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Characterization of a methionine-rich protein from the seeds of *Cereus jamacaru* Mill. (Cactaceae)

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Abstract

We describe here the isolation and characterization of a major albumin from the seeds of *Cereus jamacaru* (Cactaceae), to which we gave the trivial name of cactin. This protein has a molecular mass of 11.3 kDa and is formed by a light chain (3.67 kDa) and a heavy chain (7.63 kDa). This protein was isolated using a combination of gel filtration chromatography and reverse-phase HPLC. The amino acid composition of cactin was determined and found to resemble that of the 2S seed reserve protein from the Brazil nut, a protein remarkable for its high methionine content. The usefulness of cactin as a molecular marker in the taxonomy of the Cactaceae is discussed.

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Introduction

In the semi-arid regions of Northeastern Brazil, the succulent stems of the "mandacaru" (*Cereus jamacaru* Mill.) are used as feed for cattle (1). The plant itself, with its beautiful flowers and its fleshy, red, delicious fruits, is almost a symbol of the Brazilian Northeast. Despite the potential of the "mandacaru" as a forage crop and as a fruit crop, very few studies have been conducted in order to help exploit these potentials.

One of the main constraints of the wide acceptance of "mandacaru" as a forage crop is the low level of protein throughout the plant. The genetic manipulation of this trait is being hampered by a lack of understanding of the patterns of protein deposition and mobilization in stems and seeds. Here we present data on the characterization of a reserve protein from the seeds of "mandacaru" and show that this protein is similar to the 2S methionine-rich seed storage protein from the Brazil nut (2).

Material and Methods

Plant material and seed germination conditions

Seeds of *C. jamacaru* were obtained from mature fresh fruits collected in April 1997 in

Aiuába, Ceará, Brazil. Seeds were surface sterilized with 2.5% NaOCl for 5 min, rinsed with sterile water, and sown on wetted, sterile filter paper on Petri dishes, under dim light at 28 ± 1°C (3). After germination, seeds were transferred to the dark. Groups of 20 seedlings were collected at 0, 3, 6, 9 and 12 days of germination and kept at -20°C until needed.

Protein extraction and purification

Salt-soluble proteins were extracted from flour in 0.1 M Tris/HCl and 0.5 M NaCl, pH 8.0, for 2 h. After centrifugation (10,000 *g*, 20 min, 4°C), ammonium sulfate was added to the supernatant to a concentration of 90% and the precipitated proteins were recovered by centrifugation. After dialysis against distilled water at 4°C and centrifugation, the albumin and globulin fractions were obtained. The albumins were fractionated on a Sephacryl S-100-HR column (80 x 2.6 cm) equilibrated and eluted in 0.05 M ammonium bicarbonate at a flow rate of 30 ml/h and 4.3-ml fractions were collected. This column was previously calibrated over the range from 6.5 to 66 kDa using the MW-GF-70 kit (Sigma Chemical Co., St. Louis, MO, USA). The effluents containing the 11.3-kDa protein were pooled, freeze-dried and fractionated by molecular exclusion-HPLC on a Superdex Peptide HR 10/30 column from Pharmacia Biotech (Pharmacia Biotech, Uppsala, Sweden), linked to an HPLC system from Waters Corporation (Milford, MA, USA). The column was equilibrated and eluted in 0.05 M ammonium bicarbonate at a flow rate of 0.5 ml/min. This fractionation yielded two protein peaks.

The peak containing the 11.3-kDa protein was further fractionated by reverse phase-HPLC (RP-HPLC) on a 3.9 x 300 mm μ-Bondapak C-18 column (Waters) linked to a HPLC system from Waters Corporation. The column was equilibrated in 0.1% trifluoroacetic acid (TFA) and proteins were eluted in a linear gradient of 0-60% acetonitrile in 0.1% TFA, run in 60 min. In all of the chromatographic steps the effluents were monitored at 280 nm. Protein concentration was determined by the protein-dye binding method of Bradford (4). The homogeneity of the purified protein was ascertained by SDS-PAGE and by isoelectric focusing. For chain separation, the purified protein was reduced and carboxymethylated (5) and the reaction mixture subjected to gel permeation chromatography in a Superdex Peptide HR 10/30 column, equilibrated and eluted in 0.05 M ammonium bicarbonate at a flow rate of 0.5 ml/min.

Antibody preparation and immunoblotting analysis

Polyclonal antibodies were prepared according to Harlow and Lane (6) by injecting the purified protein subcutaneously into a 4-month-old New Zealand white female rabbit. One hundred μg of the purified protein was applied to an SDS-PAGE slab gel. After staining with Coomassie brilliant blue R-250 the protein band was cut out of the gel and sonicated in a 1.3-ml Eppendorf tube containing 500 μl of water. After sonication (Ultrasonic Homogenizer, Cole Parmer, IL, USA), 1.0 ml of Freund's complete adjuvant was added and, after emulsification, injected into the rabbit. This operation was repeated two times at 15-day intervals. Rabbits were bled and serum was prepared from the blood. The IgG fraction was purified from serum by affinity chromatography on a protein-A Sepharose column (Pharmacia Biotech) according to Harlow and Lane (6). Immunoblot analysis was performed according to Towbin et al. (7). After SDS-PAGE, proteins were transferred to a nitrocellulose membrane using a TE Series Transfor Electrophoresis Unit (Hoefer Scientific Instruments, San Francisco, CA, USA), and probed against polyclonal antibodies raised against the purified protein and detected with alka-

line phosphatase-conjugated anti-goat IgG.

SDS-PAGE and isoelectric focusing

Gel electrophoresis was performed using the tricine-SDS polyacrylamide gel electrophoresis method for the separation of low molecular mass proteins described by Schagger and Jagow (8). The proteins contained in the MW-SDS-17S kit (Sigma) for molecular mass 3.46 to 16.95 kDa were used as molecular mass markers. Isoelectric focusing was performed using a method for rapid isoelectric focusing in a vertical polyacrylamide minigel system (9).

MALDI-TOF analysis

Freeze-dried samples of the native cactin were prepared for matrix-assisted laser desorption time of flight analysis (MALDI-TOF) with a Voyager-DE STR Bioworkstation (PerSeptive Biosystems, Framingham, MA, USA) as follows: a 0.5 mg/ml solution of cactin was divided into 2 groups of 3-ml aliquots. To the first group, 9 ml of the matrix α-cyano-4-hydroxycinnamic acid (saturated solution in acetonitrile/0.1% TFA 1:1, v/v) from Sigma Chemicals was added and vortex mixed and 1 ml of the final mixture was applied onto the Voyager Bioworkstation sample plate. For the second group, prior to the addition of the matrix solution, the 3-ml samples were incubated for 10 min at room temperature with 1 ml of a 1.0 M DTT in 150 mM ammonium bicarbonate solution. Ten ml of the α-cyano-4-hydroxycinnamic acid solution was then added to the samples and 1 ml of this mixture was applied onto the sample plate. Both groups of samples were air-dried at room temperature. The spectrometer equipped with delayed-extraction system was operated in the linear mode. Sample ions were evaporated by irradiation with an N_2 laser at a wavelength of 337 nm, and accelerated at 23 kV potential in the ion source with a delay of 150 ns. Samples were ionized with 100 to 200 shots of a 3-ns pulse width laser light. The signal was digitized at a rate of 500 MHz and averaged data were presented to a standard Voyager data system for manipulation.

Amino acid analysis

Amino acid compositions of protein samples were analyzed as described previously (10). Lysozyme was also analyzed and the results were compared with published values to ensure the accuracy of the technique. Three independent determinations were performed for each sample. Selected replicates were analyzed in duplicate to ensure repeatability.

Results and Discussion

When the albumins from *C. jamacaru* were analyzed by SDS-PAGE, a major protein band was observed at a position corresponding to a molecular mass of 9.5. This protein, to which we gave the trivial name of "cactin", was purified by size exclusion chromatography and by RP-HPLC on a μ-Bondapak C-18 column. The last purification step yielded cactin in a homogeneous form, as indicated by SDS-PAGE (Figure 1A) and by isoelectric focusing (Figure 1B). This protein accounts for approximately 9% of the total salt-soluble protein.

MALDI-TOF analysis of native cactin samples revealed the presence of two major components of 11,304.87 and 5,652.83 Da (Figure 2A). The heavier component was defined as the intact cactin polypeptide, while the lighter one corresponded to its double charged form. Two minor components were also detected in the same experiment, but they showed poor resolution and not very accurate molecular masses around 7.6 and 3.6 kDa. Initially these components were considered to be sample contamination. Mass determination of the reduced cactin samples showed a series of four major peaks display-

ing the following mass values: 11,304.78 Da; 7,635.48 Da; 5,652.50 Da, and 3,677.33 Da (Figure 2B). The 11.304-kDa and 5.652-kDa peaks showed mass values very close to those found in the first experiment with the intact sample of cactin, indicating that minor quantities of cactin were not reduced by DTT in this experiment. The two new components (7,635.48 Da and 3,677.33 Da) shown in Figure 2B represent strong evidence that cactin is not a single polypeptide chain, but in fact, should be considered to be a heterodimer linked by disulfide bonds. Moreover, the resulting mass value obtained by the addition of the masses of the two new components is eight mass units higher (11,312.81 Da) than the one found for native cactin (11,304.87 Da). These findings suggest that four disulfide bonds must have been reduced and eight hydrogen atoms incorporated into the molecule. The two low resolution peaks detected in the first experiment now are interpreted as possible dissociation and/or cleavage products of the native protein yielding the two polypeptide chains of cactin. This might have occurred somewhere during sample manipulation procedures.

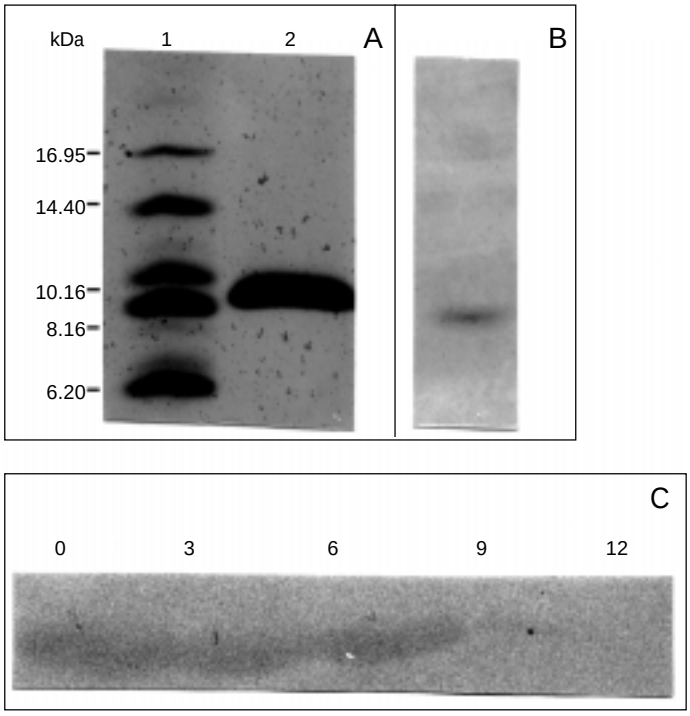

Figure 1 - *A*, Assessment of the homogeneity of the methionine-rich protein isolated from the seeds of *C. jamacaru* by SDS-PAGE. Purified protein (4 µg) was added to lane 2 and molecular weight markers were applied to lane 1. *B*, Assessment of the homogeneity of the methionine-rich protein isolated from the seeds of *C. jamacaru* by isoelectric focusing. *C*, Western blot analysis of the mobilization during germination of the methionine-rich protein isolated from the seeds of *C. jamacaru*. The samples analyzed were prepared from seeds 0, 3, 6, 9 and 12 days after germination.

The molecular mass calculated from the SDS-PAGE experiments was 9.5 kDa, while MALDI-TOF analysis indicated a molecular mass of 11.3 kDa for the native cactin. This discrepancy between the molecular mass values obtained from SDS-PAGE and MALDI-TOF data probably arises from the anomalous electrophoretic behavior which is commonly presented by basic, low molecular weight proteins.

Polyclonal antibodies were raised against purified cactin and the purified antibodies were highly monospecific. These antibodies were used to show that cactin is mobilized during germination, whereas after 12 days of germination it could no longer be detected (Figure 1C).

The production of large amounts of the small and large polypeptide chains was made possible by reduction and carboxymethylation of the purified protein and fractionation of the reaction mixture on a Superdex Peptide HR 10/30 column. This yielded two protein peaks corresponding to the large chain (peak 1, Figure 3) and small chain (peak 2, Figure 3) and two peaks at the end of the run, corresponding to the low molecular weight components of the reduction and carboxymethylation reaction mixture. The amino acid compositions of the native protein and of its large and small chains were determined by Pico Tag technology (Table 1). The overall amino acid composition of cactin resembles closely that of the 2S seed reserve protein from the Brazil nut (*Bertholletia excelsa*, Lecythidaceae), a protein remarkable for its high content of methionine (2). Another prop-

erty that cactin shares with proteins belonging to the 2S seed storage protein family is its high content of amino acids with a high nitrogen to carbon ratio (Arg, Gln and Asn) which are particularly suited for storage of nitrogen (11). The presence of two polypeptide chains kept together by disulfide bridges is another property that cactin shares with the Brazil nut seed reserve protein and with many members of the 2S seed storage protein family.

The 2S albumins have only become recognized as a major group of seed storage proteins over the last decade (12). These proteins are widely distributed in the seeds of dicots and have been most widely studied in the Cruciferae, notably oil-seed rape and *Arabidopsis* (12). The presence of two disulfide-linked polypeptide chains has been demonstrated in a number of 2S albumins, although some 2S albumins such as those from sunflower (13), prickly-pear (10) and pea (14) have a single polypeptide chain. We are currently exploring the possibilities of using cactin as a molecular marker in the taxonomy of the Cactaceae. Our preliminary results indicate that proteins cross-reacting with cactin antibodies are present in all species of the three subfamilies of the Cactaceae (Cactoideae, Opuntioideae and Pereskioideae) that we have analyzed (data not shown).

The chief interest in the 2S albumin protein family stems from the fact that many of its members are very rich in the sulfur-containing amino acids methionine and cysteine, which normally are present in very low concentration in plant-derived foodstuffs (15). Within the past decade several groups have focused their attention on using genes encoding these proteins to enhance the nutritional quality of plants (16). In the Northeastern states of Brazil, some members of the Cactaceae, notably *C. jamacaru* and *Opuntia ficus-indica*, are used as feed for farm animals. It is interesting that both *O. ficus-indica* (10) and *C. jamacaru* have these

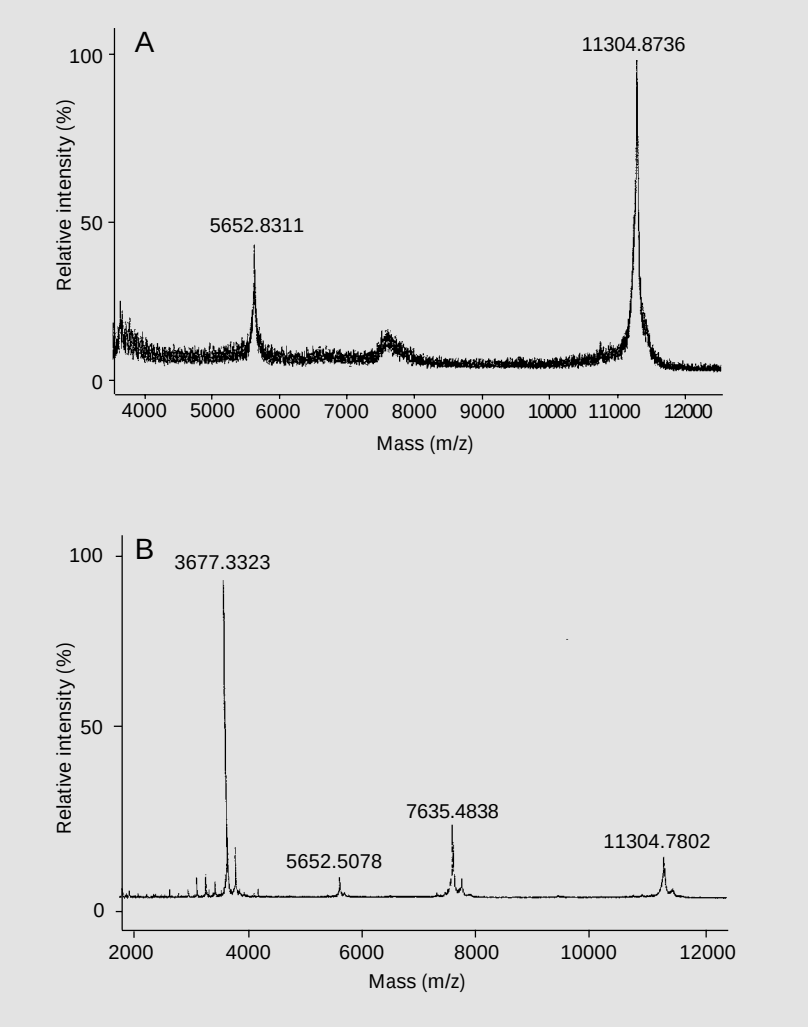

Figure 2 - *A*, MALDI-TOF mass spectrum of native cactin. *B*, MALDI-TOF mass spectrum of cactin after reduction with DTT.

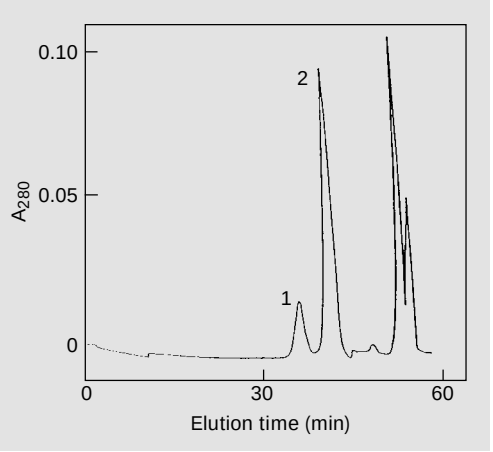

Figure 3 - Fractionation of the reduction and carboxymethylation reaction mixture of cactin on a Superdex Peptide HR 10/30 column. 1 and 2, Protein peaks corresponding to large and small chains, respectively.

Table 1 - Amino acid composition of the methionine-rich protein isolated from the seeds of *Cereus jamacaru*, as determined by the Pico Tag method.

Composition is reported as number of residues per mol of protein based on molecular masses of 11300, 7630 and 3670 kDa for the native, large and small chains, respectively. The values for cysteine and tryptophan were not determined (nd). Numbers within parentheses represent the assigned integral values.

Amino acid	Native	Large chain	Small chain
Asx	4.12 ± 0.2 (4)	4.70 ± 0.24 (5)	0.89 ± 0.10 (1)
Glx	22.60 ± 0.58 (22)	12.24 ± 0.57 (12)	8.97 ± 0.33 (9)
Ser	5.58 ± 0.20 (6)	3.94 ± 0.22 (4)	1.70 ± 0.10 (2)
Gly	7.83 ± 0.33 (8)	4.80 ± 0.17 (5)	3.60 ± 0.17 (4)
His	1.02 ± 0.00 (1)	0.99 ± 0.02 (1)	0.00 (0)
Arg	13.66 ± 0.36 (14)	9.89 ± 0.45 (10)	3.90 ± 0.14 (4)
Thr	0.90 ± 0.00 (1)	1.13 ± 0.08 (1)	0.00 (0)
Ala	5.72 ± 0.20 (6)	2.24 ± 1.13 (2)	1.42 ± 0.1 (1)
Pro	4.34 ± 0.10 (4)	1.11 ± 0.10 (1)	1.90 ± 0.00 (2)
Tyr	3.28 ± 0.17 (3)	1.27 ± 0.10 (1)	1.74 ± 0.00 (2)
Val	1.40 ± 0.6 (1)	1.45 ± 0.14 (1)	0.24 ± 0.00 (0)
Met	14.71 ± 0.87 (15)	10.51 ± 0.14 (11)	2.42 ± 0.10 (2)
Cys	nd	nd	nd
Ile	1.04 ± 0.04 (1)	1.15 ± 0.00 (1)	0.09 ± 0.03 (0)
Leu	6.40 ± 0.40 (6)	4.10 ± 0.26 (4)	1.67 ± 0.37 (2)
Phe	1.44 ± 0.10 (1)	1.54 ± 0.22 (1)	0.77 ± 0.43 (1)
Lys	1.17 ± 0.10 (1)	1.12 ± 0.00 (1)	0.00 (0)
Trp	nd	nd	nd

genes. If their expression can be enhanced, particularly in the stems, then these engineered plants would be more nutritious for farm animals. Work towards this goal is in progress in our laboratory (17,18).

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