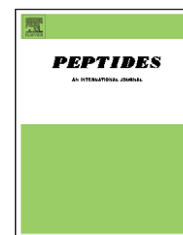


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Mapping IgE-binding epitopes of Ric c 1 and Ric c 3, allergens from *Ricinus communis*, by mast cell degranulation assay

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ABSTRACT

Ric c 1 and Ric c 3 are the major castor bean allergens. In order to identify continuous IgE-epitopes in Ric c 1 and Ric c 3, pools of sera from rats immunized with a pool of 2S albumin from these seeds, Ric c 1 and Ric c 3 overlapping synthetic peptides, were used to screen for IgE-binding epitopes. The allergenic properties were monitored by mast cell degranulation assays, histamine quantification and human-IgE binding. Large and small chains isolated from these proteins present allergenic properties. Four continuous epitopes were identified in Ric c 3 and two in Ric c 1. This knowledge may allow the induction of protective antibody responses to antagonize the IgE recognition.

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1. Introduction

The castor bean plant (*Ricinus communis*) originated in Ethiopia and gradually dispersed towards South Africa, the Mediterranean region and warm areas of Asia, until finally establishing itself as a natural species in the majority of warm climate regions of the world [12]. The castor bean contains about 50% oil, which has special characteristics such as high viscosity, stability to heat and pressure, low freezing point, and ability to

form waxy substances upon chemical treatments [7]. The modern industry uses the oil extracted from its seeds in the manufacture of explosives, varnishes, lubricants, dyes, plastics, fertilizers, leather, candles and cosmetics, as well as laxatives, antifungals and antiparasitic preparations [7]. As energy demands increase and fossil fuels are limited, researches are developed to obtain alternative renewable fuels. Biodiesel, consisting of methyl esters of fatty acids transesterified by vegetable oils with methanol, has become

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more attractive recently due to its environmental benefits and the fact that it comes from renewable resources [6,13]. Castor bean is an oleaginous candidate for such oil production [13], which will contribute to enhance the cultivation of this plant. Castor bean seeds, however, contain a strong toxin (ricin) and an allergenic protein fraction (CB-1A or 2S albumin isoforms), which severely limits the usefulness of the castor meal after oil extraction [5,12,27]. Medical problems such as conjunctivitis, rhinitis and urticaria have also been related with this kind of seed, as well as to castor bean leaves and pollen [12], described as a producer of atopic dermatitis. Recently, the atmospheric prevalence potency of castor bean pollen was demonstrated by Raju et al [21] in Chennai, India. The authors have recommended the inclusion of this pollen allergen in the diagnosis of allergy.

The mechanisms of the action of the toxin, ricin, have been established [5,22] and some treatments have been proposed to eliminate its toxicity [2]. In contrast, there is no available method for the inactivation of the allergenic fraction although the primary structure of major allergens present in the 2S albumin pool, Ric c 1 and Ric c 3 have been determined [8,15,17,24] since 1982 and 1992, respectively. A better understanding of the functional role of the castor bean allergens is fundamental to prevent allergy challenged by *R. communis*. The aim of this study was to determine the continuous IgE-epitope of the castor bean seed allergens, Ric c 1 and Ric c 3.

2. Methods

2.1. Plant material and 2S albumin purification

Castor bean (*R. communis* L., cultivar IAC-226) seeds were obtained from the Instituto Agronômico de Campinas, São Paulo/Brazil and 2S albumin fractions were isolated and identified by SDS-PAGE and immunoblotting experiments as described by Machado et al. [17,18].

2.2. Isolation of 2S albumin isoforms, Ric c 1 and Ric c 3

The pool of 2S albumin was subjected to reverse-phase liquid chromatography on a C18 column (Sephacryl peptide C18 5 μ ST 4.6/250 from Pharmacia Biotech) using a Shimadzu apparatus. The chromatography was developed at a flow rate of 0.7 mL min⁻¹ using 0.1% TFA as solvent A and 80% of acetonitrile containing 0.1% TFA as solvent B. The gradient was 0–80% of solvent B for 55 min. The elution profile was monitored by on-line measurement of the absorbance at 220 nm. Fractions containing the major peaks were dried in a speed Vac system. The UV spectrum was obtained using a photo-diode detector.

2.3. Denaturation, reduction and pyridylethylation

Ric c 1 and Ric c 3 were dissolved at 10 mg/mL in a solution containing 6 M guanidinium chloride, 0.1 M Tris-HCl buffer, pH 8.4 and 1 M EDTA for 2 h at 37 °C. After denaturation, dithiothreitol was added to a concentration of 2 mM to reduce disulphide bonds and 4-vinylpyridine (560 μ mol) to convert

the cysteine residues to stable derivatives. The mixture was incubated for 6 h at 37 °C. [11]. The reaction mixture was submitted to C18 reverse-phase HPLC for S- and L-chain separation. The chromatography was developed at a flow rate of 0.7 mL min⁻¹ using 0.1% TFA as solvent A and 80% of acetonitrile containing 0.1% TFA as solvent B. The gradient was 0–80% of solvent B for 55 min. Proteins were detected by monitoring the absorbance at 220 nm.

2.4. Circular dichroism (CD)

The native isoforms of 2S albumin, Ric c 1 and Ric c 3, and their isolated S-pyridyl-ethylated chains were dissolved in distilled water and CD spectra were obtained on 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. Spectra were the average of three scans at a speed of 50 nm/min and the water spectrum was subtracted. Only the far UV region from 190 to 260 nm was analyzed.

2.5. N-terminal amino acid sequences

The N-terminal partial amino acid sequences of the 2S albumin isoforms were obtained on a Shimadzu PPSQ-10 automated protein sequencer using the Edman degradation principle. Sequences were determined from both purified proteins. PTH amino acids were detected at 269 nm after separation on a reverse-phase C18 column under isocratic conditions, according to the manufacturer's instructions. The polypeptide sequences obtained were submitted to automatic alignment using a BLAST search system [3].

2.6. Animals and antiserum

Isogenic female R/A Tor rats weighing 150 g were obtained from the animal house of the Universidade Federal Fluminense, Niterói, RJ and all experimental procedures were approved by the animal research ethics of this University.

Rats were sub-cutaneously injected with 10 μ g of 2S albumin pool in the presence of adjuvant mixture: 5 mg of Al₂O₃ and 5 mg of nigosin. After 15 days, rats were anesthetized and bled by cardiac puncture. The sera were pooled and tested by mast cell degranulation assay.

2.7. Mast cell degranulation assay

2.7.1. Rat peritoneal mast cells

Wistar rats were bred at the animal house of the Universidade Estadual do Norte Fluminense. All experimental procedures were approved by the animal research ethics board of the Universidade Estadual do Norte Fluminense.

Rats (~250 g) were euthanized with CO₂ and a peritoneal wash performed with the injection of 20 mL of DMEM (Gibco) containing 12 U/mL of heparin. The abdomen was gently massaged for about 90 s. The peritoneal cavity was carefully opened and the fluid containing peritoneal cells was aspirated with a Pasteur pipette. Thereafter, the cells were transferred to Petri plates and incubated for 30 min at 37 °C. Two thirds of the superior layer were then aspirated and discarded. Supernatant

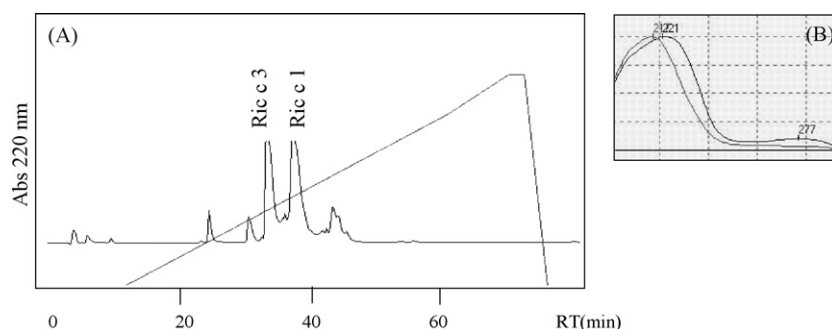


Fig. 1 – (A) Elution profile of reverse-phase chromatography on C18 column—2S albumin pool was loaded on a column pre-equilibrated with 0.1% TFA. Elution was carried out as described in Section 2 (Material and methods). The collected fractions were monitored for protein concentration at 220 nm. The line represents the acetonitrile gradient. (B) UV spectrum of the major fractions, Ric c 1 and Ric c 3.

enriched with mast cells (1.8×10^5 mast cell/mL) was separated in aliquots of 100 μ L in eppendorf tubes.

2.7.2. Mast cell activation

Mast cells (100 μ L) were incubated with Tor rat preimmune serum as a control and were initially activated for 60 min at 37 °C using a 2S albumin polyclonal anti-rat IgE. After sensitization with IgE, cells were washed twice with DMEM. Each of these experiments was carried out in the presence or absence of the 2S albumin pool (100 ng). The mast cell activation properties were also investigated for the isoallergens, Ric c 1 and Ric 3, and their small and large chains. After incubation with antibodies and allergens, histamine contents were determined, as described below, and cells (in 10 μ L) were stained for 15 min with 10 μ L of a solution containing 0.1% toluidine blue, 10% formaldehyde and 1% acetic acid, pH 2.8, allowing the visualization of degranulated mast cells. Granulated and degranulated mast cells were counted under a light microscope using the 40 $\times$ objective in a Neubauer chamber.

2.7.3. Histamine quantification

The cell suspensions, after incubation with antibodies and allergens, were centrifuged at $170 \times g$ for 10 min. An aliquot of the supernatant (20 μ L) was removed for histamine assay. The remaining cell suspensions were “sonicated” for 30 s to release the residual histamine. The histamine content was determined in both the supernatant and the cell pellets. The solutions containing histamine were fractionated by ionic chromatography on an Amino-Na column (Shimadzu) equilibrated with 0.2 M NaOH. Histamine was detected and quantified by post-column-o-phthaldialdehyde derivative, a fluorescent isoindole was formed in the presence of 2-mercaptoethanol and under alkaline conditions (Sista HS, 1986) [25].

Histamine was detected in a fluorescence detector (Shimadzu RF-535) at an excitation wavelength of 360 nm and emission wavelength of 450 nm. A histamine curve was prepared using one pmol to one nmol of histamine as standard. The release of histamine was calculated as a percentage of the total histamine content of the sample.

2.8. Human serum

Serum from an atopic patient, with allergy to castor bean IgE title, 427 KU/L as determined by chemiluminescence test, was employed as a human IgE source.

2.9. Dot-blot

Dot-blots were made on 0.2 μ m nitrocellulose membrane in triplicate with 2 μ L of 2S albumin or peptide in 0.01 M phosphate-buffered saline (PBS), pH 7.6, by applying 10 μ g per dot. After drying, the blots were blocked with 0.5% defatted milk powder/0.5% in PBS for 1 h. For antibody probing, the membrane was reacted with serum from an atopic patient, with allergy to castor bean. The membranes were incubated in this serum, diluted 1:50 in PBS, pH 7.6, during 1 h and then reacted with anti-human Biotin IgE (0.5 mg/mL) (diluted 1:2000) for 1 h. The membrane was subsequently incubated with streptavidin-biotinylated HRP complex for 1 h. The color of all probes was developed with a substrate mixture: 5 mg of DAB in 4.9 mL of water, 300 μ L of 0.1 M imidazol, 100 μ L of Tris-HCl 2 M buffer (pH 7.5) and 5 μ L of 30% H_2O_2 .

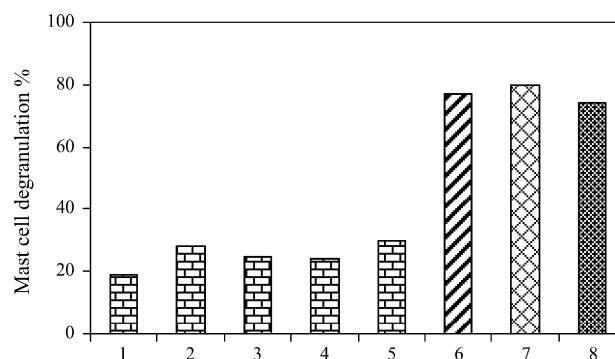


Fig. 2 – Mast cell degranulation. 1: Control cells; 2: pre-immune serum; 3: anti-2S albumin serum; 4: 2S albumin pool; 5: 2S albumin pool + pre-immune serum 6: Ric c 1 + 2S albumin antiserum; 7: Ric c 3 + 2S albumin antiserum; 8: 2S albumin pool + 2S albumin antiserum.

2.10. Peptide synthesis

To determine the amino acid sequence of the IgE-binding sites on Ric c 1 and Ric c 3, overlapping peptides with 11–16 amino acid residues that span the entire length of the small and large chain of these allergenic proteins were synthesized. Peptide separations and homogeneity were performed using a Shimadzu HPLC system with a C18 reverse-phase column.

Detection at 220 nm was achieved using a photo diode variable wavelength absorbance detector.

2.11. Peptide alignment

The amino acid sequence of major continuous IgE-epitope from castor bean, present in the Ric c 1 large chain, was submitted to alignment, which was performed using NCBI-BLAST search

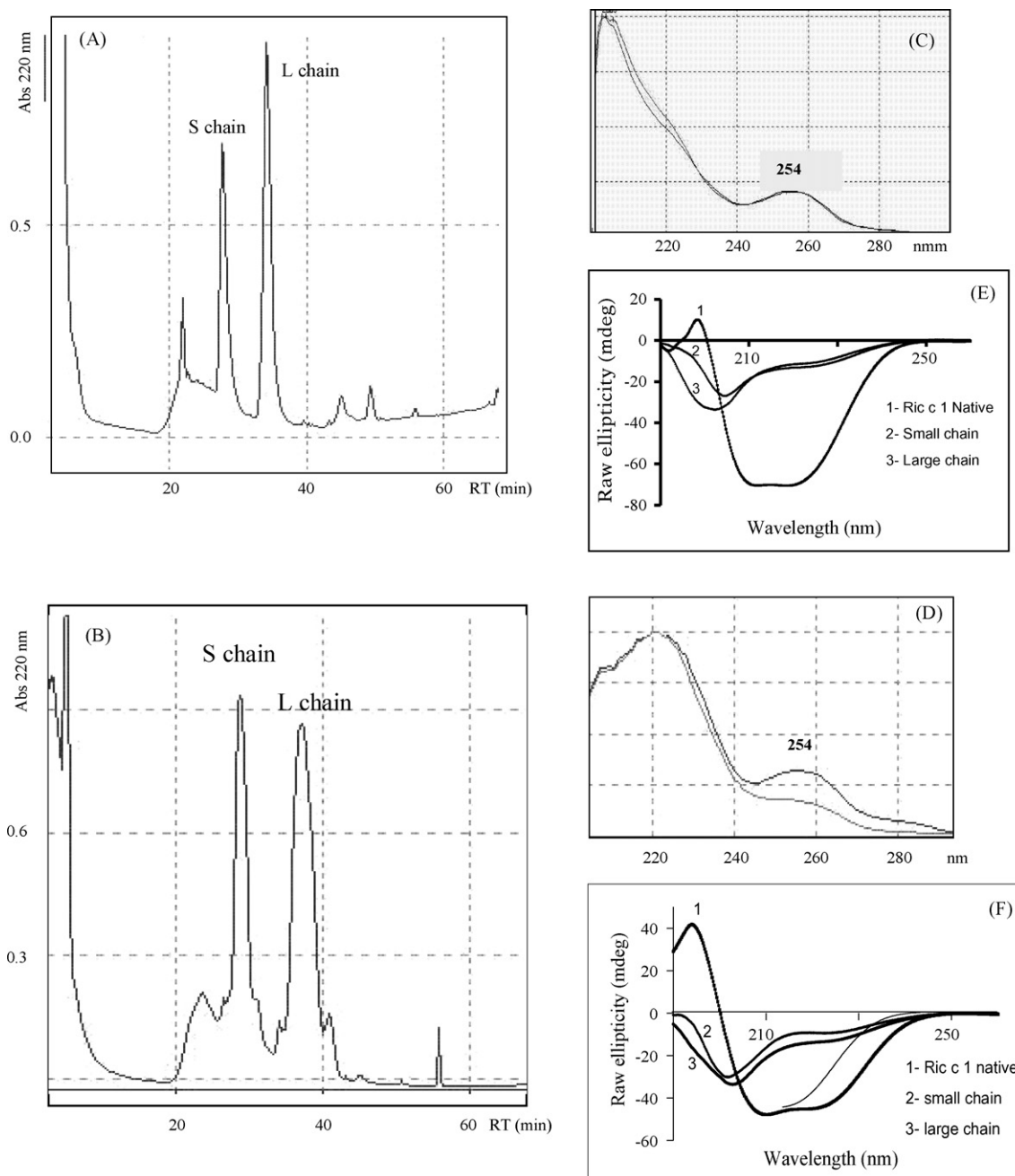


Fig. 3 – Separation of small and large chain of Ric c 1 and Ric c 3. (A) and (B) Elution profiles of reverse-phase chromatography on C18 column of Ric c 1 Ric c 3, respectively, after reduction and pyridylethylation. The reaction mixture was loaded on a column pre-equilibrated with 0.1% TFA and elution was carried out as described in the Section 2 (Material and methods) section. The collected fractions were monitored for protein concentration at 220 nm. (C) and (D) UV spectra of the isolated small and large polypeptide chains of Ric c 1 and Ric c 3, respectively. (E) and (F) CD spectra of the native and isolated small and large polypeptide chains of Ric c 1 and Ric c 3.

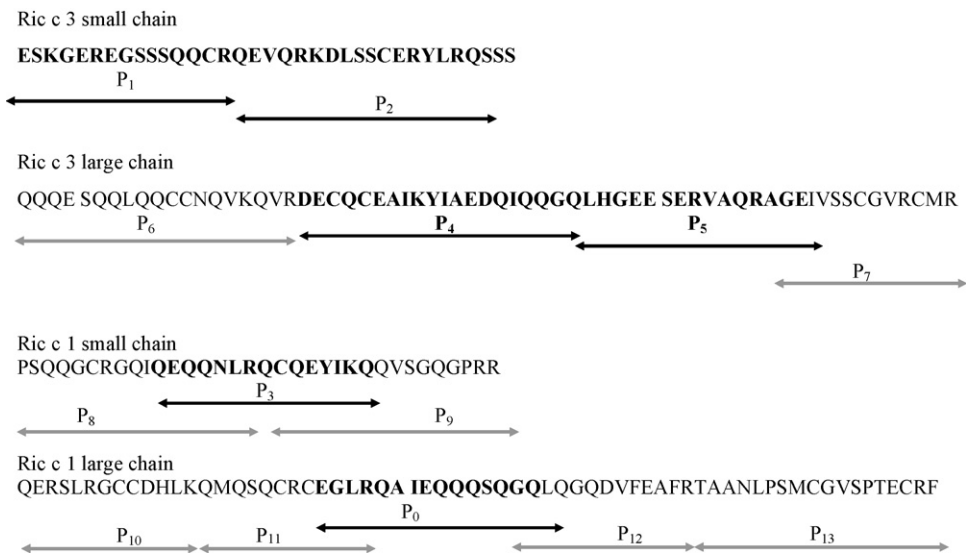


Fig. 4 – Localization of the synthetic peptides on Ric c 1 and Ric c 3 primary structures. Peptides in black induced mast cell degranulation and human IgE-binding, while peptides in gray have not induced mast cell degranulation or human IgE-binding.

system [3] on the non-redundant proteins sequences databases [4]. The search was limited in 2S albumin and allergen proteins.

3. Results

3.1. Isolation of Ric c 1, Ric c 3 and their polypeptide chains

The 2S albumin fraction from castor bean seeds was isolated after saline extraction and chromatography on Sephadex G-50. This chromatography step allowed us to separate the 12S globulin from the 2S albumin fraction. The allergenicity was demonstrated by passive cutaneous anaphylaxis in Tor rats sensitized with the sera of immunized rabbits with 2S albumin pool, as described by Machado e Silva Jr. [17] (data not shown). The pool of 2S albumin was submitted to reverse-phase chromatography (C18 column) and eight fractions were isolated. The major peaks (Fig. 1A) were dried in a SpeedVac System. These fractions were submitted to partial N-terminal sequencing (data not shown) and were identified as Ric c 3 and Ric c 1, respectively, according to Sharief and Li and daSilva et al., respectively [24,8]. The UV spectra are shown in Fig. 1B for Ric c 1 and Ric c 3. These isoallergens induced mast cell degranulation, similar to the 2S albumin pool (Fig. 2). Mast cell degranulation, promoted during the isolation of these cells, was ~25%. Similar effects were also obtained by Katayama et al. [16] during the investigation of mast cell hyperplasia, induced by multiple challenges with cedar pollen in sensitized guinea pigs. Two polypeptide chains were isolated after reduction and pyridylethylation of both Ric c 1 and Ric c 3 after reverse-phase chromatography on a C18 column (Fig. 3A and B). The UV spectra showed a new peak at 254 nm, indicating the production of a pyridylethyl cysteine (PEC) after the reduction and alkylation process (Fig. 3C and D) [10]. The isolated polypeptide chains were freeze-dried and submitted to N-terminal sequencing. The N-terminal sequence of the

most hydrophilic peak from Ric c 3 was ESKGEREG and it was identified as a Ric c 3 small chain, whilst the other peak was identified as the Ric c 3 large chain [8]. After fractionation of reduced and alkylated Ric c 1 isoallergen on the C18 column, the first peak was also identified as the Ric c 1 small chain (N-terminal sequence: PSQQGCRGQI) and the most hydrophobic peak was identified as the large chain [15]. The circular dichroism spectra (Fig. 3E and F) indicated that the denaturation with 6 M guanidinium chloride and pyridylethylation reaction produced a unfolding of the Ric c 1 and Ric c 3 molecules.

All of the polypeptide chains isolated induced ~70% of mast cell degranulation when these cells were incubated in the presence of IgE, as presented in Table 1, indicating the presence of continuous IgE epitopes in these polypeptide fractions.

3.2. Peptide synthesis and allergenic reactivity

To determine the amino acid sequence of the IgE-binding sites, we synthesized 14 overlapping peptides that span the entire Ric c 1 and Ric c 3 allergenic proteins (Fig. 4). All synthesized peptides were initially examined by means of reverse-phase HPLC and found to be $97 \pm 1.5\%$ pure. The pre-immune serum

Table 1 – Percent of mast cell degranulation induced by *Ricinus communis* allergen isoforms

Sample	Mast cell degranulation (%)
2S albumin pool	74
Ric c 1 small chain	72
Ric c 1 large chain	67
Ric c 3 small chain	73
Ric c 3 large chain	68
Mast cell control	25

did not recognize any of the peptides synthesized (data not shown). Table 2 shows that six peptides, P₀, P₁, P₂, P₃, P₄ and P₅ along the length of the small and large chain of Ric c 1 and Ric c 3 were recognized by the pool of sera and induced the mast cell degranulation. In contrast, mast cell degranulation was not induced by the other peptides (P₆–P₁₃) under the same conditions. Furthermore, the mast cell was not degranulated in the absence of the IgE source (without serum). These data were confirmed by the content of histamine released (Table 2).

To confirm the results of the IgE epitope mapping by mast cell degranulation and to demonstrate that the identified

epitopes are relevant to humans, serum from a patient with IgE antibodies against Ric c 1, or Ric c 3 were used for immunoblotting assays. As observed in Fig. 5A, all peptides (P₀–P₅) that induced rat mast cell degranulation were positive in dot blot assays using anti-human IgE. In contrast, the other peptides (P₆–P₁₃) from Ric c 1 and Ric c 3 were not recognized by this antibody (Fig. 5B).

Six IgE-epitopes on Ric 1 and Ric c 3, major allergens in castor bean seeds, were characterized and the P₀ polypeptide appears to be immunodominant in IgE-binding epitopes, since the serum samples are best recognized in terms of intensity of

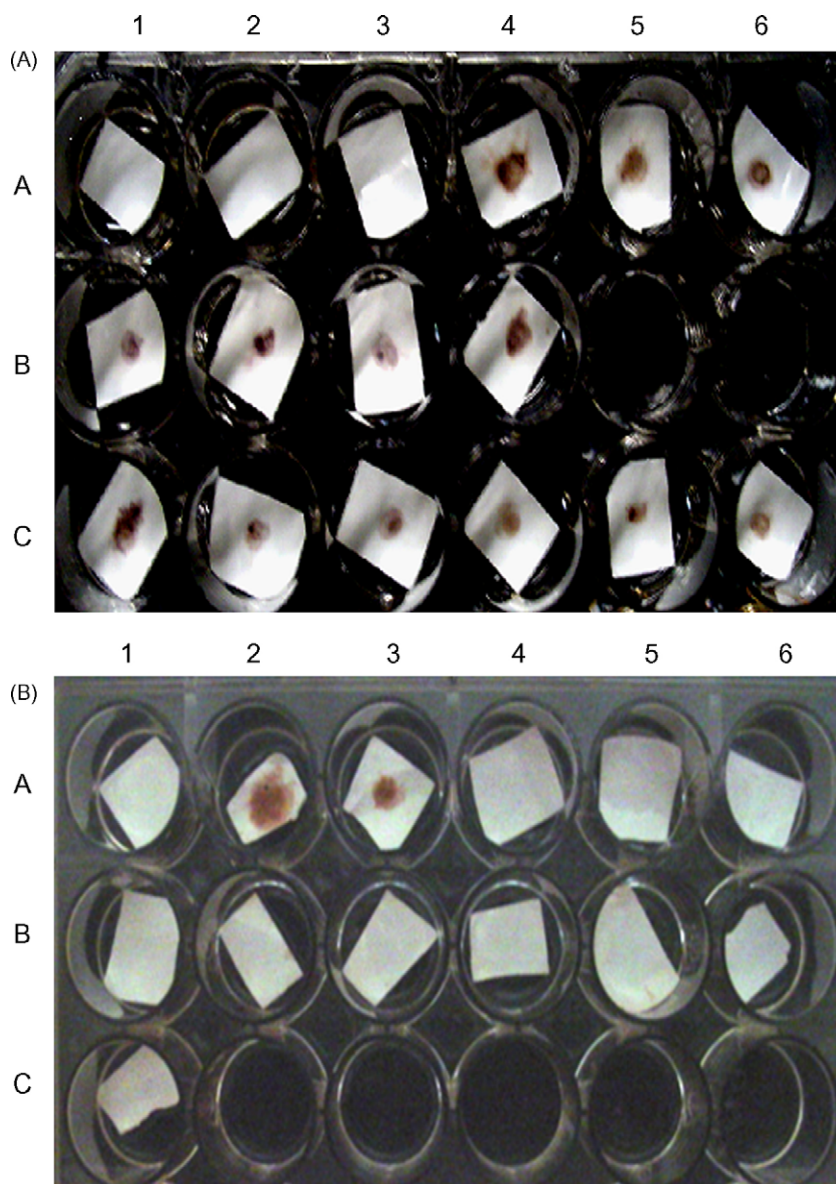


Fig. 5 – (A) Dot-blot assay using anti human IgE of peptides that induced rat mast cell dgranulation. 1a: Water (blank); 2a: 2S albumin without serum (negative control); 3a: chymotrypsin + serum (negative control); 4a: 2S albumin with serum (positive control); 5a: Ric c 1 with serum; 6a: Ric c 3 with serum; 1b: Ric c 1 small chain 1 with serum; 2b: Ric c 1 large chain with serum; 3b: Ric c 3 small chain with serum; 4b: Ric c 3 large chain with serum; 1c: P₀ with serum; 2c: P₁ with serum; 3c: P₂ with serum; 4c: P₃ with serum; 5c: P₄ with serum; 6c: P₅ with serum. (B) Dot-blot assay using anti human IgE of peptides that did not induce rat mast cell dgranulation, 1a: water (blank); 2a: 2S albumin with serum (positive control); 3a: P₄ with serum (positive control); 4a: 2S albumin without serum (negative control); 5a: P₆ with serum; 6a with serum: P₇ with serum; 1b: P₈ with serum; 2b: P₉ with serum; 3b: P₁₀ with serum; 4b: P₁₁ with serum; 5b: P₁₂ with serum; 6b: P₁₃ with serum; 1c: chymotrypsin + serum (negative control).

Table 2 – Percent of mast cell degranulation induced by *Ricinus communis* synthetic peptides

Peptide no	Amino acid sequences in the peptide	Mast cell degranulation (%)	Histamine release (%)
Control	–	33	2
2S albumin pool	–	70	64
P ₀	EGLRQAIEQQQSQGQ	74	80
P ₁	ESKGEREGSSSQCR	64	58
P ₂	QEVQRKDLSSCERYLRQSSS	70	64
P ₃	QEQQLNRQCQEYIKQ	65	58
P ₄	DECQCEAIKYIAEDQQGQ	67	60
P ₅	LHGEESERVAQRAGE	54	56
P ₆	QQQESQQLQCCNQVKQVR	23.8	<2
P ₇	AGEIVSSCGVRCMR	24.9	<2
P ₈	PSQQGCRGQIQEQQLNR	24.3	<2
P ₉	QCQEYIKQQVSGQGPRR	28.7	<2
P ₁₀	QERSLRGCCDHLK	22.8	<2
P ₁₁	QMMSQCRCEGLR	28.2	<2
P ₁₂	GQLQGQDVFEAFR	28.3	<2
P ₁₃	TAANLPSMCGVSPTECRF	27.8	<2

response. This peptide, part of the Ric c 1 large chain, was submitted to blast search (Fig. 6). The amino acid sequence alignment shows a high degree of homology with other 2S albumin allergenic fractions, reinforcing the possibility of involvement of these epitopes in cross reactions in patients allergic to several sources.

4. Discussion

The castor bean has become one of the common seed allergens in workers. Various studies performed over the last few years have elucidated the identity of several allergens in *R. communis* seeds, [18]; however, despite this interest, there are, to date, no reports on the characterization of the epitopes of these allergens.

In type-1 allergy, the allergic reaction is mediated by allergen-specific IgE. Cross-linking of the allergen by allergen-specific IgE bound to high affinity receptors on mast cells and basophiles leads to cell degranulation and the release of a variety of mediators of the allergic reaction, including histamine. The release of these mediators requires at least two binding sites on the surface of the allergen and antibodies specific for each of these epitopes [20]. Continuous epitopes

from several sources have been mapped using immunoblotting [1,19,23,26,30], however, in these experiments the cross-linking reactions were not investigated. We, herein, identify IgE binding epitopes from *R. communis* major allergens, using the mast cell degranulation assay that demonstrates this cross-linkage. This approach was also employed by Katayama et al. [16] to investigate the allergic conjunctivitis caused by cedar pollen.

Continuous epitopes are generally known to be resistant to heat, chemical denaturation and proteolysis. They tend to retain their allergenic reactivity, whereas many conformational epitopes of native proteins are modified or disrupted by heat, chemical treatments, or both, thus losing their IgE-binding potential [28]. Our study focused on identification of continuous IgE-binding epitopes, since both the small and large chain, isolated after the denaturation, reduction and alkylation process of isoallergens Ric c 1 and Ric c 3, unfolding molecules, demonstrate allergenic properties. Short overlapping peptides can be synthesized and then probed with serum samples from animals sensitized with these allergens. We have demonstrated, both by rat mast cell degranulation and by human IgE binding, at least two different IgE-recognition sites on Ric c 1 and four epitopes on Ric c 3. The identification of multiple epitopes on a single allergen is not a

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A 1   QERSLRGCCDHLKQMMSQCRCEEGLRQAIEQQQSQGQLQGQDVFEAFRTAA...
B 73   .RQHFRQCCQQLSQMEEQCCEGLRQAVRQQQEEGIRGEEMEEMVQCAS...
C 91   .RQHFRQCCQQLSQIEEQCCCEGLRQAVRRQQQQLRGEEMEEMVQSAR...
D 69   .RQHFRQCCQQLSQMDEQCCEGLRQVRRQQQQLRGEEMEEMVQSAR...
E 75   .EPHMECEQLEGMDESCRCEGLRRMMRMQQKEMQPRGEMRRMRLA...
F 72   .QQLLQCCQELQNIIEGQCCEAVKQVVRDAQRHEQQRPVFPQGSQSQ...
G 85   .EQSLRDCCQQLRNVDERCCEAIRQAVRQQQEGGYQEGSQQVYQRRAR...
H 94   .SQHQRCCNELNEFENNQRMCCEALQQIMENQSDRLQGRQQEQQFKREL...

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Fig. 6 – Alignment of amino terminal sequence of Ric c 1 large chain with 2S albumin allergenic proteins from several sources (numbers represent the initial position of alignment) A: Ric c 1 large chain. In this sequence, the P₀ peptide sequence is underlined; B: putative allergen from *Carya illinoensis*; C: allergen from *Juglans nigra*; D: allergen from *Juglans regia* Jug r 1; E: allergen from *Bertholletia excelsa* Ber e 1; F: 2S albumin from *Helianthus annuus*; G: (-globulin from *Sesamum indicum*; H: allergen from *Arachis hypogae* Ara h 2.

novel finding, since there are reports of multiple IgE-binding epitopes on allergens from many foods that cause immediate hypersensitivity reactions, as in allergens from cow's milk [28], hazel [9,29], and soybean [14].

The identification of these IgE epitopes may allow the induction of protective antibody responses to antagonize the IgE recognition of allergens by use of synthetic peptides that are directly derived from IgE epitopes of major castor bean allergens.

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