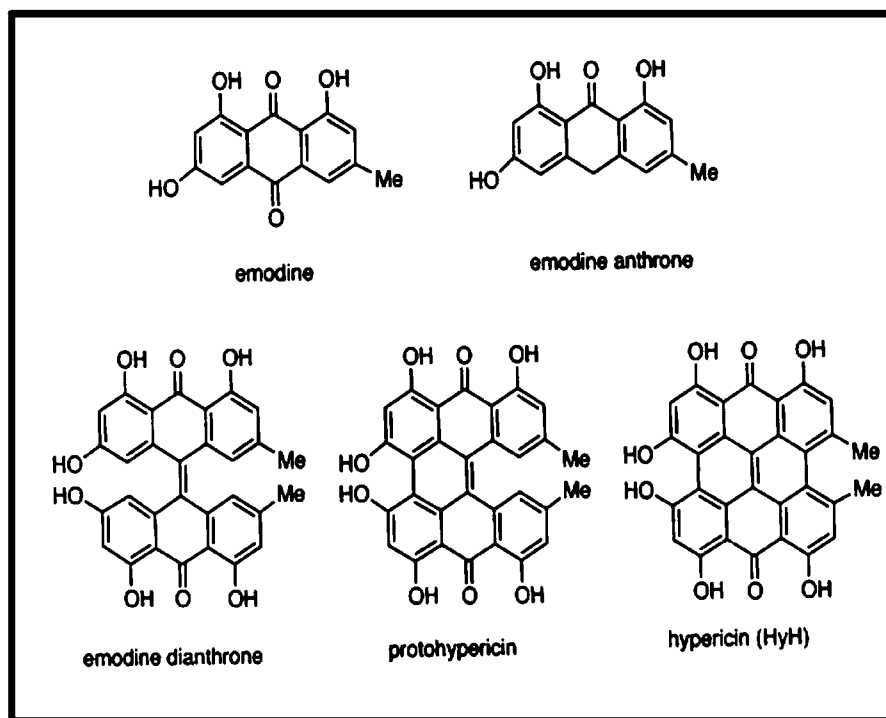


FIG. 1. Structures of hypericin, emodin, and other possible biosynthetic intermediates involved in hypericin biosynthesis in *H. perforatum*.

We developed an approach to identify the gene(s) responsible for the biosynthesis of HyH using a cDNA library constructed from *H. perforatum* cell cultures, supplemented with emodin (a possible precursor for HyH) and using a novel color-based screening methodology. Biochemical characterization of the recombinant protein revealed saturation kinetics for different concentrations of emodin. Other physiological factors such as the effect of divalent ions, ATP, temperature, and pH were also analyzed for their influence on enzyme activity. Our results also show variable product inhibition patterns by different structure analogues of emodin. Our studies show that the gene involved in HyH biosynthesis from emodin has a close homology to Bet.v.1 class allergens and that its enzymatic activity may involve a condensation followed by dehydration and two oxidative coupling reactions.

EXPERIMENTAL PROCEDURES

Plant Material

Seeds of *H. perforatum* L. (S5195) were obtained from Richters, Ontario, Canada.

Chemicals

Plant growth regulators were obtained from Sigma Co. (St. Louis, MO). Emodin structure analogues, rhein and rhein methyl ester were from the Stermitz laboratory, Department of Chemistry, Colorado State University, Fort Collins, CO, and DNA3-55-1 was obtained from Dr. J. Romagni, University of St. Thomas, Texas. All other chemicals were of analytical and HPLC grade and were obtained from Sigma Co. unless specified.

Callus and Suspension Culture Initiation

Callus cultures were initiated using leaves from a 10-day-old *H. perforatum* plant grown *in vitro* as previously described by Bais *et al.* (12). Cell suspension cultures were established from the callus cultures and were maintained in 125-ml Erlenmeyer flasks with 50 ml of nutrient MS (13) medium containing 2,4-dichlorophenoxyacetic acid (0.90 μ M), kinetin (0.11 μ M), and sucrose (30 g liter⁻¹) by biweekly sub-culturing on a rotary shaker at 90 rpm maintained at 25 $\pm$ 2 $^{\circ}$ C.

Total Protein Isolation and Incubation Experiments

Cell cultures of 1-month-old *H. perforatum* plants were collected and the total soluble proteins from the roots were extracted as described before by Vivanco *et al.* (14). Different concentrations of total proteins

(0.5–30 μ g ml⁻¹) and emodin (0.5–30 μ g ml⁻¹) were incubated with a buffer comprised of 100 mM Tris-HCl, 167 mM KCl, and 100 mM MgCl₂, pH 7.2 for 24 h at 4 $^{\circ}$ C under dark conditions. The incubation mixture was extracted with hexane overnight, and the hexane solutions were concentrated to dryness under N₂.

Estimation of HyH using Liquid Chromatography-Mass Spectrometry (LC-MS)

The dried powder obtained after incubation of emodin with the total cell culture proteins was further extracted using 5 ml of absolute ethanol (Fisher Co.) and was sonicated (Branson Co., ~60 Hz) for 10 min. The solution was centrifuged at 10,000 rpm for 10 min. The supernatant was further concentrated by freeze-drying (Model 25LL, Virtis, Genesis), and the powder was dissolved in 1 ml of methanol (Fisher). The samples were prepared for LC analysis after filtration through a 0.2- μ m filter (Gelman Sciences). Samples were chromatographed by gradient elution on a reverse-phase, 5- μ m, C₁₈ column (25 cm $\times$ 4.6 mm, Supelco). The chromatographic system consisted of P₅₈₀ pumps (Dionex) connected to an ASI-100 Automated Sample Injector (Dionex). The visible absorbance at 590 nm was measured by a PDA-100 photodiode array variable UV-visible detector (Dionex). Mobile phase solution A consisted of a 70% solution of 1% ammonium phosphate (adjusted to 7.0 pH with NaOH) and 30% acetonitrile (Fisher); solution B was 70% acetonitrile and 30% water. A multistep gradient was set up for HyH estimation as described by Bais *et al.* (12), with an initial injection volume of 15 μ l and a flow rate of 1 ml min⁻¹. HyH was quantified at 590 nm. Authentic HyH was purchased from Sigma Co., and samples were compared with the HyH standard on the basis of retention time and peak area. Chromelon software (Dionex) was used for peak integration analyses. The LC eluant was injected into an API-2000 mass spectrometer (Dionex) at a flow rate of 0.25-ml min⁻¹. The MS parameters were optimized using HyH as the reference standard compound. Subsequent high gas temperature (200 $^{\circ}$ C) and gas flow (50 p.s.i.) were applied to the LC-MS parameters. HyH [(M-H)⁻ 504.5] (minus one proton after proton-proton splitting) was used for negative mode monitoring to produce a negatively charged molecule. Scan ranges of 100–750 atomic mass units (milliabsorbance units) were used for negative ions. A step size of 1 atomic mass unit and a dwell time of 1 ms was used during the analysis.

RNA Extraction and cDNA Library Construction from Cell Cultures of *H. perforatum*

Our earlier studies indicate that cells of *H. perforatum* grown in dark conditions result in efficient production of HyH as compared with cells grown under light conditions (12, 15); hence for the present study dark-grown cells of *H. perforatum* were used for cDNA library construc-

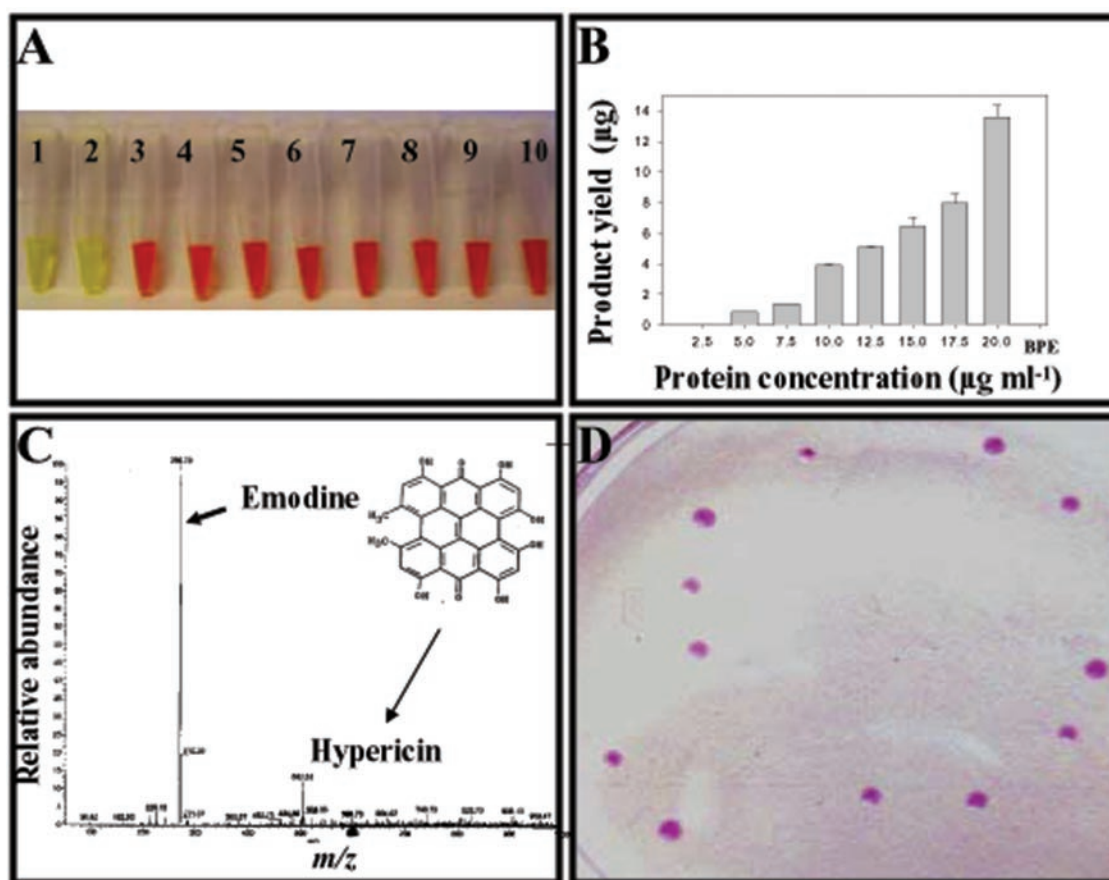


FIG. 2. Hypericin accumulation and incubation of emodin with cell-free total proteins isolated from cell cultures of *H. perforatum*. A, interconversion of emodin to HyH. Emodin was added at the $0.5\text{--}30\ \mu\text{g ml}^{-1}$ level to the total protein extracts ($0.5\text{--}30\ \mu\text{g ml}^{-1}$) from cell suspension cultures of *H. perforatum* grown under dark conditions. Lane 1, standard emodin; lane 2, standard emodin with the incubation buffer; lanes 3–10, refers to the variable dilutions of emodin and total protein extract concentration. B, production of hypericin upon incubation of emodin with total protein extracts isolated from cell cultures of *H. perforatum* grown *in vitro*. Experiments were performed by incubating total proteins extracted from cell cultures of *H. perforatum* ($0.5\text{--}30\ \mu\text{g ml}^{-1}$) with emodin ($15\ \mu\text{g ml}^{-1}$). The incubation mixture was extracted with ethanol, concentrated under N_2 , and subjected to HPLC-MS. (BPE in the inset depicts boiled protein extracts as a control to confirm the enzymatic involvement for conversions.) C, typical mass spectrometric profile of bioconverted HyH under negative ion splitting mode (inset shows the structure of HyH). D, interconverted *E. coli* (BM 25.8) cell lines showing instant and unique color-change-based colony formations upon emodin ($20\ \mu\text{g ml}^{-1}$) administration.

tion. RNA was extracted from 21-day-old cell suspension cultures of *H. perforatum*. Dark-grown *H. perforatum* cells were powdered in liquid nitrogen, and the total RNA was extracted using an RNeasy kit (Qiagen, Valencia, CA). $1\ \mu\text{g}$ of total RNA was used for cDNA synthesis using reverse transcriptase (RT) (Clontech, Powerscript™ RT). The cDNA was amplified by 20 cycles of long distance PCR. The amplified DNA was phenol-extracted, digested with *Sfi*I, and ligated into a λ -Triplex2 vector using the SMART™ cDNA construction kit (Clontech, PTR30001). The λ -Triplex2 vector was packed into an SBET phage using the Packgene® Lambda DNA packaging system (Promega).

Red Color-based Screening for Enzyme(s) Involved in the Synthesis of HyH

A λ -Triplex2 vector containing the *H. perforatum* cDNA library was plated with *Escherichia coli* (XL-1 blue cell line) for screening. The percentage of recombinant clones was determined by blue/white screening in the presence of isopropyl-1-thio- β -D-galactopyranoside and 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal). Primary screening of the λ -Triplex2 library for genes involved in the biosynthesis of HyH was performed after 16-h incubation at 37°C under dark conditions by spreading emodin ($20\ \mu\text{g ml}^{-1}$) on the bacterial lawns and selecting the phage plaques that turned pale yellow emodin into red-colored HyH. Twelve positive plaques were selected and re-screened after amplification. Secondary screening of amplified plaques was performed identically to the primary screening by addition of $20\ \mu\text{g ml}^{-1}$ emodin to the overnight grown plaque lawn. Colonies, which turned red instantly, were picked with a micro-tip and then converted to pTriplex2 in *Escherichia coli* (BM 25.8) by *in vivo* excision and circularization into a complete plasmid from the recombinant phage. For the large scale

conversion of emodin to HyH, selected *E. coli* clones were cultured overnight under dark conditions in LB (16) broth media (50 ml in a 150-ml Erlenmeyer flask). Subsequently, emodin was added ($20\ \mu\text{g ml}^{-1}$) to visualize and quantify the bioconverted HyH using LC-MS as described previously. Interconverted bacterial cells (BM 25.8) and selected plaques from secondary and tertiary screening were picked using a micropipette tip. The pTriplex2 sequencing primers provided with the SMART™ cDNA construction kit (Clontech, PTR30001) were used to perform PCR for the determination of insert sizes. The bacterial cells, cell-free media filtrate, and plaques, obtained from secondary and tertiary screening, were extracted as per the methodology described earlier and analyzed by HPLC-MS for HyH identification.

Sequencing

Plasmid DNA was prepared using a Miniprep kit (Qiagen) and used for sequencing (Molecular Structure Facility, University of California, Davis, CA). The sequence was analyzed, followed by amino acid alignment to compare homologous proteins using VectorNTI Suite software (Informax, Bethesda, MD).

Probe Preparation

The template for the probe was obtained by PCR with gene-specific primers deduced from a full-length sequence from the interconverted BM 25.8 bacterial clones. The PCR product was separated on agarose and extracted from the gel using a Quantum Prep Gel Slice kit (Bio-Rad). The DNA was then labeled with [^{32}P]ATP using random primers from the DECAprime II Random Priming DNA labeling kit (Ambion). This labeled sequence was then used as a probe for Northern analysis.

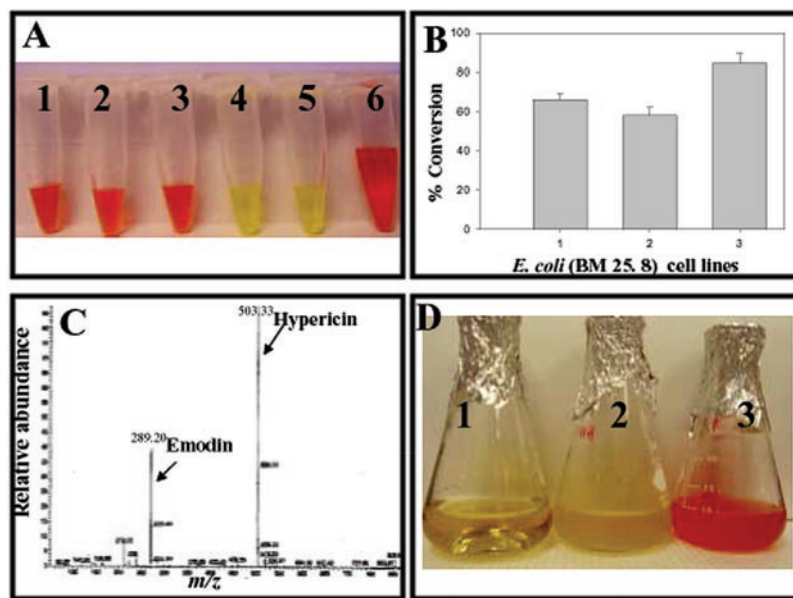


FIG. 3. Functional analysis of positive clones and conversion to HyH. A, positive clones in *E. coli* (BM 25.8) cell lines were tested for bioconversion efficacy. All three clones showed an efficient conversion from precursor to HyH as compared with the untreated control. Lanes 1–3, positive clones exhibiting formation of HyH upon emodin ($20 \mu\text{g ml}^{-1}$) administration in LB medium; lane 4, emodin ($20 \mu\text{g ml}^{-1}$); lane 5, emodin with *E. coli* (BM 25.8) cell lines without the insert; lane 6, standard HyH (in ethanol). B, percentage conversion of emodin to HyH in the interconverted *E. coli* cell lines. C, mass spectrometric profile of bioconverted HyH under negative ion splitting mode *in vivo* exhibiting increased yield of HyH. D, large scale conversion of HyH in LB medium after administration of emodin ($20 \mu\text{g ml}^{-1}$). Treatments: 1, plain LB media with emodin ($20 \mu\text{g ml}^{-1}$); 2, *E. coli* (BM 25.8) cells without the insert in presence of emodin ($20 \mu\text{g ml}^{-1}$); 3, *E. coli* (BM 25.8) cells with the insert in the presence of emodin ($20 \mu\text{g ml}^{-1}$).

Southern Blots

Total genomic DNA was extracted from *in vitro* grown plants using a DNeasy kit (Qiagen). $10 \mu\text{g}$ of total genomic DNA was digested overnight using three different restriction enzymes (RE): *EcoRI*, *HindIII*, and *BamHI*. The digests were separated on 1% agarose gel, transferred to nylon membranes (Biodyne, Pall Gelman Laboratory), and probed with a ^{32}P -labeled sequence obtained by PCR.

RT-PCR Analysis

Using an RNeasy® Plant Mini kit (Qiagen), we extracted the total RNA from leaves, roots, and shoots grown *in vitro*, and from light- and dark-grown cell suspension cultures. 500 ng of RNA was used for first strand cDNA synthesis using a SMART™ cDNA synthesis kit (Clontech, PTR30001). Reverse transcription was performed according to the instructions for the First-Strand cDNA Synthesis kit (Clontech, PTR30001) using a set of primers specific to the internal control gene, *Hyp-1* (5'-ATGGCGCGTACACTATTGTTAAGGAGG-3'; 5'-GGATTAGCAGCAAGGTATCTTCCAC-3'), and a set of primers specific to the housekeeping gene for β -tubulin in the different reaction mixture, because they had the same size constraint ($\sim 500 \text{ bp}$). An aliquot ($5 \mu\text{l}$) of each PCR reaction mixture was subjected to electrophoresis through an agarose-ethidium bromide gel. Ethidium bromide intensities in each band were quantified using an Alpha Imager 1220 digital imaging system (Alpha Inotech Corp., San Leandro, CA).

Heterologous Expression of Hyp-1 in E. coli

Recombinant Hyp-1 protein was expressed with His₆ affinity tag linked to its N terminus region using the expression vector pET-28a (Novagen, Madison, WI). pET-Hyp-1, carrying the full-length *Hyp-1* gene, was constructed by PCR amplification using the primers: F5'-TTCATATGGCGCGTACACTATTGTTAAGG incorporating a *NdeI* site and R5'-TTGGATCCGGATTAGCAGCAAGGTATCTTCCAC incorporating a *BamHI* site. The PCR product was cloned into pGEM®-T Easy (Promega), and the plasmid was isolated using a plasmid mini-prep from a single colony. The *Hyp-1* sequence was cleaved from the pGEM®-T Easy plasmid with *NdeI* and *BamHI* and cloned into pET-28a digested with the same enzymes. The pET-Hyp plasmid was transformed into BL21(DE3)pLysS (Novagen) for protein expression studies. Freshly grown BL21(DE3)pLysS-containing pET-Hyp-1 was induced for gene expression with 1 mM isopropyl-1-thio- β -D-galactopyranoside for 3 h, and the bacterial proteins were subsequently extracted by sonication. The recombinant Hyp-1 protein was purified using Ni^{2+} -chelate chromatography using an His-Trap kit (Amersham Biosciences,

Piscataway, NJ). The bacterial extract was applied to the column dissolved in 20 mM phosphate, 0.5 M NaCl, and 20 mM imidazole buffer (pH 7.5) and washed with the same buffer to remove unbound proteins. The column was then washed with 20 mM phosphate, 0.5 M NaCl, and 100 mM imidazole to remove proteins that were bound nonspecifically. Finally, the recombinant Hyp-1 protein was eluted from the column with 20 mM phosphate, 0.5 M NaCl, and 300 mM imidazole. The purity of the recombinant Hyp-1 protein was analyzed by silver staining of the eluted fraction separated on 12% SDS-PAGE gel. The recombinant Hyp-1 protein was incubated with emodin using the methodology described earlier, and HyH biosynthesis was analyzed by HPLC-MS for HyH identification.

Biochemical Characterization of Recombinant Protein (Hyp-1)

Precursor Concentration—Activity for HyH formation was checked in $100 \mu\text{l}$ of 0.05 M of phosphate buffer, pH 7.0, containing different concentrations of precursor (emodin, 0.55 – $22.2 \mu\text{M}$) at 25°C . The enzymatic activity was measured after the addition of $10 \mu\text{g ml}^{-1}$ of recombinant Hyp-1 protein over an assay time of 10 min. The substrate conversion to HyH was quantified by a spectrophotometric detection (Bio-Rad SmartSpec-3000™) at 588 nm. The specific activity was expressed in micrograms per milliliter of HyH produced per minute ($\mu\text{g ml}^{-1} \text{ min}^{-1}$). Enzymatic activity measurements were conducted in triplicate. The K_m and V_{max} values for the HyH were determined under the conditions described above. All K_m and V_{max} values were calculated by non-linear regression analysis and represented in Lineweaver-Burk plot.

Metal Elicitation and ATP Dependence Studies—The effect of divalent metal ions for spiking HyH formation at varying concentrations of precursor emodin was studied by supplementing 0.05 M phosphate buffer (pH 7.0) with CaCl_2 (10 – $200 \mu\text{M}$), MgCl_2 (10 – 60 mM), and MnSO_4 (10 – $60 \mu\text{M}$) to the incubation mixture. For ATP dependence studies 100 – $600 \mu\text{M}$ ATP was included in the incubation mixture and incubated at 25°C for 10 min.

Enzyme Stability Assays—The thermal stability profile of recombinant Hyp-1 enzyme was obtained by measuring the enzymatic activity in $100 \mu\text{l}$ of 0.05 M of phosphate buffer, pH 7.0, containing different concentrations of precursor (emodin, 0.55 – $22.2 \mu\text{M}$) with varying temperature conditions (25 – 60°C). Standard measurements were carried out as described above. The pH stability profile of the recombinant enzyme was obtained by measuring the enzymatic activity in $100 \mu\text{l}$ of 0.05 M of phosphate buffer, containing different concentrations of emo-

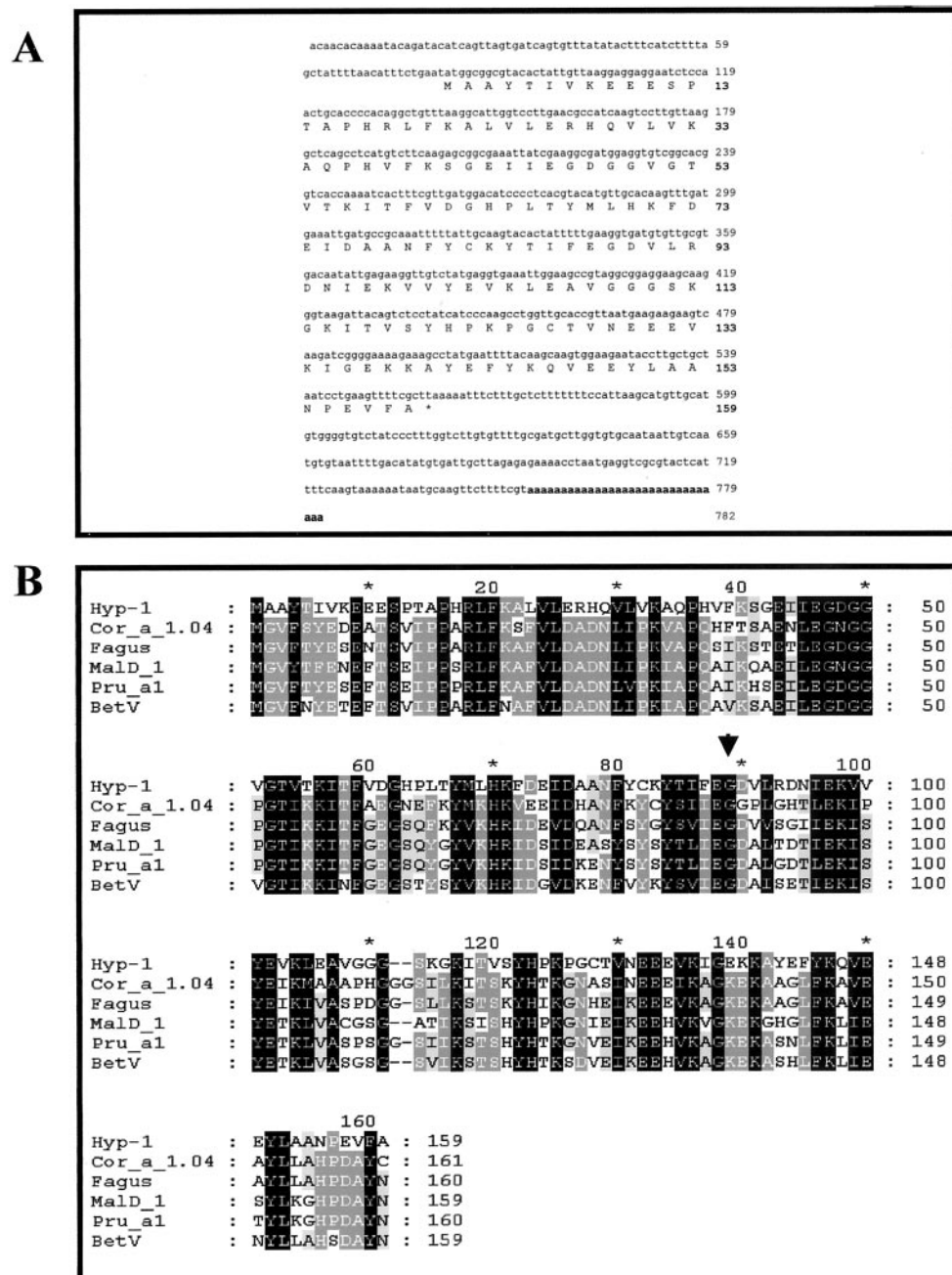


FIG. 4. **Comparison of cDNA sequence of Hyp-1 with Mal d1 and Bet.v. allergens.** A, nucleotide sequence and deduced amino acid sequence of Hyp-1. Nucleotides (*above*) and deduced amino acid residues (*below*) of the sequence are numbered. N-terminal sequence as determined by microsequencing. *Bold letters* stand for poly(A) tail. B, deduced amino acid sequence comparison of Hyp-1 with Mal d1 (Major apple allergen) (accession no. T17004), Bet.v. (White Birch) (accession no. JC4276), Pru-a-1 (*Prunus* sp.) (accession no. O24248), Cor-a-1.04. (hazelnut) (accession no. AAG40331), and *Fagus* (*Fagus* sp.) (accession no. CAA10235). *Numbers to the right* of the sequences indicate the amino acid residue locations. *Shaded portions* indicate that the residue is identical to the column consensus; *no shading* indicates that the residue is neither similar nor identical to the column consensus.

din (0.55–22.2 μM) with varying pH conditions (5.0–9.0). Product estimation was carried as described previously.

Product Inhibition Assay—Structure analogues for emodin, DNA3-55-1, rhein, and rhein methyl ester were prepared at stock concentrations of 0.92–36 μM in 0.05 M phosphate buffer, pH 7.0. The inhibitory effect of the analogues was studied at fixed concentrations of emodin (0.42 μM) by including increasing concentrations of the analogues. All assays with DNA3-55-1, rhein, and rhein methyl ester were conducted with 10 $\mu\text{g ml}^{-1}$ of recombinant Hyp-1 protein, with an assay time of 10 min. The product formation was quantified as described previously.

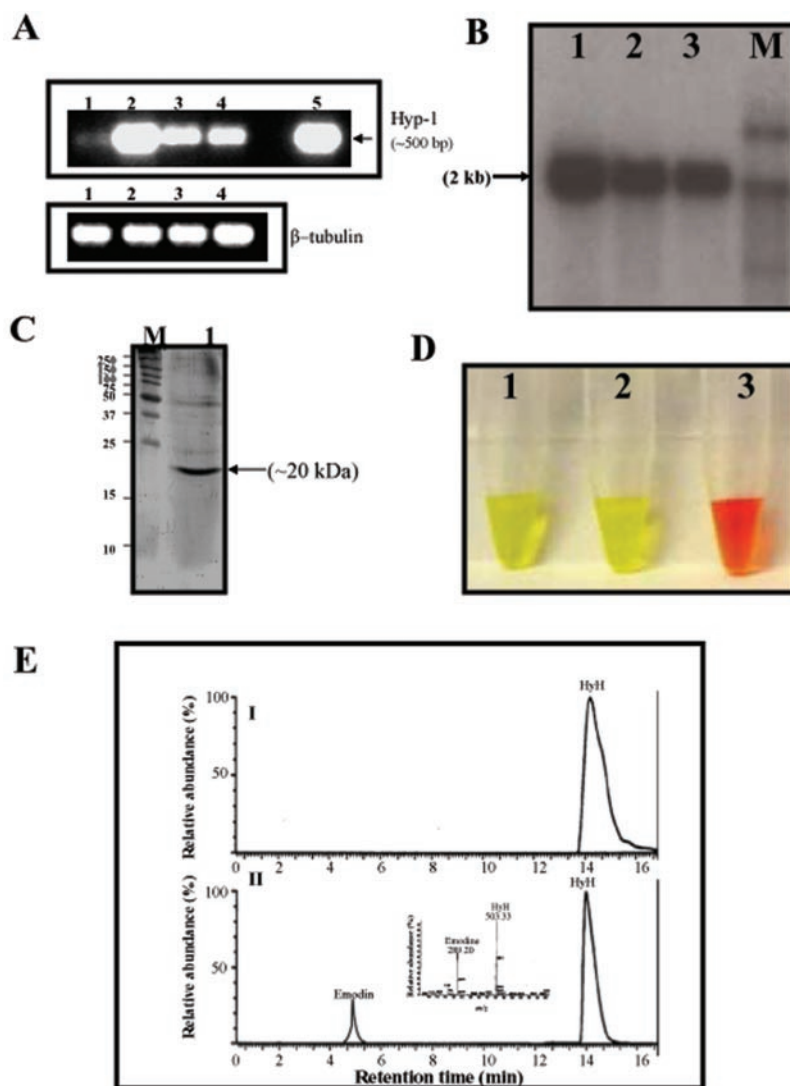
RESULTS

Hypothetical Pathway for HyH Production in Cell Cultures of H. perforatum—The initial steps in the biosynthesis of HyH are

derived from acetyl-CoA and malonyl-CoA conversions (10). These steps are followed by cyclization steps leading to the formation of an anthrone derivative, which may further bifurcate to form emodin and emodin anthrone (Fig. 1). Early laboratory syntheses involved many steps and low yields, but HyH could be prepared with a 63% yield from emodin dianthrone, which is available synthetically from commercially available emodin (10). We hypothesized that in *H. perforatum* conversion of emodin to HyH may be due to an enzymatic dimerization reaction. In support of this hypothesis, we found traces of emodin in *H. perforatum* cell cultures (data not shown).

Incubation of Emodin with Cell-free Total Proteins Isolated from Cell Cultures of *H. perforatum*—We conducted *in vitro*

FIG. 5. Tissue-based expression of *Hyp-1*. A, transcript levels in different plant tissues and cell cultures grown under different sets of conditions. Total RNA was extracted from *in vitro* grown roots, stem, and leaves of a 2-month-old *H. perforatum* plant for RT-PCR analysis. 500 ng of RNA was used for first strand cDNA synthesis. An aliquot (5 μ l) of each PCR reaction mixture was subjected to electrophoresis; intensities in each band were quantified using an Alpha Imager 1220 digital imaging system. Lanes 1, cell cultures grown under light conditions; 2, cell cultures grown under dark conditions; 3, leaves; 4, roots; 5, positive control from recombinant *E. coli* line plasmid; below the panel are depicted transient levels of β -tubulin gene in different tissues of *H. perforatum* such as light (1), dark-grown cell cultures (2), leaves (3), and roots (4). B, Southern hybridization. DNA was extracted from a callus culture of *H. perforatum* and 10 μ g of DNA was digested with *Eco*RI, *Hind*III, and *Bam*HI separately overnight. The digests were separated on a 1% agarose gel and transferred to nylon membranes. The blot was hybridized with a ^{32} P-labeled *Hyp-1* cDNA sequence. C, SDS-PAGE of recombinant *Hyp-1* protein; recombinant *Hyp-1* protein has an approximate size of $\sim$ 20 kDa. D, recombinant *Hyp-1* protein was tested for bioconversion efficacy. Lane 1, standard emodin; 2, standard emodin (20 μ g ml $^{-1}$) with bovine serum albumin (BSA) (5 μ g ml $^{-1}$); lane 3, recombinant *Hyp-1* protein (5 μ g ml $^{-1}$) exhibiting formation of HyH upon emodin (20 μ g ml $^{-1}$) administration. E, HPLC-mass spectrometric profile of bioconverted HyH using precursor emodin and recombinant protein *Hyp-1*; mass spectrometric analysis was conducted under negative ion splitting mode *in vivo* exhibiting increased yield of HyH.



feeding experiments with emodin and total proteins from dark-grown cell cultures of *H. perforatum*. Emodin (17.5 μ g ml $^{-1}$) incubated with 12.5 μ g ml $^{-1}$ of total protein extracts resulted in its maximum bioconversion to HyH (Fig. 2, A and B). We varied the amount of precursors and total protein extracts, with the time of incubation remaining constant. Fig. 2B shows the results of feeding emodin (0.5–30 μ g ml $^{-1}$) to the total protein extracts (0.5–30 μ g ml $^{-1}$) from cell cultures. Emodin (15 μ g ml $^{-1}$) incubated overnight with 15 μ g ml $^{-1}$ of total protein extracts resulted in the maximum bioconversion to HyH (Fig. 2B). The presence of biosynthesized HyH was further confirmed by MS analysis (Fig. 2C). These results confirmed the presence of enzymatic activity in the protein extracts, which catalyzed HyH biosynthesis from emodin under dark conditions.

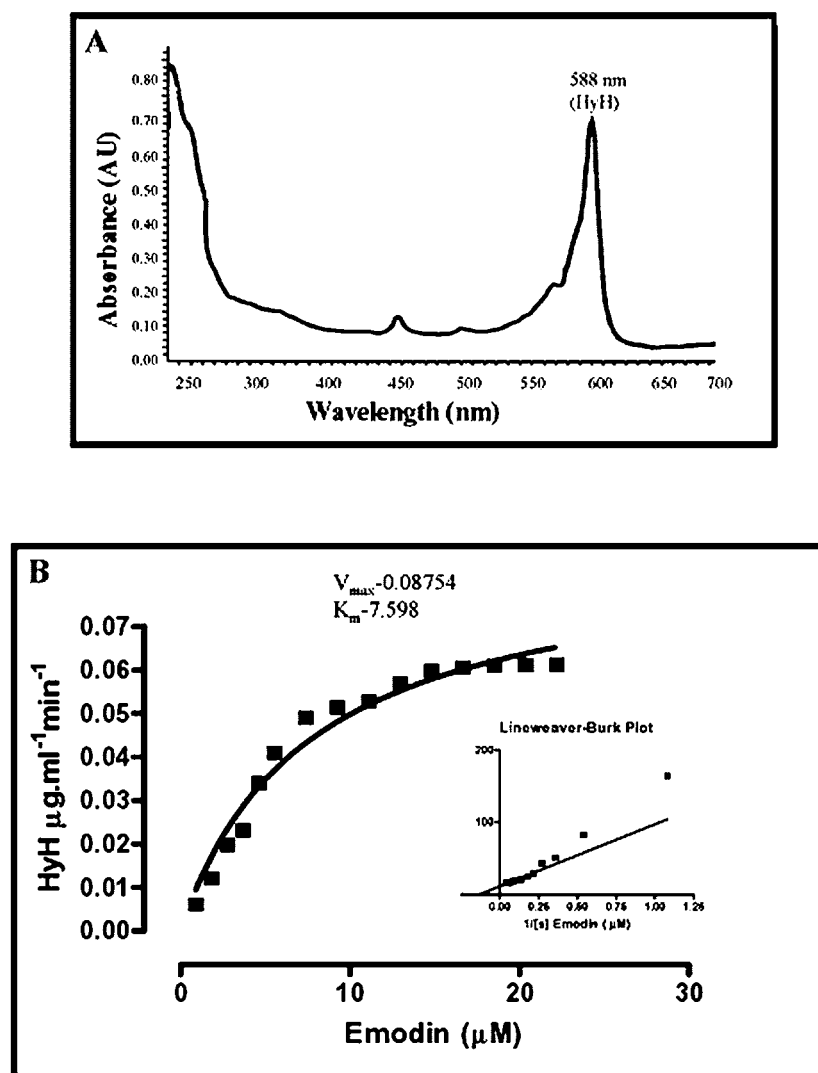
cDNA Cloning and Precursor-based Screening of λ -Phage—A cDNA library was constructed by isolating RNA from dark-grown cell suspension cultures of *H. perforatum* using the λ -Triplex2 vector. Primary screening of a cDNA library was performed from the unamplified library using *E. coli* (XL-1 blue cell line). After a 16-h incubation at 37 $^{\circ}$ C, positive plaques were selected by white/blue color screening to obtain stable transfectants and selected white clones turned red post-emodin administration. The plaques were amplified and used for inter-conversion in *E. coli* (BM 25.8) cell lines.

Functional Analysis of Positive Clones and Conversion to HyH—The interconverted BM 25.8 cell lines formed unique

red-colored colonies instantly upon emodin (20 μ g ml $^{-1}$) administration under dark conditions (Fig. 2D). The red colonies were isolated and checked for insert size using the pTriplex2 primers. Most of the positive clones showed a fragment of $\sim$ 1 kb upon PCR analysis (data not shown), and all positive clones in BM 25.8 were tested for bioconversion efficacy using HPLC-MS analysis. It was observed that three clones showed an efficient conversion from emodin to HyH compared with the control BM 25.8 cell line without the insert (Fig. 3, A and B). Clone 3 showed the maximum HyH conversion (84.6%) efficiency compared with the other positive clones (Fig. 3B). Additionally, it was observed that the interconverted clone 3 resulted in the conversion of emodin (20 μ g ml $^{-1}$) to HyH at large scale levels under dark conditions (Fig. 3C). *E. coli* (BM 25.8) cells without the insert and basal LB medium did not convert to HyH post-emodin (20 μ g ml $^{-1}$) administration (Fig. 3D). The plasmid DNA containing cDNA inserts was prepared using a Miniprep kit (Qiagen) and was used for dideoxynucleotide sequencing.

Sequence Analysis and Homology to *Bet.v.1* Class Allergen—The cDNA of *Hyp-1* sequence has 725 bp with an open reading frame of 480 nucleotides (including stop codons), thus coding for a protein of 159 amino acid residues (Fig. 4, A and B). All positive clones analyzed contained a 3' non-coding region of 188 nucleotides between the stop codon and a poly(A) tail. Because the cloned gene is considered responsible for biosynthesis of HyH, it was termed *Hyp-1*. A BLAST search using the deduced amino acid sequence of *Hyp-1* produced significant alignments

FIG. 6. *A*, absorbance spectrum of bio-converted HyH using emodin in phosphate buffer, pH 7.0, depicting the maximum absorption ($\lambda_{\max}$) at 588 nm. *B*, kinetic studies with recombinant Hyp-1 ($10 \mu\text{g ml}^{-1}$) protein using different concentrations of emodin ($0.55\text{--}22.2 \mu\text{M}$) in phosphate buffer (pH 7.0), incubated for 10 min at 25°C . The product conversion to HyH was quantified by a spectrophotometric detection at 588 nm. The specific activity is expressed in $\mu\text{g ml}^{-1} \text{min}^{-1}$ of HyH. All K_m and $V_{\max}$ values were calculated by non-linear regression analysis and represented in a Lineweaver-Burk plot. Specific activity measurements were done in triplicate.



with the Bet.v.1/PR-10 family of proteins found in a wide variety of species (Fig. 4B). Protein sequence comparisons revealed significant homology between Hyp-1 and a major apple allergen (Mal d1) with a 45.1% homology, followed by Cor-a-1.04, a major hazelnut pollen allergen (17) with 43.4% identity in amino acid sequence to Hyp-1 (Fig. 4B). Additionally, Hyp-1 showed significant homology with various other classes of allergens, including Pru-a-1, a major cherry allergen (43.1%) (18), Bet.v.1 (41.5%) from hornbeam (19), and a *Fagus saluatica* allergen (44.3%) (20) (Fig. 4B). The amino acid sequence analysis indicates that Hyp-1 has a molecular mass of 17.8 kDa with a pI of 5.54. The motif scan for the sequence of Hyp-1 shows similarity with the Bet.v.1 family (pathogenesis-related proteins) signature motif. The signature G-X₂-[LIVMF]-X₄-E-X₂-[CSTAEN]-X_{8,9}-[GND]-G-[GS]-[CS]-X₂-K-X₄-[FY], which is a characteristic of Bet.v.1, is positioned at 89–120 amino acid residues in Hyp-1.

Expression of Hyp-1 Gene in *H. perforatum*—RT-PCR analysis of the total RNA from different parts of *H. perforatum*, including roots, stems, leaves, and cell cultures, indicates that this gene is present throughout the tissues with elevated transcript levels in dark-grown cell cultures of *H. perforatum* (Fig. 5A). The recombinant *E. coli* line also showed the higher titers of transcripts for Hyp-1 (Fig. 5A). As observed previously, *Hyp-1* gene expression was higher in cells cultured under dark conditions compared with light-grown cells and *in vitro* grown leaves (Fig. 5A) (12, 15).

Southern Analysis—To study the number of genes homologous to *Hyp-1* in *H. perforatum*, genomic DNA was digested with *EcoRI*, *HindIII*, and *BamHI*, resolved on agarose gel, and blotted. None of the enzymes used for digestion cut the *Hyp-1* cDNA sequence. The blot was probed with ^{32}P labeled *Hyp-1* sequence. A single band appeared in all these digestions. All the lanes show one band with stronger hybridization, suggesting the thick band could be due to hybridization with the *Hyp-1* sequence. The southern hybridization of *H. perforatum* with gene-specific probes showed a single band in each lane indicating that just one *Hyp-1* gene exists in *H. perforatum* (Fig. 5B).

Heterologous Expression of Hyp-1 in *E. coli*—The recombinant Hyp-1 protein expressed in *E. coli* was affinity-purified and checked for purity on a 12% SDS-PAGE ("Experimental Procedures"), which showed a single protein band of approximately ~20 kDa (Fig. 5C). This protein size is in accordance with the amino acid sequence analysis that hypothetically indicated that Hyp-1 has a molecular mass of ~17.8 kDa. The possible increase in Hyp-1 size could be due to the incorporation of the His₆ tag (20 additional amino acids) at the N-terminal region of Hyp-1 protein from the pET-28a vector. It was observed that the pure recombinant Hyp-1 protein showed an efficient conversion from emodin to HyH under *in vitro* conditions as visualized and analyzed by HPLC-MS analysis (Fig. 5, D and E).

Biochemical Characterization of Recombinant Hyp-1—The enzymatic activity of the purified recombinant enzyme was

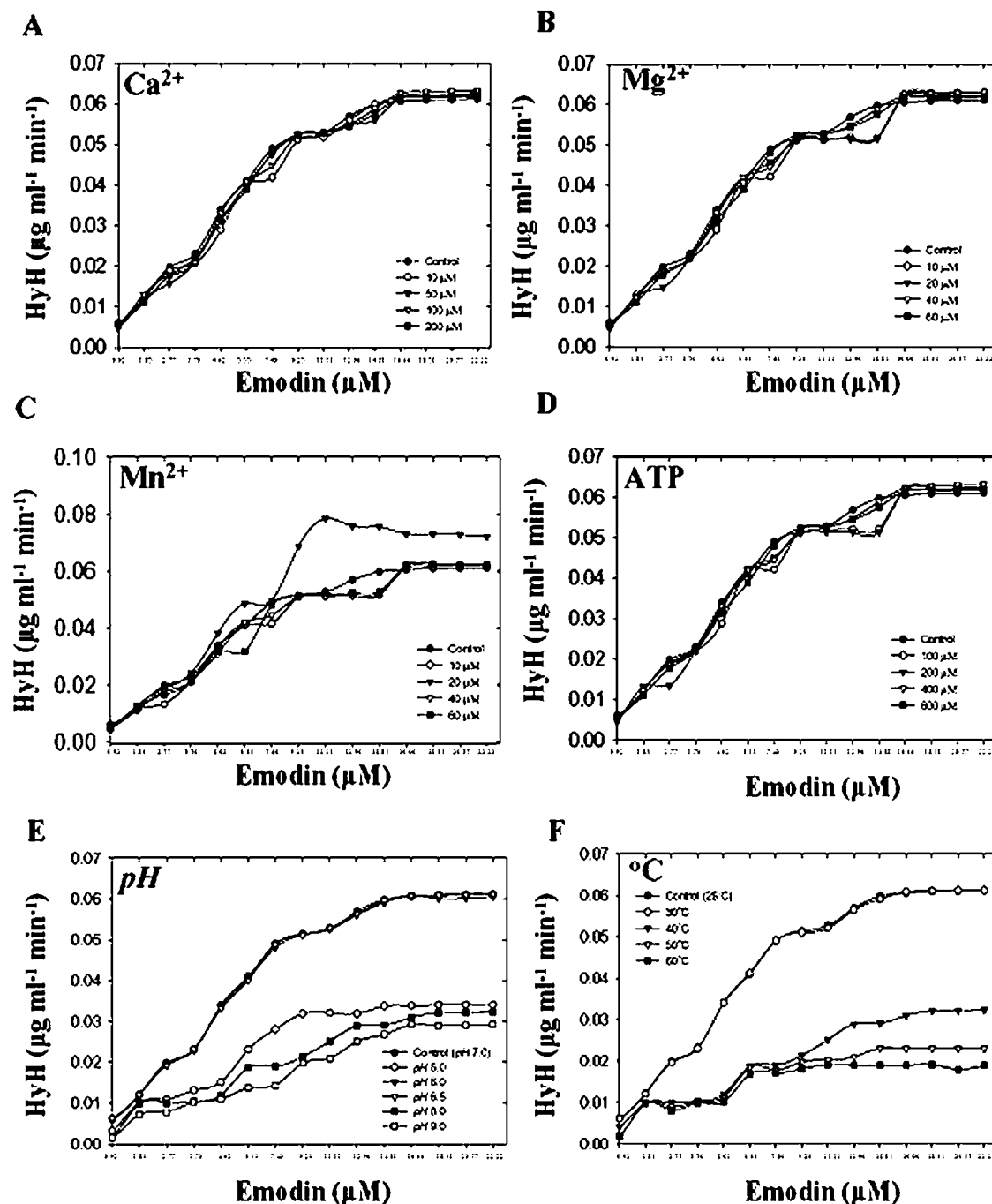


FIG. 7. Effect of various physiological factors on recombinant Hyp-1 activity using different concentrations of emodin (0.55–22.2 μM) in phosphate buffer (pH 7.0), incubated for 10 min at 25 °C. The conversion to HyH was quantified by a spectrophotometric detection at 588 nm. The specific activity is expressed in $\mu\text{g ml}^{-1} \text{ min}^{-1}$ of HyH. A, effect of different Ca^{2+} concentrations (1–200 μM) supplemented with known concentrations of emodin and recombinant Hyp-1. B, effect of different Mg^{2+} levels (1–60 μM) supplemented with emodin and recombinant Hyp-1. C, effect of different Mn^{2+} levels (1–60 μM) supplemented with known concentrations of emodin and recombinant Hyp-1. D, effect of different ATP concentrations (100–600 μM) supplemented with known concentrations of emodin and recombinant Hyp-1. E, effect of different pH conditions (pH 5.0–9.0) supplemented with known concentrations of emodin and recombinant Hyp-1. F, effect of different temperature conditions (25–60 °C) supplemented with known concentrations of emodin and recombinant Hyp-1. The values obtained are mean of three experiments.

checked by varying the concentrations of precursor emodin. A spectrophotometric assay was standardized for quantitative analysis of HyH formation. The UV-visible spectrum of HyH had a strong absorbance at 588 nm (Fig. 6A). The rate of enzymatic activity as judged by product formation (HyH) was directly proportional to the concentrations of the precursor (Fig. 6B). The K_m and $V_{\max}$ values were obtained by non-linear regression of HyH formation with emodin as a substrate. The recorded K_m and $V_{\max}$ values were 7.598 μM and 0.08754 $\mu\text{g ml}^{-1} \text{ min}^{-1}$, respectively (Fig. 6B). The product formation

using emodin at different incubation temperature conditions showed an optimal activity at 25–30 °C (Fig. 7). The enzyme retained ~50% of its activity at 40 °C (Fig. 7). The enzymatic activity of recombinant Hyp-1 was optimum at pH 6.5–7.0 and half-maximal at pH 6.0 (Fig. 7), indicating a rapid decline in enzyme function with increasing acidity. However, the recombinant enzyme was also relatively sensitive to pH increases in the basic range (>pH 8.0) (Fig. 7). Recombinant protein Hyp-1 showed high efficiency conversions to form HyH at temperatures between 25 and 30 °C (Fig. 7) and shows only half the

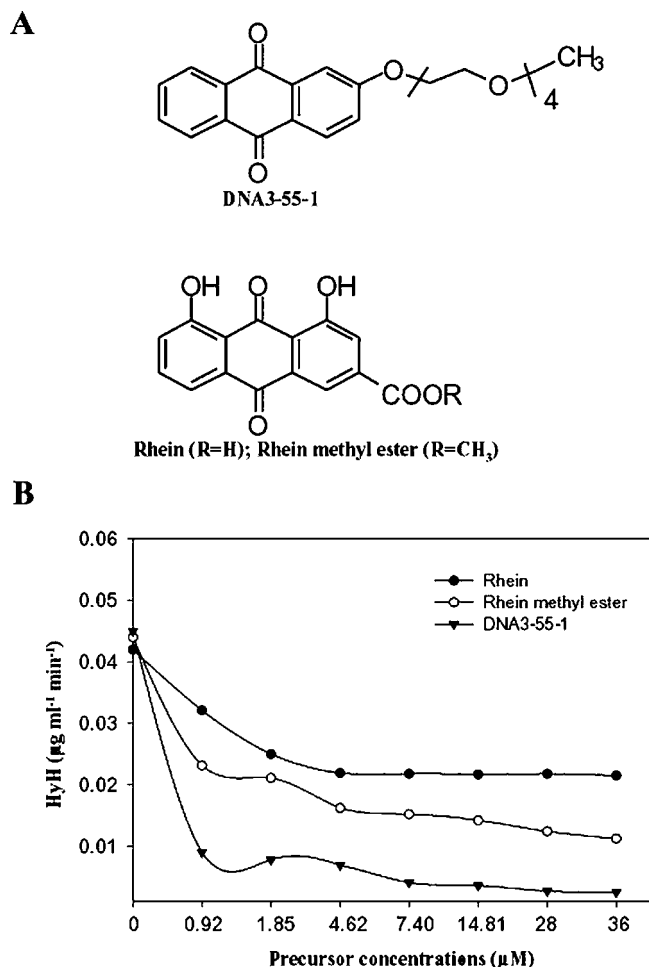


FIG. 8. A, structure analogues of emodin. B, effect of different structure analogues of emodin on production (HyH) inhibition. Structure analogues of emodin, DNA3-55-1, rhein, and rhein methyl ester were prepared at stock concentrations of (0.92–36 μM) in 0.05 M phosphate buffer, pH 7.0, with equivalent concentrations of emodin (0.42 μM). All assays were performed with 10 μg ml⁻¹ of recombinant Hyp-1 protein, with an assay time of 10 min. The product formation was quantified spectrophotometrically.

maximal activity at 40 °C (Fig. 7). The recombinant protein Hyp-1 was unaffected by the inclusion of divalent ions Ca²⁺/Mg²⁺ and ATP in the standard assay (Fig. 7). Hyp-1 showed some dependence on Mn²⁺ for its activity to form HyH (Fig. 7). The optimum activity was obtained at 20 μM Mn²⁺ concentration in a standard assay (Fig. 7).

Product Inhibition of Recombinant Hyp-1—The specific activities of the purified recombinant protein Hyp-1 was determined with structure analogues of emodin such as rhein, rhein methyl ester, and DNA3-55-1 (Fig. 8A). The maximum product inhibition of structure analogues of emodin was observed with the non-polar anthraquinone DNA3-55-1 (Fig. 8B) with 80% inhibition of HyH formation observed with 0.92 μM DNA3-55-1 (Fig. 8B). Total inhibition (~94.5%) of HyH formation occurred when 36 μM DNA3-55-1 was added to the reaction mixture (Fig. 8B). The product inhibition was reduced with polar anthraquinones, rhein, and rhein methyl ester than with DNA3-55-1 (Fig. 8B). Concomitantly, rhein, and rhein methyl ester showed 50% product inhibition at 1.85 and 0.92 μM concentrations, respectively (Fig. 8B). Higher concentrations of rhein and rhein methyl ester (36 μM) resulted in 52.5 and 25% decreases in product formation, respectively (Fig. 8B).

DISCUSSION

The isolation of HyH from *H. perforatum* is not feasible on a large scale because of lengthy procedures caused by other intervening metabolites and the difficulty of separating it from the accompanying pseudohypericin (10, 11). Here we show the direct conversion of emodin to HyH by Hyp-1, a gene isolated from dark-grown cell cultures of *H. perforatum*.

The amino acid sequence of Hyp-1 has a close homology with the Bet.v.1 allergen family. Bet.v.1 is a major allergen from the white birch tree (*Betula verrulosa*). Proteins belonging to the Bet.v.1 family are the main cause of pollen-related food allergies (21, 22), and this protein family shows significant (35–60%) homology to PR (pathogenesis-related)-10 type proteins (23–25), which are expressed in plant tissues upon microbial infection and chemical treatment (26, 27). The deduced amino acid sequence for Hyp-1 comprises several of the signature features of Bet.v.1 class allergens (19). A conserved motif present in Hyp-1 is common throughout all the allergen-coding sequences from the Bet.v.1 family as well as in the PR-10 intracellular PR proteins. PR-10 proteins have been reputed to have kinase and RNase activities (28, 29).

In a recent study it was shown that Bet.v.1 has an affinity to a number of ligands, which include the plant pigments flavone and 4',5,7-trihydroxyflavone (naringenin) (30). It was documented that the Bet.v.5 class allergen later referred to as Bet.v.6 showed close homology to phenylcoumaran benzylic ether reductase as well as to isoflavone reductase (31, 32). Recent studies on recombinant Bet.v.6 and Pyr-c-5, a related protein from pear (*Pyrus americana*), have shown that these proteins convert dehydroadiconiferyl alcohol to isodihydrodehydrodiconiferyl alcohol by reductase activity (31). Phenylcoumaran benzylic ether reductase and isoflavone reductase catalyze a single step, the NADPH-dependent reaction from (±)-dehydrodiconiferyl alcohol and 2-hydroxyformononetin to form (±)-isodihydrodehydrodiconiferyl alcohol and (3R)-vestitone in the isoflavonoid and lignan biosynthetic pathways (31, 32). Additionally, Mal d1 as well as Bet.v.1 class allergens have exhibited a role in cell wall expansion, but no enzymatic activity has been assigned to this function (33). The expression of the Bet.v.1 class allergens is mainly constitutive, and the functional role of these proteins is not yet known.

HyH was detected at a level of 16% (dry weight) in cultured *H. perforatum* cell cultures (12, 15). However, the HyH pool was shown to be localized within a vacuolar compartment in cultured *H. perforatum* cells (12) suggesting that the subcellular trafficking of emodin, and its availability to Hyp-1, represent additional levels of metabolic regulation. Recombinant Hyp-1 exhibited optimum catalytic activity between pH 6.5 and 7.0, did not require Mg²⁺ or Ca²⁺ as reported for other enzymes (31, 32), and displayed a narrow temperature optimum between 25 and 40 °C, suggesting that the enzyme is unstable thermally. Mn²⁺ supplementation resulted in substantial induction of the enzymatic activity compared with other tested divalent ions. The enzyme specificity and interference of anthrone ring compounds in the emodin conversion was tested using structure analogues of emodin. The maximum product inhibition activity of DNA3-55-1 suggests that the large side chain of this emodin analogue and its lack of phenolic (-OH) groups would make it fairly non-polar compared with other analogues. This property could be important for significant inhibition.

The fact that the Hyp-1 sequence showed a close homology to Bet.v.1 class allergens indicates a novel enzymatic function for this group of proteins. Hyp-1 shows a direct and specific bio-conversion of emodin to HyH in the dark. This may involve a condensation reaction followed by dehydration and a sequence

of oxidative cyclizations to yield HyH. We hypothesize that Hyp-1 catalyzes an initial condensation reaction between emodin and emodin anthrone followed by dehydration to form emodin dianthrone. Emodin dianthrone may subsequently undergo phenolic oxidation to protohypericin, which in turn yields HyH. This last reaction is usually photoactivated, but, because our experiments were performed under dark conditions, it rather seems that Hyp-1 may be involved in this oxidative process. Based on our results we concluded that Hyp-1 may catalyze at least four serial reactions, possibly a condensation followed by dehydration and two phenolic oxidative coupling reactions.

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