Heterologous Expression, Isolation, and Characterization of Versicolorin B Synthase from Aspergillus parasiticus

A KEY ENZYME IN THE AFLATOXIN B₁ BIOSYNTHETIC PATHWAY*  

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Jeffrey C. Silva and Craig A. Townsend†‡

From the Department of Chemistry, The Johns Hopkins University, Baltimore, Maryland 21218

Aflatoxin B₁, a potent environmental carcinogen produced by certain strains of Aspergillus, Central to the biosynthesis of this mycotoxin is the reaction catalyzed by versicolorin B synthase (VBS) in which a racemic substrate, versiconal hemiacetal, is cyclized to an optically active product whose absolute configuration is crucial to the interaction of aflatoxin B₁ with DNA. Attempts to over-produce VBS in Escherichia coli led principally to protein aggregated into inclusion bodies but also small amounts of soluble but catalytically inactive enzyme. Comparisons to wild-type VBS by SDS-polyacrylamide gel electrophoresis and after N-glycosidase F treatment revealed that extensive glycosylation accounted for the mass discrepancy (7,000 ± 1,500 Da) between the native and bacterially expressed proteins. Several over-expression systems in Saccharomyces cerevisiae were surveyed in which one that incorporated a secretion signal was found most successful. VBS of indistinguishable mass on SDS-polyacrylamide gel electrophoresis and kinetic properties from the wild-type enzyme could be obtained in 50–100-fold greater amounts and whose catalytic behavior has been examined. The translated protein sequence of VBS showed three potential N-glycosylation sites (Asn-Xaa-Ser/Thr) consistent with the modifications observed above and unexpectedly revealed extensive homology to the ADP-binding region prominently conserved in the glucose-methanol-choline (GMC) family of flavoenzymes. Overproduction of VBS in yeast marks the first aflatoxin biosynthetic enzyme to be so obtained and opens the way to direct study of the enzymology of this complex biosynthetic pathway.

The study of secondary metabolism has advanced rapidly in the last decade from whole-cell experiments to the purification and characterization of individual biosynthetic enzymes. In fewer instances the genes that encode not only these proteins but also all or part of the biosynthetic machinery required to synthesize a fully elaborated natural product have been detected and sequenced. Among these is aflatoxin B₁ (AFB₁), ¹,²

Scheme 1, ⁹, a potent environmental carcinogen produced by the common molds Aspergillus flavus, Aspergillus parasiticus, and Aspergillus nomius. Its oxidative activation and induction of mutations, notably in the p53 gene, has brought into sharp focus the relation of exposure to this mycotoxin and the incidence of human disease (1–3). AFB₁ is synthesized by a complex pathway involving many chemical steps requiring up to 20 different enzymes (4, 5).

Contrary to earlier reports (6–8), the genes in A. parasiticus responsible for AFB₁ formation appear to be clustered (9, 10). Two of these have been localized by complementation of classically derived blocked mutants and confirmed by gene disruption (11, 12). Similarly, an O-methyltransferase has been purified and its corresponding gene cloned (13). All three of these genes have been linked to within 45 kilobase pairs of each other (9). Homology data from the genomic DNA regions flanking these genes has revealed among other things a pair of yeast-like fatty acid synthase subunits (12) and a putative polyketide synthase (14). An important tool has been developed to help determine the function of these encoded proteins by gene disruption, allowing the insertion of DNA at specific genomic sites to generate rationally blocked mutants (15). Recent application of this method identified the pair of fatty acid synthase subunits identified above as composing a specialized synthase whose function is to prepare an unusual hexanoyl starter unit (Scheme 1, bold in 1) for further elaboration by the proposed polyketide synthase (10, 14–16). Biochemical experiments have both foreshadowed (17) and confirmed these implications (18).

In this paper we report a yeast expression system for the over-production of proteins involved in AFB₁ biosynthesis. While inactivation of biosynthetic genes affords important information about the function of the encoded proteins in the study of natural product biosynthesis, these observations alone may not be sufficient to completely understand their biosynthetic role or the extent of their association, if any, required for catalysis. A case in point is VER-1, a classical A. parasiticus mutant that accumulates versicolorin A (7) (19). Complementation has identified the gene ver-1 as the site of lesion in this mutant (11). Insertional inactivation of this gene leads to the accumulation of versicolorin A (7), confirming this identification. The apparent function of VER-1 in the synthesis of demethylstigmatisocystin (8, R = R’ = H) may be assumed from a 56% amino acid sequence identity to skytalone reductase, an enzyme well-documented to catalyze an aryl dehydroxylation in the biosynthesis of melanin (20). The conversion of anthra-phosphate isomerase; PCR, polymerase chain reaction; bp, base pair(s); PMSF, phenylmethylsulfonyl fluoride; FPLC, fast protein liquid chromatography; PNGase F, N-glycosidase F; bg, bovine growth hormone; MES, 4-morpholineethanesulfonic acid; MOPS, 4-morpholinepropane-sulfonic acid; HMPA, hexamethylphosphoramide.

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† To whom correspondence should be addressed: Dept. of Chemistry, The Johns Hopkins University, 3400 N. Charles St., Baltimore, MD 21218, Tel.: 410-516-7444; Fax: 410-516-8420; E-mail: Townsend@jhunix.hcf.jhu.edu.
‡ The abbreviations used are: VBS, versicolorin B synthase; GMC, glucose-methanol-choline; PAGE, polyacrylamide gel electrophoresis; EDC, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride; DEPC, diethyl pyrocarbonate; IEF, isoelectric focusing; TPI, triose-phosphate isomerase; PCR, polymerase chain reaction; bp, base pair(s); PMSF, phenylmethylsulfonyl fluoride; FPLC, fast protein liquid chromatography; PNGase F, N-glycosidase F; bg, bovine growth hormone; MES, 4-morpholineethanesulfonic acid; MOPS, 4-morpholinepropane-sulfonic acid; HMPA, hexamethylphosphoramide.
quione 7 to xanthone 8 involves ring cleavage, rearrange-
ment, deoxygenation, and decarboxylation and very well may
require several enzymes as has been presumed to be the case in
A. nidulans (21). The degree to which these proteins interact to
achieve catalysis remains to be established. In the event that
6-deoxyversicolorin A (7, 6-OH → 6-H) is not incorporated into
AFB1 (9) strongly suggests that aryl dehydroxylation is not the
first step in xanthone formation (contrary to the implications
drawn from the previously described homology data) (20).
Therefore, study of these individual proteins and, if necessary,
in association with one another may be required to arrive at a
complete understanding of their true catalytic roles in the
pathway (9, 18). The development and application of an over-
production system suitable for these studies is described for the
first time for VBS, an enzyme central to the biosynthesis of
AFB1 (22).

VBS has recently been isolated from A. parasiticus as a
dimer with an apparent monomeric molecular mass of 78 kDa
as estimated by SDS-PAGE (22). In the absence of metals and
cofactors, VBS catalyzes the dehydrogenation of racemic
versicolorin hemiacetal (6) to optically active (−)-versicolorin B (6/22–24). In this reaction the intrinsic stereochemical liab-
ility of the substrate (the chiral center is benzylic and adjacent
to a masked aldehyde) provides a mechanism of enantiomeric in-
version of a specific configuration with a t½ of ~45 min (22). VBS efficiently binds the correct antipode of 5 (Kₘ = 1 μM) and catalyzes tetrahy-
drobenzofuran ring formation with concomitant loss of water.
The absolute configuration of the tetrahydrobenzofuran set in this
reaction is critical to the DNA interactive properties of meta-
bolically activated AFB₁ (9) ultimately produced (25–27).

The VBS gene has been cloned and sequenced from both
cDNA and gDNA libraries of A. parasiticus and has been found
to bear significant similarity at the transcribed amino acid
level to several flavin-dependent oxidases and dehydrogenases,
notably glucose oxidase from Aspergillus niger (38% identity
and 58% similarity) (9). This was a surprising finding since
VBS does not catalyze a redox reaction and does not bind FAD
or FMN despite an apparent, albeit truncated, FAD binding
domain (9). These observations have led us to over-produce this
protein for the purpose of further mechanistic study.

Isolable amounts of the native enzyme are low, between 15
and 30 μg of VBS per liter of A. parasiticus culture (22, 28).
Attempts to over-produce catalytically active VBS in Esche-
richia coli were unsuccessful due to the formation of inclusion
bodies and low amounts of soluble but inactive protein. At-
tempts to re-fold and reactivate the solubilized VBS failed.
Reports of expression of glucose oxidase cloned from A. niger
into Saccharomyces cerevisiae encouraged our efforts to use
yeast as a host for the heterologous expression of versicolorin B
synthase (29). S. cerevisiae has been used with increasing fre-
quency for the secretion of heterologous proteins (30–35) in
expression vectors having various secretion signals to facilitate
the active transport of the over-produced proteins (29, 36–38).
Secretion of an over-produced protein simplifies isolation and
eliminates exposure to endogenous proteolytic enzymes allowing
a greater yield and is thought to yield more uniform co-
and post-translational modifications (39). This strategy has also
been exploited with the development of “super-secreting” S.
cerevisiae strains that provide the added advantage of a 5–250-
fold enhancement in protein accessibility compared with that of
wild types (38, 40–43). In this paper we report the purification
and characterization of recombinant VBS from A. parasiticus
over-produced in S. cerevisiae in a protocol of general utility to
enable understanding of aflatoxin biosynthesis at the enzyme-
level.

EXPERIMENTAL PROCEDURES

Materials—The following were obtained from Sigma: benzamidine
hydrochloride, ammonium sulfate (grade III), 1,4-dithio-
threitol, sodium dodecyl sulfate (SDS), ampicillin (sodium salt), N-ethylmale-
ime, isocitric acid, 4-chloromercuribenzoate acid, O-methy-
lorea, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC),
diethyl pyrocarbonate (DEPC), and all metal salts. Bacto-agar, yeast
extract, yeast nitrogen base without amino acids or ammonium sulfate,
and Bacto-tryptone were purchased from Difco. CSM (− URA) dropout
mix was obtained from BIO-101 (Natick, MA). The collodian apparatus
and membranes were purchased from Schleicher & Schuell. PNGase F
was obtained from New England BioLabs (Beverly, MA). The following
were obtained from Stratagene (La Jolla, CA): Pfu DNA polymerase, T4
DNA ligase, ethidium bromide, pBluescript-II SK(−), E. coli XLI Blue
cells. Restriction enzymes were obtained from both Stratagene and New
England Biolabs. The following were purchased from Life Technologies Inc.: ultra-pure urea, acrylamide, N,N'-methylenebisacrylamide, and
calf alkaline phosphatase. Opti-fluoro® O Scintillation Mixture was ob-
tained from Packard (Meriden, CT). PCR experiments were performed
using an Eppendorf Micrcycler (Fremont, CA). The CH2-Ultrafiltration
System was purchased from Amicon, Inc. (Beverly, MA). Kaleido-
scope Prestained Standard molecular mass standards were purchased
from Bio-Rad. Chemiluminescence detection was carried out using
Expression of VBS in S. cerevisiae

CSPD<sup>®</sup> development reagents for alkaline phosphatase, and biotinylated SDS-PAGE molecular weight markers were all purchased from Tropix (Bedford, MA). Western blots were performed using a Bio-Rad Trans-Blot Electroblotter and Tropix, polyvinylidene fluoride (Tropifluor) membranes. Mouse monoclonal antibody, 12CA5, for the influenza hemagglutinin epitope tag, 3xHis, was purchased from Boehringer Mannheim.

Strains and Vectors—Both the E. coli expression vector pT7-7 and the E. coli host strain K-38 carrying pGPI-2 were gifts from Professor S. Tabor at Harvard Medical School, Dept. of Biological Chemistry, Boston, MA. The S. cerevisiae expression vector pCGS861 was kindly provided from Dr. J. T. Moir at Collaborative Research (Mannheim). The vector immediately downstream of the signal sequence. However, the existence of a second NcoI site in the expression vector complicated its utility. The VBS secretion vector pCGSVBS was thus constructed by introducing the source plasmid pCGS861 into two fragments because of the second NcoI site within the URA3 selectable marker. The plasmid pCGS861 was treated with EcoRI and SalI, and the resulting two fragments, named the TPI/BGH fragment and the pCGS fragment, were purified from low melting agarose (51). The TPI promoter-containing fragment TPI/BGH was subcloned into the EcoRI and SalI sites of pBSIISK<sup>®</sup>, which contains no NcoI site, to make pBSTPI/BGH. The linearized yeast episomal fragment of pCGS861, the pCGS fragment (with EcoRI and SalI overhangs), was purified with low melting agarose and used later to make the final construct. The carrier vector, pBSTPI/BGH, was treated with both NcoI and SalI to remove the BGH cDNA insert from the TPI promoter region. The pBSTPI fragment, containing the SUC2 signal sequence immediately downstream of the TPI promoter, was purified from the digestion mixture through a 1% low melting agarose gel and used for further cloning. The vbs insert for the yeast expression vector was prepared from two fragments owing to the presence of two NcoI sites within the coding DNA. The first vbs fragment, the 5'-end, was produced by PCR, and the second vbs fragment, the 3'-end, was obtained as a restriction fragment from the cDNA clone, c241e (9). A restriction site was introduced by PCR into the first vbs fragment, the 5'-end of the vbs gene, using a 42-bp oligonucleotide primer (5'-CCATGGGACGAAACTGGTAGATGGCGGTTGG-3') as the upstream primer and an 18-bp internal vbs oligonucleotide primer, 31NC (5'-ACGGCAGCATTGTGTTCC-3'), as the downstream primer. The downstream primer was homologous to a region 606 bases downstream of the start site of the vbs gene. The upstream primer was designed to engineer a single NcoI site at the translational initiation codon (ATG). This change was effected through incorporation of a cytosine just upstream of the start codon (bold) and through alteration of the nucleotide C297 (bold/underlined nucleotide) within the coding sequence to remove an NcoI site immediately downstream of the start codon but not change the amino acid encoded at this site. The PCR reaction was carried out under normal conditions as described by Lundberg et al. (44) using 30 cycles of 1 min, 95°C denaturation step, a 30-s, 53°C annealing step, and a 3-min and 30-s, 74°C polymerization step. The vbs PCR product was treated with dCTP using terminal deoxynucleotidyltransferase following the procedure outlined by Smith et al. (45). The modified vbs PCR product was subcloned into dCTP-tailed PsiI cut plasmid pBSIISK<sup>®</sup> vector. The N-terminal primer contained an NdeI restriction site (underlined nucleotides) which was introduced into the vbs gene and was then treated with calf intestinal alkaline phosphatase. The linearized pBSIISK<sup>®</sup> vector, pBSIISK(GC)<sub>1900</sub>, was digested with NdeI and XhoI to excise the vbs fragment, purified by electrophoresis as described by the manufacturer (Bio-Rad), and subcloned into the NdeI and SalI sites of pT7-7 (47, 48) to give the E. coli vbs expression vector pT7-7-vbs. The sequence of vbs in the pT7-7-vbs expression vector was verified using either commercially available or custom-synthesized oligonucleotide primers.

Expression of E. coli-derived VBS— Cultures of E. coli K-38 cells (100 ml) containing both pGPI-2 and pT7-7vbs (47, 48) were prepared by inoculating a single colony into 500 ml of LB medium (90 µg/ml ampicillin and 50 µg/ml kanamycin) and grown on a rotary shaker (200 rpm) at 37°C to ~3-2 x 10<sup>8</sup> cells/ml (A<sub>600</sub>nm = 0.40). Expression of the E. coli-derived VBS was induced by placing the flask of cells in a 42°C water bath for 20 min. Expression of VBS was then allowed to cool, and the cells were harvested by centrifugation at 5,000 g for 10 min, frozen in liquid nitrogen, and stored at −80°C.

Partial Purification of E. coli-derived VBS Inclusion Bodies— Cells (1 g wet weight) were suspended in 10 ml of breaking buffer (50 mM potassium phosphate (pH 7.4), 20% glycerol, 1 mM EDTA, and 0.25 mM PMSF) and incubated for 30 min on ice. Acid-washed glass beads (~2 g, 0.5-10 mm diameter) were added, and the cell suspension was vortexed five times for 1-min intervals and set on ice for 2-min intervals. The cell debris and inclusion bodies were collected by centrifugation (10,000 x g) for 45 min and washed twice with breaking buffer supplemented with 0.25% Triton X-100 and 10 mM EDTA. The precipitate containing the inclusion bodies was solubilized for 12 h at room temperature in 10 ml of breaking buffer supplemented with 6 M urea. The solution was centrifuged (10,000 x g) for 45 min, and then the supernatant was dialyzed overnight (2 h) against low salt buffer (5 mM potassium phosphate (pH 7.4), 20% glycerol, 1 mM EDTA, and 0.25 mM PMSF) containing 3, 1.5, 0.75, and 0 M urea (49, 50).

Construction of S. cerevisiae Expression Vector, pCGSVB53xf—A NofI site was engineered into the cDNA clone c241e just upstream of the stop codon of vbs. Two PCR primers, NotITag1R (5'-GGCGCGCG- CCTGCGCGAGCCATCTTC-3') and NotITag2R (5'-GGCGCG- CCGTAGCACGGTGGTGCAGTCCG-3'), were designed to incorporate a NotI site just upstream of the stop codon and an additional nucleotide (cytosine) upstream of the NotI site. These primers were used in separate PCR reactions with T3 and T7 primers and used for limiting the size of the vector, T3 and T7 primers, to amplify two fragments of vbs from c241e. The two PCR products were ligated after treatment with NotI and then purified from low melting agarose. The ligated PCR product (VBS(NofI)) was then cut with EcoRI and SalI, allowing the TIP/VBS fragment (~2900 bp) to be purified using low melting agarose (51). Finally, the secretion vector pCGSVBS (Fig. 1) was completed by ligating the EcoRI/SalI cut TPI/VBS fragment from pBSTPI/VBS to the EcoRI/SalI cut pCGS fragment isolated from the pCGS861 plasmid. The sequence of vbs in the pCGSVBS expression vector was verified using either commercially available or custom-synthesized oligonucleotide primers.

Construction of S. cerevisiae Expression Vector, pCGSVBS3xf—A NofI site was engineered into the cDNA clone c241e just upstream of the stop codon of vbs. Two PCR primers, NotITag1R (5'-GGCGCGCG- CCTGCGCGAGCCATCTTC-3') and NotITag2R (5'-GGCGCG- CCGTAGCACGGTGGTGCAGTCCG-3'), were designed to incorporate a NotI site just upstream of the stop codon and an additional nucleotide (cytosine) upstream of the NotI site. These primers were used in separate PCR reactions with T3 and T7 primers and used for amplifying two fragments of vbs from c241e. The two PCR products were ligated after treatment with NotI and then purified from low melting agarose. The ligated PCR product (VBS(NofI)) was then cut with EcoRI and XhoI for ligation into EcoRI/XhoI cut pUC19, to produce pUC19VBS/(C/NotI). The plasmid pUC19VBS/(C/NotI) was linearized with NotI and the 3xf NotI cassette (epitope tag) was ligated into the vector to create pUC19VBS3xf. The plasmid pUC19VBS3xf was linearized with KpnI and filled-in using Klenow fragment. The blunt-ended, linearized pUC19VBS3xf was then digested with EcoRI, and the 3'-end of the modified vbs gene fragment containing the epitope tag was purified from the partially digesting agarose. The restriction vector was linearized with KpnI and filled-in using Klenow fragment. The blunt-ended linearized pCGSVBS was then digested with EcoRI and, the YEp portion of pCGSVBS (EcoRI/blunt) vector was purified using low melting agarose. The 3xf SalI fragment (EcoRI/blunt) was then ligated into the YEp portion of pCGSVBS (EcoRI/blunt) to create the epitope-tagged secretion expression vector, pCGSVBS3xf.
Expression of VBS in S. cerevisiae

Yeast Transformation and Growth—Yeast transformations were carried out according to Hinnen et al. (52). All transformants were obtained on (URA) selective plates containing 3% glucose. Inocula were grown in uracil-selective medium containing 3% glucose for 2 days and diluted 1:10 into uracil-selective medium containing 3% glucose and potassium phosphate (pH 7.4) with 20% glycerol and 1 mM EDTA. Secreted VBS activity was determined after diluting media 10-fold in 50 mM potassium phosphate (pH 7.4) with 20% glycerol and 1 mM EDTA.

Western Analysis of VBS—For Western blot analyses, epitope-tagged fusion protein VBSxFk, an aliquot of the expression medium (1 ml) was denatured in SDS Gel Loading Buffer (50 mM Tris (pH 6.8), 2% β-mercaptoethanol, 2% SDS, 10% glycerol, and 0.1% bromophenol blue) and subsequently analyzed by SDS-PAGE. Prestained and biotinylated molecular mass standards were simultaneously electrophoresed. The proteins were then transferred onto a polyvinylidene fluoride membrane and were detected using mouse monoclonal antibody 12CA5 for the influenza hemagglutinin epitope tag, 3x (Figs. 6 and 7).

Purification of S. cerevisiae-derived VBS—All steps were carried out at 0–4°C. The yeast expression vector encoding the sucl2 gene sequence fused to the coding sequence of VBS from A. parasiticus under the control of the TPI promoter was used to express secreted VBS in S. cerevisiae. Functional VBS was easily detected in the culture medium after 72–96 h of growth. The cells were removed by centrifugation, and the clarified medium was concentrated ~20-fold by ultrafiltration using the CH2-System from Amicon equipped with a fluoride membrane and were detected using mouse monoclonal antibody 12CA5 for the influenza hemagglutinin epitope tag, 3x (Figs. 6 and 7).

Mono Q FPLC—A Mono Q HR5/5 FPLC anion exchange column (5 × 50 mm; Pharmacia) was equilibrated at room temperature with 50 mM potassium phosphate (pH 7.5) and 20% glycerol (0.5 ml/min). One ml of active Rotofor sample was loaded, and the column was rinsed with ~50 ml of buffer. A 2-h linear gradient (60 ml) was then initiated from 0 to 300 mM sodium chloride, and the eluent was monitored at λ = 280 nm. Fractions were collected between 155 and 145 ml NaCl (Table I and Fig. 4). Deglycosylation of VBS—VBS stock solutions (5 mg/ml) were made in 50 mM potassium phosphate (pH 7.4) with 20% glycerol. Reactions were conducted in 50 mM sodium phosphate (pH 7.5) containing 0.5% SDS, 1% β-mercaptoethanol, 1.0% Nonidet P-40, and 10% of Millions of P-Nase G. Samples were denatured at 100°C for 10 min before adding PNGase F, and incubations were carried out at 37°C for 15 h. Samples were analyzed by SDS-PAGE (Fig. 51). Enzyme Assay—VBS assays were performed in a volume of 0.1 ml in 50 mM potassium phosphate (pH 7.4) containing 30 μM [3H]versicolanic acid (specific activity of 9.3 × 10^12 dpm/mmol) as described elsewhere (22). Determination of K_m and V_max for Yeast-derived VBS—from a solution of [3H]versicolanic hemiacetal dissolved in assay buffer (~50 mM, 1% HMPA), serial dilutions were carried out to obtain six different substrate concentrations, each of which was determined radiochemically and found to be 71.6, 6.7, 2.9, 1.5, 1.2 and 1.0 μM, respectively. These were incubated in quadruplicate for 12.5, 12, 11.5, 11, 10.5, and 10 min, respectively, with and without purified VBS (6.0 μg/ml). The results from these assays were averaged and calculated as μM versicolin B produced per min from incubations with enzyme with different substrate concentrations. Enzyme activity was calculated using a Lineweaver-Burk analysis of the data (55) and found to be 6.5 ± 0.6 μM and 0.096 ± 0.006 μM/min, respectively; the K_m for expressed VBS was ~2.1 ± 0.2 s^-1.

Evaluation of the Effects of Metals on Enzyme Activity—VBS (5 μg/ml) was preincubated at 37°C for 20 min with metal ions at 1.0 mM in 0.05 ml of 10 mM potassium phosphate buffer (pH 7.0) containing 20% glycerol. Tubes containing only VBS and phosphate buffer were used as controls. After preincubating for 20 min, an equal volume of 30 μM [3H]versicolanic hemiacetal (specific activity 9.3 × 10^12 dpm/mmol) in 20 mM potassium phosphate buffer with 20% glycerol was added. VBS activity was determined as described above. The effect of metal ions on VBS activity was determined at concentrations of 1.0 mM. All metal ions were added to the reaction mixture, and VBS was preincubated with VBS (5 μg/ml) for 30 min at 37°C before assaying for VBS activity. In protection experiments, the preincubation mixture contained radioinactive versicolanic hemiacetal (10 μM) in addition to the chemical modifying reagent, before 10-fold dilution in Assay Buffer (50 mM potassium phosphate (pH 7.4) with 20% glycerol and 1 mM EDTA) to test for the remaining activity in the presence of 30 μM [3H]versicolinic acid (Figs. 6 and 7).

VBS was inactivated with DEPC diluted with ethanol. The ethanol concentration in the reaction mixture never exceeded 1% (v/v). Concentration-dependent inactivation of VBS (3 μg/ml) with DEPC was carried out in 50 mM potassium phosphate (pH 6.0) with 20% glycerol by preincubating at 25°C for 30 min with increasing concentrations of DEPC (1.0, 3.0, and 10.0 mM). Time-dependent inactivation of VBS with DEPC was carried out for 30 min at 25°C in the presence of 10 mM DEPC, whereas aliquots were removed at 3, 12.5, 25, and 30 min to test remaining VBS activity (Fig. 6). Concentration-dependent inactivation of VBS (2 μg/ml) with EDC was carried out in 150 mM MOPS (pH 6.0) containing 50 mM glycine ethyl ester, 20% glycerol, and 1 mM EDTA by preincubating at 25°C for 30 min with increasing concentrations of EDC (1.0, 3.0, and 10.0 mM) (56, 57). Time-dependent inactivation of VBS by EDC was carried out using 1 mM EDC hydrochloride, whereas aliquots were withdrawn at 3, 12.5, 25, and 30 min to test remaining VBS activity (Fig. 7). Inhibition Studies—Radiochemical assays were performed as described above using purified VBS (3.0 μg/ml). Initial inhibition experiments with VBS were conducted using radiochemical assays containing either 2-hydroxyphenylacetic acid lactone (10, 20 μM), 2,5-dihydroxyphenylacetic acid lactone (11, 20 μM), emodin (12, 75 μM), benzo-furan diol (2,3-dihydroxy-3-benzo furan ether (13, 50 μM), or versicolin B (6, 15 μM) to determine the relative rates of versicolin B formation (Table III). The K_m of benzofuran diol (13) was carried out at varying concentrations of inhibitor (0, 100, and 200 μM), whereas the versicolinic acid (15) concentrations were varied from 1.25 to 30 μM (1.25, 2.50, 10.0, and 30.0 μM, Fig. 10). The enzyme assays were carried out for 10 min at 37°C. Kinetic data were test-fitted to the equations appropriate for competitive, noncompetitive, and uncompetitive inhibition (59).

Effect of Flavin Cofactors on VBS Activity—In 1.5-ml microcentrifuge tubes, protein (10 μg) of the corresponding cofactor solution (FAD, FMN, or riboflavin; 100 μM), 80 μl of [3H]versicolinic hemiacetal dissolved in assay buffer (~100 μM, 1% HMPA), and 10 μl of active, purified VBS (5 μg/ml). The assays were carried out with both native and yeast-derived VBS. Each assay was performed in duplicate and compared with reactions containing no cofactor (assay buffer), with and without enzyme. The reactions were quenched with ether:hexanes:acetic acid (1:1.0:0.01) after 15 min at 37°C, and the results were reported in terms of the amount of [3H]versicolinic B produced per min above background levels.

Effect of Glucose on VBS Activity—Serial dilutions of a 2.0 M glucose solution (10 mM potassium phosphate (pH 7.4) and 20% glycerol; “assay buffer”) were performed to obtain 200.0, 20.0, and 2.0 mM concentrations of sugar. In 1.5-ml microcentrifuge tubes were mixed 50 μl of a glucose solution, 40 μl of assay buffer, 2 μl of [3H]versicolinic acid (10 μCi), and 10 μl of active, purified VBS (5 μg/ml). The assays were conducted with both native and yeast-derived VBS. Each glucose concentration (100.0, 1.0, and 0.1 M) was performed in duplicate and compared with reactions containing no glucose (assay buffer), with and without enzyme. The reactions were quenched with ether:hexanes:acetic acid (1:1.0:0.01) after 15 min at 37°C, and the
Expression of VBS in *S. cerevisiae*

Results were reported in terms of the amount of \[^{3}H\]versicolorin B produced per min above background levels.

Other Methods—UV/VIS spectrophotometry employed a Beckman DU 70 Spectrophotometer (Fullerton, CA). Protein concentration determination assays were performed by the method of Bradford (60) using bovine serum albumin as a standard. SDS-PAGE employed the buffer system of Laemmli (61) in a Hoeffer SE 400 Slab Gel Electrophoresis Unit (San Francisco, CA).

RESULTS

Utilization of the pTT7-7/pGP1-2 dual plasmid expression system (47, 48) allowed the tightly regulated expression of inactive VBS in *E. coli* at levels between 30 and 40% of total *E. coli* protein. Although the levels of expressed protein were impressive, we were unable to obtain cell-free extracts with catalytically active VBS. Further analysis of the protein extracts revealed the substantial production of inclusion bodies and small amounts of soluble protein upon heat-shock induction. Attempts were made to denature and refold the aggregated protein by dialysis and elution through a Sephadex G-25 desalting column (49, 62). While catalytically active protein was never obtained, despite several attempts, it was observed that the *E. coli*-derived VBS has an apparent molecular mass of ~70 kDa, similar to that predicted by translation of the gene sequence, but significantly less than the 78-kDa mass found for the wild-type enzyme (22). Three potential N-glycosylation sites had been noted earlier from the deduced protein sequence of VBS (Asn-Xaa-Ser/Thr) (9) suggesting that the discrepancy in molecular masses between bacterially expressed protein and native VBS owed to post-translational carbohydrate attachment. Wild-type VBS was treated with (PNGase F) to test this hypothesis and found to give a protein whose behavior on SDS-PAGE was indistinguishable from the *E. coli*-produced protein (see below and Fig. 2). An alternative host for heterologous expression of VBS which was capable of the apparently necessary post-translational modification was sought.

*S. cerevisiae* has been used as a host for the heterologous expression of eukaryotic genes including those from *Aspergillus*. Tatsumi and co-workers (33) have expressed functional alkaline protease in *S. cerevisiae* identical to the native protein using cDNA encoding prepro-alkaline protease from *Aspergillus oryzae*. Further evidence of its utility has been demonstrated by Hata and co-workers (31) who have successfully expressed glucoamylase from *A. oryzae* in an active form. Most promising among these, however, was a report by Frederick and co-workers (29) who were able to express glucose oxidase from *A. niger* in *S. cerevisiae*. This success was particularly relevant because we had previously determined that VBS was most homologous to glucose oxidase of the GMC oxidoreductase whose gene sequences are known (9). In addition, glucose oxidase shares many physical characteristics to those of VBS. 1) Both proteins are dimers of similar molecular weight, 2) both proteins have similar degrees of N-glycosylation; 3) both proteins have prominent amino acid identities in the highly conserved FAD binding motif characteristic of FAD-dependent oxidoreductases; 4) both proteins have a similar pl and, last, 5) both proteins come from the closely related *Aspergillus* species.

The plasmid pCGSVBS1 is a 2-μm-based *S. cerevisiae* plasmid carrying the URA3 auxotrophic selectable marker (37, 39). It carries the ColE1 origin of replication from pBR322 and the ampicillin resistance marker for *E. coli* propagation of the plasmid. Expression is controlled by the constitutive *S. cerevisiae* TPI promoter, which has the suc2 (sucrase) secretion signal downstream of the promoter. Heterologous expression and secretion of VBS was accomplished by subcloning the coding cDNA of *vbs* into the YEp portion of the pCGSVBS1 yeast expression vector to make pCGS VBS, thus fusing the Suc2 secretion signal sequence to the ATG-start of the versicolorin B synthase gene (Fig. 1).

In order to facilitate monitoring small amounts of heterologously expressed protein in *S. cerevisiae*, an epitope tag was engineered into the 3'-end of the VBS coding sequence just before the stop codon to make a second expression plasmid, pCGSVBS3xf (Fig. 2). The epitope tag 3xf was derived from the influenza hemagglutinin protein and possessed the repeating amino acid sequence of YPYDVPDYA. The monoclonal antibody 12CA5 recognizes this primary amino acid sequence and was used for Western analysis of the protein derived from the yeast expression plasmid pCGSVBS3xf. The epitope tag was subcloned into the VBS sequence of the expression plasmid as an infram 119-bp NotI cassette (Fig. 2). The fragment of DNA (3xf) provided the coding DNA sequence for the epitope tag, repeated three times. Western analysis of the epitope-tagged expression system revealed the constitutive expression of VBS (Fig. 3). However, VBS activity was not obtained from the epitope-tagged expression system, presumably due to C-terminal modification of the protein. However, catalytic activity was realized using the nonepitope-tagged expression vector pCGS-VBS (Fig. 1).

Heterologous VBS secretion was measured from a variety of yeast strains to select one that optimally produced VBS. Together with three wild-type yeast strains, four super-secreting mutants were tested (39, 40). It was found that the super-secreting mutant CGY2998 (39) produced approximately 25-fold more VBS than any of the wild-type strains of *S. cerevisiae*. This significant increase in VBS production made the detection of active VBS in the crude expression shake flasks noticeably easier and was the first step toward successfully isolating
Expression of VBS in *S. cerevisiae*

In unbuffered shake flask cultures no active VBS was isolable due to the natural pH decrease in growing *S. cerevisiae* cultures. To minimize this effect, two separate buffered (−URA) dropout media were developed which also allowed determination of the optimal pH for heterologous secretion of VBS. Over 90 h of expression the pH of both buffered media was stable to within 0.4 pH units. It was observed that the optimal pH for expression was 6.5 and that there was no significant difference in active protein expression using either of the two buffering systems. There was approximately a 2-fold increase in expression levels of active VBS when the expression of VBS was carried out at pH 6.5 versus pH 5.75–6.25.

The effect of growth temperature on VBS secretion was also investigated in shake flask experiments. When normalized for activity (units/g (lyophilized dry cell weight)), expression conditions were optimized at 25 °C. The increase in yield was approximately 30% compared with either 22.5 or 27.5 °C. Utilizing the super-secreting *S. cerevisiae* strains under the optimal conditions for VBS production (25 °C and pH 6.5), we have been able to produce 2–8 mg of active VBS per liter of culture.

Purification of the *S. cerevisiae*-derived VBS was carried out employing ultrafiltration followed by preparative isoelectric focusing (IEF) and Mono-Q anion exchange FPLC (Fig. 4 and Table I). The generated protein extract of the medium was concentrated and filtered through a 30,000 molecular mass cut-off spiral wound membrane before being ultradialyzed. The resulting protein extract was prepared for isoelectric focusing by further concentration before subsequent dialysis. The yeast-derived VBS was efficiently separated from the majority of the contaminating secreted yeast proteins. In an electric potential the majority of these proteins precipitated between pH 5.0 and 7.0. IEF experiments indicated that the yeast-derived VBS possessed a pI of 4.6 ± 0.1 (native pI = 4.7 ± 0.1) (22). Homogeneous yeast-derived VBS was afforded by Mono-Q FPLC anion exchange chromatography. After the column was equilibrated in 50 mM potassium phosphate with 20% glycerol (pH 7.5), a linear gradient from 0 to 300 mM NaCl gave the active protein at a chloride concentration between 135 and 145 mM. An average of 1.2 mg of heterologously expressed VBS was obtained per liter of yeast expression culture (50 g of cells/liter, wet mass). The purification of VBS was carried out in two steps with an overall 185-fold purification (Fig. 4 and Table I). Homogeneity was determined by visualization of a single 78,000 ± 1,500-Da protein band in a Coomassie Blue-stained SDS-PAGE gel, experimentally indistinguishable from the wild-type enzyme (Fig. 5).

Treatment of both native and yeast-derived VBS with PNGase F resulted in deglycosylated proteins of indistinguishable molecular mass as determined by SDS-PAGE (70,000 ± 1,500 Da). The molecular mass shift of 7,000 ± 1,500 Da is believed to reflect similar patterns of glycosylation. This shift in apparent molecular mass is not seen when *E. coli*-derived VBS is treated with PNGase F. In fact, the molecular mass observed with both PNGase-treated and nontreated *E. coli*-derived VBS appears to be that expected from the translated cDNA of *vbs* (~70,000 Da) (9).

A radiochemical assay was developed to accurately monitor the production of versicolorin B (6) by VBS. The ability to incorporate acetate into AFB1 (9) was exploited as an efficient means to obtain radiolabeled substrate. A simple method of separating the substrate and product from the VBS assay mixture was developed using a 1:1 mixture of diethyl ether and hexanes with 0.05% acetic acid (v:v) to selectively elute versicolorin B (6), whereas the considerably more polar unreacted versicolinal hemiacetal (5) remained bound to the silica gel. As long as proper controls were simultaneously performed, the amount of enzymically derived product could be accurately distinguished from that resulting from trivially acid-catalyzed cyclization (22).

![Western blot of the yeast Invsc2 cell-free extracts containing the heterologically expressed, epitope-tagged VBS grown in Buffered Medium A (*lanes A–F*) and Buffered Medium B (*lanes G–L*) at various time points (see “Experimental Procedures”).](image)

**FIG. 3.** Western blot of the yeast Invsc2 cell-free extracts containing the heterologically expressed, epitope-tagged VBS grown in Buffered Medium A (*lanes A–F*) and Buffered Medium B (*lanes G–L*) at various time points (see “Experimental Procedures”). A, Invsc2 without pCGSVBS3xf at 30 h; B, in versusc2 with pCGSVBS3xf at 30 h; C, Invsc2 without pCGSVBS3xf at 60 h; D, Invsc2 with pCGSVBS3xf at 60 h; E, Invsc2 without pCGSVBS3xf at 90 h; F, Invsc2 with pCGSVBS3xf at 90 h; G, Invsc2 without pCGSVBS3xf at 30 h; H, Invsc2 with pCGSVBS3xf at 30 h; I, Invsc2 without pCGSVBS3xf at 60 h; J, Invsc2 with pCGSVBS3xf at 60 h; K, Invsc2 without pCGSVBS3xf at 90 h; L, Invsc2 with pCGSVBS3xf at 90 h; and M, molecular mass markers.

![SDS-PAGE monitoring the yeast-derived VBS purification protocol.](image)

**FIG. 4.** SDS-PAGE monitoring the yeast-derived VBS purification protocol. A, molecular mass markers; B, molecular mass markers; C, cell-free extract of pCGSVBS-expressed VBS from CGY2998; D, preparative isoelectric focusing fractionation (pH 4.0–6.0); E, Mono-Q HR 5/5 anion exchange chromatography; F, molecular mass markers.

| Purification step       | Protein (mg/ml) | Protein (mg) | T. A. (μM/min) | S. A. (μM/min/mg) | % Yield | Purification fold |
|-------------------------|----------------|-------------|---------------|-------------------|---------|------------------|
| Cell-free extract       | 5.47           | 820         | 27508         | 34                | 100     | 1                |
| Rotorfor-IEF            | 0.18           | 3.50        | 8803          | 2513              | 32      | 75               |
| Mono-Q                  | 0.15           | 1.20        | 3301          | 2751              | 12      | 185              |
Expression of VBS in S. cerevisiae

Analysis of the effect of changing substrate concentrations on initial velocities was used to determine the kinetic parameters of \( V_{\text{max}} \) and \( K_m \) for the yeast-derived VBS. The selection of substrate concentrations was made after preliminary experiments gave a rough estimate of the \( K_m \) and these were assayed in triplicate with dilute active solutions of purified yeast-derived VBS; D, S. cerevisiae-derived VBS treated with PGase F; E, untreated S. cerevisiae-derived VBS; F, E. coli-derived VBS treated with PGase F; G, untreated E. coli-derived VBS.

Table II. Effect of metals and chemical modifying reagents on VBS

| Metals or chemical modifying reagent | Concentration | Relative activity (% of control) |
|-------------------------------------|---------------|---------------------------------|
| None (control)                      | 0.0           | 100                             |
| B(OH)\(_3\)                         | 1.0           | 105                             |
| Na\(_2\)B\(_4\)O\(_7\)-10H\(_2\)O\) | 1.0           | 110                             |
| Mg\(_2\)NH\(_2\)O\(_2\)-4H\(_2\)O\) | 1.0           | 92                              |
| Fe\(_2\)(SO\(_4\))\(_3\)-6H\(_2\)O\) | 1.0           | 94                              |
| FeCl\(_2\)                           | 1.0           | 100                             |
| CuSO\(_4\)                           | 1.0           | 93                              |
| MnSO\(_4\)-H\(_2\)O                  | 1.0           | 104                             |
| ZnCl\(_2\)                           | 1.0           | 109                             |
| HgCl\(_2\)                           | 1.0           | 0                               |
| Co\(_2\)(NH\(_3\))\(_6\)Cl\(_3\)     | 1.0           | 111                             |
| CoCl\(_2\)                           | 1.0           | 97                              |
| MgSO\(_4\)                           | 1.0           | 112                             |
| EDTA                                | 1.0           | 111                             |
| NEM                                 | 10.0          | 87                              |
| Iodoacetamide                       | 10.0          | 103                             |
| 4-Mercuribenzoic acid               | 10.0          | 100                             |
| O-Methylisourea                     | 10.0          | 97                              |
| DEPC                                | 10.0          | 41                              |
| DEPC                                | 3.0           | 57                              |
| DEPC                                | 1.0           | 97                              |
| EDC                                 | 10.0          | 12                              |
| EDC                                 | 3.0           | 42                              |
| EDC                                 | 1.0           | 68                              |

*The enzyme activity in the absence of any metals or chemical modifying reagent was expressed as 100%. Assay error was ~ 5%.*

Based on the pH-rate profile of VBS (22), we designed chemical modification studies to provide further evidence for the identities of the VBS active-site amino acid residues. Initial studies utilizing N-ethylmaleimide, iodoacetamide, and O-methylisourea were not supportive of the involvement of cysteine as an active-site amino acid residue (Table II). While the effect of Hg\(^{2+}\) on VBS activity suggested the involvement of cysteine in the active site, these results were not paralleled with the effects of Cu\(^{2+}\) or any of the cysteine-specific chemical modifying reagents. Other metals were tested to determine whether the dehydrative cyclization required metal ion. The presence of metals did not seem to enhance the activity of VBS, ruling out this possibility.

While the concentrations required were high, further chemical modification studies with yeast-derived VBS gave tentative evidence of the involvement of both histidine and aspartate and/or glutamate (Figs. 6 and 7). Both in the presence of DEPC and EDC expressed VBS activity was diminished relative to control reactions. Protection studies employing unlabeled versicol- }

FIG. 5. SDS-PAGE monitoring the deglycosylation of native, yeast-derived, and E. coli-derived VBS. A, molecular mass markers; B, native VBS treated with PGase F (34 kDa); C, untreated native VBS; D, S. cerevisiae-derived VBS treated with PGase F; E, untreated S. cerevisiae-derived VBS; F, E. coli-derived VBS treated with PGase F; G, untreated E. coli-derived VBS.

![DEPC Inactivation of VBS](image)

Fig. 6. DEPC chemical modification of the yeast-derived VBS with and without substrate protection. □, control; ◯, with substrate protection; ○, without substrate protection.

and 7.3 ± 0.1 in agreement with the active-site amino acid residues suggested by the chemical modification data. Careful analysis of the pH/activity curve suggests one or two acidic residues play a role in catalysis consistent with the mechanistically similar enzyme glycosidation reactions in which these enzymes typically have one to two acidic amino acid residues at the active site to carry out the general acid-base chemistry of glycosidic bond formation (63). While these data must be interpreted with caution, one could visualize up to two acidic amino acid residues conducting the same type of catalysis and possibly the presence of a histidine residue (Fig. 8).

With further evidence for the identities of the active-site residues of versicolorin B synthase in hand, efforts were made to test simple substrate analogues to aid the design of future mechanism-based inactivators. Among those analogues examined (Fig. 9) were 2-hydroxyphenylacetic acid lactone (10), 2,5-dihydroxyphenylacetic acid lactone (11), emodin (12), versicol-
orin B (6), and the benzofurandiol 13, a close structural analogue of the right-hand half of the normal substrate. The compounds were rapidly surveyed for their relative extents of inhibition as a function of their concentration. Most of these had no effect on the cyclization reaction rate. Notably, emodin (12), containing the trihydroxylated anthraquinone analogous to the substrate, showed no inhibition (Table III), whereas the product of the reaction, versicolorin B (6), did. However, most revealing among these experiments, which mimics the C-ring and side chain of the substrate 5, was found to decrease the efficiency of the normal reaction. More detailed investigation of the inhibitory behavior of 13 clearly indicated a competitive pattern with a $K_i$ of 96.0 ± 18.0 μM (Fig. 10).

Further insight into the nature of the active site was sought in the striking amino acid sequence similarity between VBS and glucose oxidase from A. niger, as well as other members of the GMC family (64) of flavin-dependent oxidoreductases (9). While the dehydrative cyclization catalyzed by VBS requires no redox cofactor, the structural similarity between the tricyclic anthraquinone of versiconal (5) and the flavin nuclei of FAD, FMN, and riboflavin was nonetheless intriguing. That glucose oxidase also binds the substrate, glucose, in the vicinity of the flavin compound our interest as it size and composition was not unlike the side chain of versiconal. Thus, despite the more than 20-amino acid deletion evident from alignment of the apparent flavin-binding domains of VBS and glucose oxidase and other GMC flavoproteins, the thought was entertained that these protein structural modifications could direct this region to versiconal binding and the comparatively simple catalytic task of acid-promoted cyclization chemistry.

Inhibition experiments were conducted with FAD, FMN, riboflavin, and glucose, and, to our disappointment, none of these, even at quite high concentrations (for example, glucose at 100 mM), gave any detectable inhibition of the cyclization reaction. This outcome is consistent with the observations above made with emodin (13) and suggest that the anthraquinone nucleus is not of primary importance to substrate binding (a finding perhaps not unexpected as progress through the biosynthetic pathway involves six other anthraquinone-containing intermediates).

**DISCUSSION**

Versicolorin B synthase, VBS, plays a pivotal role in the biosynthesis of the mycotoxin aflatoxin B$_1$ (9) by catalyzing the cyclodehydration of (±)-versiconal hemiacetal (5) to (−)-versicolorin B (6) to establish the absolute configuration of the dihydrobisfuran ringsystem essential to DNA interaction (25, 65, 66). Initial attempts to over-produce this enzyme in E. coli gave mainly inclusion bodies and small amounts of catalytically inactive protein. Recognizing by SDS-PAGE and treatment with PNGase F that ~10% of the apparent mass of the wild-type enzyme owed to post-translational glycosylation, over-production of VBS in S. cerevisiae was attempted. Of several systems surveyed, one incorporating a secretion signal gave the best yield of protein and whose molecular mass and kinetic properties were virtually identical to the wild-type en-
zyme (22).

The gene encoding VBS has been cloned and sequenced from gDNA and cDNA libraries of A. parasiticus (9). The translated sequence of VBS gave three potential sites of N-glycosylation (Asn-Xaa-Ser/Thr) in keeping with the post-translational modifications observed above and shows striking similarities to the GMC family of flavin-dependent oxidoreductases (64). The extent of identity was greatest with glucose oxidase (35, 67) (GGC BESTFIT analysis: 38% identical, 58% similar) among the available protein sequences. A partially deglycosylated derivative of this enzyme has been crystallized (68), and its x-ray structure shows the presence of carbohydrate residues at the dimer interface. Like glucose oxidase, VBS forms a homodimer, and it may be suggested, but not proved, that glycosidation of the protein could be important to catalytic activity by promoting dimer formation or facilitating correct folding of the protein.

In the event, it is curious that VBS, which does not catalyze a redox reaction, should show such high homology to the flavin binding domain of the GMC family of flavoproteins. In this connection, it is interesting to note that computer-assisted (BESTFIT) sequence alignments also reveal 33% identity and 61% similarity to mandelonitrile lyase, which takes part in the enzymatic degradation of cyanogenic glycosides (69). This enzyme catalyzes the decomposition of the cyanohydrin to benzaldehyde and hydrogen cyanide, a reaction, like that carried out by VBS, that does not involve oxidation/reduction. A number of mandelonitrile lyase enzymes have been isolated and studied (70). Interestingly, some require oxidized flavin (71) (FAD) for activity, whereas others do not (72). Unfortunately, only one primary protein sequence is currently available from this class, one of the FAD-requiring lyases (73). As more gene/protein sequences become available, it will be of interest to see whether those that do not bind flavin retain, like VBS, vestigial structural elements consistent with evolution from a flavin-binding ancestor. Of those mandelonitrile lyase enzymes that do bind flavin, it does not participate in catalysis (74), and its role is unclear. It has been proposed to be important to proper folding of the protein to achieve a catalytically active form (75).

The low pI and broad pH stability of both the wild-type and yeast-derived VBS have allowed its substantial purification by preparative isoelectric focusing in tandem with conventional ion exchange chromatography steps. Chemical modification experiments are consistent with the participation of carboxylic acid residues and a possible histidine in catalysis, an observation in accord with pH-rate profile data and isotopic labeling studies (76). The S. cerevisiae secreting system described has made possible 50–100-fold improvement in the production of VBS compared with the wild-type A. parasiticus and confers advantages of ease of isolation and uniformity of post-translational glycosylation. This first example of eukaryotic over-expression of an individual biosynthetic gene opens the way to direct study of other enzymes of aflatoxin biosynthesis and will complement the information available from insertional inactivation studies (11, 15) to generate blocked mutants of the pathway.

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Expression of VBS in S. cerevisiae

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