Structures of Alkaloid Biosynthetic Glucosidases Decode Substrate Specificity

Liqun Xia,^{1*} Martin Ruppert,^{2*} Meitian Wang,^{3#} Santosh Panjikar,^{4#} Haili Lin,¹ Chitra Rajendran,³ Leif Barleben,⁵ Joachim Stöckigt,^{1#}

¹Institute of Materia Medica, College of Pharmaceutical Sciences, Zhejiang University, 866 Yu Hang Tang Road, Hangzhou 310058, P.R. China

²Lehrstuhl für Pharmazeutische Biologie, Institut für Pharmazie und Biochemie, Johannes Gutenberg-Universität, Staudinger Weg 5, D-55099 Mainz, Germany

³Swiss Light Source, Paul Scherrer Institute, CH-5232 Villigen, Switzerland

⁴Australian Synchrotron, 800 Blackburn Road, Clayton VIC, Australia 3168

⁵Department of Medical Biochemistry and Biophysics, Karolinska Institute, SE-17177 Stockholm, Sweden

*these authors contributed equally to this work

#correspondence should be addressed to M. W. (meitian.wang@psi.ch); S. P. (panjikar.santosh@embl-hamburg.com) and J. S. (joesto2000@yahoo.com)

Inventory of Supplemental Information:

Figure S1, The Role of RG as Tool for Facile Production of Small Alkaloid Libraries Including Novel Alkaloids with Un-natural C-2 Configuration by Chemo-enzymatic Approaches.

Figure S2A, Structure Based Alignment of β -Glucosidases of Family 1 from Higher Plants.

Figure S2B, Sequence-based Phylogenetic Tree of 11 Structurally Characterized Plant GH1 Enzymes.

Figure S3, Stereoview of Raucaffricine Binding Site of RG.

Figure S4, The Different Conformations of Glu476 in RG and Glu472 in SG.

Figure S5, Hydrogen Binding Network Between Secologanin and Residues in a Distance of $\leq 3.8\text{\AA}$ in the Ligand Structure of His₆-RG Inactive Mutant E186Q.

Table S1, Physical and Biochemical Properties of RG and SG from *R. serpentina*.

Table S2, Kinetic Constants of His₆-tagged RG Wild-type, Mutant W392A.

Table S3, Data Collection and Refinement Statistics of RG.

Supplemental Experimental Procedures

Figure S1, The Role of RG as Tool for Facile Production of Small Alkaloid Libraries Including Novel Alkaloids with Un-natural C-2 Configuration by Chemo-enzymatic Approaches.

RG η1
22

RGMATQSSAVI.....DND.....AT

SGMDNTQAEPLVVAIVPKPNAST.....EHTNSHLIPVT

CBGIHAFKPLP.....ISFDDFS.....

Os3bglu12MAAAGAMPGLLLTFLLLAVV.....ASGAYNG.....

Os3bglu7MAARRANCALVVLALALLAARDAG.....AAAVPK.....P

Os3bglu6MGRICKSSSGRCSTARLEAVAVLVVVFVG.....ASSSLRGCIAQ

Zm-p60.1 MAPLLAAAMNHAAHPGLRSHLVGPNNESFSRHHLPSSSPQSSKRRCNLSFTTRSARVGSQ.NG..VQML

Glu1 MAPLLAAAMNHAAHPGLRSHLVGPNNESFSRHHLPSSSPQSSKRRCNLSFTTRSARVGSQ.NG..VQML

Dhr1 MALLLASAINHTAHPLGRSH...PNNESFSRHHLCSSPONISKRRSNLSFRPRAQTISESAG..IHLRL

TaGlu1b MALLAAATINPTTHLS.LRSR.AGRNSENWLRLSTASS.QKSKGRFCNLITIRAGTSPSKPAEPIGPVFTKL

ScGlu MALLVGGTLNPTTHLS.LRSR.AGRNSENWLRSAASS.QTSKGRFCNLITVRAGTSPSKPSEPIGPVFTKL

RG η2 β1 α1 TT α2 η3 η4 α3

RG R...ISRSDFPADFIMGTGSSAYQICGARDGGRGPSTIWDFTTHRRPDMIRGGTNGDVAVDSYHLYKED

SG RSKIVVHRDFPQDPIFGAGGSAYQCCAYNEGNGRPSIWDFTTHRRPAKISDGSNGNQAINCYHYMYKED

CBG ...DLNRSCFAPGVFGTASSAYQYGAADFEGKGPSTIWDFTTHRRPEKIKDRTNGDVAIDYHRYKED

Os3bglu12 AGEPPVSRSRFPKGFITGTASSAYQYEGAAEGGRGPSTIWDFTTHRRPEKIDRSDGVDVSDSYHLYKED

Os3bglu7 NWLGGLSRAAFPKRFVFGTASSAYQYEGMAASGGRGPSIWDFAFAHT.PGNVAGNQNGDVATDYHRYKED

Os3bglu6 QSGGGLTRGSFPEGVFGTASSAYQYEGAVKEDGRGQTIWDFTFAHT.FGKITDFSNADVAVDQYHRYKED

Zm-p60.1 SPSEIPQRDWFPSTDFGAATSAYQIEGAWNEDGKESNWDHFCNNHPERILDGSSNDIGANSYHYMYKTD

Glu1 SPSEIPQRDWFPSTDFGAATSAYQIEGAWNEDGKESNWDHFCNNHPERILDGSSNDIGANSYHYMYKTD

Dhr1 SPWEIPQRDWFPSTDFGAATSAYQIEGAWNEDGKESNWDHFCNNHPERILDGSSNDIGANSYHYMYKTD

TaGlu1b KPWQIPKRDWFDKDFLFGASTSAYQIEGAWNEDGKESNWDHFCNNHPERILDGSSNDIGANSYHYMYKTD

ScGlu KPWQIPKRDWFDKDFLFGASTSAYQIEGAWNEDGKESNWDHFCNNHPERILDGSSNDIGANSYHYMYKTD

RG β2 α4 η5 α5 β3 α6

RG VNILKNLGLIAVYFSTISWSRVLPGRRLSGGVNKEGINVYNNLIDGLLANGIKPFVTLFWWDVPGALEDEY

SG IKIMKQGLIESYFSTISWSRVLPGRRLSGGVNKEGINVYNNLIDGLLANGIKPFVTLFWWDVPGALEDEY

CBG IGLMKDMMNLDAVYFSTISWSRVLPGRRLSGGVNKEGINVYNNLIDGLLANGIKPFVTLFWWDVPGALEDEY

Os3bglu12 VRLLKEMMGMDAVYFSTISWSRVLPGRRLSGGVNKEGINVYNNLIDGLLANGIKPFVTLFWWDVPGALEDEY

Os3bglu7 VNLKMSLNDVAVYFSTISWSRVLPGRRLSGGVNKEGINVYNNLIDGLLANGIKPFVTLFWWDVPGALEDEY

Os3bglu6 IQLMADMGMDAVYFSTISWSRVLPGRRLSGGVNKEGINVYNNLIDGLLANGIKPFVTLFWWDVPGALEDEY

Zm-p60.1 VRLLKEMMGMDAVYFSTISWSRVLPGRRLSGGVNKEGINVYNNLIDGLLANGIKPFVTLFWWDVPGALEDEY

Glu1 VRLLKEMMGMDAVYFSTISWSRVLPGRRLSGGVNKEGINVYNNLIDGLLANGIKPFVTLFWWDVPGALEDEY

Dhr1 VRLLKEMMGMDAVYFSTISWSRVLPGRRLSGGVNKEGINVYNNLIDGLLANGIKPFVTLFWWDVPGALEDEY

TaGlu1b VKALKDMGMKVYFSTISWSRVLPGRRLSGGVNKEGINVYNNLIDGLLANGIKPFVTLFWWDVPGALEDEY

ScGlu VKALKDMGMKVYFSTISWSRVLPGRRLSGGVNKEGINVYNNLIDGLLANGIKPFVTLFWWDVPGALEDEY

RG η6 α7 β4 α8 TT

RG GGFSPRI...VDDFCEYAEICFWEFGDRVKHMMTLNEPHTFSVHCYATGLYAPGRGRTSPHEVNHPTVQ

SG GGFSPRI...VDDFCEYAEICFWEFGDRVKHMMTLNEPHTFSVHCYATGLYAPGRGRTSPHEVNHPTVQ

CBG RGFSPRI...VDDFCEYAEICFWEFGDRVKHMMTLNEPHTFSVHCYATGLYAPGRGRTSPHEVNHPTVQ

Os3bglu12 NGFLSPNI...INDPKDYAEICFWEFGDRVKHMMTLNEPHTFSVHCYATGLYAPGRGRTSPHEVNHPTVQ

Os3bglu7 GGFSPRI...VDDFCEYAEICFWEFGDRVKHMMTLNEPHTFSVHCYATGLYAPGRGRTSPHEVNHPTVQ

Os3bglu6 KGFSPRI...VDDFCEYAEICFWEFGDRVKHMMTLNEPHTFSVHCYATGLYAPGRGRTSPHEVNHPTVQ

Zm-p60.1 GGFSPRI...VDDFCEYAEICFWEFGDRVKHMMTLNEPHTFSVHCYATGLYAPGRGRTSPHEVNHPTVQ

Glu1 GGFSPRI...VDDFCEYAEICFWEFGDRVKHMMTLNEPHTFSVHCYATGLYAPGRGRTSPHEVNHPTVQ

Dhr1 GGFSPRI...VDDFCEYAEICFWEFGDRVKHMMTLNEPHTFSVHCYATGLYAPGRGRTSPHEVNHPTVQ

TaGlu1b GGFSPRI...VDDFCEYAEICFWEFGDRVKHMMTLNEPHTFSVHCYATGLYAPGRGRTSPHEVNHPTVQ

ScGlu GGFSPRI...VDDFCEYAEICFWEFGDRVKHMMTLNEPHTFSVHCYATGLYAPGRGRTSPHEVNHPTVQ

RG α9 α10 β5

RG HRCSTVAPQICSTENPGETPYVTHHLLLAHAAVELYKNKFQRCQEGQIGISHATQWMEPWDENSASD

SG HRCSTVAPQICSTENPGETPYVTHHLLLAHAAVELYKNKFQRCQEGQIGISHATQWMEPWDENSASD

CBG HRCSTVAPQICSTENPGETPYVTHHLLLAHAAVELYKNKFQRCQEGQIGISHATQWMEPWDENSASD

Os3bglu12 HRCSTVAPQICSTENPGETPYVTHHLLLAHAAVELYKNKFQRCQEGQIGISHATQWMEPWDENSASD

Os3bglu7 HRCSTVAPQICSTENPGETPYVTHHLLLAHAAVELYKNKFQRCQEGQIGISHATQWMEPWDENSASD

Os3bglu6 HRCSTVAPQICSTENPGETPYVTHHLLLAHAAVELYKNKFQRCQEGQIGISHATQWMEPWDENSASD

Zm-p60.1 HRCSTVAPQICSTENPGETPYVTHHLLLAHAAVELYKNKFQRCQEGQIGISHATQWMEPWDENSASD

Glu1 HRCSTVAPQICSTENPGETPYVTHHLLLAHAAVELYKNKFQRCQEGQIGISHATQWMEPWDENSASD

Dhr1 HRCSTVAPQICSTENPGETPYVTHHLLLAHAAVELYKNKFQRCQEGQIGISHATQWMEPWDENSASD

TaGlu1b HRCSTVAPQICSTENPGETPYVTHHLLLAHAAVELYKNKFQRCQEGQIGISHATQWMEPWDENSASD

ScGlu HRCSTVAPQICSTENPGETPYVTHHLLLAHAAVELYKNKFQRCQEGQIGISHATQWMEPWDENSASD

RG α11 α12 α13 η7 α14 β6

RG VEAARALDFMLGWFMPIITSCDYPKSMKKFVGSRLPKFSEFQSKMLKGSYDFVGLNYTASVYTNASTN

SG VEAARALDFMLGWFMPIITSCDYPKSMKKFVGSRLPKFSEFQSKMLKGSYDFVGLNYTASVYTNASTN

CBG VEAARALDFMLGWFMPIITSCDYPKSMKKFVGSRLPKFSEFQSKMLKGSYDFVGLNYTASVYTNASTN

Os3bglu12 VEAARALDFMLGWFMPIITSCDYPKSMKKFVGSRLPKFSEFQSKMLKGSYDFVGLNYTASVYTNASTN

Os3bglu7 VEAARALDFMLGWFMPIITSCDYPKSMKKFVGSRLPKFSEFQSKMLKGSYDFVGLNYTASVYTNASTN

Os3bglu6 VEAARALDFMLGWFMPIITSCDYPKSMKKFVGSRLPKFSEFQSKMLKGSYDFVGLNYTASVYTNASTN

Zm-p60.1 VEAARALDFMLGWFMPIITSCDYPKSMKKFVGSRLPKFSEFQSKMLKGSYDFVGLNYTASVYTNASTN

Glu1 VEAARALDFMLGWFMPIITSCDYPKSMKKFVGSRLPKFSEFQSKMLKGSYDFVGLNYTASVYTNASTN

Dhr1 VEAARALDFMLGWFMPIITSCDYPKSMKKFVGSRLPKFSEFQSKMLKGSYDFVGLNYTASVYTNASTN

TaGlu1b VEAARALDFMLGWFMPIITSCDYPKSMKKFVGSRLPKFSEFQSKMLKGSYDFVGLNYTASVYTNASTN

ScGlu VEAARALDFMLGWFMPIITSCDYPKSMKKFVGSRLPKFSEFQSKMLKGSYDFVGLNYTASVYTNASTN

Figure S2A. Structure Based Alignment of β -Glucosidases of Family 1 from Higher Plants.

RG, raucaffricine glucosidase from *Rauvolfia serpentina*; SG, strictosidine glucosidase from *Rauvolfia serpentina*; CBG, cyanogenic β -glucosidase from *Trifolium repens*; Os3bglu12, Os3bglu7 and Os3bglu6 are β -glucosidases from *Oryza sativa Japonica Group*; Zm-p60.1 and , zeatin β -glucosidase from *Zea mays*; Glu1, β -glucosidase 1 from *Zea mays*; Dhr1, dhrurrinase 1 from *Sorghum bicolor*; TaGlu1b, β -glucosidase from *Triticum aestivum*; ScGlu, β -glucosidase from *Secale cereale*. The green color indicates the conserved catalytic residues: Glu186, Glu420. The cyan color shows the five residues with hydrophilic interaction to the glucose part. The residues influencing the conformation of the aglycone part are marked in yellow. The residues colored in red are highly conserved residues of all 11 β -glucosidases.

Figure S2B. Sequence-based Phylogenetic Tree of 11 Structurally Characterized Plant GH1 Enzymes.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 1.94776883 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000) and are in the units of the number of amino acid differences per site. The analysis involved 11 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 458 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011). Enzymes were named according to their abbreviations used in Figure S2A.

Figure S3. Stereoview of Raucaffricine Binding Site of RG.

Figure S4. The Different Conformations of Glu476 in RG and Glu472 in SG.

The 3-D structure of RG-WT (magenta), RG-E186Q-dihydroucaucaffricine complex (green), RG-E186Q-secologanin complex (cyan), SG-WT (yellow), SG-E207Q (salmon) was superimposed.

Figure S5. Hydrogen Binding Network Between Secologanin and Residues in a Distance of $\leq 3.8\text{\AA}$ in the Ligand Structure of His₆-RG Inactive Mutant E186Q.

Table S1. Physical and Biochemical Properties of RG and SG from *R. serpentina*.

	RG			SG		
Amino Acids	540			532		
Theoretical pI / rel. MW (Da)	6.07 / 60934.19			6.01 / 60880.59		
CAZY-Family	GH-1			GH-1		
Fold	(β/α) ₈ barrel fold			(β/α) ₈ barrel fold (Barleben et al., 2007)		
Sequence-Identity / Similarity	55% / 69%					
Amino acid composition,	Ala (A) 32	5.9%		Ala (A) 35	6.6%	
	Arg (R) 29	5.4%		Arg (R) 25	4.7%	
	Asn (N) 33	6.1%		Asn (N) 24	4.5%	
	Asp (D) 36	6.7%		Asp (D) 33	6.2%	
	Cys (C) 5	0.9%		Cys (C) 7	1.3%	
	Gln (Q) 14	2.6%		Gln (Q) 17	3.2%	
	Glu (E) 27	5.0%		Glu (E) 39	7.3%	
	Gly (G) 47	8.7%		Gly (G) 41	7.7%	
	His (H) 16	3.0%		His (H) 16	3.0%	
	Ile (I) 28	5.2%		Ile (I) 28	5.3%	
	Leu (L) 37	6.9%		Leu (L) 34	6.4%	
	Lys (K) 26	4.8%		Lys (K) 38	7.1%	
	Met (M) 12	2.2%		Met (M) 9	1.7%	
	Phe (F) 24	4.4%		Phe (F) 28	5.3%	
	Pro (P) 25	4.6%		Pro (P) 23	4.3%	
	Ser (S) 44	8.1%		Ser (S) 30	5.6%	
	Thr (T) 29	5.4%		Thr (T) 28	5.3%	
	Trp (W) 14	2.6%		Trp (W) 12	2.3%	
	Tyr (Y) 28	5.2%		Tyr (Y) 30	5.6%	
	Val (V) 34	6.3%		Val (V) 35	6.6%	
pH- / Temperature- Optimum	5.0 / 28°C (Warzecha et al., 2000)			5.0 / 30°C (Gerasimenko et al., 2002)		
Km-value (strictosidine)	1.8 mM (Warzecha et al., 2000)			0.12 mM (Gerasimenko et al., 2002)		
Km-value (raucaffricine)	1.3 mM (Warzecha et al., 2000)			No product detectable		

Table S2. Kinetic Constants of His₆-tagged RG Wild-type, Mutant Trp392Ala.

Enzyme	Substrate	K _m (mM)	K _{cat} (s ⁻¹)	K _{cat} /K _m (1/(mM s ⁻¹))*
RG-WT	Raucaffricine*	0.78 ± 0.08	47.11 ± 8.70	59.94
	Strictosidine*	1.70 ± 0.03	1.26 ± 0.28	0.72
RG-Trp392Ala	Raucaffricine	3.10 ± 1.49	1.11 ± 0.86	0.35
	Strictosidine	3.10 ± 0.6	3.74 ± 1.31	1.17

Measurements of RG-WT and RG-Trp392Ala mutant for conversion of the substrates raucaffricine and strictosidine (average data from 2* or 3 different measurements) are displayed.

Table S3. Data Collection and Refinement Statistics of RG.

Measurement temperature	Cryo-temperature (100 K)	Room temperature (295 K)		
Data set	RG-WT-glycerol Complex	RG-E186Q	RG-E186Q-1,2- dihydro- raucaffricine Complex	RG-E186Q- secologanin Complex
Wavelength (Å)	0.8150	1.0000	1.0000	1.0000
Unit cell (Å)	<i>a</i> =102.77	<i>a</i> =105.56	<i>a</i> =104.89	<i>a</i> =104.93
(Space group <i>I</i> 222)	<i>b</i> =127.33	<i>b</i> =129.52	<i>b</i> =129.55	<i>b</i> =129.20
	<i>c</i> =215.84	<i>c</i> =216.64	<i>c</i> =216.92	<i>c</i> =216.80
Total reflections	1169406	254774	279283	332690
Unique reflections	76299	75161	55224	65973
Redundancy	15.3	3.4	5.1	5.1
Mosaicity	0.43	0.13	0.18	0.30
Resolution (Å)	20-2.15	94.89-2.20	65.16-2.43 (2.56- 2.32-2.20)	47.68-2.30 (2.42- 2.30)
Completeness (%)	99.3 (98.8) ^a	99.5 (98.8)	98.4 (91.0)	99.6 (97.6)
<i>I</i> /σ(<i>I</i>)	24.6 (4.4)	10.0 (2.7)	11.7 (2.9)	16.1 (3.4)
<i>R</i> _{merge} (%) ^b	11.7 (69.4)	7.0 (39.6)	8.5 (48.5)	6.5 (49.0)
Refinement				
Resolution (Å)	20-2.15	94.89-2.20	65.16-2.43	47.68-2.30
<i>R</i> _{cryst} / <i>R</i> _{free} (%) ^c	16.5/20.4	22.0/26.7	20.6 (23.8)	19.1 (23.1)
Av. B (Å ²) for protein	36.8	39.0	34.6	40.9
Av. B (Å ²) for water	26.1	41.2	32.4	42.2
Av. B (Å ²) for substrate ¹	35.7	-	59.2	76.0
Number of atoms				
Non-hydrogen	8016	7686	7719	7737

Water	443	153	93	153
R.m.s.deviations				
Bond (Å)	0.023	0.016	0.013	0.012
Angles (°)	1.69	1.69	1.43	1.34
Ramachandran plot				
Residues in most favorable regions (%)	90.3	92.0	93.5	94.2
Residues in additional allowed regions (%)	8.7	6.5	5.5	4.4
Residues in generously allowed regions (%)	1.0	1.5	1.0	1.4
PDB code	4A3Y	3U5U	3U57	3U5Y

Supplemental Experimental Procedures

Expression, Purification, Activity Assay, Kinetic Data and Mutagenesis of RG

In order to obtain recombinant RG [AAF03675], the cDNA of RG (AF149311), isolated by Warzecha et al. (2000) from *R. serpentina*, was cloned into the pQE-2 vector as a fusion protein with an N-terminal His₆-tag (Ruppert et al., 2006). The pQE-2 plasmid construct was transformed into *E. coli* strain M15 [pREP4] (Qiagen, Hilden, Germany), expressed, isolated and purified as described by Ruppert et al. (2006). RG concentration was determined by the Bradford method (1976) except the protein concentration for RG enzyme activity assays was determined at A_{280nm} with Warburg equation and the purity of RG was analyzed by SDS-PAGE.

For determination of relative enzyme activity, the consumption of raucaffricine, strictosidine, secologanin and arbutin were monitored at 258 nm, 280 nm, 236 nm, 284 nm respectively. Quantitative RG kinetic assays were performed at the optimal pH of the enzyme (pH 5, sodium citrate). The standard curve of raucaffricine and strictosidine was determined at concentrations of 0.4, 0.8, 1.2, 1.6, 2.0 mM, and the R^2 values were up to 0.99. In a total volume of 100 µl, 0.11 µg wild-type RG was incubated with raucaffricine (1.6 mM) at 30°C for 30 min. Under these conditions enzyme activity was set at 100%; an average from three measurements were used for each ligand. For RG mutants with a low activity and incubation with other putative substrates, enzyme amounts were increased to 35 µg RG, leading to a detection limit of < 0.02% relative activity. K_m values were determined in a similar way, with enzyme concentrations depending on the activity of the mutant from 0.035 to 7 µg and substrate concentrations between 0.015 and 2 mM. Kinetic data, K_m and V_{max} were determined by HPLC with a

LiChrospher 250 x 4 mm, 5 μ m 60 RP-select B column, injection volume of 0.05 ml and flow rate of 1.0 ml x min⁻¹.

Site-directed mutants were constructed using the QuickChangeTM Site-Directed Mutagenesis Kit (Stratagene, La Jolla, USA) and RG-pQE-2 plasmid as a template. Mutants were verified by sequencing, expressed and purified in the same way as the wild type enzyme. The primer pairs used for mutations are shown in the following table.

Primer Pairs used for Generating 12 Different Mutants.

Mutant	Primer pairs
E186D	Glu186Asp-for: 5'-GATGACATTGAACGACCCATGGACATTTAGC-3' Glu186Asp-rev: 5'-GCTAAATGTCCATGGGTCGTTCAATGTCATC-3'
E186Q	Glu186Gln-for: 5'-GATGACATTGAACAGCCATGGACATTTAGC-3' Glu186Gln-rev: 5'-GCTAAATGTCCATGGCTGGTTCAATGTCATC-3'
E420Q	Glu420Gln-for: 5'-CATATATGTCACACAGAATGGAGTTGACG-3' Glu420Gln-rev: 5'-CGTCAACTCCATTCTGTGTGACATATATG-3'
E186Q/E420Q	Glu186Gln-for: 5'-GATGACATTGAACAGCCATGGACATTTAGC-3' Glu186Gln-rev: 5'-GCTAAATGTCCATGGCTGGTTCAATGTCATC-3' Glu420Gln-for: 5'-CATATATGTCACACAGAATGGAGTTGACG-3' Glu420Gln-rev: 5'-CGTCAACTCCATTCTGTGTGACATATATG-3'
W392A	Trp392Ala-for: 5'-CCAGTCTGGTTCAGATGCGCTACTTATTTATCCAG-3' Trp392Ala-rev: 5'-CTGGATAAATAAGTAGCGCATCTGAACCAGACTGG-3'
H193A	His193Ala-for: 5'-CATGGACATTTAGCGTTGCTGGATATGCAACAGGC-3' His193Ala-rev: 5'-GCCTGTTGCATATCCAGCAACGCTAAATGTCCATG-3'
Y200A	Tyr200Ala-for: 5'-GATATGCAACAGGCCTTGCAAGCAGCCGTCGAGGTC-3' Tyr200Ala-rev: 5'-GACCTCGACCGGTGCTGCAAGGCCTGTTGCATATC-3'
T189A	Thr189Ala-for: 5'-GAACGAGCCATGGGCATTTAGCGTTCATGG-3' Thr189Ala-rev: 5'-CCATGAACGCTAAATGCCCCATGGCTCGTTC-3'
F485Y	Phe485Tyr-for: 5'-GGTATGGAGTGCGTTATGGTATCATCCACATCG-3' Phe485Tyr-rev: 5'-CGATGTGGATGATACCAACGCACTCCATACC-3'
S390G	Ser390Gly-for: 5'-GGTCCCCAGTCTGGTGGAGATTGGCTACTTATTTATC-3' Ser390Gly-rev: 5'-GATAAATAAGTAGCCAATCTCCACCAGACTGGGGACC-3'
E476L	Glu476Leu-for: 5'-CATTATTGGATAATTTCTATGGGGTGAAGGGTATGG-3' Glu476Leu-rev: 5'-CCATACCCTTCACCCCATAGAAAATTATCCAATAATG-3'
E476A	Glu476Ala-for: 5'-CATTATTGGATAATTTGCAATGGGGTGAAGGGTATGG-3' Glu476Ala-rev: 5'-CCATACCCTTCACCCCATGCAAAAATTATCCAATAATG-3'

Structural Analysis

Structure-based sequence alignment was performed using the program STAMP (Russell and Barton, 1992) and the picture of the sequence alignment was made using the program ESPRIPT (Gouet et al, 1999). All figures were produced using MOLSCRIPT (Kraulis, 1991), RASTER3D (Merrit and Murphy, 1994), PyMol (DeLano, 2002) and LIGPLOT (Wallace et al., 1995). The 2D diagram was prepared using the program CHEMDRAW and edited using Microsoft Power point.

Supplemental References

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248-254.

DeLano, W.L. (2002). The PyMOL Molecular Graphics System (San Carlos, CA: DeLano Scientific).

Felsenstein J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39, 783-791.

Gerasimenko, I., Sheludko, Y., Ma, X. and Stöckigt, J. (2002). Heterologous expression of a *Rauvolfia* cDNA encoding strictosidine glucosidase, a biosynthetic key to over 2000 monoterpenoid indole alkaloids. *Eur. J. Biochem.* 269, 2204-2213.

Gouet, P., Courcelle, E., Stuart, D.I. and Metoz, F. (1999). ESPript: multiple sequence alignments in PostScript. *Bioinformatics* 15, 305-308.

Kraulis, P.J. (1991). *Molscript*: A program to produce both detailed and schematic plots of protein structures. *J. Appl. Crystallogr.* 24, 946-950.

Merrit, E.A. and Murphy, M.E. (1994). Raster3D Version 2.0. A program for photorealistic molecular graphics. *Acta Crystallogr. D Biol. Crystallogr.* 50, 869-873

Nei M. and Kumar S. (2000). *Molecular Evolution and Phylogenetics*. Oxford University Press, New York.

Ruppert, M., Panjikar, S., Barleben, L. and Stöckigt, J. (2006). Heterologous expression, purification, crystallization and preliminary X-ray analysis of raucaffricine glucosidase, a plant enzyme specifically involved in *Rauvolfia* alkaloid biosynthesis. *Acta Crystallogr. Sect. F* 62, 257-260.

Russell, R.B. and Barton, G.J. (1992). Multiple protein sequence alignment from tertiary structure comparison: Assignment of global and residue confidence levels. *Protein Struct. Funct. Genet.* 14, 309-323.

Saitou N. and Nei M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4, 406-425.

Tamura K., Peterson D., Peterson N., Stecher G., Nei M., and Kumar S. (2011). MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution* (In Press).

Wallace, A.C., Laskowski, R.A. and Thornton, J.M. (1995). LIGPLOT: A program to generate schematic diagrams of protein-ligand interactions. *Protein Eng.* 8, 127-134.

Warzecha, H., Gerasimenko, I., Kutchan, T.M. and Stöckigt, J. (2000). Molecular cloning and functional bacterial expression of a plant glucosidase specifically involved in alkaloid biosynthesis. *Phytochemistry* 54, 657-666.