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ScienceDirect
Phytochemistry 68 (2007) 802–810
PHYTOCHEMISTRY
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Expression of the sucrose binding protein from soybean: Renaturation and stability of the recombinant protein

Carolina S. Rocha [a,1], Dirce F. Luz [a,1], Marli L. Oliveira [a], Maria C. Baracat-Pereira [a], Francisco Javier Medrano [b], Elizabeth P.B. Fontes [a,*]

[a] *Departamento de Bioquímica e Biologia Molecular/BIOAGRO, Universidade Federal de Viçosa, 36571.000 Viçosa – MG, Brazil*
[b] *Laboratório Nacional de Luz Syncroton – 13084-971, Campinas – SP, Brazil*

Received 7 June 2006; received in revised form 12 September 2006
Available online 12 January 2007

Abstract

The sucrose binding protein (SBP) belongs to the cupin family of proteins and is structurally related to vicilin-like storage proteins. In this investigation, a SBP isoform (GmSBP2/S64) was expressed in *E. coli* and large amounts of the protein accumulated in the insoluble fraction as inclusion bodies. The renatured protein was studied by circular dichroism (CD), intrinsic fluorescence, and binding of the hydrophobic probes ANS and Bis-ANS. The estimated content of secondary structure of the renatured protein was consistent with that obtained by theoretical modeling with a large predominance of β-strand structure (42%) over the α-helix (9.9%). The fluorescence emission maximum of 303 nm for SBP2 indicated that the fluorescent tryptophan was completely buried within a highly hydrophobic environment. We also measured the equilibrium dissociation constant (K_d) of sucrose binding by fluorescence titration using the refolded protein. The low sucrose binding affinity ($K_d = 2.79 \pm 0.22$ mM) of the renatured protein was similar to that of the native protein purified from soybean seeds. Collectively, these results indicate that the folded structure of the renatured protein was similar to the native SBP protein. As a member of the bicupin family of proteins, which includes highly stable seed storage proteins, SBP2 was fairly stable at high temperatures. Likewise, it remained folded to a similar extent in the presence or absence of 7.6 M urea or 6.7 M GdmHCl. The high stability of the renatured protein may be a reminiscent property of SBP from its evolutionary relatedness to the seed storage proteins.

Keywords: Glycine max; Soybean; Legumes; Heterologous expression; Protein renaturation; Cupin structure; Sucrose binding protein

1. Introduction

The cupin superfamily corresponds to a functionally diverse family of proteins that share a β-barrel structural core domain to which the term cupin (Latin *cupa*, barrel) was given (Dunwell et al., 2000). In spite of having very low levels of sequence identity, members of the cupin superfamily have highly conserved structures and include enzymes as well as proteins that bind different sugars. The cupin superfamily also comprises the major globulin storage proteins mainly from nuts and legumes from which the three-dimensional structures have been solved (Ko et al., 1993; Lawrence et al., 1990, 1994; Maruyama et al., 2001). The main feature of the cupin domain is a two-motif conserved sequence [motif 1, PG(X)5HXH(X)4E(X)7G and motif 2, G(X)5PXG(X)2H(X)3N] with a variable intermotif sequence ranging from 15 residues in many bacterial enzymes up to more than 50 residues in some storage proteins. The members of this family can be organized into a single domain (monocupin) or a two-domain cupin (bicupin) structure (Dunwell et al., 2000).

The sucrose binding protein (SBP) belongs to the bicupin family of proteins and is structurally related to the vicilin-like storage proteins (Dunwell et al., 2004). Despite the evolutionary relatedness between SBPs and seed storage

Abbreviations: CD, circular dichroism; GdmHCl, guanidinium chloride; GmSBP1, *Glycine max* sucrose binding protein isoform 1; GmSBP2/S64, *Glycine max* sucrose binding protein isoform 2; VfSBP, *Vicia faba* sucrose binding protein.

* Corresponding author. Tel.: +55 31 3899 2949; fax: +55 31 3899 2864.
E-mail address: bbfontes@ufv.br (E.P.B. Fontes).

[1] These authors contributed equally to this work.

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doi:10.1016/j.phytochem.2006.11.036

proteins, they exhibit distinct biochemical activities and diverge functionally. The *Glycine max* SBP (GmSBP) binds sucrose and exhibits a low sucrose transport activity in the yeast heterologous system (Elmer et al., 2003; Grimes and Overvoorde, 1996; Overvoorde et al., 1996, 1997). Furthermore, GmSBP interacts with GTP in a sucrose traslocation-independent manner and has been shown to be involved in sucrose-dependent physiological processes in transgenic tobacco plants (Delú-Filho et al., 2000; Pedra et al., 2000; Pirovani et al., 2002). However, characterized SBPs from other species do not share these biochemical and functional properties and, therefore, a functional signature for the members of the SBP family is still a matter of debate.

Further complications to assign a function to SBP are derived from the observation that its deduced amino acid sequence does not possess structural motifs of typical membrane transporters and hence the underlying mechanism for a SBP-mediated sucrose transport remains elusive and controversial (Elmer et al., 2003; Grimes et al., 1992; Grimes and Overvoorde, 1996; Overvoorde et al., 1996, 1997; Pirovani et al., 2002). Topological characterization of SBP in purified plasma membrane vesicles has demonstrated that a proportion (25%) of SBP is tightly associated with the external leaflet of the plasma membrane and behaves as a type II membrane protein, which spans the bilayer once and has the bulk of the protein exposed to the extracellular environment (Overvoorde and Grimes, 1994). According to this topological model, the oligomerization properties of the protein would provide the means for assembling protein conduits across the membrane to mediate sucrose transport. This possibility has been considered with the observation that SBP is structurally related to vicilin-like storage proteins (Overvoorde et al., 1996; Braun et al., 1996), which assemble into trimers to form an 86–88 Å toroid complex with an internal hole of 18 Å (Ko et al., 1993; Braun et al., 1996). Conserved residues involved in stabilizing the three-dimensional structure of vicilin-like proteins are found at similar positions in the SBP sequence (Pirovani et al., 2002). However, a high-resolution three-dimensional structure of SBP is still missing. The primary reason for the lack of progress in this matter is the difficulty to purify large amounts of SBP from soybean seeds due to the low abundance of the protein in a highly concentrated storage protein environment. In this study the sucrose binding protein was expressed in *Escherichia coli* and large amounts of the protein accumulated as inclusion bodies. The renaturation and stability of the protein were monitored by CD and spectrofluorimetric studies.

2. Results and discussion

2.1. Expression of SBP in *E. coli* and renaturation of the recombinant protein

After induction with IPTG, the pUFV120 transformed cells expressed the SBP recombinant protein, which predominantly accumulated in the insoluble fraction as inclusion bodies (Fig. 1a). The SBP recombinant protein purified under denaturing conditions is shown in Fig. 1b. The protein, in the presence of 8 M urea, was diluted 50-fold to favor intramolecular interactions (self-organization) as opposed to aggregation, and refolding was achieved by progressive removal of urea in the renaturation buffer. The renaturation buffer contained a redox shuffling system (2 mM reduced glutathione and 0.2 mM oxidized glutathione) and 5% glycerol in H_2O (v/v) as a protein-stabilizing agent. The renatured protein (0.15 mg protein from 1 L culture of *E. coli*) was dialyzed against storage buffer (1 mM Tris–Cl, pH 7.5) and concentrated to 5 mg/mL by lyophilization.

In order to obtain a rough estimate of the secondary structure of the renatured protein, CD spectra were taken (Fig. 2). The far-UV CD spectrum of the renatured protein SBP2 showed a negative band with a shoulder in the 223–225 nm region and a broad minimum at around 204 nm, as well as a positive band in the 186–190 nm range, consistent with predominantly β-sheet structure proteins (Manavalan and Johnson, 1983). The near-UV spectrum presented a minimum around 263 nm. The secondary structure content was estimated using the program CDNN (CD spectra deconvolution, Bohm et al., 1992) and showed a large predominance of β-sheets (antiparallel, 38.6% and parallel,

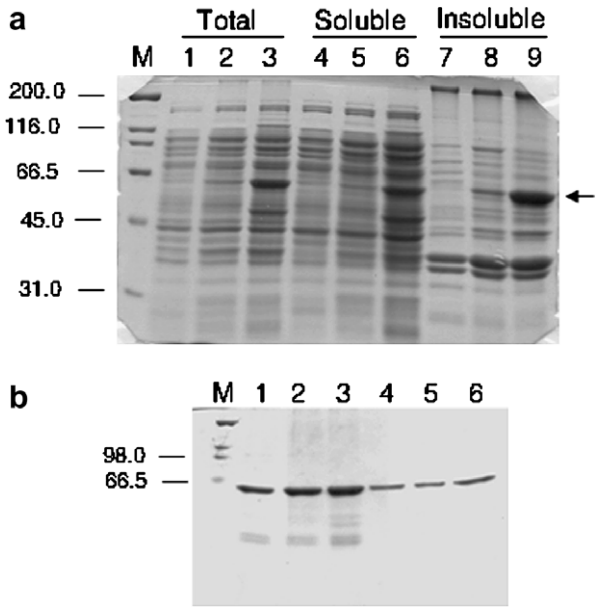

Fig. 1. Expression of S64/SBP2 in *E. coli*. (a) SBP2 accumulates predominantly in the insoluble fraction. Protein extracts from untransformed BL21(DE_3) (lanes 1, 4, 7), transformed BL21(DE_3) with pUFV120 in the absence (lanes 2, 5, and 8) or presence of IPTG (lanes 3, 6 and 9) were fractionated by SDS–PAGE and visualized using Coomassie Brilliant Blue. Total protein extracts, soluble and insoluble fractions, are shown. The arrow indicates the position of the S64/SBP2 recombinant protein. (b) Purification of SBP2 recombinant protein from the *E. coli* protein insoluble fraction. N-Terminal His-tagged S64/SBP2 fusion protein was produced in *E. coli*, affinity-purified and separated by SDS/PAGE. Lanes 1, 2 and 3 are the result of the first elution and lanes 4, 5 and 6 of the second elution from a Ni^{2+}NTA-agarose resin. Molecular mass markers (kDa) are shown on the left.

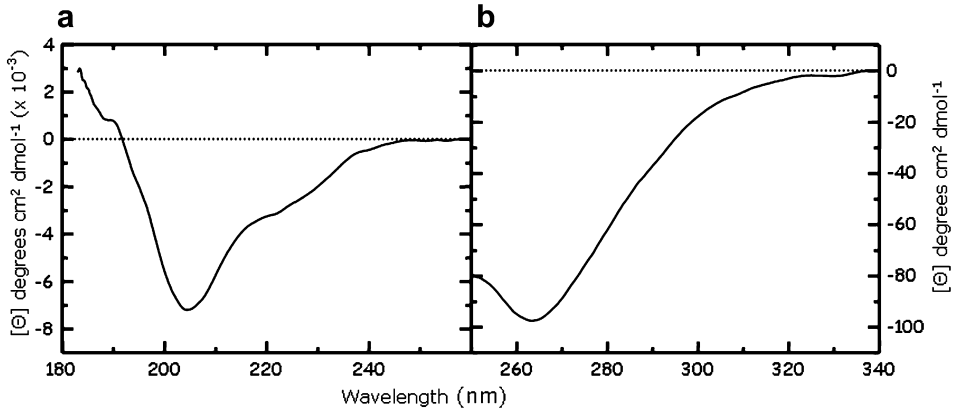

Fig. 2. CD spectra of the SBP2 recombinant protein. (a) Far-UV CD spectra of the renatured SBP2 protein. The spectra were measured in an 0.1-mm pathlength cell at a protein concentration of 0.12 mg/mL and averaged over four scans. The data are represented in mean residue ellipticities. The spectra were corrected for the buffer. (b) Near-UV CD spectra of the renatured protein. For the near-UV CD spectra, the protein concentration was 1.16 mg/mL and the cell path length was 1 mm. The measurements were performed in 10 mM Tris–HCl, pH 8.0. The data represented the average of four determinations.

3.4%) over α-helix (9.9%) in addition to 24.7% of β-turn and 29.5% of random coil. These values are consistent with the secondary structure content of several members of the bicupin group of vicilin-like proteins with known three-dimensional structures, such as β-conglycinin (14.1% α-helix, 36.7% β-sheet; Maruyama et al., 2001), canavalin (11.0% α-helix, 36.9% β-sheet; Ko et al., 2000) and phaseolin (16.6% α-helix, 36.0% β-sheet; Lawrence et al., 1994).

From the intrinsic fluorescence emission spectra, the renatured SBP2 protein has an emission wavelength maximum at 303 nm (Fig. 3a). This indicates that the aromatic residues are completely buried in an highly hydrophobic environment. Similar fluorescence λ_{max} (307 nm) has only been found for azurin, a 14-kDa blue-copper electron-transfer protein with a single tryptophan residue (Ladokhin, 2000). This SBP2 λ_{max} differs largely from that of other bicupin-related proteins, such as the seed storage proteins, phaseolin and concavalin, that exhibit an λ_{max} at 329–332 nm and at 332 nm, respectively (Deshpande and Damodaran, 1991; Chatterjee and Mandal, 2003). Such a low emission wavelength maximum at 303 nm for GmSBP2/S64 may be explained by the predicted localization of its four tryptophan residues into the canonical three dimensional models for vicilin-like protein. The overall tertiary structure of SBP can be predicted by comparison to phaseolin and canavalin (Fig. 4). Alignment of GmSBP2 with canavalin from *Canavalia ensiformis*, phaseolin from *Phaseolus vulgaris* and β-conglycinin from *Glycine max* showed that SBP comprises a duplicated, two-domain, structure (residues 108–283, N-terminal domain and 303–490, C-terminal domain). The N-terminal and C-terminal tandem domains basically contain a core β-barrel subdomain of a Swiss-roll topology with an extended loop subdomain that contains helices 1, 2 and 3 (Ko et al., 2000). Like in phaseolin, a fourth helix interconnects the N-terminal and C-terminal domains in SBP, which are tightly associated via an extensive hydrophobic interface. The loop subdomains are responsible for assembly into trimers. The assembly of SBP into trimers has been assessed by model building (Ko et al., 2000) and by analytical ultracentrifugation of membrane preparations from soybean cotyledons under nonreducing conditions (Overvoorde et al., 1997). While these results confirmed that SBP fractionated as multimeric and trimeric complexes, they did not rule out the possibility that other proteins were present in these higher order complexes. Here we also conducted dynamic light scattering (DLD) studies, thereby confirming the oligomerization state of the purified and renatured recombinant protein. The hydrodynamic radii of gyration measured for the renatured protein by DLS were 7.3 nm and 4.9 nm at a concentration of 0.56 mg/ml, which correspond to a molecular mass of 351 kDa (hexamer) and 141 kDa (trimer), respectively. In the three-dimensional model of GmSBP2, the tryptophan residues are located in the interior of the protein, in a highly hydrophobic environment and protected from the bulk solvent. In fact, two adjacent tryptophan residues (positions 288–289) of GmSBP2/S64 are positioned into the N- and C-terminal domain-interconnecting sequence and hence in a highly hydrophobic environment. The residue 243 is located at the N-terminal loop subdomain that promotes subunit assembly and residue 326 in the rigid C-terminal β-barrel motif. In contrast, the tryptophan residues of phaseolins are located in a more exposed and flexible tertiary domain upstream of the N-terminal repeated domain.

The renatured SBP2 was tested for the existence of hydrophobic pockets with the probes ANS and Bis-ANS (Fig. 3b and c). A slight increase in the intensity of ANS fluorescence at 480 nm (excitation wavelength of 390 nm) was observed that saturated at 48 μM. Likewise, the intensity of Bis-ANS fluorescence (excitation wavelength of 398 nm) was slightly increased and saturated at 24 μM concentration. These results indicate the absence of hydrophobic pockets located in the surface of the protein due to the low increase in the fluorescence intensity of probes. Either a highly ordered and rigid tertiary structure in which the

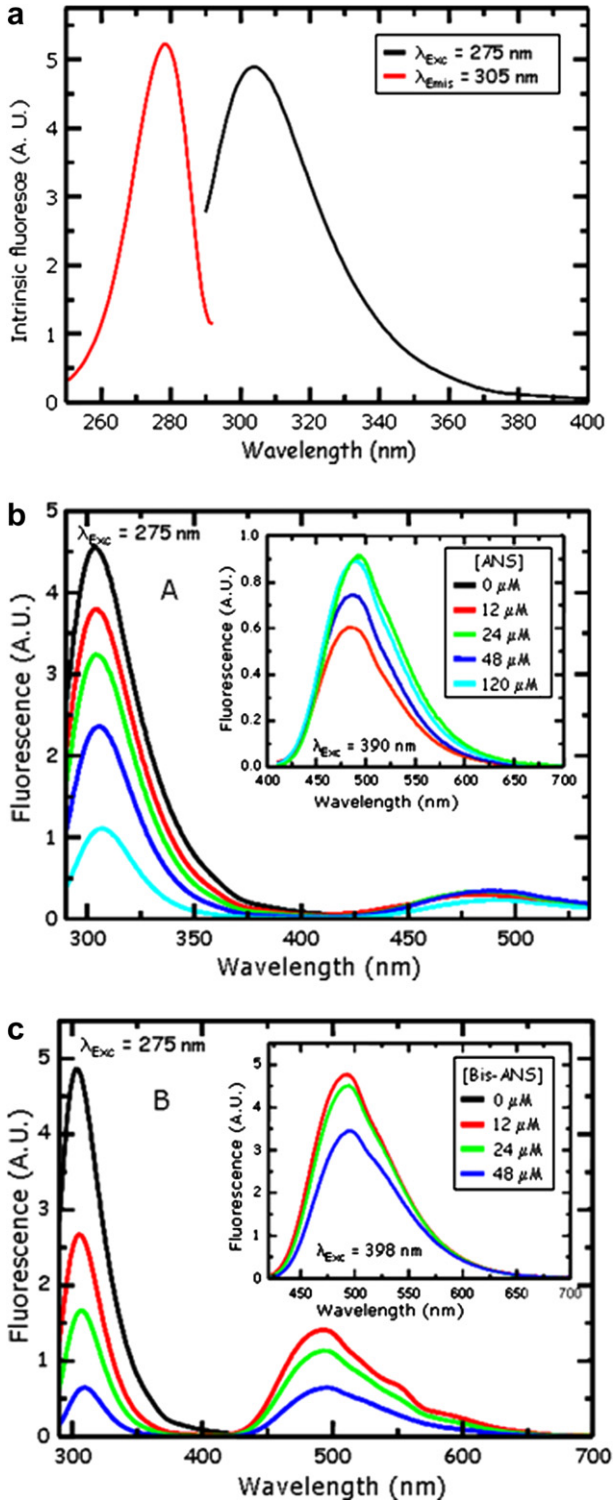

Fig. 3. Fluorescence spectra at 25 °C of the renatured SBP2/S64 protein in the absence (a) and presence of ANS (b) and Bis-ANS (c). (a) Intrinsic fluorescence of SBP2/S64. The curve in red corresponds to the excitation spectrum at an emission wavelength of 305 nm and in black, the emission spectrum is shown at an excitation wavelength of 275 nm. (b) ANS binding to SBP2/S64. Fluorescence spectra at 25 °C of SBP2/S64 in the presence of the indicated concentrations of ANS. The inset represents the extrinsic fluorescence of ANS at an excitation wavelength of 390 nm. (c) Bis-ANS binding to SBP2/S64. Fluorescence spectra at 25 °C of SBP2/S64 in the presence of the indicated concentrations of Bis-ANS. The inset shows the extrinsic fluorescence of Bis-ANS at excitation wavelength of 398 nm.

hydrophobic core is well protected from solvents, or a fully unfolded protein that has no structure, would fit the prediction for the low affinity binding of these hydrophobic probes. The CD data of the renatured protein is consistent with the first possibility. To further distinguish between these two possibilities, we measured the capacity of the refolded protein to bind sucrose, as a functional state of folding (see below).

2.2. Sucrose binding properties of the recombinant protein

The sucrose binding protein was first identified in soybean by its capacity to bind to a sucrose analog, 6′-deoxy-6′-(4-azido-2-hydroxy)-benzamido-sucrose, which competitively inhibited the influx of radiolabeled sucrose into protoplasts from developing soybean cotyledons (Ripp et al., 1988). Despite the lack of structural similarity between SBP and other known membrane transport proteins, subsequent progress in characterizing SBP has implicated the protein as the low affinity component of the sucrose uptake system in plants (Grimes et al., 1992; Grimes and Overvoorde, 1996; Overvoorde et al., 1996, 1997). We have thus used fluorescence quenching assays to test whether the renaturation of the recombinant GmSBP2/S64 protein restored the sucrose binding capability (Fig. 5a). Incubation of renatured GmSBP2 with increasing concentrations of sucrose promoted a quenching in the fluorescence emission spectrum of the protein but kept the maximum emission wavelength at 303 nm. This indicates that even high concentrations of ligand (100 mM) did not cause perturbations of the tertiary structure. The argument that sucrose binding did not cause any significant conformational change in GmSBP2 was further strengthened by the results of the CD spectra of the renatured protein in the presence of different sucrose concentrations (Fig. 5c). Except at 80 mM sucrose that promoted a slight increase in the ellipticity of the renatured protein spectrum at wavelengths lower than 205 nm, no significant change was observed in the far-UV CD spectra in the presence of sucrose. Likewise, the near-UV CD spectra was not modified by sucrose (Fig. 5d), indicating that the modifications observed at 80 mM sucrose in the far-UV region might be a direct result from the high dichroic signal of sucrose. The CD and fluorescence data together suggest that the conformational state of the renatured protein is not altered by sucrose binding.

The calculated sucrose dissociation constant, $K_d = 2.79 \pm 0.22$ mM (Fig. 5b), indicated that the renatured protein exhibited a low affinity binding for sucrose, as compared to the maltose dissociation constant of maltose binding proteins that vary in the micromolar range (Szmelcman et al., 1976; Miller et al., 1983; Hall et al., 1997; Guntas et al., 2005). This result validates previous observations regarding the affinity of SBP for sucrose and is consistent with the low rate of sucrose transport mediated by GmSBP1 in yeast (Ripp et al., 1988; Elmer et al., 2003). Furthermore, they demonstrate for the first

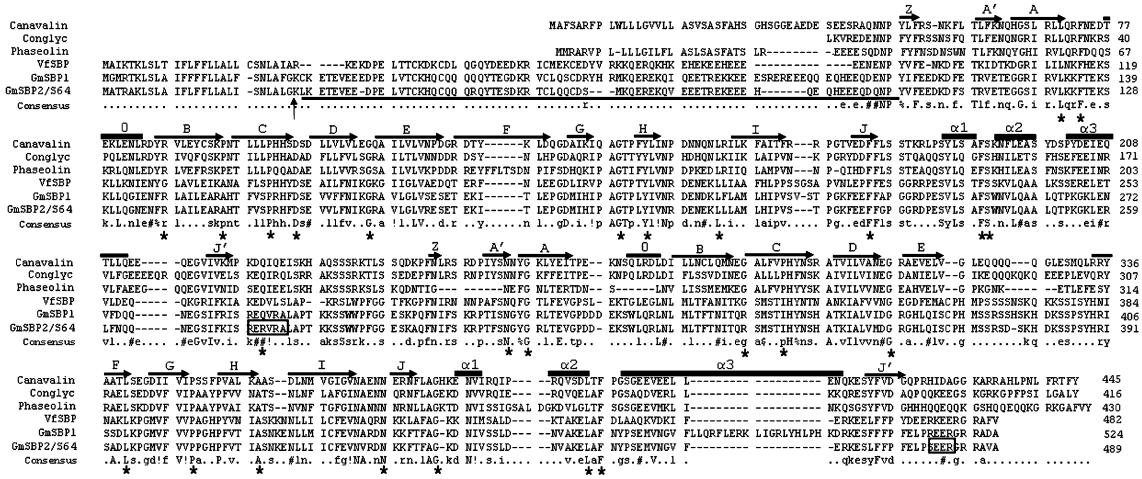

Fig. 4. Homology-based molecular modeling of GmSPB2/S64. A multiple sequence alignment of the deduced amino acid sequence of soybean GmSPB2/S64 (GenBank Accession No. AF191299), soybean GmSBP1 (GenBank Accession No. L0603), and *Vicia faba* SBP homologue (VfSBP, GenBank Accession No. VFA292221) with canavalin, phaseolin and β-conglycinin was obtained with the MultAlin software. The common secondary structural elements of strands and helices are shown above the sequences. The helix 4 is shown in the GmSBP2/S64 sequence by open boxes, and it is also present at a similar position in the phaseolin sequence. The putative signal peptide cleavage site of GmSPB2 is indicated by the arrow and the N-terminal hydrophilic segment is underlined in the SBP sequences. Conserved residues in equivalent positions of vicilin-like protein sequences, that are important in maintaining their three-dimensional structures are indicated by asterisks. The tryptophan residues in the GmSBP2/S64 sequence are in bold.

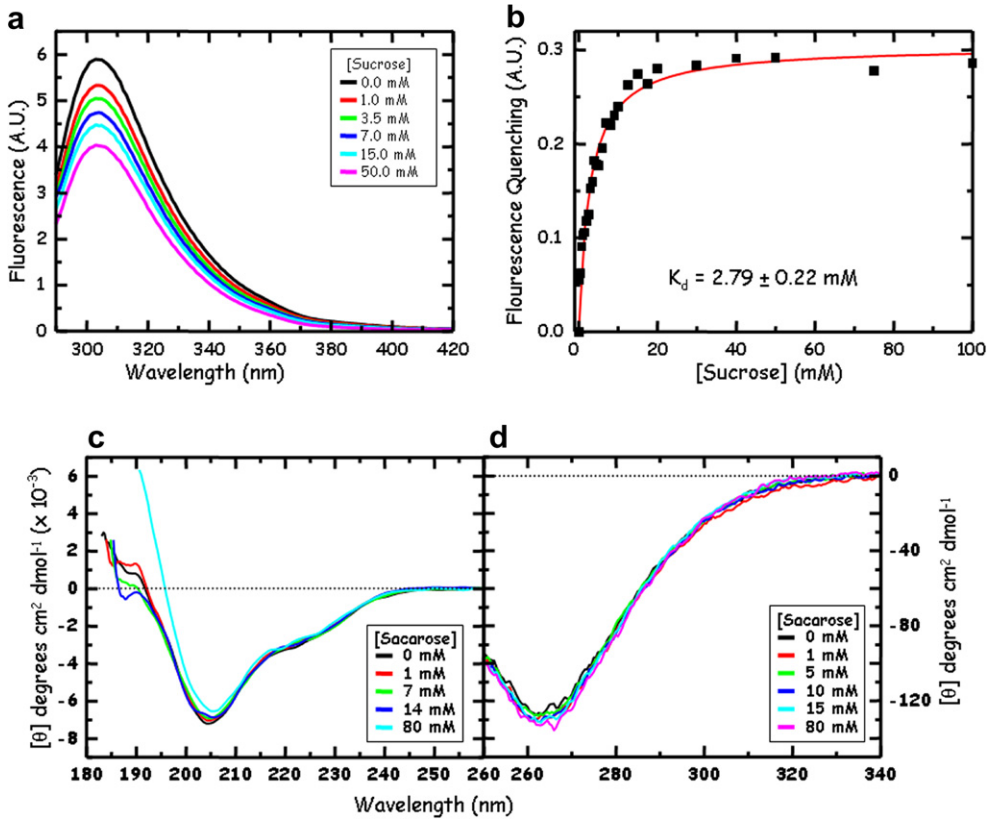

Fig. 5. Sucrose binding affinity of the renatured SBP2/S64 protein. (a) Sucrose binding induced-fluorescence quenching of SBP2. The emission spectra of the renatured protein were monitored in the presence of increasing concentrations of sucrose. (b) Fluorescence quenching titration. Corrected data were adjusted to a hyperbolic equation using non-linear regression. (c) Far-UV CD spectra of the renatured SBP2 protein in the presence of increasing concentrations of sucrose. (d) Near-UV CD spectra of the renatured SBP2 protein in the presence of increasing concentrations of sucrose.

time that the recombinant sucrose binding protein isoform 2 (GmSBP2/S64), which has been shown to facilitate sucrose uptake in yeast (Pirovani et al., 2002) and in plant cells (Delú-Filho et al., 2000), binds sucrose. Despite the tertiary structure conservation of the vicilin-like proteins, it has been previously pointed out that SBP differs from the vicilins by the presence of a large N-terminal extension and two insertions at the C-terminal domain between

strands E and F and between helix 3 and strand J′ (just for the GmSBP1 isoform) in addition to a C-terminal extension (Fig. 4). The effect of the 19 amino acid insertion at the C-terminal loop sub-domain has not yet been investigated, but it has been proposed to be relevant for the functional divergence of SBP from vicilins (Overvoorde et al., 1997). However, it is not a conserved feature of sucrose binding proteins as it is absent in the GmSBP2/S64 isoform and in the VfSBP.

Another structural discrepancy from the vicilin-like proteins is the presence of an extensive N-terminal region in the GmSPB2/S64 sequence, which is conserved in the GmSBP1 isoform and in VfSBP. However, as this region has no equivalent in vicilin-like proteins used as template proteins, it could not be modeled and, as the three-dimensional structure of SBP has not been determined, the effects of this segment remain unknown (Fig. 4). This N-terminal extension contains a hydrophobic sequence of amino acids at the SBP N-terminus that may function as a signal sequence followed by a highly charged hydrophilic segment. In the *E. coli*-expressed recombinant protein, the putative signal sequence was replaced by an extension of histidine residues provided by the expression vector that could make salt bridge contacts with the glutamate residues present in the immediately downstream region of the recombinant protein. While this histidine tag could contribute to some conformational perturbation in the recombinant protein, it did not cause a global misfolding upon renaturation, as the renatured protein regained its capacity to bind sucrose and exhibited CD and fluorescence spectra consistent with a folded cupin tertiary structure. It would be interesting, however, to analyze the effect of the histidine extension on the capacity of the recombinant protein to transport sucrose in yeast.

2.3. Stability of the renatured recombinant SBP protein

As a member of the bicupin family of proteins which includes highly stable seed storage proteins, it was of interest to determine the structural stability of SBP. Thermal denaturation of the protein was examined by monitoring changes in the CD spectra of the renatured protein. As the temperature rises, it promoted a gradual decay in the intensity of the minimum at 204 nm, and upon reaching 95 °C, the minimum was shifted to 202 nm (Fig. 6a). These changes were accompanied by a reduction in the shoulder at 223–225 nm. The estimation of secondary structure at each temperature (Fig. 6b) demonstrated a slight reduction in α-helix content, β-turn and random coil structure as the temperature rises, whereas a slight increase in the antiparallel β-sheet was observed at high temperature. Based on the CD spectra as a function of temperature, the renatured SBP2 recombinant protein was thermally stable and only minor conformational alterations were observed at 95 °C. This behavior resembles that of representative members of the bicupin family, such as the thermal stable storage protein phaseolin (Deyer et al., 1992). In fact, the thermal denaturation of phaseolin has been shown to require more than one simultaneous denaturing agent, such as the presence of either GdmHCl or pH changes. Presumably, it was the thermo tolerance and stability of the cupin tertiary structure that dictated its evolutionary capture by plants as storage proteins. Thus, the thermal stability of the renatured SBP2/S64 isoform may be considered as indicative that the recombinant protein folded properly into a cupin structure.

We have also monitored the stability of the renatured protein by GdmHCl and urea-induced denaturation. The fluorescence emission spectra of the recombinant protein in 6.7 M GdmHCl and in 7.6 M urea are shown in Fig. 7a. Some perturbation of the native tertiary structure was observed in the presence of the chemical treatments, markedly by the shift of the wavelength from 303 to 307 nm in 6.7 M GdmCl and to 305 nm in 7.6 M urea. Nevertheless, in place of the expected shift in wavelength from 303 to 350 nm on complete unfolding, fluorescence intensity remained virtually constant in this wavelength region, suggesting that considerable heterogeneity in the aromatic residues environments persisted in 6.7 M GdmHCl and 7.6 M urea. Thus, it seems that a significant fraction of the aromatic residues remained shielded in an apolar environment even in the presence of 6.7 M GdmHCl or 7.6 M urea.

The extent of SBP2/S64 denaturation was also monitored by measuring fluorescence as a function of the pH (Fig. 7b). Upon pH exposure of the renatured SBP2, a

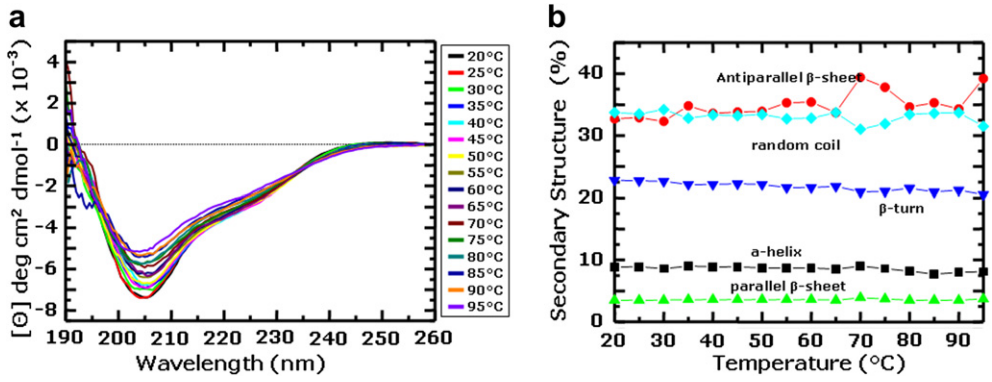

Fig. 6. Thermal stability of the renatured SBP2/S64 protein. Far-UV CD spectra were taken at different temperatures, as indicated. The percentage of secondary structure was calculated as the temperature was increased from 20 to 95 °C by 5° increments.

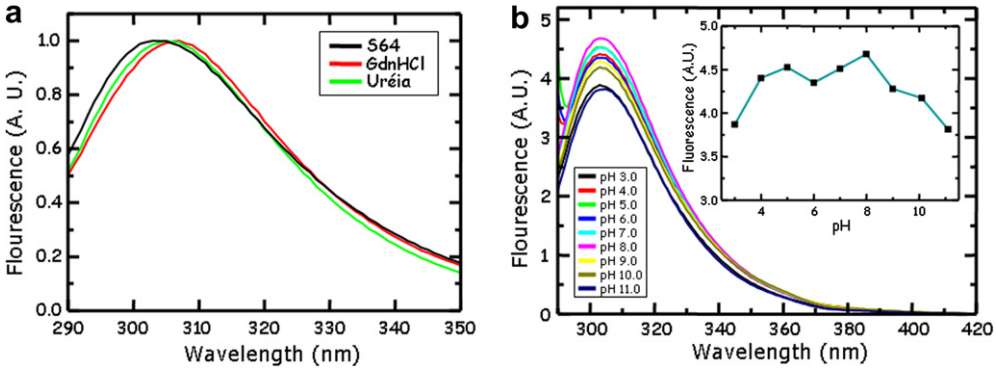

Fig. 7. Chemical denaturation of the renatured SBP2/S64 protein. (a) Fluorescence emission spectra of renatured SBP2 in the absence and presence of 6.7 M GndHCL and 7.8 M urea. (b) Effect of pH on the intrinsic fluorescence of renatured GmSBP2/S64. Fluorescence emission spectra of renatured SBP2 were recorded at different pHs, as indicated. The excitation wavelength was 275 nm. The inset shows the fluorescence emission values at 305 nm over the pH range from 3 to 11.

stable conformational state seemed to persist over the pH range from 4 to 8, as the intrinsic fluorescence was not significantly altered in this range of pH. However, at alkaline (pH > 8) and acidic (pH < 4) pHs, the fluorescence intensity was reduced to an extent that indicates a certain degree of exposure of the tryptophan residues to a more polar environment. The effect of pH on the fluorescence intensity of members of the bicupin family of proteins, such as concanavalin and phaseolin, has been previously investigated (Deyer et al., 1992; Khan et al., 2005). In general, the variation of pH caused a more drastic conformational effect on the stability of phaseolin and concanavalin than on SBP2.

Concluding remarks: In addition to structural homology, the similarities between vicilin-like seed storage proteins and SBP extend to the subcellular compartimentalization and expression. In fact, a large proportion of GmSBP is co-localized in seed protein bodies and exhibits cotyledon-specific expression that is temporally coordinated with the soybean vicilin-like storage protein, β-conglycinin, during seed development (Heim et al., 2001; Contim et al., 2003; Elmer et al., 2003; Waclawovsky et al., 2006). In general, the high stability of vicilin-like storage proteins may be a result of evolutionary pressure towards conservation of structural tertiary motifs that would promote tolerance to desiccation during seed maturation, packing of the storage proteins into protein bodies, etc. Thus, the high stability of SBP2 recombinant protein may be a reminiscent property of SBP from its evolutionary relatedness to the seed storage proteins and is clearly connected to its capacity to fold into a cupin tertiary structure.

3. Experimental

3.1. DNA construct

The S64/SBP2 protein was expressed as a fusion protein using the pET-16b vector (Novagen), which provides an N-terminal His tag. The cloning of the *S64*/*SBP2* cDNA into pET-16b yielding pUFV120 has been previously described (Pirovani et al., 2002).

3.2. Expression of SBP in E. coli

LB medium (1 L) plus ampicillin (100 mg) was inoculated with an overnight cell culture of transformed BL21 (DE3) cells containing the plasmid pUFV120. The cells were grown under shaking at 30 °C to an $A_{600\,nm}$ of 0.4–0.6, with the expression of the *SBP2* gene induced by 0.4 mM isopropyl-β-D-thiogalactopyranoside and growth continued for 4 h at 37 °C. The cells were harvested and stored at −20 °C. Accumulation of recombinant protein was monitored by SDS–PAGE in whole cell extracts, as well as in soluble and insoluble fractions. SDS–PAGE was carried out as described previously (Laemmli, 1970) and proteins were visualized by Coomassie staining.

3.3. Purification of the recombinant protein

Cells were pelleted by centrifugation, resuspended in lysis buffer (10 mL, 100 mM sodium phosphate, pH 7.9, 10 mM Tris–HCl, 10 mM imidazole, 0.3 M NaCl, 8 M urea, 1 mM PMSF) disrupted by sonication, and centrifuged at 39,000*g* for 20 min. The induced protein was affinity-purified using a Ni^{2+}NTA-agarose resin (Qiagen, 0.5 mL resin/L culture). The resin was previously equilibrated with lysis buffer, and then incubated with cell extract at 4 °C for 2 h. After washing with buffer A (5 × 10 mL, 50 mM sodium phosphate, pH 7.9, 10 mM Tris–HCl, 0.3 M NaCl, 8 M urea containing 60 mM imidazole), the bound proteins were eluted twice with buffer A (500 μL) containing 250 mM imidazole during 1 h at 4 °C. The protein concentration was estimated using the Beer–Lambert equation. The molar extinction coefficient for SBP2 (32080 M^{-1} cm^{-1} at 280 nm) was calculated on the basis of the tryptophan and tyrosine content of the protein (Gill and Von Hippel, 1989) using the program ProtParam (http://us.expasy.org/tools/protparam.htlm).

3.4. Renaturation of the recombinant protein

The purified protein was dialyzed against 20 mM Hepes, pH 7.5, 250 mM NaCl, 2 mM $MgCl_2$, 0.2 mM oxidized glutathione, 2 mM reduced glutathione, 5% in H_2O glycerol (v/v), and 0.1% (v/v) NP-40 with a decreasing series of urea concentration ranging from 7 to 0 M, for 45 min at 4 °C for each urea concentration. After renaturation, the purified protein was dialyzed against 1 mM Tris–HCl, pH 7.5.

3.5. Circular dichroism (CD) measurements

CD spectra were recorded in a Jasco J-810 spectropolarimeter equipped with a Peltier-Type temperature control system PFD425S and thermostatic bath. Far-UV (180–250 nm) CD spectra were measured at a protein concentration of 0.12 mg/mL with a 0.1 cm path length cell, whereas for the near-UV (250–340 nm) CD spectra, the protein concentration was 1.16 mg/mL and the cell path length was 1 cm. All measurements were performed in 10 mM Tris–HCl, pH 8.0. The data represent the average of four measurements. The observed ellipticities were converted to mean residue ellipticity $[\theta]$ based on a mean molecular mass per residue of 115. The secondary structure was estimated from the far-UV spectra using the program CDNN (CD spectra deconvolution) (Bohm et al., 1992) for the five components of the secondary structure: α-helix, antiparallel β-sheet, parallel β-sheet, β-turn and random-coil.

3.6. Fluorescence spectroscopy

Fluorescence measurements were made using an Amino BOWMAN series 2 spectrofluorometer. The excitation and emission bandwidths were 8 nm and 16 nm, respectively, and a 0.5×1 cm path length quartz cuvette was used for all measurements. The temperature was kept constant at 25 °C with a circulating bath. All measurements were carried out in 10 mM Tris–HCl buffer at pH 8.0. The measured fluorescence data were corrected for the contribution of the buffer. The protein concentration used was 58 μg/mL. Thermal unfolding experiments were performed by increasing the temperature from 20 to 95 °C, allowing temperature equilibration for 5 min before recording each spectrum. To monitor ANS and Bis-ANS binding to SBP2, the excitation wavelength was 390 nm and 398 nm, respectively. The samples were incubated for 15 min at 25 °C with various concentrations of ANS and Bis-ANS, as indicated in the legend of Fig. 3. The intrinsic tryptophanyl fluorescence quenching was monitored using excitation wavelength of 275 nm and the emission spectrum was recorded between 290 and 600 nm.

3.7. Sucrose binding assay

The binding of sucrose was followed by fluorescence quenching measurements using an Aminco BOWMAN series 2 spectrofluorometer. Excitation and emission bandwidths were 8 and 16 nm, respectively. The fluorescence cell (0.5×1 cm) was mounted on a thermostatted holder. Protein fluorescence was measured with an excitation wavelength of 275 nm and the emission spectra were recorded between 290 and 420 nm. All measurements were performed in 10 mM Tris–HCl, pH 8.0, at 25 °C with the protein at concentration of 58 μg/ml. Sucrose was added to the protein solution and the emission spectrum recorded at the sensitivity range of the binding curve (1–100 mM). The fraction of sucrose–protein complex was determined by the decrease of the intrinsic fluorescence. The measured fluorescence data were corrected for dilution and inner filter effects using the graphical method described by Mertens and Kägi (1979). The corrected data were fitted to a hyperbolic equation using non-linear least-square regression using the program ORIGIN assuming one binding site per monomer.

3.8. Chemical denaturation

Denaturation experiments were carried out in 6.7 M GdnHCl and 7.6 M urea. The protein sample and the denaturant were incubated at 25 °C for 24 h to ensure that equilibrium had been achieved. The GmSBP2/S64 concentration was 58 μg/mL and the fluorescence spectra were recorded as described above. To determine the effect of pH on GmSBP2 structure, the pH and buffers used were: 3.0 (sodium citrate), 4.0 and 5.0 (sodium acetate), 6.0 and 7.0 (sodium cacodylate), 8.0 (Hepes–Na), 9.0 (Tris–HCl), 10.0 and 11.0 (CAPS–Na).

3.9. Sequence analysis

Sequence comparison analysis was performed against the Protein Data Bank using the BALSTp program (Altschul et al., 1990) at http://www.ncbi.nlm.nih.gov/BLAST/. For sequence alignment, the MultAlin (multiple alignment) Network Protein Sequence Analysis software (http://prodes.toulouse.inra.fr/mulalin/multalin.html) was used and the secondary structure prediction was performed with the PSIPRED software (http://bioinf.cs.ucl.ac.uk/psiform.html).

Acknowledgments

This research was supported by CNPq Grants 50.6119/2004-1 and 470787/2004-0 (to E.P.B.F.) and FAPEMIG Grants EDT 560/05 and CBB 1493/05 (to E.P.B.F.). C.S.R. was a recipient of a CNPq undergraduate scholarship and D.F.L. is supported by a CNPq graduate fellowship. This study was part of the Brazilian National Structural and Molecular Biology Network coordinated by the Laboratorio Nacional de Luz Síncrotron.

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