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In Silico Structural Characteristics and α-Amylase Inhibitory Properties of Ric c 1 and Ric c 3, Allergenic 2S Albumins from *Ricinus communis* Seeds

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ABSTRACT: The major *Ricinus communis* allergens are the 2S albumins, Ric c 1 and Ric c 3. These proteins contain a trypsin/α-amylase inhibitor family domain, suggesting that they have a role in insect resistance. In this study, we verified that Ric c 1 and Ric c 3 inhibited the α-amylase activity of *Callosobruchus maculatus*, *Zabrotes subfasciatus*, and *Tenebrio molitor* (TMA) larvae as well as mammalian α-amylase. The toxicity of 2S albumin was determined through its incorporation in *C. maculatus* larvae as part of an artificial diet. Bioassays revealed that 2S albumin reduced larval growth by 20%. We also analyzed the tridimensional structures of Ric c 1 and Ric c 3 by (a) constructing a comparative model of Ric c 1 based on Ric c 3 NMR structure and (b) constructing the theoretical structure of the Ric c 1−TMA and Ric c 3−TMA complexes. Our biological and theoretical results revealed that Ric c 1 and Ric c 3 are a new class of α-amylase inhibitors. They could potentially be used to help design inhibitors that would be useful in diverse fields, ranging from diabetes treatment to crop protection.

KEYWORDS: castor bean, amylase inhibitor, 2S albumin, *Ricinus communis*, α-amylase, Ric c 1, Ric c 3, *Zabrotes subfasciatus*, *Callosobruchus maculatus*, *Tenebrio molitor*

INTRODUCTION

Castor oil and ricinoleate have many important industrial uses as sources of lubricants, coatings, plastics, and fungicides.[1,2] However, castor cultivation and processing may expose workers to potent allergens that elicit an immunoglobulin E (IgE) response, leading to serious medical consequences.[3,4] The 2S albumin proteins are the primary allergenic components of castor seed meal.[5,6] These proteins are a family of storage proteins present in a wide range of dicotyledonous plant seeds. They belong to the prolamin superfamily,[7] with members that include 2S albumins, α-amylase and trypsin inhibitors, puroindolines, grain softness proteins from cereal seeds, hydrophobic soybean proteins, and nonspecific lipid-transfer proteins. Members of the 2S albumin family (12−15 kDa) contain eight conserved cysteine residues (C...C...CC...CXC...C...C) and are evolutionarily related.[8] They are generally composed of two polypeptide chains linked by two disulfide bonds, and their final 3D-structure conserves a similar fold with bundles of α-helices stabilized by four disulfide bridges.[8]

Clearly, 2S albumins primarily function as storage proteins *in vivo*.[9] However, the conserved nature of castor 2S albumins suggests that their intrinsic features could play a role other than that related to nutrient reserves for seed germination. Previous reports suggest that 2S albumins have a role in plant defenses based on their antifungal activity,[10−12] serine protease inhibition,[13,14] and allergenic properties.[3,15] In fact, about 33% of well-known food allergens are trypsin/α-amylase inhibitor family members, according to the Food Allergy Research Resource Program (FARRP) database (http://www.allergenonline.com[8]).

The 2S albumins, major *Ricinus communis* allergens, are encoded as a 258 amino acid precursor protein. This precursor generates two heterodimeric proteins: Ric c 1, 11.2 kDa in size, and Ric c 3, 12 kDa in size.[3,5,6] As part of a genetic approach to eliminate the allergenic properties of the castor bean, Chen et al. investigated the gene expression of 2S albumins in castor seeds.[8] Analysis of the protein domains revealed that castor 2S albumins contain a domain belonging to the trypsin/α-amylase inhibitor family. α-Amylases (α-1,4-glucan-4-glucanohydrolase; EC 3.2.1.1) constitute a family of hydrolases that cleave α-D-(1,4)-glucan linkages in starch components, glycogen, and several related carbohydrates. These widely distributed enzymes play an important role in the carbohydrate metabolism of microorganisms, animals and plants. α-Amylases are the most important digestive enzymes in several insects that feed exclusively on seed products during their larval and/or adult life.[16] The presence of an α-amylase inhibitory domain in castor 2S albumins suggests that they have a role in plant resistance against insects. The Ric c 1 and Ric c 3 2S albumins also contain a trypsin/α-amylase domain and therefore may act as trypsin inhibitors, which are thought to

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confer some insect resistance, and as storage proteins during seed development.[8] One example is the seeds of *B. juncea*, which normally express the trypsin inhibitor and are naturally resistant to insects. Despite their wide diversity, some trypsin inhibitors share sequence homology with seed storage proteins.[17]

Based on a study by Ahn and Chen,[18] we investigated the ability of Ric c 1 and Ric c 3 to inhibit the cowpea weevil (*Callosobruchus maculatus*), Mexican bean weevil (*Zabrotes subfasciatus*), and yellow meal worm (*Tenebrio molitor*) larval gut and mammalian α-amylases. In bioassays using artificial seeds containing 2S albumin α-amylase inhibitor-rich fractions (0.1%, 0.2%, 0.5% and 2.0%), we found that the *R. communis* seed α-amylase inhibitors were able to reduce the larval growth of *C. maculatus*.

Molecular modeling is reported in the literature as being a useful tool for studying agriculture related issues. These reports include the use of homology modeling and docking techniques to (i) design new fungicides;[19] (ii) understand insecticide resistance [20] and (iii) help in genetic engineering for designing plants with specific resistance to herbicides.[21] Thus, since the 3D-structure of these proteins may help to establish significant relationships between structure, allergenicity, and α-amylase inhibition, we constructed a comparative model of the Ric c 1 3D-structure using the NMR structure of Ric c 3 as a template.[22] We also constructed the theoretical structures of the Ric c 1−TMA and Ric c 3−TMA complexes. The knowledge about the inhibitory effects of Ric c 1 and Ric c 3 on α-amylase and the characterization of their structure−activity relationship may help in the design of new α-amylase inhibitors with potential applications for crop protection in agriculture.

MATERIALS AND METHODS

Purification of α-Amylase Inhibitors, Ric c 1 and Ric c 3. Castor bean seeds (cultivar IAC-226) were obtained from the Instituto Agronômico de Campinas, São Paulo, Brazil. The proteins were extracted in phosphate buffer to obtain the 2S albumin pool, according to the method by Machado et al., 2003.[23] The isolation of Ric c 1 and Ric c 3 was described in Felix et al., 2008.[4]

Insects. *Callosobruchus maculatus* (Coleoptera: Bruchidae), *Z. subfasciatus* (Coleoptera: Bruchidae), and *T. molitor* (Coleoptera: Tenebrionidae) were obtained from a colony maintained in the Laboratório de Química e Função de Proteínas e Peptídeos, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense, Campos dos Goytacazes, RJ, Brazil. *Zabrotes subfasciatus* insects were reared in common bean (*Phaseolus vulgaris*) seeds, *C. maculatus* insects in *Vigna unguiculata* seeds, and *T. molitor* insects in wheat flour at 28 °C, 60−80% relative humidity with a L12:D12 photoperiod. Human salivary α-amylase was purchased from Sigma Co., USA.

Callosobruchus maculatus (16−17 days old), *Z. subfasciatus* (16−17 days old), and *T. molitor* (30−35 days old) fourth instar larvae were dissected in ice-cold iso-osmotic solution containing 25 mM NaCl, ground using a pestle and mortar, homogenized, and centrifuged at 10000*g* for 20 min at 4 °C to remove the walls of the gut and cellular debris. Midgut supernatants were used for enzyme and inhibitory enzyme assays. In the larvae of cowpea weevil (*Callosobruchus maculatus*), used for the bioassay, the pH of the gut contents was in the range of pH 5−7.[24]

α-Amylase Assays. The α-amylase activity assay (EC 3.2.1.1) was performed according to the method described by Bernfeld, 1955.[25] Enzymes (25 μg mL^{-1}) were assayed using a 1% starch substrate. After the addition of 3.5 dinitrosalicylic acid (DNS), the reaction was stopped at 100 °C and monitored at 540 nm. Each assay contained 10 U of α-amylase activity. All assays were performed in triplicate. One unit of α-amylase activity was the concentration of enzyme that increased the 540 nm absorbance by 0.1 absorbance unit during 25 min of the assay.

α-Amylase inhibitory Assays. α-Amylase inhibitory assays using *T. molitor*, *C. maculatus*, *Z. subfasciatus*, and human salivary α-amylases were carried out as described above with serially diluted concentrations of 2S albumin (10−50 μg mL^{-1}) added to the preincubation mixture. The Ric c 1 or Ric c 3 2S albumin was incubated in a water bath for exactly 15 min at 37 °C before addition of the substrate solution (1% starch) for a 15 min enzyme−substrate reaction (human amylase) and a 30 min enzyme−substrate reaction (insect amylases). All inhibition assays were performed in triplicate.

Bioassays. In order to test the potentially deleterious effects of seed coat flour on larval development, we used an artificial seed system.[26] Artificial seeds (final mass of 400 mg) were made by using a finely ground decorticated cowpea seed meal from an insect-susceptible *Vigna unguiculata* genotype (cv. Fradinho). The 2S albumin pool, obtained according to the method of Machado et al., 2003,[23] was added to the cowpea meal in 0.1, 0.2, 0.5 and 2.0% concentrations. Then, the mixture was pressed with a hand press, thus preparing the artificial seed. The artificial control seeds consisted exclusively of *V. unguiculata* (cv. Fradinho) seed meal. The artificial seeds were exposed to 3-day-old fertilized females for 24 h at 28 °C and 70% relative humidity. After this time, the females were removed and three eggs were left per seed (excess eggs laid on the seeds were removed). After 20 days, the infested seeds were opened and the weight and number of larvae were recorded. Experiments consisted of three seeds per assay (total of nine eggs per dose tested). Dose−response curves were drawn using the number and average weight of the surviving larvae found for each dose tested compared to larvae grown in the artificial control seeds. The results were analyzed using Student's *t* tests, and significant differences were determined at $P < 0.05$.[27] The dose−response curve values were used to calculate the dose at which the larval weight was reduced by 50% (WD_{50}).

Structural Studies. *Circular Dichroism (CD).* The native 2S albumin Ric c 1 and Ric c 3 isoforms were dissolved in distilled water, and CD spectra were obtained using a Jasco spectropolarimeter model J-715 (Jasco Corporation, Tokyo, Japan). The spectra were collected at 25 °C using a 0.1 cm path length quartz cell. The spectra were the average of three scans at a speed of 50 nm min^{-1}, and the water spectrum was subtracted. Only the far UV region, from 190 to 260 nm, was analyzed.

Homology Modeling of 2S Albumin Isoforms. Multiple sequence alignments and secondary structure predictions of the 2S albumin isoforms, Ric c 1 and Ric c 3, and other members of related families were performed using the Clustal-W [28] (http://www.ebi.ac.uk/clustalw) and the Jpred servers [29] (http://www.compbio.dundee.ac.uk/www-jpred). Homology modeling of processed Ric c 1 was performed using the Swiss-Model server (http://swissmodel.expasy.org/SWISS-MODEL) and the Swiss-PDB Viewer program (http://www.expasy.org/spdbv).[30,31] The model was constructed using the NMR structure of the 2S albumin isoform, Ric c 3 (PDB entry code 1PSY),[22] as a template due to its degree of structural and biological similarities. Blocks of structurally conserved regions were identified, and the structural alignment of the 2S albumins was generated. Coordinates for all residues were transferred to the Ric c 1 sequence, and loops were constructed in a single round. Several cycles of constrained energy minimization regularized the structure and its geometrical parameters. The model was minimized using the Gromos96 parameter set available in the Swiss-PDB Viewer program. The energy minimization of the models was performed in a vacuum without a reaction field and with a cutoff point of 10,000 Å. After evaluating the energy of each structure and repairing the distorted geometries through energy minimization, the models were submitted to analysis by Ramachandran plots and 3D profile scoring.

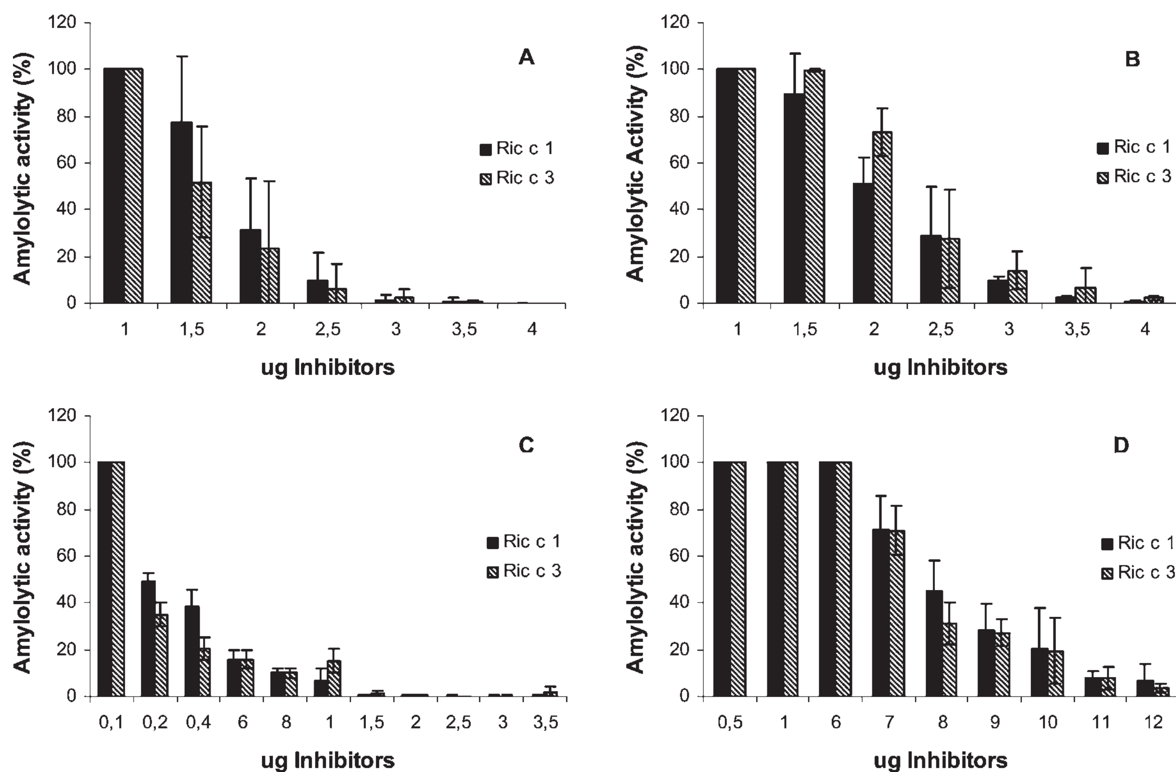

Figure 1. Inhibitory profiles of the 2S albumin isoforms, Ric c 1 and Ric c 3, on insect α-amylases and the human salivary α-amylase. (A) *C. maculatus* α-amylase. (B) *T. molitor* α-amylase. (C) *Z. subfasciatus* α-amylase. (D) Human salivary alpha-amylase. Enzymes and inhibitors were preincubated for 15 min prior to the addition of the substrate (1% starch) to measure enzyme activity. Inhibition (%) is relative to the control assay (without seed extract). Each point is the average of three measurements.

We also constructed two docking complexes, Ric c 1−TMA and Ric c 3−TMA, using the RBI-TMA complex X-ray crystal structure as a template (PDB entry code 1TMQ).[32] Initially, the complex crystal structure template was superimposed with Ric c 1 on the Swiss-PDB Viewer program,[31] guided by the structurally conserved regions using the Cα atoms. Then, the original inhibitor from the crystal (RBI) was deleted. The other docking complex (Ric c 3−TMA) was constructed using the procedures already described. The energy minimization of the models was performed in a vacuum without a reaction field, and with a cutoff point of 10,000 Å. Important interactions were analyzed, such as hydrogen bonds, electrostatic interactions, and hydrophobic interactions. The stereochemical quality of the models was checked using the PROCHECK program and the Profile 3D available at the PARMODEL web server.[33,34]

RESULTS AND DISCUSSION

Evaluation of 2S Albumins as α-Amylase Inhibitors. Insect α-amylases have an essential role in carbohydrate metabolism. Knowledge of α-amylase inhibitor activity and specificity is an important strategy for pest control.[35] The α-amylase inhibitory assays showed that Ric c 1 and Ric c 3 significantly inhibited the insect α-amylases tested, including the cowpea weevil (*C. maculatus*), Mexican bean weevil (*Z. subfasciatus*) and yellow meal worm (*T. molitor*) larval gut α-amylases. The Ric c 1 and Ric c 3 2S albumins also inhibited the human salivary α-amylase (Figure 1). These proteins proved to be potent inhibitors when compared to the inhibitors from other sources.[36,37] With an inhibitor concentration of 10−4 μg mL^{-1} we observed 100% inhibition of 10 units of α-amylase from *C. maculatus*, *Z. subfasciatus* and *T. molitor*. At the same concentration, the salivary α-amylase was not inhibited, but about 50% inhibition was observed when 80 μg mL^{-1} of both Ric c 1 and Ric c 3 were used (Figure 1).

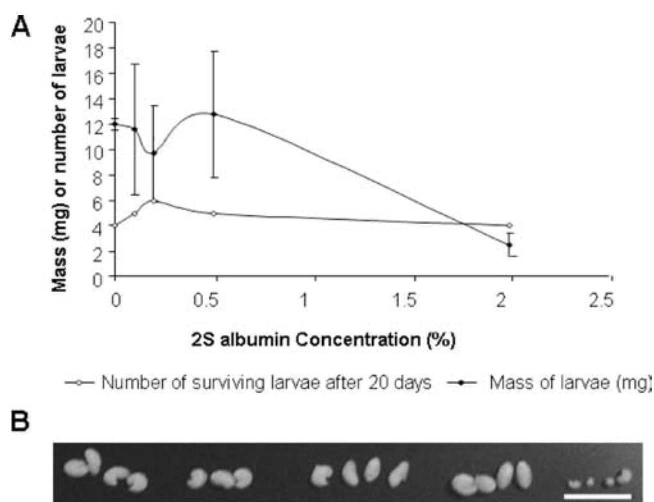

Figure 2. A) Performance and survival of *Callosobruchus maculatus* on artificial seeds containing different concentrations (0, 0.1, 0.2, 0.5 and 2.0 %) of a 2S albumin pool. Experiments were done in triplicate and the data SHOWN are the average of these results. B) Picture of developing *C. maculatus* control larvae and larvae grown on artificial seeds containing different concentrations (0.1, 0.2, 0.5 and 2.0%) of a 2S albumin pool. Bar = 1 cm.

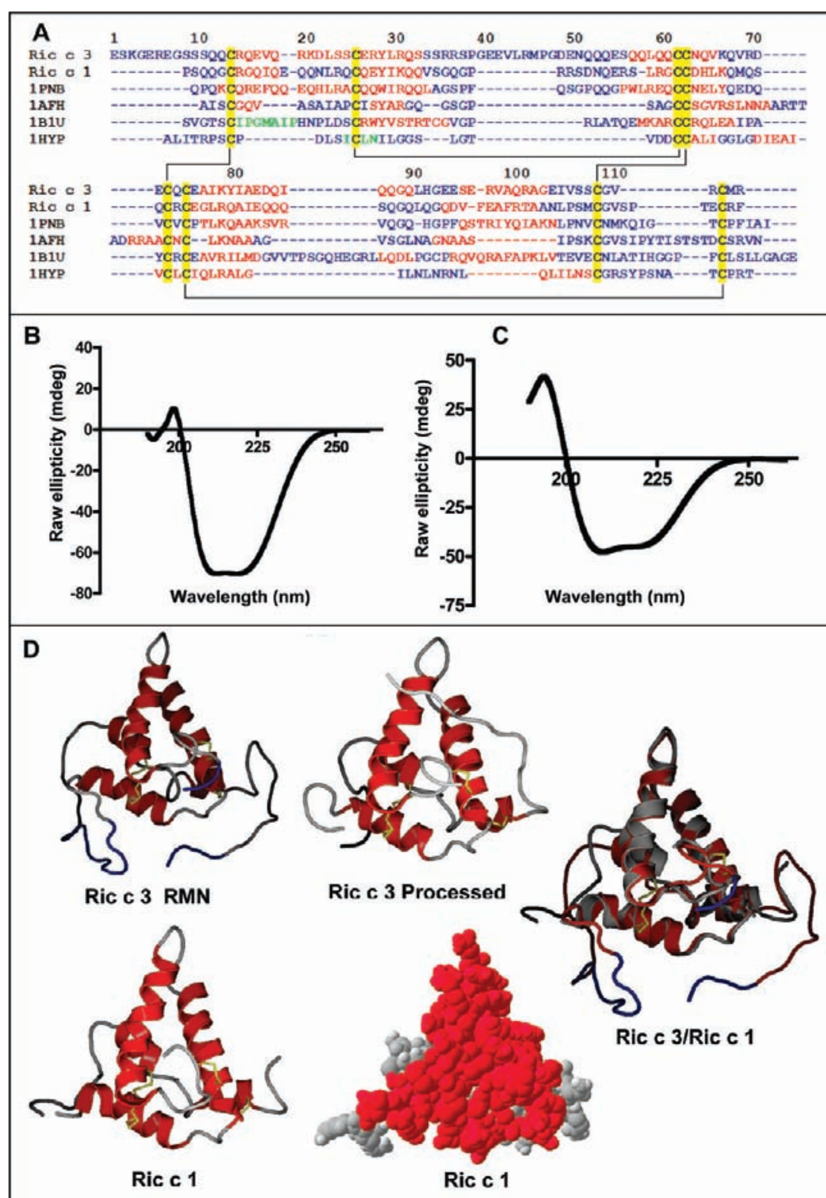

Figure 3. A) Primary sequence alignment of Ric c 1 with Ric c 3 and representative members of four related protein families (with structures deposited at the Protein Data Bank) including 2S albumin (Napin Bnib from *Brassica napus*, PDB = **1PNB**)[41], nonspecific lipid-transfer proteins (nsLTP from *Zea mays*, PDB = **1AFH**)[38], a-amylase inhibitor (Inhibitor ragi from *Eleusine coracana gaertneri*, PDB = **1B1U**)[39], and hydrophobic soybean protein (HPS from *Glycine max*, PDB = **1HYP**)[40]. The alignment was performed as described by Pantoja-Uceda *et al.*, 200242. Secondary structure prediction using the JPred server is represented in red (a-helices), green (b-sheets) and blue (loops). B) Dichroism spectra of Ric c 1 and Ric c 3. (C) Comparison of the NMR structure of unprocessed Ric c 3 (N-terminal, C-terminal and linker peptide shown in blue), processed Ric c 3, and Ric c 1 model. cpk representation of Ric c 1 shows the light (gray) and heavy (red) chains, and superimposition of the Ric c 1 model (gray) with the unprocessed Ric c 3 (red) shows the differences between them, including the longer N-terminal region (blue), the peptide linker (blue) and C-terminal (blue) in the Ric c 3 NMR structure. a-Helices are in red, unstructured loops in gray, and disulfide bonds in yellow.

Several insect pests synthesize at least two isoforms of α-amylase in their digestive tract.[38,39] Sivakumar et al. 2006 [40] demonstrated, under nondenaturing PAGE conditions, that more than one isoform of α-amylase can be detected in the midgut crude extracts from eight insect pests. Nevertheless, a single major form of α-amylase was observed in *T. molitor*,[41] whereas several isozymes were detected in *C. maculatus* and *Z. subfasciatus*.[39] According to Silva and co-workers, the presence of several α-amylase isozymes is an efficient strategy for insects to escape from inhibitor toxicity.[39] Apparently, the 2S albumins from *R. communis* tested here are able to bypass this insect survival strategy because they completely inhibit the activity of all α-amylase isoforms present in these insects' midguts. These results reinforce the potential use of these molecules in biotechnology, as they may serve as prototypes for new high affinity/selectivity α-amylase inhibitors.

Toxicity of the Seed Coat Flour to Larvae. According to Youle and Yang,[9] the 2S albumins of castor bean are localized within the protein bodies and constitute 40% of the total seed protein. In order to investigate the potential effect of 2S albumins

Table 1. Statistic Maps

	Ric c 3 NMR	Ric c 3 processed structure	Ric c 1 model
residues in most favored regions	62.9%	58.8%	67.1%
residues in additional allowed regions	26.8%	30.7%	26.8%
residues in generously allowed regions	10.3%	10.5%	4.9%
residues in disallowed regions	0%	0%	1.2%

in insect protection, the artificial seeds were made with a concentration of 2S albumin lower than endogenous concentrations, since these proteins, as described by the same authors, are rapidly degraded during germination.

The toxicity experiments (Figure 2) showed that the 2S albumin from *R. communis* seeds interfered with the normal development of *C. maculatus* larvae, considerably reducing the weight of 20 day old larvae at a 2% concentration. However, statistically significant alterations were not observed in the number of larvae that survived after 20 days of development. Although a high percentage of larvae survived from the seeds containing 2.0% 2S albumin, these larvae, after 20 days of development, had only about 20.8% (2.5 mg) the mass of control larvae that developed in artificial seeds containing only *V. unguiculata* seed flour (12 mg).

A dose−response curve was drawn using the weight of the surviving larvae and used to calculate the dose that reduced larval weight by 50% (WD_{50}). The values of WD_{50} to 2S albumin were about 1.3%.

Sequence Alignment and Construction of Ric c 1 Models. A primary structure alignment between Ric c 1 and Ric c 3 revealed a low identity (31%) but a significant degree of similarity (79%) when conserved and semiconserved substitutions were accounted for (Figure 3A). Sequence analysis revealed several common features, such as the conservation of eight cysteine residues that may be important in the formation of four disulfide bonds (Cys14−Cys73, Cys26−Cys62, Cys63−Cys109, and Cys75−Cys113) in the final structure (Figure 3A). Conservation of these cysteine residues has also been observed in representative members of four related protein families, including nonspecific lipid transfer proteins (nsLTP from *Zea mays*),[42] α-amylase inhibitors (inhibitor Ragi from *Eleusine coracana gaertneri*),[43] hydrophobic soybean protein (HPS from *Glycine max*),[44] and 2S albumins (Napin Bnib from *Brassica napus*).[45] This implies that these disulfide bridges are important for the stability of the final structure of these molecules (Figure 3A).

The secondary structures of Ric c 1, Ric c 3, and the other related family members were predicted using the Jpred program and suggested the conservation of an α-helix and unstructured loops among these molecules (Figure 3A). This result is in accordance with the experimental determination of Ric c 1 and Ric c 3 secondary structures (Figure 3B and C, respectively) using UV CD and far-UV CD spectra, which showed a positive band at 208 nm, a negative band at 216 nm, and the predominance of an α-helix structure. Overall, these data suggested that conservation of the eight cysteine residues, the presence of specific disulfide bond patterns, and the presence of the α-helix structure are the main structural features among 2S albumins, nsLTPs, α-amylase inhibitors, and hydrophobic soybean proteins, as found in the current literature.[46]

Both Ric c 1 and Ric c 3 are composed of two polypeptide chains linked by two disulfide bridges.[5] The analysis of biological and *in silico* structural features of these 2S albumins suggested that Ric c 3 can be used as a good template for constructing a molecular model of Ric c 1. The structure of Ric c 3 was determined by nuclear magnetic resonance (NMR) [22] with a 16-residue linker between the two polypeptide segments. This 16-residue linker is lost in the wild-type protein due to post-translational processing by endopeptidases and carboxypeptidases (Figure 3D). Here, we simulated the native wild-type protein structure using the Ric c 3 NMR structure as a template (Figure 3D). Because Ric c 1 and Ric c 3 apparently conserve similar features, and a similar processing mode, we also modeled the native structure of Ric c 1 based on the processed Ric c 3 structure. The model of the processed Ric c 1 structure is a bundle of five α-helices folded in a right-handed superhelix, similar to that observed in the Ric c 3 NMR structure (Figure 3D).[22] A similar fold was observed in other 2S albumins,[47] nonspecific lipid transfer proteins (nsLTPs),[42,48] hydrophobic soybean proteins,[49] and α-amylase inhibitors,[50] but with significantly different intrinsic features. Despite their similar overall folding pattern, there were still some small variations between Ric c 1 and Ric c 3 that led to a final rms deviation of 2.04 Å after superimposition, with a deviation of 7.19 Å in some other regions. These rms variations and the particular differences in the overall folding structure may be responsible for these intrinsic immunobiological differences in the 2S albumins (Figure 3B).

The evaluation of the quality of the processed Ric c 3 and Ric c 1 structures is an important part of the process and is a crucial step in homology modeling that guarantees the reliability of the generated structure. Our analysis using Ramachandran plots revealed a good stereochemistry of the models (Table 1). Standard deviations of all of the main chain and side chain stereochemical parameters were within, or better than, the expected values. Most of the residues adopted the most favored main chain torsion angles (67.1%) compared to the processed structure of the Ric c 3 template and the Ric c 3 NMR structure, which were 58.8% and 62.9%, respectively, in the most favorable regions. In agreement, the 3D profile score *S* of the Ric c 1 model, which evaluates the compatibility of the 3D protein structure with the sequence, was within the limits of acceptable values and better than the template. The energy distribution analysis for each amino acid residue showed that no negatively scored regions occurred in either of the processed Ric c 1 or Ric c 3 structure models, nor in the template Ric c 3 NMR structure.

Docking Simulation. A balance between structural and electronic properties is essential for protein−protein interactions, including inhibitor−enzyme interactions.[33,50,51] The evaluation of the Ric c 1 electrostatic potential map revealed several positive regions on this molecule's surface that may serve as anion-binding sites. In addition, the neutral and negatively charged regions of this inhibitor may help orient this binding event (Figure 4B). Interestingly, according to our experimental results, these electrostatic features are complementary to the features of *Tenebrio molitor* (TMA) α-amylase, making TMA a potential target for 2S albumin. The structure of TMA is available at PDB (code 1TMQ) and its electrostatic potential map revealed a predominantly negative surface that may interact with the positive regions of Ric c 1.

In the theoretical complexes, Ric c 1−TMA and Ric c 3−TMA, the inhibitors bind to the active site of the enzyme in the N-terminal region (Figure 4), a behavior also observed in the template.[33] The active site of the enzyme lies in a V-shaped

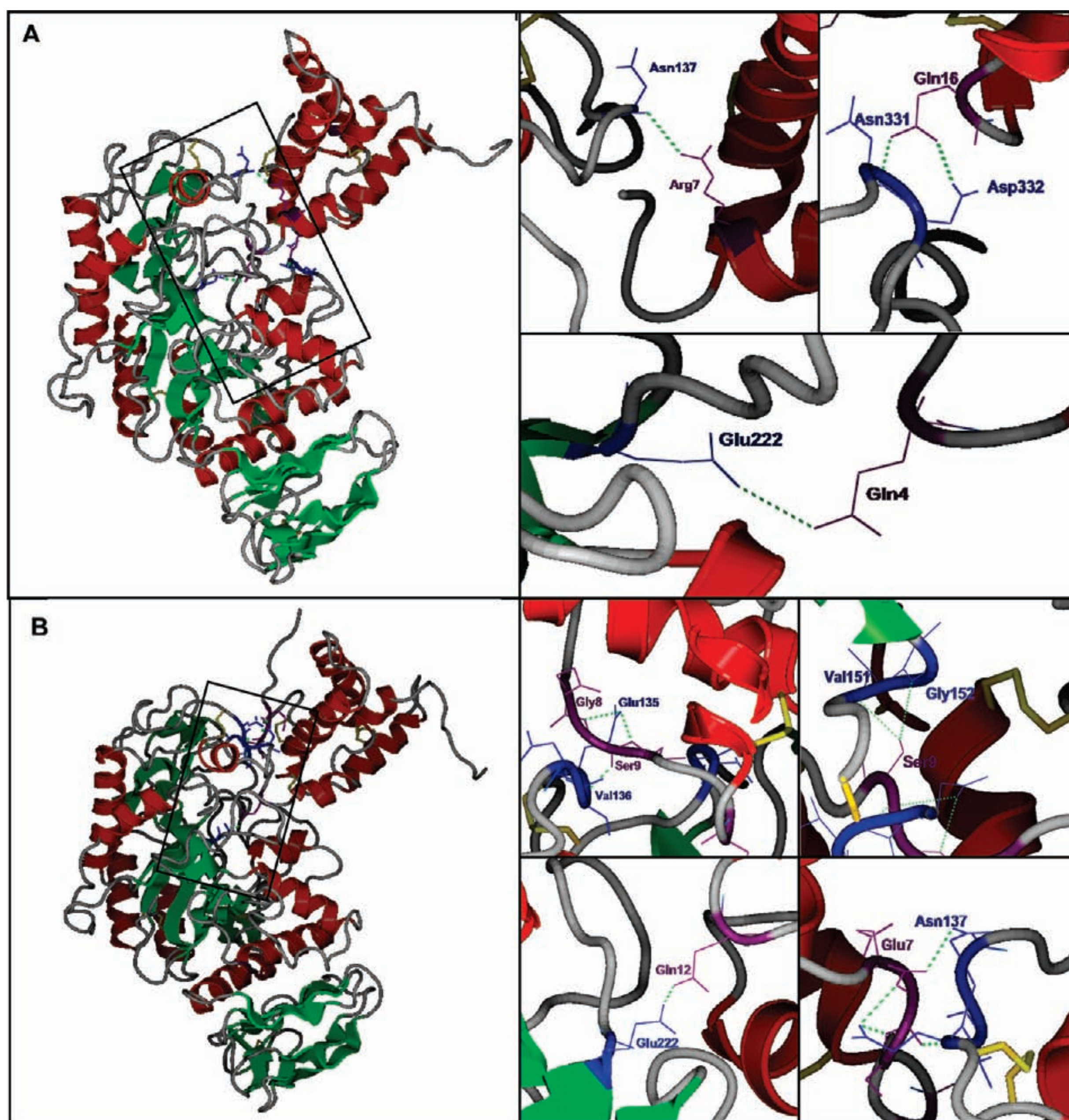

Figure 4. (A) Interaction and three-dimensional structure between Ric c 1 and TMA. Hydrogen bond interactions (green) between inhibitor residues (Arg7, Gln4 and Gln16) and TMA residues (Asn137, Glu222, Asn331 and Asp332). Inhibitor residues are shown in purple and enzyme residues in blue. (B) Interaction and three-dimensional structure between Ric c 3 and TMA. Hydrogen bond interactions (green) between inhibitor residues (Glu7, Gly8, Ser9, and Gln12) and TMA residues (Asn137, Glu135, Val136, Val151, Gly152 and Glu222). Inhibitor residues are shown in purple and enzyme residues in blue.

depression at the interface of domains A and B, and the inhibitors interact with residues of both domains. The catalytic site of TMA has three important residues for catalysis, Asp185, Glu222, and Asp287. The structural analysis of the complexes revealed that the residues Gln4 in Ric c 1−TMA and Gln12 in Ric c 3−TMA interact by hydrogen bonding to Glu222, blocking the catalytic residue (Figure 4 A and B). Other interactions via hydrogen bonding were also observed in Ric c 1−TMA (Gln16−Asn331, Gln16−Asp332, Arg7−Asn137) and Ric c 3−TMA (Glu7−Asn137, Gly8−Glu135, Gly8−Val136,Ser9−Glu135, Ser9−Val151, Ser9−Gly152) (Figure 4A and B, respectively).

Interestingly, proteinaceous α-amylase inhibitors may present different polypeptide scaffolds and are grouped based on their 3D structures into six classes: lectin-like, knottin-like, cereal-type, Kunitz-like, γ-purothionin-like and thaumatin-like.[52] Our data suggest that the 2S albumin isoforms, Ric c 1 and Ric c 3, represent a new group of α-amylase inhibitors (i.e., a 2S albumin-like group).

In conclusion, our biological data reinforced the theory that Ric c 1 and Ric c 3, members of the prolamin superfamily and major allergen storage proteins found in *R. communis* seeds,[7] could also present protective roles in castor seeds since they

significantly inhibit insect α-amylase activity. Knowledge of the 3D structure of Ric c 3 and Ric c 1, potent inhibitors of α-amylases of insects, associated with the identification of sites of interaction with α-amylase, taken together with the mapping of allergenic epitopes that was also a target of our studies,[4] are bases for the design of genes that may be used in biotechnology processes where the defense activity could be preserved and allergenic activity could be eliminated, thus producing plants resistant to insects and safe regarding human health.

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