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Bactericidal Activity Identified in 2S Albumin from Sesame Seeds and In silico Studies of Structure–Function Relations

Simone Maria-Neto · Rodrigo V. Honorato · Fábio T. Costa · Renato G. Almeida · Daniel S. Amaro · José T. A. Oliveira · Ilka M. Vasconcelos · Octávio L. Franco

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Abstract Pathogenic bacteria constitute an important cause of hospital-acquired infections. However, the misuse of available bactericidal agents has led to the appearance of antibiotic-resistant strains. Thus, efforts to seek new antimicrobials with different action mechanisms would have an enormous impact. Here, a novel antimicrobial protein (*Si*AMP2) belonging to the 2S albumin family was isolated from *Sesamum indicum* kernels and evaluated against several bacteria and fungi. Furthermore, in silico analysis was conducted in order to identify conserved residues through other 2S albumin antimicrobial proteins (2S-AMPs). *Si*AMP2 specifically inhibited *Klebsiella* sp. Specific regions in the molecule surface where cationic (RR/RRRK) and hydrophobic (MEY-WPR) residues are exposed and conserved were proposed as being involved in antimicrobial activity. This study reinforces the hypothesis that plant storage proteins might also play as pathogen protection providing an insight into the mechanism of action for this novel 2S-AMP and evolutionary relations between antimicrobial activity and 2S albumins.

Keywords Antimicrobial proteins · 2S albumins · Phylogenetic analysis · Homology modeling · Structure–function relationship

Abbreviations

AMP	Antimicrobial peptide/protein
RF	Protein rich fraction
NRP	Non-retained proteins
RP	Retained proteins
MIC	Minimum inhibitory concentration
IPG	Immobilized pH gradient
*Si*AMP2	Antimicrobial protein from *Sesamum indicum*
CFU	Colony forming units
MP	Maximum Parsimony method
2S-AMP	Antimicrobial 2S albumin

S. Maria-Neto · R. V. Honorato · F. T. Costa · R. G. Almeida · D. S. Amaro · O. L. Franco (✉)
Centro de Análises Proteômicas e Bioquímicas, Pós-Graduação em Ciências Genômicas e Biotecnologia, Universidade Católica de Brasília, SGAN Quadra 916, Modulo B, Brasília, Distrito Federal 70790-160, Brazil
e-mail: ocfranco@pos.ucb.br; ocfranco@gmail.com

S. Maria-Neto
e-mail: simoneneto@gmail.com

R. V. Honorato
e-mail: rvhonorato@gmail.com

F. T. Costa
e-mail: fubu03@gmail.com

R. G. Almeida
e-mail: renatogda@gmail.com

D. S. Amaro
e-mail: amarods@gmail.com

D. S. Amaro · O. L. Franco
Pós-graduação em Patologia Molecular, Universidade de Brasília, Campus Universitário Darcy Ribeiro, Brasília, Distrito Federal 70910-900, Brazil

J. T. A. Oliveira · I. M. Vasconcelos
Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Ceará, Av. da Universidade 2853, Benfica, Fortaleza, Ceará 60020-181, Brazil

O. L. Franco
Universidade Federal de Juiz de Fora, Rua José Lourenço Kelmer s/n, Campus Universitário Bairro São Pedro, Juiz de Fora, Minas Gerais 36015-260, Brazil

1 Introduction

Bacterial infectious diseases directly affect the world's population, particularly in developing countries. These infections have been partially controlled by antibiotic therapies [3]. However, the misuse of available antibiotics has led to the appearance of resistant strains that are able to survive in the presence of almost all current antibacterial drugs. Under these circumstances, hospital infections pose an enormous challenge as they cause numerous lethal occurrences and economic losses [44]. Bacteria from the *Enterobacteriaceae* family are responsible for 95% of hospital outbreaks in Latin America [43] and hospital-acquired pulmonary infections and other related diseases have been ascribed to infection by *Klebsiella* sp. [61].

The worldwide emergence of bacterial resistance to antibacterial agents demands continuous efforts to seek new antimicrobials with different action mechanisms to combat bacterial infections [23]. In this regard, antimicrobial peptides and proteins (AMPs) have been isolated from several sources including bacteria [38], invertebrates [16], amphibians [46], fish [14], fowl [72], mammals [71] and plants [58, 59]. Although leaves, flowers and roots are able to synthesize AMPs [59, 77], seeds have usually been the plant organ of choice to isolate new AMPs, owing to their relative higher abundance and ease of extraction and purification in seeds. Thus, various seed-derived antibacterial peptides that hamper the development of a broad spectrum of fungi and/or bacteria have been reported [17, 30]. These include ribosome-inactivating proteins [50], lipid transfer proteins [48], chitin-binding proteins [17], plant defensins [30], thaumatin-like proteins [54], hevein-like proteins [39], and proteins derived from seed-storage proteins, such as glycine-rich proteins and 2S albumins [56, 58, 59, 64].

2S albumins are the second major storage proteins of cereal kernels. Usually this protein family originates from a single precursor that is cleaved, during its post-translational processing, by an asparaginyl endopeptidase. The resulting heterodimer is composed of two subunits stabilized by two disulfide bonds and with molecular masses of approximately 4 and 9 kDa [34], but some variation in these values has been reported [59]. Although the main biological role for 2S albumins is thought to be storage, several other physiological functions have been attributed to this protein family. Accordingly, the ability of 2S albumins to stabilize oil-in-water emulsions [12], inhibit proteolytic enzymes [45] and their antifungal activity [1, 56, 59, 78] have been suggested. However, most 2S albumins have never been evaluated for other activities and were reported only as storage proteins.

Currently, huge amounts of biotechnological data are widely available as well as many bioinformatic tools with which to analyze these data [13, 33]. Investigation of the protein structure–activity relationship frequently resort to phylogenetic analysis and three-dimensional structure elucidation. Establishing evolutionary relations within a protein family is useful to describe function distribution on the phylogenetic tree and set up a primary sequence pattern that is important for the function [21]. Understanding the three-dimensional structure of the molecule and its characteristics is a powerful tool in the elucidation of essential amino acid residues, their behavior and interactions in the environment [7].

In this present paper, a new 2S albumin isolated from *Sesamum indicum* kernels, named *Si*AMP2, was described and its theoretical three-dimensional structure was proposed. The biochemical and biological properties of *Si*AMP2 able to inhibit the growth of human pathogenic bacteria are here presented. With the aid of bioinformatics tools, such as comparative modeling, phylogenetic reconstruction and structural analysis, the potential of other 2S albumins as antibacterial agents was discussed, as well as specific residues that are probably involved in the antimicrobial activity, in addition to the real importance of 2S albumin as a multifunctional family.

2 Materials and Methods

2.1 General In vitro Experimental Procedures

2.1.1 Materials

Sesamum inidicum (cv. Guatemala) were bought at local market. Phytopathogenic microorganisms were provided by EMBRAPA Recursos Genéticos e Biotecnologia collection. Human pathogenic microorganisms were obtained from Hospital Universitário de Brasília (HUB). Reagents for SDS–PAGE were from Sigma Chemical Co. (St Louis, MO, USA). Chemicals and IPG strips for isoelectric focusing and Red-Sepharose CL-6B matrix were purchased from GE Healthcare (Uppsala, Sweden). Vydac C-18TP analytical column was from Separation Group (Hesperia, CA., USA). Amino acid sequencing reagents were from Wako (Japan). All other chemicals were of analytical grade and purchased from VETEC (Rio de Janeiro/Brazil).

2.1.2 Extraction and Isolation of SiAMP2

Kernels of white *S. indicum* were washed with a bleach (30% NaCl solution) for 5 min followed by distilled water for another 5 min. Sesame kernels were extracted with a solution containing 0.6 M NaCl and 0.1% HCl, in a proportion of 1:4 (w/v), for 6 h at 4 °C. This crude extract was centrifuged at 4.500×*g* for 90 min, at 4 °C. Proteins in

supernatant were precipitated with $(NH_4)_2SO_4$ at 100% saturation with constant stirring. After centrifugation (in equal conditions), the precipitate was resuspended and dialyzed (3.0 kDa cut off) against distilled water. The resulting protein rich fraction (RF) was lyophilized and protein concentration was determined by the Bradford method [8]. Subsequent chromatography of the RF onto Red-Sepharose CL-6B was carried out at 0.5 mL·min^{-1} flow rate and the eluted fractions [39] monitored at 280 nm. Non-retained proteins (NRP) were eluted with 0.1 M Tris–HCl, pH 7.0, equilibration buffer. Bound proteins (RP) were eluted with 3.0 M of NaCl dissolved in the equilibration buffer. After dialysis (3.0 kDa cut off) against distilled water and lyophilization, 1.0 mg of RP was dissolved in 0.1% CF_3CO_2H acid (TFA) and applied onto a reversed-phase Vydac C-18TP analytical column equilibrated with 0.1% of TFA and coupled to a HPLC system. Proteins were eluted at a flow rate of 1.0 mL·min^{-1} with differential CH_3CN concentrations (0% for 5 min; 0–20% for 5 min; 20–35% for 25 min; 35–50% for 15 min; 50–100% for 5 min). Protein detection was carried out at 216 nm.

2.1.3 SDS–PAGE Analysis

Protein fractions eluted from HPLC were analyzed by 15% SDS–PAGE under reducing conditions [41]. Bromophenol blue was used as tracking dye and gels were silver stained. Invitrogen-BenchMark™ Pre-stained Protein Ladder was used as molecular weight standard.

2.1.4 Isoelectric Focusing

Isoelectric focusing of *Si*AMP2 was conducted according to Gorg et al. [31] and performed with 13 cm immobilized non-linear gradient (IPG) strips with a pH range of 3–11 on Multiphor II electrophoresis system (GE HealthCare). 50 µg of *Si*AMP2 was dissolved in 250 µL of 2% CHAPS, 8 M urea, 7 mg·mL^{-1} dithiothreitol and 2% IPG Buffer (GE Healthcare). Strips were rehydrated in the above solution for 16 h. IEF was carried out in gradient mode for 30 min at 500 V, 30 min at 1,000 V, 90 min at 3,500 V and 380 min at 3,500 V, at 2 mA and 5 W. After running, strips were stained with Coomassie brilliant blue G250.

2.1.5 Antimicrobial Activity Assays

Pathogenic bacteria were cultured in 2.0 mL LB (Luria–Bertani) broth (10 g L^{-1} NaCl, 5 g·L^{-1} yeast extract and 45 g·L^{-1} bactopeptone) for 18–24 h, at 37 °C. RF and the purified peptides were re-suspended in distilled water, filtered through 0.22 µm nylon membranes, and incubated at several *Si*AMP2 concentrations, respectively, with 5×10^6 CFU·mL^{-1} of each bacterium tested, for 4 h, at 37 °C. The minimal inhibitory concentrations (MIC) were obtained according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [18]. The Gram-negative bacteria assayed were *Klebsiella* sp., *Proteus* sp., *Escherichia coli*, *Salmonella* sp. and *Xanthomonas* sp., and the Gram-positive bacteria were *Streptococcus pyogenes*, *Staphylococcus aureus* and *Rathayibacter* sp. Sterile distilled water and chloramphenicol (40 µg·mL^{-1}) were used as negative and positive controls, respectively. Bacterial growth was determined spectrophotometrically at 595 nm every hour within the period of incubation. The assay of antifungal activity [55] was conducted in 85 × 10 mm sterile Petri dishes containing 15 mL of Potato Dextrose Agar medium. Fungal mycelia of *Rhizoctonia solani*, *Aspergillus awamori*, *Aspergillus flavus*, *Aspergillus nidulans*, *Aspergillus fumigatus* and *Fusarium oxysporum* from freshly grown culture were inoculated at the center of the Petri dishes and 0.5 cm diameter sterile paper disks were placed on the periphery of the growing mycelia. Then 10 µL aliquots of RF, RP (100 µg·mL^{-1} each) and *Si*AMP2 (200 µg·mL^{-1}) were added individually to each paper disk. Equal aliquots of 0.5% fungizone and sterile distilled water were used as positive and negative controls, respectively. Experiments were carried out in triplicate.

2.1.6 Amino Acid Sequencing

Initially, the partial amino acid sequence of *Si*AMP2 was determined on a Shimadzu PPSQ-23A Automated Protein Sequencer performing Edman degradation [25]. PHT-amino acids were detected at 269 nm after separation on a reversed phase C18 column (4.6 × 2.5 mm) under isocratic conditions, according to the manufacturer's instructions. Afterwards, purified *Si*AMP2 were incubated with 25 µL of 100 mM NH_4HCO_3 and 10 mM DTT at 56 °C, for 30 min. Thereafter, 55 mM of iodoacetamide was added to peptide solution and incubated in the dark, at room temperature, for 90 min. The protein digestion was carried out with Sequencing Grade Modified Trypsin (Promega) according to Shevchenko [70], with minor modifications. Briefly, 600 ng of buffered trypsin was added to peptide solution and incubated on ice for 30 min. Sequentially, 40 µL of 50 mM NH4HCO3 was added and incubated at 37 °C, for 22 h. Digestion supernatant was collected and storage at −20 °C. The peptides derived from tryptic digestion were analyzed by using an UltraFlex III MALDI TOF/TOF (BrukerDaltonics), precisely calibrated with peptide calibration standard II (BrukerDaltonics). A sample of 1 µL was mixed in 3 µL of matrix solution (1% (w/v) α-cyano-4-hydroxycinnamic acid, 3% (v/v) trifluoroacetic

acid and 50% (v/v) acetonitrile) and applied onto a MALDI target plate (1 μL in duplicate). After crystallization at room temperature, samples were analyzed by mass spectrometry, operated in reflector mode for MS acquisitions and LIFT mode for tandem MS (MS/MS). The MS/MS spectra of tryptic peptides were manually sequenced.

2.2 General In silico Experimental Procedure

2.2.1 Identification of SiAMP2 Protein Family

Sequenced protein was directly correlated to *S. indicum* proteins (taxid: 4182) from non-redundant protein database (NR) of NCBI for sequence confirmation using Blastp algorithm [4].

2.2.2 Structural Analysis and Molecular Modeling

The theoretical three dimensional structure of *Si*AMP2 was built from a primary structure composed by the sequenced portion of *Si*AMP2 (86%) and the non-sequenced portion was supplied from its closer related Sesin (GenBank ID: AAK15088; UniProtKB ID: Q9AUD1) [74]. The structural model was generated by Modeller *9v7* [27] through comparative modeling using 1psy (PDB ID) [57] as the atomic coordinates template. One hundred models were obtained and the best model was chosen considering both DOPE and GA341 scores [69]. Energy minimization and charge count were carried out using GROMACS [40] with steepest descent method until a maximum force of 1,000.0 $kJ \cdot mol^{-1} \cdot nm^{-1}$ was observed, and further validated on PROCHECK [42]. The electrostatic maps generated with APBS [5]. Structural analysis of conserved 2S-AMPs residues also exposed on *Si*AMP2 was performed on *PyMol* 1.1r1 [22] and UCFS Chimera [60].

2.2.3 2S Albumin Data Set

The correlation of function, amino acid composition and phylogenetic divergences between *Si*AMP2 and the other 2S albumins was evaluated through a sequence data set. The data set was built considering all protein sequences of 2S albumin deposited in the NR maintained by NCBI, with a cut off of 85% threshold of redundancy on JalView [83], and was manually curated. Protein sequences were inserted according to their original annotation in the databank, without previous removal of signal peptides and pro-peptides. Antimicrobial proteins present in the 2S albumins dataset (2S-AMPs) were also considered for a further analysis of amino acid conservation throughout the 2S-AMPs. The 2S-AMPs were submitted to a multiple alignment on ClustalW [79] with the gap opening penalty set to 5 and blosum32 as matrix. The alignment was manually edited to match the cysteine bridge pattern.

2.2.4 Phylogenetic Relations

Phylogenetic analysis was carried out using the Maximum Parsimony (MP) method [24]. The consensus tree was inferred from the 21 most parsimonious trees. Branches corresponding to partitions reproduced in less than 50% of trees were collapsed. The initial trees were obtained with the random addition of sequences (10 replicates). The MP tree was obtained using the Close-Neighbor-Interchange algorithm with search level 3 in initial trees. All alignment gaps were treated as missing data. Phylogenetic analyses were conducted in MEGA4 [76]. The bootstrap statistical test [28] was used to confirm the reliability of the inferred tree.

3 Results and Discussion

3.1 Isolation and Functional Characterization of *Si*AMP2

In order to identify a novel AMP from *S. indicum*, the protein rich fraction (RF) extracted from sesame kernels was submitted to antimicrobial screening assays towards different pathogenic Gram-negative (*Klebsiella* sp., *Proteus* sp., *E. coli*, *Salmonella* sp. and *Xanthomonas* sp.), Gram-positive bacteria (*S. pyogenes*, *S. aureus*, *Rathayibacter* sp.) and fungi (*R. solani*, *A. awamori*, *A. flavus*, *A. nidulans*, *A. fumigatus* and *F. oxysporum*). Surprisingly, the RF specifically inhibited only the growth of *Klebsiella* sp. None of the other bacteria or fungi tested was affected (data not shown). This remarkable peculiarity led us to purify *Si*AMP2 and study its properties. To this end, the RF was applied onto a Red-Sepharose column, generating two different fractions (Fig. 1a). The non-retained proteins (NRP) were eluted with the equilibrium buffer, and the retained proteins (RP) were displaced from the matrix with 3 M of NaCl dissolved in the equilibrium buffer. RP were pooled, dialyzed against distilled water, lyophilized and loaded onto a reversed phase HPLC analytic column, yielding eleven peaks. *Si*AMP2 was eluted at 26% acetonitrile concentration (Fig. 1b). Purified protein was evaluated on all bacteria and fungi listed on Sect. 2.1.5. However, both crude extract and purified *Si*AMP2 were able to inhibit only the growth of *Klebsiella* sp., being no other activity observed toward the multiple microorganisms tested. A lower MIC of 3.1 μM was observed against this bacterium. Other AMPs isolated from plants also showed similar MICs. For example, a glycine-rich peptide isolated from *Psidium guajava* seeds, named Pg-AMP1, showed clear bactericidal activity, where MIC values

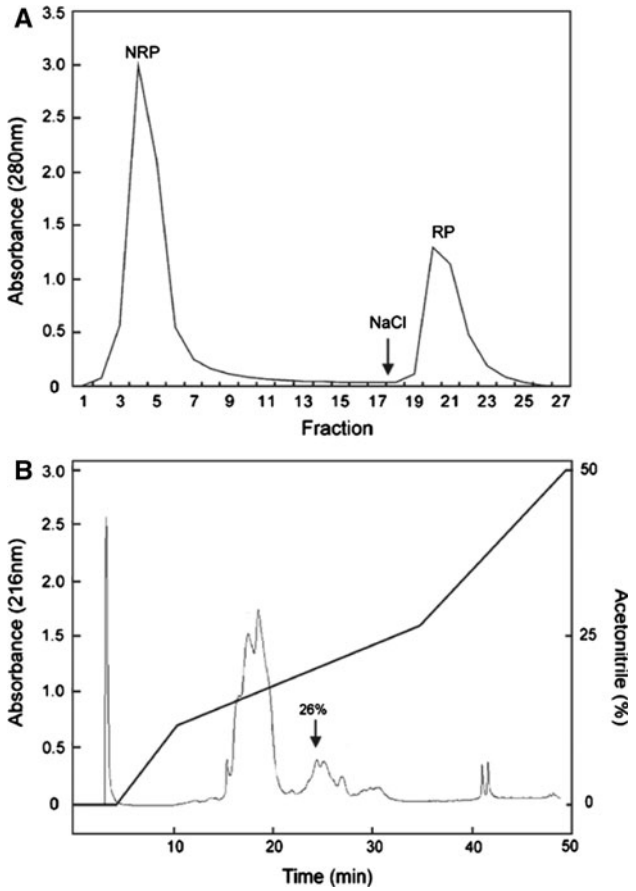

Fig. 1 Chromatography profiles of protein fraction of *S. indicum* kernels. **a** The protein rich fraction of *S. indicum* was applied to a Red-Sepharose CL-6B column equilibrated with 0.1 M Tris–HCl buffer, pH 7.0, containing 0.05 M $CaCl_2$. *NRP* denotes non-retained protein and *RP* the retained protein. RP was displaced from the column with 3.0 M of NaCl dissolved in Tris buffer (*black arrow*). **b** Vydac C-18TP reversed-phase chromatogram profile of distilled water dialyzed and lyophilized RP. *Diagonal lines* indicate non-linear acetonitrile gradient up to 50% concentration. Elution of *Si*AMP2 with 26% acetonitrile is indicated by *arrow*

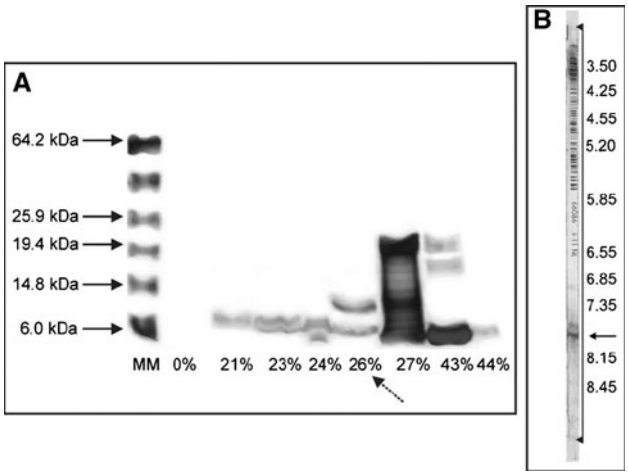

Fig. 2 **a** SDS–PAGE analysis of *Si*AMP2. The protein molecular weight markers (*MM*) and the fractions obtained by fractionation of RP on Vydac C-18TP reversed-phase analytic column eluted with different concentrations of acetonitrile are shown. *Si*AMP2 is indicated by *dotted arrow*. **b** The isoelectric focusing of *Si*AMP2 is evidenced (*black arrow*) in relation to p*I* protein markers

obtained for this peptide were 9.8 μM for *E. coli* and 4.3 μM for *Klebsiella pneumoniae* [58]. Moreover, a defensin named Cp-thionin2 isolated from *Vigna unguiculata* seeds also showed similar MICs when evaluated against Gram-positive and Gram-negative bacteria [30].

3.2 Identification of *Si*AMP2

Analyses of *Si*AMP2 sequence by Edman degradation and mass spectrometry allowed its coverage of 86% the full sequence. The partial sequence of *Si*AMP2 showed different degrees of similarity with 2S albumin proteins, including sesin (GenBank ID: AAK15088; UniProtKB ID: Q9AUD1), a 2S albumin of 153 amino acid residues from seeds of the same plant species, *S. indicum* [74], from which *Si*AMP2 was also purified. As expected, since 2S albumins are encoded by a multigenic family, the data presented here indicate the syntheses of different 2S albumin isoforms in *S. indicum* seeds [74]. The protein pattern obtained by SDS–PAGE of *Si*AMP2 revealed two distinct bands, one with approximately 6 kDa and the other below 14 kDa (Fig. 2a). This band pattern is commonly observed for proteins belonging to the 2S albumin family, such as napins [65]. These were first described as having only the large subunit, and today are used almost as a synonym for 2S albumins. The MS data confirmed the monoisotopic mass of the two subunits of *Si*AMP2 as 5,874 and 8,374 Da (data not shown). IEF analysis demonstrated that *Si*AMP2 possesses a slightly cationic p*I* of approximately 7.8 (Fig. 2b). Most AMPs are cationic, a feature that probably facilitates their interaction with anionic cell walls and/or cell membrane phospholipids of pathogenic bacteria [10, 88]. For instance, a napin with a p*I* of 9.5 does have antimicrobial activity [65]. The observed selectivity of *Si*AMP2, able to inhibit specifically *Klebsiella* sp., might be related to the presence of a capsular complex formed by acidic polysaccharides, which is essential for *Klebsiella* sp. virulence, but could be seen in other species of the *Enterobacteriaceae* family [61]. A current example is a 2S albumin from *Raphanus sativus* seeds with deleterious activity toward *Bacillus megaterium*, a non-pathogenic Gram-positive organism [89]. In fact, only a low number of antimicrobial 2S albumins able to inhibit the growth of pathogenic bacteria have ever been described. Instead, most of them inhibit pathogenic fungi. The same refers to 2S-AMPs from seeds of *Brassica napus* [65], *Malva parviflora* [82], *P. edulis* [1, 59] and *Capsicum annuum* [64].

Organism	ID		Sequence		Reference
S. indicum	*Si*AMP2		QEGCEWE--SRPC-NRGC--WLPRQWESVHMRFGVLVLWR		This report
S. indicum	AAK15088	22	----TTYTTTVTTTAIDDEANQQSQQCRQQ--LQGRQFRSCQRYL-SQGRSPYGGEEDEVLEMS------	79	[74]
S. indicum	Q9XHP1	22	----SAHKTVVTTSVAEEGEEENQRGCEWE--SRQCQMRHCMQWM-RSMRGQY---EESFLRSA------	73	[73]
L. japonicas	AAW66631	01	------------------------------MLQGRQFRSCQSYL-RQ--------RGNVLEM-------	24	[87]
H. annus	CAC81359	34	------HTTIITTTIEDENPLSEQRQCIQQVQGQR--LNQCRMFL--QQGQRGQQHQ------------	81	[63]
H. annus	CAC81359	158	-----------IDIPFRDRPFGRSQQCS-ETEIQR-PVSQCQRYV-KQQMQSPMPYIRRPGQ--------	206	[63]
M. charantia	CAD32938	23	------YRTTITTVEVDEDNQGRHERCHHIRPREQ--LRSCESFL-RQS--------RGYLEMRGVE---	73	[80]
R. sativus	CAA01774	22	SIYRTVVEFDEDDATNPAGPF-RIPRCRKEFQQAQ-HLRACQQWLHKQAMQVRGGPSL-ALDGEFDFEDD	89	[9]
M. jalapa	CAA01775	28	----------------SAGPF-RIPRCRREFQQAQ-HLRACQQWLHRQARQSG----------------	63	[9]
R. communis	1PSY	01	---------AEFMESKGEREGSSSQQCRQEVQRKD--LSSCERYL-RQSSSRRSTGEE-VLRMPGD----	53	[57]

S. indicum	*Si*AMP2		APQFEHFRECCNELR CEALRCMMR MRMEYWPRGLQDQQVYQPRRDLEPRRH-CGMSYPVECRMPR		This report
S. indicum	AAK15088	80	TGNQQSEQSLRDCCQQLR-NVDER--CRCEAIRQAVR----------QQQQEGGYQEGQS--QQVYQRARDL-PRR--CNMR-PQQCQFRVIFV-------	132	[74]
S. indicum	Q9XHP1	74	EANQGQFEHFRECCNELR-DVKSH--CRCEALRCMMR----------QMQQEYGMEQ-EMQQMQQMMQYL-PRM--CGMSYPTECRMRPIFA-------	148	[73]
L. japonicus	AAW66631	25	TGNPQS-QTVEECCESLKDIERKQQQCGCEAIKHAMR----------QMQGGQSE--EVYRKARML-PRT--CGLR-SQQCQFNVIFV-------	95	[87]
H. annus	CAC81359	82	--QQQEQQLLQQCCQEL-QNIDQQ--CQCEAVKQVFR----EAQQQVQQQQGRQSVPFRSSQQT-QQ-LQKAQIL-PNV--CNLQ-SRRCEIGTITTTVVTESN	157	[63]
H. annus	CAC81359	207	--QQQEPE-LQQCCNQL-QNVNRE--CQCEAVQEVARRVMRQPQQH---QQQQRRRGQFGGQEM-DIARRVIQNL-PNQ--CDLE-VQQCNIPY----------	285	[63]
M. charantia	CAD32938	74	ENQWERQQGLEECCRQLR-NVEEQ--CRCDALQEIAREVQRQ----------ERGQEG-SQMLQKARML-PAM--CGVR-PQRCDF----------	140	[80]
R. sativus	CAA01774	90	MENPQRPFLLQQCCNELHQEEPL---CVCPTLKGASKAVK--------QQIQQQQGQQQAHQRMVSRIYQTATHL-PRV--CNIPQVSVCPFQKTMPGPYY---	175	[9]
R. communis	1PSY	54	ENQQQESQQLQQCCNQVKQ-VRDE--CQCEAIKYIAE----------DQIQQGQLHGEESERVAQRAGEI---VSSCGVR----CMRQTRTN-------	125	[57]

Fig. 3 Multiple alignment of *Si*AMP2 and the closest 2S-AMPs performed on ClustalW. Amino acids with partial conservation and three-dimensional exposition that might be involved with antimicrobial activity are indicated by *squares*

3.3 Relations Between Antimicrobial Structure and Function in the 2S Albumin Family

The alignment between *Si*AMP2 and related 2S albumin (Fig. 3) suggests that despite of wide amino acid variation and sequence divergences into 2S albumin family [34], the correlation of cysteine scaffolds is extremely conserved leading to a relative conserved structure among several 2S albumins. Indeed, the conservation of multiple residues, in addition to preserved cysteines, indicates a possible sequence correlation with antimicrobial function. Among the hydrophobic and cationic residues are included leading to the inference that sesin as well the other 2S albumins might be able to inhibit bacterial growth just like *Si*AMP2. Initially, a multiple alignment was performed with nine full sequences of 2S albumin (Fig. 3). Among them is the 2S albumin isolated from *Ricinus communis*, which was used as template for *Si*AMP2 model (PDB code: 1psy) [57] and further eight 2S-AMPs [9, 63, 73, 74, 80, 87] obtained from 2S albumin data set. In prior analysis the high content of glutamine in all sequences became evident. However, there is no conservation in glutamine positions on the alignment or in the length of the glutamine domain. This observation, as expected, is also valid for storage proteins since it is involved in the seed's metabolic processes, being one of the major sources of NH_4 [34, 49], a fundamental macronutrient for seedling development. Therefore, the storage function no longer depends on a standard amino acid position in the sequence. Since the amino acid content is released, only the nutrient concentrations matter to the metabolic processes. In a further evaluation, the conserved residues throughout the alignment were identified and a structural analysis was carried out to elucidate which of the conserved residues are exposed and might be involved in the antimicrobial activity.

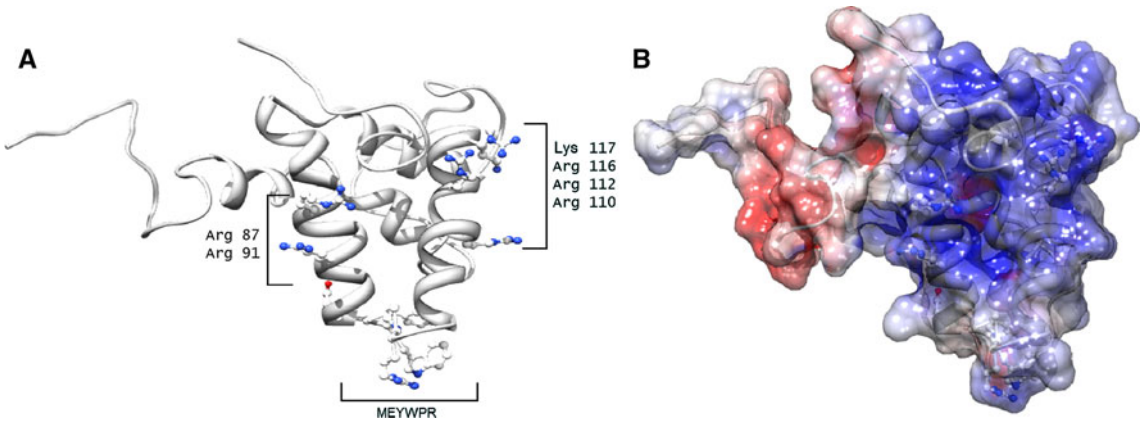

Fig. 4 **a** Three-dimensional model of *Si*AMP2 (GA341: 0.99991 DOPE: −8376.60059), obtained by comparative modeling using Modeller 9v7 with 1psy as template and 589 steps of energy minimization. **b** The electrostatic potential of the three-dimensional structure of *Si*AMP2. *Light gray* (*red*) indicated the surface region negatively charged; *dark gray* (*blue*), the positively charged. The total charge calculated was +2. Amino acids residues with partial conservation and three-dimensional exposition that might be involved with antimicrobial activity have their sequence position identified (Color figure online)

3.3.1 Analysis of Three-Dimensional Structure of 2S Albumin from Sesame

By using comparative modeling, a valid three-dimensional model of *Si*AMP2 (Fig. 4a) was obtained (GA341: 0.99991 DOPE: −8,376.60059) by using 1psy [57] as a template. *Si*AMP2's model consists of five helices arranged in a right-handed super helix, a folding motif common to most 2S albumins. *Si*AMP2 possess eight cysteines, six involved in the formation of three disulfide bridges Cys^{11}–Cys^{57}, Cys^{23}–Cys^{46}, Cys^{47}–Cys^{94}. The first two of these make the link between small and large subunits, while the third is an intra-chain disulfide bridge present in the large subunit. Energy minimization was carried out using GROMACS default parameters, and took 576 steps to reach the maximum force of 1,000.0 $kJ \cdot mol^{-1} \cdot nm^{-1}$. The final model was used for further analysis. The electrostatic potential generated with APBS (Fig. 4b) is mostly cationic with only a few negative spots due to the low content of aspartate and glutamate residues. Among the conserved residues in the 2S-AMPs previously identified, the cationic residues found on positions 87, 91, 110, 112, 116 and 117 appear exposed in the three-dimensional structure of *Si*AMP2 (Fig. 4a). A topology file was generated on GROMACS to investigate *Si*AMP2 total charge, and the data showed a total charge of +2 (qtot: 2).

Through meticulous study of the structural model of *Si*AMP2 and its close relationship with the other 2S-AMPs, it is possible to make conjectures about the antimicrobial activity of the 2S albumin family. It is clear that the electrostatic potential present and conserved in these 2S-AMPs has been significant for antimicrobial dynamism. The strong positive electrostatic potential might result in an attractive electrostatic interaction of the 2S-AMP with the negative part of the cytoplasmic membrane or still acts synergistically with a specific membrane receptor. The effects of electrostatic interaction would lead to a change in the overall electrostatic potential property of the bacterial membrane, occasioning ion influx and/or membrane permeation. It has been reported by Jean-François [35] that arginine plays an important role in interaction with negatively charged lipid head groups. Specifically, the side chain of arginine is capable of establishing both electrostatic and hydrogen bonds with the negative portion of membranes [68] such as dioleoylphosphatidylglycerol (DOPG), found in Gram-negative bacteria [26] like *Klebsiella* sp. When anionic lipids are associated with the cationic charges of a polymer or oligomer the electrostatic binding will work to wrap the membrane around the positively charged protein, leading to maximal contact and entropy gain from the counter-ion release [68].

Indeed, the major cationic electrostatic surface of *Si*AMP2 is not likely to be alone in its responsibility for the AMP action. Apparently the strong electrostatic interactions are tightly stabilized by Van der Waals forces. Additionally to positive charge of the conserved arginines, the aromatic portion of hydrophobic residues is also involved (Fig. 4). The amino acid residues exposed between helices IV and V (MEYWPR) form a small salient loop, with predominance of hydrophobic residues (Fig. 4b), could be useful for the stabilization of the peptide-membrane complex. The addition of an arginine beside the hydrophobic domain of *Si*AMP2 further increases the potential of interaction with bacterial membrane. The actual mechanism involved in 2S albumin antimicrobial activity is not yet clear. However, these data suggest that *Si*AMP2 exhibits a mechanism in which the protein binds to the surface of the membrane, predominantly by electrostatic interactions, providing this 2S albumin with its antimicrobial activity.

3.4 Phylogenetic Analysis

As previously emphasized here, 2S albumins are a protein family with a well-established nutritional properties, providing sulfur, nitrogen and carbon to plants in development [49, 51]. However, other biological activities have been observed over time and could be considered as secondary functions of these macromolecules. Studies with 2S albumins have already mentioned multiple functional properties, such as enzyme inhibiting [45], ribosome inactivating, allergenic and antifungal activities [78, 81]. With the intention of establishing an evolutionary relationship between the different biological functions already described for 2S albumin proteins, a phylogenetic analysis was performed. A data set was built gathering all 2S albumin proteins deposited in the NCBI's Protein Data Bank with less than 85% redundancy to avoid the isoforms. The information contained in the FASTA file header line of each sequence was also included in the data set. The main annotation consists of a single description according to its entry in the databank: biological activity, accession number in NR and organism of origin. The complete data set was composed of 102 amino acid sequences of 2S albumin. Among them, 69 sequences are annotated as storage proteins; seventeen as allergenic; two as trypsin inhibitors; two as α-amylase inhibitors; two as ribosome inactivators; and the last 10 as antimicrobial proteins. Our attention has been focused especially on antimicrobial activity since *Si*AMP2 has been identified as belonging to a 2S albumin family. The data set was submitted to phylogenetic reconstruction for amino acids using the MP method [24] (Fig. 5). In phylogenetic tree analysis it is evident that a closer phylogenetic relationship exists between proteins of a related genus than with those that have a related biological function. Accordingly, antimicrobial activity is freely distributed throughout the 2S albumin tree, apparently without

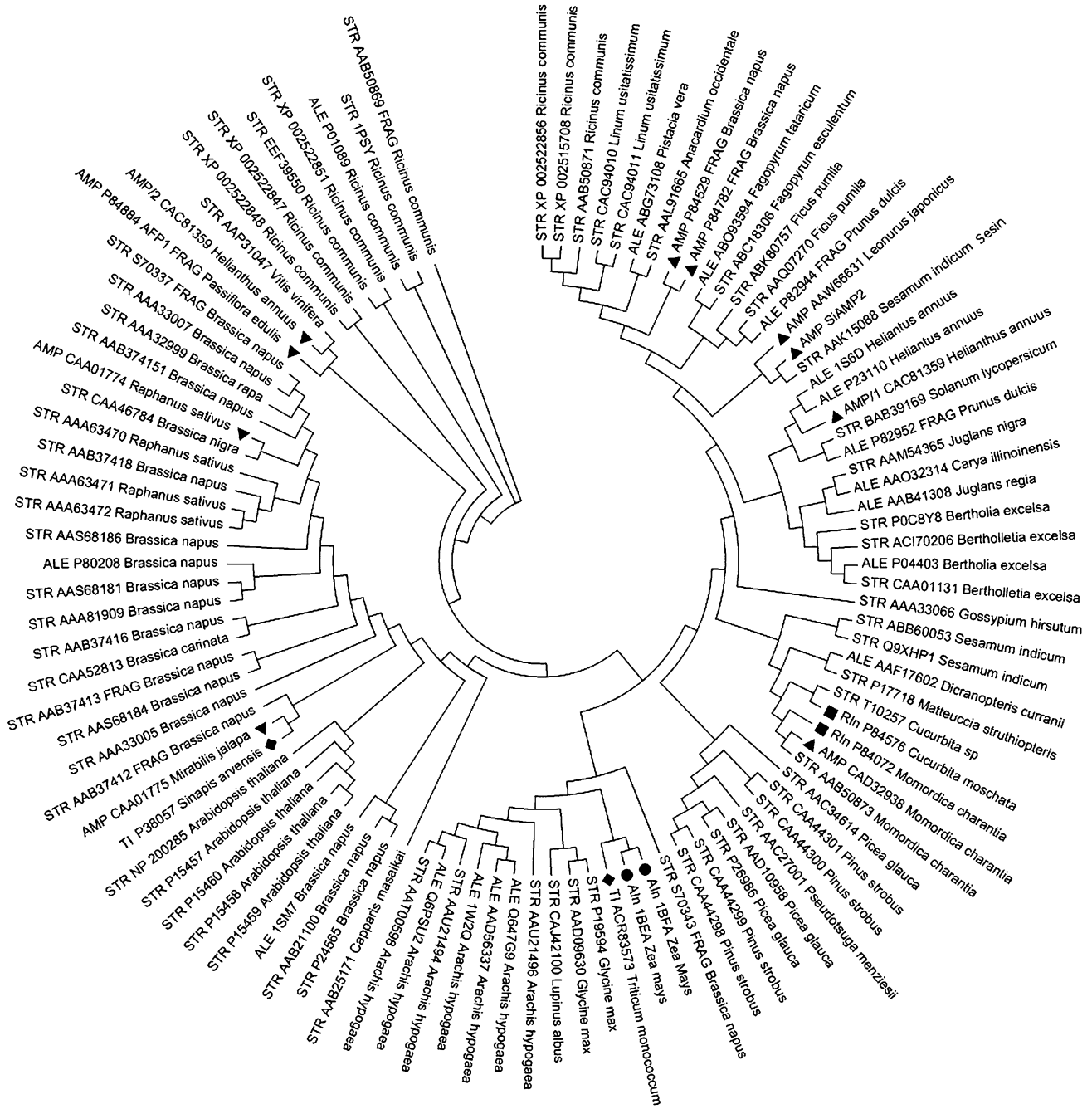

Fig. 5 Evolutionary relationship tree of 2S Albumins. The evolutionary history was inferred using the Maximum Parsimony method. All alignment gaps were treated as missing data. There were a total of 297 positions in the final dataset, out of which 199 were parsimony informative. Phylogenetic analyses were conducted in MEGA4. 2S albumins are marked with *triangles* for antimicrobial activity, *squares* for ribosome inhibitor activity, *circles* for α-amylase inhibitor and a diamond for trypsin inhibitor

being related to an amino acid sequence pattern (Fig. 5). The absence of a pattern of primary structure common to 2S-AMPs led us to raise some hypotheses about the evolution of antimicrobial activity in 2S albumin proteins. Firstly, the development of antimicrobial activity in each protein would be an independent event, without any phylogenetic relationship at all. Therefore, different mechanisms of action would be expected. It is not unusual to find reports of AMPs from the same organisms which diverge in primary and tertiary structure, acting by opposite mechanisms, as occurs with cationic [29] and anionic AMPs [32].

Despite the frequency of sequence divergence widely observed in some AMP families, high conservation has been detected in specific amino acids and motifs,

apparently significant to the function. A recent study with the α-defensin multigene family in primates [21] evidences its division into three distinct phylogenetic classes with the functional importance of defensins. It suggests that evolutionary forces acted on this family in two ways: one, to preserve the functional and structural proprieties, even among its subclasses; and two, to enlarge the range of microorganisms affected by those molecules with an extensive variation of sequences. Therefore, it is would be plausible that one primitive storage protein could be a common ancestor of all 2S albumins with antimicrobial activity and a standard mechanism of action may be common to most of those proteins. A similar observation had already been made by Tamang and Saier [75] in the cecropin superfamily, which comprises all toxic peptides of cecropin, pleurocidin and dermaceptin/ceratotoxin families. The literature supplies information about several protein families exerting physiologic functions beyond that of direct host defense. The β-defensin family, for instance, plays an important role in directly killing bacteria [37] and fungi cells [36]. It also acts in inhibiting viral infection [84], interacting with Toll-like receptors and presenting chemotactic activity [66], besides being important for the initiation of sperm maturation [90]. The cyclotides are another peptide family largely characterized by multiple functionality. It has been suggested that the main role of cyclotides is in plant defense [20]. However, in addition to insecticidal [6] and antimicrobial activities [62], cyclotides have a broad range of biologic activities, including anthelmintic [19], anti-HIV [15], uterotonic [67], toxicity and anti-tumor [11] and inhibition of neurotensin binding activities [85]. Cathelicidins, in turn, a multifunctional family of peptides found in neutrophils and epithelia of mammals, act essentially as defensive [47, 53] and immunomodulatory molecules [2]. It seems probable that during the evolutionary process plants evolved an efficient way to save cell energy by synthesizing a unique molecule comprising two distinct and essential physiological functions, such as storage and defense, during seed germination and plant development. Moreover, it is essential to have in mind that among all the 2S albumin sequences analyzed in this study; only a few have already been evaluated in search of a secondary biological role. Therefore, it is possible that several other 2S albumin proteins may also show another function besides storage.

The sequences analyzed in this work correspond to all 2S albumins which have so far been described with secondary roles. Indeed, since the unique analysis described below it has been possible to infer that there is a high degree of similarity between proteins present in small clusters and their position in the phylogenetic tree (Fig. 5). The protein isolated in the present work, *Si*AMP2, is found in the same branch of a storage protein that also comes from *S. indicum* (GenBank ID: AAK15088) [74]. *Si*AMP2 is also closely related to a 2S-AMP from *Leonurus japonicus* (GenBank ID: AAW66631) [86] described as showing antifungal and antibacterial activities besides having a ribosome inactivation property. As expected, the two 2S-AMPs from *B. napus* (UniProtKB ID: P84529, P84782) [51, 52], well-described napins, share a common position in the tree and probably also share characteristics of their antimicrobial activity. The data shown on the phylogenetic tree here indicates that it is impossible to connect antimicrobial activity with a specific cluster of 2S albumins, because 2S-AMP sequences are not positioned in related branches throughout the tree. This information motivates the theory that antimicrobial activity might potentially occur in 2S albumins with the same patterns.

4 Conclusion

In this work we purified a 2S albumin from *S. indicum* (*Si*AMP2) with specificity toward *Klebsiella* sp. This protein family is already known for having a significant role in seedling development. In the last decade, recent research has shown 2S albumins to be strong antimicrobial proteins. However, no phylogenetic relationship could be established between the functions of 2S albumins in storage, antimicrobial activity and other functions already described. Further in silico investigation of *Si*AMP2 and other antimicrobial 2S albumins resulted in the identification of conserved and exposed cationic amino acid residues as well as a hydrophobic domain that might be responsible for the antimicrobial activity. Based on this new knowledge, insights about the mechanism of action of 2S albumins were possible, predominantly by electrostatic interactions in cell membrane. Indeed, the actual endogenous role of *Si*AMP2 in sesame seeds could not be fully elucidated in this present study. However, it is clear that 2S albumins are multifunctional molecules and, considering the peculiar biological property of *Si*AMP2, these proteins could be studied as prototypes for new antibiotic drugs. In the near future they could, therefore, be useful in reducing hospital-acquired infections through biotechnological approaches.

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