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Molecular cloning and protein characterization of a heme-binding globin predicted in a sugar cane EST database

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ABSTRACT

A very large and representative sugar cane expression sequence tag (EST) library (SUCEST) was sequenced by a Brazilian consortium, opening the possibility to study important proteins, such as hemoglobins, which are largely present across the plant kingdom. The widespread presence and long evolutionary history of plant hemoglobins suggest a major role for this protein family in plants; however, little is known about their functional roles. In this study, we report the identification and characterization of a putative non-symbiotic hemoglobin cDNA clone that was identified in SUCEST. The cDNA was cloned, and the recombinant protein was purified and folded, as shown by its circular dichroism and emission fluorescence spectra. The expressed globin protein was able to bind hemin, as a characteristic Soret band was observed in the absorbance spectrum and increases were seen in the amount of secondary structure and in the stability of the protein. A model for the structure of the sugarcane hemoglobin was created using the crystal structure of a rice hemoglobin, and this model showed a conserved globular conformation.

Keywords: Sugar cane; Hemoglobin; Purification; Spectroscopy .

Abbreviations

Sc: Sugar cane; **Hb:** Hemoglobin; **EST:** expression sequence tag; **A:** absorbance; **CD:** circular dichroism; **PCR:** polymerase chain reaction; **SDS–PAGE:** sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

1. Introduction

Globins are respiratory proteins that bind oxygen molecules through the iron ion of the porphyrin ring and a histidine in the polypeptide chain [1-3]. Globins are among the best-studied proteins when structure, function, and evolution are considered [1-3]. As a matter of fact, hemoglobin has been found in bacteria, plants, fungi, and animals. The first description of a plant hemoglobin was provided by Kubo [4], and since then, a large number of plant hemoglobins have been described [2,3,5]. Although hemoglobins were first identified in plant species that fix nitrogen via symbiosis with bacteria, it is likely that all plants have a hemoglobin gene, and in addition to the symbiotic leghemoglobins found in legumes, there may be another gene or gene family in legumes that encodes hemoglobins that function in non-symbiotic plant tissues [6]. In support of this notion, non-symbiotic hemoglobin genes were identified in both nitrogen - and non-nitrogen-fixing dicot species and in monocot species [2,3,5-8].

The widespread presence and long evolutionary history of plant hemoglobins suggest a major role for these proteins in plants [2], and thus, it is important to search for and characterize new proteins of this class to increase our general knowledge on this subject. Due to its major role in ethanol production, sugar cane has become of great importance in bioenergy studies, and to learn more about this plant, a Brazilian scientific consortium produced and sequenced a repre-

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sentative expression sequence tag (EST) library from sugar cane (SUCEST) [9]. To gain information about hemoglobins in sugar cane, SUCEST was searched for a putative hemoglobin cDNA. Its cloning and protein purification and characterization are reported here. The recombinant protein was purified and folded, as shown by its circular dichroism and emission fluorescence spectra, and was able to bind hemin, as confirmed by the existence of a characteristic Soret band in the absorbance spectrum. This sugar cane hemoglobin showed an increase in secondary structure and stability when bound to heme, as is characteristic of globin family proteins. A structural model of the sugar cane hemoglobin was created using the crystal structure of a rice hemoglobin and showed a conserved globular conformation. These findings suggest that, like many plants, sugar cane expresses a heme-binding globin.

2. Material and Methods

2.1. *Cloning, expression and purification*

The cDNA library was constructed from a sugarcane cultivar that was a hybrid of crossing *Saccharum officinarum* with *Saccharum spontaneum* [9]. Blast searches of the database of expressed sequence tags SUCEST (http://sucest.lad.ic.unicamp.br/public) using the amino acid sequence of sperm whale myoglobin (GenBank accession number GI: 209563) as query revealed the sugar cane globin EST clone SCMCRZ3064B09.g (SUCEST). Sugar cane globin was amplified from the EST clone by PCR using primers 5´CAGTAGGTACATATGGGGTTC3´ and 5´CAGCCGGA TCCTTAGTCACGC3´ introducing *Nde*I and *Bam*HI restriction sites. The PCR product was digested and then inserted into the pET3a expression vector (Stratagene) to generate the pET3aScHb vector, which was confirmed by DNA sequencing. For protein expression, the construct was transformed into *Escherichia coli* BL21(DE3). The cells were grown in Luria Bertani medium at 37°C. When the A_{600} reached 0.8, 0.4 mM IPTG was added to induce protein expression [10] and the temperature was increased to 42°C for the production of inclusion bodies as previously described [11]. Four hours after expression was induced, the cells were harvested by centrifugation at 2,600 g and 4°C for 10 min, resuspended in 20 mL lysis buffer per L of culture, sonicated 12 times for 10 s each at 35 watts of output power with 2 min intervals and centrifuged at 13,000 g and 4°C for 10 min. The pellet, which contained the inclusion bodies, was washed 5 times with lysis buffer, resuspended in solubilization buffer by gentle agitation (60 min at room temperature) and centrifuged at 13,000 g and 4°C for 10 min. The supernatant, which contained the solubilized protein, was diluted twice with the same volume of equilibration buffer A and centrifuged as described above. The final supernatant was exhaustively dialyzed with equilibration buffer A. The sample was then loaded onto a Q Sepharose column (Pharmacia) equilibrated with 5 bed volumes of equilibration buffer A and recovered with increasing concentrations of NaCl (0 to 1 M). The fractions that contained the protein were pooled, exhaustively dialyzed with equilibration buffer B, loaded onto a HiLoad Superdex 200pg 26/60 molecular exclusion column (Pharmacia Biotech) equilibrated with 5 bed volumes of equilibration buffer B and recovered with the same buffer. Protein purification profiles were analyzed by SDS-PAGE as described by Laemmli [12]. The purified samples were exhaustively dialyzed in 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 100 mM NaCl (buffer conditions for the experiments described below) and frozen for storage. The compositions of the working buffers were as follows. Lysis buffer: 100 mM Tris-HCl (pH 8.0), 100 mM KCl, 1 mM EDTA, 0.1 mM PMSF, 0.1 mM lysozyme, and 0.2% β-mercaptoethanol. Solubilization buffer: 100 mM Tris-HCl (pH 8.0) and 8 M GdnCl. Equilibration buffer A: 20 mM Tris-HCl (pH 8.0), 1 mM DTT and 1 mM EDTA. Equilibration buffer B: 20 mM Tris-HCl (pH 7.5), 1 mM DTT, 1 mM EDTA and 100 mM NaCl. The chemicals used were of analytical grade. All solutions were filtered, and their pH was checked before and after filtration.

2.2. *Concentration measurements and hemin binding*

The concentration of *Saccharum spp*. hemoglobin (ScHb) in the apo form was measured with a JASCO model 530 UV/VIS spectrometer using either the calculated extinction coefficient for denatured proteins [13,14] or the Bradford protein assay [15] using a commercial kit (Bio-Rad). For the preparation of the protein in the holo form, hemin was mixed with the protein solution at a 1:2 ratio (protein:hemin) and incubated at 4°C for 30 min, followed by centrifugation at 90,000 g and 4°C for 20 min to eliminate any of the remaining apo form, as previously described for recombinant sperm whale myoglobin [11]. The concentration of holoScHb was measured at the Soret band in 20 mM sodium phosphate (pH 7.0), as described by Antonini and Brunori [16].

2.3. *Spectroscopic experiments*

A JASCO model J-810 Circular Dichroism (CD) spectropolarimeter equipped with a thermoelectric sample temperature controller (Peltier system) was used to record the CD spectra. The data were collected from 260 nm to 200 nm and accumulated at least 16 times for the spectral measurements. The data were collected at 222 nm for the stability measurements, and each plotted CD signal at 222 nm was the average of 5-min recordings. The CD measurements were made in cuvettes with a 1-mm path length at 20°C, and the thermal-induced unfolding and refolding was recorded every 1°C at a scan rate of 60°C/h. The average of at least three unfolding experiments was used to build each curve profile. The fluorescence measurements were collected using an Aminco Bowman Series 2 luminescence spectrometer (SLM Aminco) and a 1 x 1-cm path length cuvette with 2 μM apoScHb at 20°C. Excitation was at 280 nm with a bandpass of 2 nm, and emission was measured from 300 to 400 nm with a bandpass of 8

nm. The intrinsic emission fluorescence data were analyzed by either their emission maxima wavelength or their spectral center of mass (<λ>), as described by the equation below:

$$<\lambda> = \sum\lambda_i F_i / \sum F_i \qquad \text{[Equation 1]}$$

where λ_i is each wavelength and F_i is the fluorescence intensity at λ_I [17]. Curve fitting was accomplished with Origin (Microcal Software). Unless stated otherwise, the experimental error was less than 5%.

2.4. *Structural modelling*

The structure of *Oryza sativa* non-symbiotic hemoglobin 1 (PDB 1D8U chain A), which shares 66% sequence identity with ScHb (Table 1), was used as a template to model the structure of ScHb. First, the HHpred server (toolkit.tuebingen.mpg.de/hhpred) [18] was used to generate a homology model for ScHb, and then, the stereochemical quality of the model was assessed using the PROCHECK server (swissmodel.expasy.org/workspace/index.php?func=tools_structureassessment1) [19].

3. Results and Discussion

3.1. *Sugar cane hemoglobin*

Proteins are important biomolecules that are involved in the majority of the physiological functions of a cell. Thus, to learn how a cell functions, information can be gathered about the proteins that are expressed during the life of the cell and how these proteins are chemically modified. This large amount of information is called the cell proteome and requires tremendous effort to complete. Proteins are produced by the ribosomal machinery from the information encoded in mRNAs, and thus, even the information in the mRNA alone is important to gain information about the cell proteome. As a consequence, expressed sequence tag (EST) projects have been used as low-cost alternatives to complement genome and proteome projects [20]. An EST project for sugar cane was completed by a Brazilian scientific consortium (SUCEST), and the resulting database is currently available [9]. We searched this database for a putative heme-binding globin and identified a candidate (Fig. 1) that has high identity with heme-binding globins from several organisms (Table 1). To further characterize this gene as a hemoglobin, we cloned the cDNA, purified the recombinant protein, characterized its folded state and tested whether the globin was able to bind heme.

Fig. 1 shows the nucleotide sequence and the deduced amino acid sequence of the sugar cane hemoglobin (ScHb). The amino acid sequence of ScHb was submitted to Pfam (http://pfam.sanger.ac.uk/), a database with a large collection of protein families, which found a significant (e-value of 8.8e-21) globin domain match starting at residue 7 and ending at residue 118. ScHb was 188 residues long and had a high identity (approximately 83%) with a hemoglobin from *Zea mays*, which is 191 residues long (Table 1). ScHb is more closely related to *Arabidopsis* AHb1 (GLb1) than AHb2 (GLb1). AHb1 has a sequence and oxygen-binding characteristics that are typical of stress-induced hemoglobins, and its overexpression protects *Arabidopsis thaliana* from the effects of severe hypoxia [21], whereas AHb2 has greater similarity to symbiotic hemoglobins in both its sequence and oxygen-binding characteristics [22]. Whether ScHb functions in the stress response requires further investigation. However, because most genes with high identity to ScHb were classified as non-symbiotic hemoglobin class 1 (Table 1), we suggest that the sugar cane gene investigated here also belongs to this class.

Table 1. Amino acid sequence homology between sugar cane hemoglobin and other globins.

Protein identification	Gen bank accession number	Organism	Number of residues	Identity (%)
hemoglobin	GI:74058375	*Zea mays*	191	83
unknown protein with globin domain	GI:50932383	*Oryza sativa*	145	79
non-symbiotic hemoglobin class 1	GI:17366135	*Oryza sativa*	166	66
non-symbiotic hemoglobin class 1	GI:15809394	*Citrus unshiu*	183	65
hemoglobin	GI:2071976	*Hordeum vulgare*	162	63
hemoglobin Hb1	GI:27085255	*Triticum aestivum*	162	63
non-symbiotic hemoglobin 1 GLB1	GI17432970	*Arabidopsis thaliana*	160	63
non-symbiotic hemoglobin 2 GLB2	GI:17432971	*Arabidopsis thaliana*	158	54
myoglobin	GI:209563	*Physeter catodon*	153	20

ATG	GGG	TTC	AGT	GAG	GCA	CAG	GAA	GAG	CTT	GTC	ATC	CGT	TCA
M	G	F	S	E	A	Q	E	E	L	V	I	R	S
TGG	AAA	GCC	ATG	AAG	AAC	GAC	TCC	GAG	TCA	ATC	GCT	CTT	AAG
W	K	A	M	K	N	D	S	E	S	I	A	L	K
TTC	TTC	CTC	AGG	ATC	TTT	GAG	ATC	GCG	CCG	GAT	GCC	AAG	CAG
F	F	L	R	I	F	E	I	A	P	D	A	K	Q
ATG	TTC	TCC	TTC	CTG	CGC	GAC	GAC	GCC	GGC	GAC	GCG	ACC	CTG
M	F	S	F	L	R	D	D	A	G	D	A	T	L
GAG	AAC	CAC	CCC	AAG	CTC	AAG	GCG	CAC	GCC	GTC	ACC	GTC	TTC
E	N	H	P	K	L	K	A	H	A	V	T	V	F
GTC	ATG	GCT	TGC	GAG	TCC	GCG	ACG	CAG	CTG	AGG	AGC	ACC	GGC
V	M	A	C	E	S	A	T	Q	L	R	S	T	G
GAC	GTG	AAG	GTG	AGG	GAG	GCC	ACC	CTG	AAG	CGG	CTG	GGC	GCG
D	V	K	V	R	E	A	T	L	K	R	L	G	A
ACG	CAC	GTC	AAG	GCG	GGC	GTC	GCC	GAC	GCG	CAT	TTC	GAG	GTC
T	H	V	K	A	G	V	A	D	A	H	F	E	V
GTA	AAG	ACG	GCG	CTG	CTG	GAC	ACC	ATC	AGG	GAC	GCG	GTC	CCG
V	K	T	A	L	L	D	T	I	R	D	A	V	P
GAC	AGG	TGG	ACG	CCG	GAA	ATG	AAG	GCG	GCG	TGG	GAG	GAG	GCC
D	R	**W**	T	P	E	M	K	A	A	**W**	E	E	A
TAC	GAC	CAG	CTG	GCC	GCC	GCC	ATC	AAG	GAG	GAG	ATG	AAG	AAC
Y	D	Q	L	A	A	A	I	K	E	E	M	K	N
GGC	GCC	GTC	AAG	GAG	GAG	ATG	AAG	AAC	GGC	GCC	GTC	AAG	GAG
G	A	V	K	E	E	M	K	N	G	A	V	K	E
GAG	ACG	AAG	GAC	GCC	GCC	GCG	GCT	CGA	TGG	TTC	CTA	TGC	TCC
E	T	K	D	A	A	A	A	R	**W**	F	L	C	S
TCC	GCT	AGC	TCG	CGT	GAC	TAA							
S	A	S	S	R	D	**STOP**							

Figure 1. DNA nucleotide sequence and deduced amino acid sequence of the sugar cane hemoglobin gene. Nucleotides and amino acid residues are represented by an one-letter code. The initial ATG codon and the STOP codon are in bold, and the Trp residues (W) are in red.

As a matter of fact, non-symbiotic hemoglobin genes have already been identified in several plants [5-8].

3.2. *Protein production*

Protein expression was induced at 42°C to induce the formation of inclusion bodies, from which it is possible, after a few steps, to purify a folded globin protein in the apo form, as previously reported [11]. Under this expression condition, apoScHb was the major protein present in the inclusion body fraction and was solubilized by the addition of a chemical denaturant. Thereafter, it was dialyzed and then loaded onto a Q Sepharose column (Fig. 2, lane 1) and purified (Fig. 2, lane 2). The fraction from the last step, which contained apoScHb, was dialyzed and then loaded onto a High Load Superdex 200pg 26/60 column for the final chromatographic step (Fig. 2, lane 3). The final protein product was pure, with a yield of approximately 24 mg/mL (Table 2).

The folded state of apoScHb was first investigated by fluorescence. The fluorescence of Trp is very sensitive to the polarity of the environment, thus allowing insight on whether the residue is buried in the apolar interior of the protein or exposed to the solvent. ScHb has four Trp residues (Fig. 1), and its emission fluorescence spectroscopy spectrum is shown in Fig. 3A. The emission fluorescence spectrum has a maximum intensity at 337 nm with center of mass of 342 nm (Table 2), indicating that the Trp residues were well buried in the globin protein. This result was characteristic of a well-folded protein in which the hydrophobic Trp residues are buried and the hydrophilic residues are at the surface. Because the observed fluorescence spectrum is the sum of each Trp fluorescence spectrum, only an average evaluation of the Trp environment is possible. However, further indication of our purified apoScHb as a well-folded protein was also provided by circular dichroism (CD) studies (Fig. 3B). Circular dichroism is a fast and reliable tool to evaluate the secondary structure of a protein and, thus, its folded state [23]. The far-UV spectrum of apoScHb was characteristic of an α-helical protein with minima at 208 and 222 nm (Fig. 3B), and the secondary structural prediction indicated that it was approximately 68% α-helical (Table 2). These results are in good agreement with a globin fold, in which a large α-helical content is characteristic even for the apo form of the protein [24].

3.3. *Heme binding*

Because the cDNA annotated as a hemoglobin encoded a protein with a fold that is characteristic of the globin family, we tested whether this protein is able to bind heme and if this binding caused conformational changes in the protein. Hemin binding was evaluated as previously described for recombinant sperm whale myoglobin [11] using the absorbance spectra of apo and holoScHb from 250 to 600 nm (Fig. 3C). ApoScHb showed absorption at approximately 280 nm due to its aromatic residues and showed no significant absorption in the visible region (Fig. 3C, Table 2), whereas holoScHb also showed absorption at approximately 280 nm due to its aromatic residues and had an absorption peak at 416 nm, the Soret absorption peak of bound hemin (Fig. 3C,

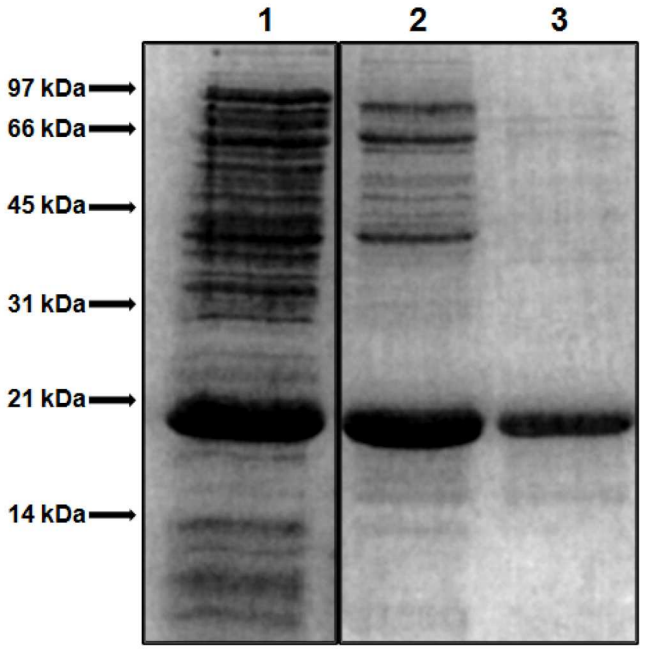

Figure 2. SDS-PAGE showing the ScHb purification steps (see Material and Methods). Lane 1, Sample prior to loading onto a Q Sepharose column. Lane 2, pool of ScHb purified from the Q Sepharose column. Lane 3, pool of ScHb purified from the High Load Superdex 200pg 26/60 column. The resulting ApoScHb was pure, with a yield of approximately 24 mg/mL (Table 2). The molecular mass standards are shown on the right (arrows).

Table 2. ScHb parameters.

	Apo ScHb	Holo ScHb
Yield (mg/L)	24	-
Emission fluorescence maximum wavelength (nm)	337	-
Emission fluorescence spectral center of mass (nm)	342	-
A_{416}/A_{280}	0.06	2.63
α-helical content (%)	68	87

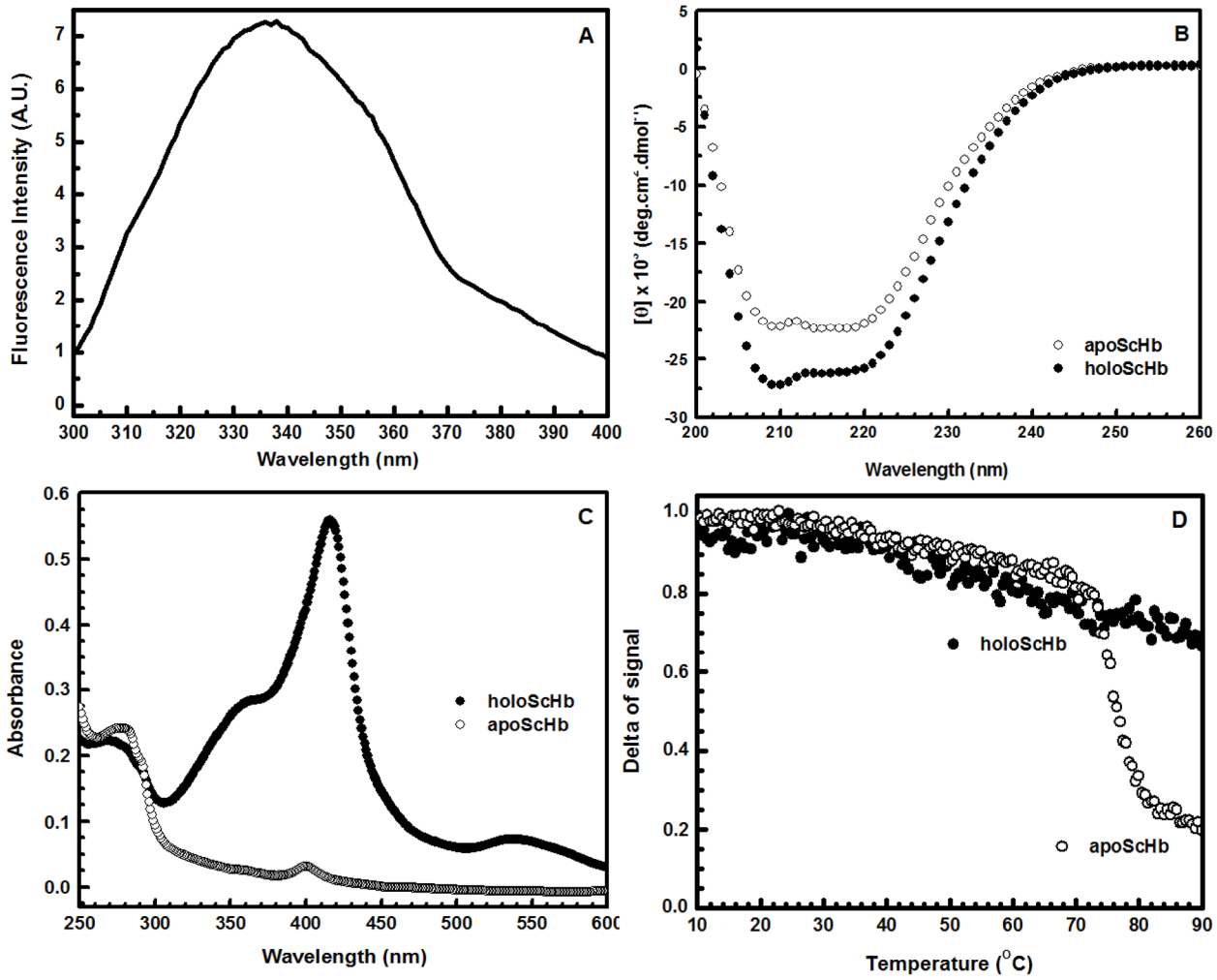

Figure 3. Spectroscopic experiments. A) Fluorescence. The emission fluorescence spectrum was measured from 300 to 400 nm and had a maximum intensity at 337 nm with a center of mass of 342 nm. B) Circular dichroism. The circular dichroism (CD) spectra of apoScHb (open circles) and holoScHb (filled circles) were measured from 200 to 260 nm. The CD spectra of both forms showed minima at 208 and 222 nm, a characteristic of α-helical proteins. C) Absorbance. The absorbance of untreated (apo, open circles) and treated (holo, filled circles) ScHbs were measured in the UV and Soret regions (250-600 nm). Peaks at 280 nm, due to aromatic residues, and at 540 and 416 nm, which are indicative of native-like heme coordination, were present in the spectrum of holoScHb. D) Thermal-induced unfolding followed by CD. Thermal-induced unfolding was monitored by CD at 222 nm from 10 to 90°C. ApoScHb (open circles) had an unfolding transition starting at approximately 72°C with a midpoint at approximately 75°C, while holoScHb (filled circles) showed no apparent transition even at 90°C.

Table 2). Another absorption peak at 540 nm was present in the holoScHb spectrum (Fig. 3C), which is also considered to be characteristic of specific heme binding [25]. These absorption peaks are not caused by free hemin because the spectrum of free hemin is characterized by two large maxima of the same magnitude at approximately 340 and 400 nm and by a small minimum at approximately 600 nm (data not shown). Additionally, holoScHb had typical characteristics of hologlobins. HoloScHb had both a higher amount of secondary structure than the apo form (87% and 68%, respectively) (Fig. 3B and Table 2) and a higher stability, as shown by thermal-induced unfolding experiments (Fig. 3D). Thermal-induced unfolding was monitored by CD at 222 nm. ApoScHb had an unfolding transition starting at approximately 72°C, with a midpoint at approximately 75°C, while holoScHb showed no apparent transition even at 90°C (Fig. 3D). Increases in both secondary structure and stability as a consequence of heme binding have been reported for several globin proteins [26-29].

The above results provide strong support to the hypothesis that the gene predicted as a hemoglobin in the sugar cane EST database does indeed code for a protein with a globin fold. Therefore, the three-dimensional structure of ScHb is likely to be similar to that of other hemoglobins because globins are strikingly similar to each other despite extensive variations in amino acid sequence. Additionally, the array of α-helices that constitutes the globin protein fold has been conserved throughout the evolution of plants and animals [30]. The structure of the *Oryza sativa* non-symbiotic hemoglobin GLB1a (PDB 1D8U chain A; Fig. 4, top A), which shares 66% sequence identity with sugar cane Hb (Table 1 and Fig. 4, bottom), was used as a template to model the structure of ScHb (Fig. 4, top B). The final model of ScHb had good stereochemical quality according to the Ramchandran plot (Supplementary Fig. 1). The residues in most favored regions, residues in additional allowed regions, residues in generously allowed regions, and residues in disallowed regions were 89.4%, 7.0%, 2.8%, and 0.7%, respectively (Supplementary Fig. 1), confirming the geometrically acceptable quality of the model. The globular fold of the ScHb model and the relative position of the α-helices were very similar to those of GLB1a (Fig. 4). The Sc hemoglobin length of 188 amino acids exceeds the lengths of vertebrate myo- and hemoglobins and results from a C-terminal protein extension, whose functional relevance is unclear. However, the globin fold was conserved, as shown in Fig. 4 (top B), and the proximal and distal histidines in positions E7 and F8 as well as the phenylalanine at the CD1 corner were present in ScHb (Fig. 4, top B). Thus, the key residues that are important for the function of ScHb as a typical oxygen binding protein are strictly conserved. The class 1 and 2 Hbs [31] of plants are similar to vertebrate Hbs in that all of these molecules have an E7 histidine residue, which is necessary for binding oxygen and other ligands in the distal pocket of the molecule. Altogether, these results also support the hypothesis that the protein studied here is indeed a hemoglobin.

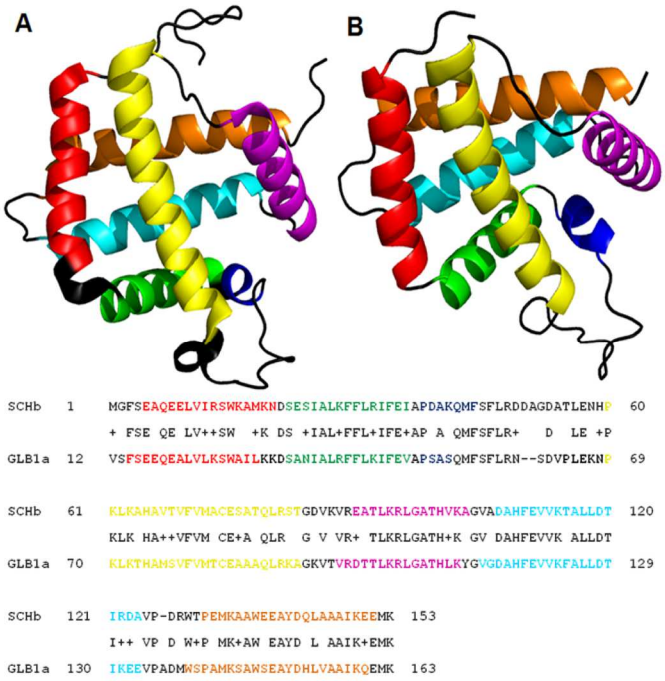

Figure 4. The structure of ORYsa GLB1a (PDB: 1D8U, top A) was used as a template to model the structure of ScHb (top B) by homology. The HHpred server [18] was used to generate a homology model for the *Saccharum officinarum* globin protein ScHb. The images were produced using PyMol (PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC). The amino acid alignment is shown at the bottom (only amino acids 1-153 of ScHb are shown), and colors were used to label the α-helices in both the amino acid sequence and the structures.

4. Concluding Remarks

We report the identification of a heme-binding globin in sugar cane. A gene coding for hemoglobin in sugarcane was predicted from an EST database [9] and mRNA expression data [32]. Sugar cane hemoglobin was purified as a soluble protein and in its apo form, i.e., without heme bound. The apoprotein was able to bind heme, as seen by the existence of a characteristic Soret band and an increase in secondary structure, as is expected for its holo form. The widespread presence and long evolutionary history of plant hemoglobins suggest a major role for these proteins in plants; however, little is known about their function. The identification of this gene in sugar cane supports the hypothesis that non-symbiotic hemoglobin genes are present in all plants.

5. Supplementary Material

Supplementary data and information is available at: http://www.jiomics.com/index.php/jio/rt/suppFiles/156/0

Supplemental Figure 1. Ramachandran plot of the ScHb 3D model, which was based on the structure of ORYsa GLB1a (PDB: 1D8U-A). The plot shows the acceptability of the model.

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References

[1] R.E. Dickerson, I. Geis, Hemoglobin: structure, function, evolution, and pathology, Benjamin/Cummings Pub. Co., 1983.

[2] J.A. Hoy, M.S. Hargrove, Plant Physiol Biochem, 46 (2008) 371-379. doi: 10.1016/j.plaphy.2007.12.016

[3] K.J. Gupta, K.H. Hebelstrup, L.A. Mur, A.U. Igamberdiev, FEBS Lett, 585 (2011) 3843-3849. doi: 10.1016/j.febslet.2011.10.036

[4] H. Kubo, Acta Phytochim., 11 (1939) 195-200.

[5] C. Dordas, J. Rivoal, R.D. Hill, Ann Bot, 91 Spec No (2003) 173-178. doi: 10.1093/aob/mcf115

[6] D. Bogusz, C.A. Appleby, J. Landsmann, E.S. Dennis, M.J. Trinick, W.J. Peacock, Nature, 331 (1988) 178-180. doi: 10.1038/331178a0

[7] E.R. Taylor, X.Z. Nie, A.W. Macgregor, R.D. Hill, Plant Molecular Biology, 24 (1994) 853-862. doi: Doi 10.1007/Bf00014440

[8] B. Trevaskis, R.A. Watts, C.R. Andersson, D.J. Llewellyn, M.S. Hargrove, J.S. Olson, E.S. Dennis, W.J. Peacock, Proc Natl Acad Sci U S A, 94 (1997) 12230-12234. doi: 10.1073/pnas.94.22.12230

[9] A.L. Vettore, F.R. da Silva, E.L. Kemper, P. Arruda, Genetics and Molecular Biology, 24 (2001) 1-7. doi: 10.1590/s1415-47572001000100002

[10] F. William Studier, A.H. Rosenberg, J.J. Dunn, J.W. Dubendorff, 185 (1990) 60-89. doi: 10.1016/0076-6879(90)85008-c

[11] E.A. Ribeiro, Jr., W.C. Regis, L. Tasic, C.H. Ramos, Protein Expr Purif, 28 (2003) 202-208. doi: 10.1016/s1046-5928(02)00651-4

[12] U.K. Laemmli, Nature, 227 (1970) 680-685. doi: 10.1038/227680a0

[13] H. Edelhoch, Biochemistry, 6 (1967) 1948-1954. doi: 10.1021/bi00859a010

[14] S.C. Gill, P.H. von Hippel, Anal Biochem, 182 (1989) 319-326. doi: 10.1016/0003-2697(89)90602-7

[15] M.M. Bradford, Anal Biochem, 72 (1976) 248-254. doi: 10.1016/0003-2697(76)90527-3

[16] E. Antonini, M. Brunori, eds., Hemoglobin and Myoglobin in their Reactions with Ligands, American Elsevier, New York, 1971.

[17] J.L. Silva, E.W. Miles, G. Weber, Biochemistry, 25 (1986) 5780-5786. doi: 10.1021/bi00367a065

[18] J. Soding, A. Biegert, A.N. Lupas, Nucleic Acids Res, 33 (2005) W244-248. doi: 10.1093/nar/gki408

[19] R.A. Laskowski, J.A. Rullmannn, M.W. MacArthur, R. Kaptein, J.M. Thornton, J Biomol NMR, 8 (1996) 477-486. doi: 10.1007/bf00228148

[20] J. Parkinson, M. Blaxter, Methods Mol Biol, 533 (2009) 1-12. doi: 10.1007/978-1-60327-136-3_1

[21] P.W. Hunt, E.J. Klok, B. Trevaskis, R.A. Watts, M.H. Ellis, W.J. Peacock, E.S. Dennis, Proc Natl Acad Sci U S A, 99 (2002) 17197-17202. doi: 10.1073/pnas.212648799

[22] R. Arredondo-Peter, M.S. Hargrove, J.F. Moran, G. Sarath, R.V. Klucas, Plant Physiol, 118 (1998) 1121-1125. doi: 10.1104/pp.118.4.1121

[23] D.H.A. Corrêa, C.H.I. Ramos, Afr J Biochem Res 3(2009) 9.

[24] W.C. Regis, J. Fattori, M.M. Santoro, M. Jamin, C.H. Ramos, Arch Biochem Biophys, 436 (2005) 168-177. doi: 10.1016/j.abb.2005.01.016

[25] C.S. Craik, S.R. Buchman, S. Beychok, Proc Natl Acad Sci U S A, 77 (1980) 1384-1388. doi: 10.1073/pnas.77.3.1384

[26] E. Breslow, S. Beychok, K.D. Hardman, F.R. Gurd, J Biol Chem, 240 (1965) 304-309.

[27] Y.Q. Feng, S.G. Sligar, Biochemistry, 30 (1991) 10150-10155. doi: 10.1021/bi00106a011

[28] C.H. Ramos, M.S. Kay, R.L. Baldwin, Biochemistry, 38 (1999) 9783-9790. doi: 10.1021/bi9828627

[29] D.A. Landfried, D.A. Vuletich, M.P. Pond, J.T. Lecomte, Gene, 398 (2007) 12-28. doi: 10.1016/j.gene.2007.02.046

[30] D. Bashford, C. Chothia, A.M. Lesk, J Mol Biol, 196 (1987) 199-216. doi: 10.1016/0022-2836(87)90521-3

[31] P.W. Hunt, R.A. Watts, B. Trevaskis, D.J. Llewelyn, J. Burnell, E.S. Dennis, W.J. Peacock, Plant Molecular Biology, 47 (2001) 677-692. doi: 10.1023/a:1012440926982

[32] R. Linacero, M.G. Lopez-Bilbao, A.M. Vazquez, Protoplasma, 217 (2001) 199-204. doi: 10.1007/BF01283401