

Structural Studies on Allergen RC-13 from *Ricinus communis* L.: Isolation and Characterization of a Major Glycopeptide

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ABSTRACT

A glycoprotein, RC-13, isolated from *Ricinus communis* seeds was reduced, S-alkylated and cleaved by trypsin. The tryptic digest was fractionated by ion-exchange chromatography and a glycopeptide was isolated and purified by high-voltage paper electrophoresis. When submitted to amino acid and carbohydrate analyses this major glycopeptide showed the following chemical composition: Lys₁, Asp₁, Thr₂, Ser₄, Glu₁, Pro₂, Gly₂, Ala₂, Val₂, GlcN₆, Man₆ and Gal₈. Hydrazinolysis positioned Ser as the C-terminal residue. It is postulated that this glycopeptide belongs to the C-terminal region of the allergen.

Key words: Castor bean allergen, RC-13 glycopeptide characterization

I - INTRODUCTION

Well defined chemical structures of atopic allergens are useful in designing new approaches for immediate hypersensitivity immunotherapy. For example, it is known that the allergen reaction is triggered by cross-linking, promoted by the specific polyvalent allergen, of IgE antibodies fixed to mast cells. Inhibition of this reaction could be achieved by univalent, hapten-like components of the allergen which bind to the F_{ab} regions in the place of the multivalent allergen (Chakrabarty *et al.*, 1980). Haptens have also been conjugated with nonimmunogenic carriers such as the synthetic copolymer of D-glutamic and D-lysine, and this therapeutic modality tested in animal systems (Tse *et al.*, 1978). Establishment of

the chemical structure of determinants of allergenic molecules is important in both cases.

In previous paper we reported on the isolation and characterization of CB-1A, a complex mixture of low molecular weight allergenic glycoproteins, from castor bean seed (Trugo *et al.*, 1984). Fractionation of CB-1A by ion-exchange chromatography on SP-Sephadex led to the isolation of RC-13, and allergenic fraction, homogeneous by re-chromatography and isoelectric focusing (Trugo *et al.*, 1985). Characterization of RC-13 (Silva-Júnior *et al.*, 1987) showed it to be a glycoprotein rich in glutamic/glutamine (characteristic feature of seed storage proteins), poor in aromatic amino acids, acidic in nature (pI 4,2) and containing mannose, galactose and glucosamine. Results indicated RC-13 to consist of a single polypeptide chain.

Sharief and Li (1982) determined the complete amino acid sequence of another glutamine-rich storage protein isolated from the seeds of

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Ricinus communis. This protein was shown to possess two polypeptide chains linked by disulfide bond(s). The smaller chain is composed of 34 amino acids, with proline as NH₂-terminus and the larger chain contains 61 amino acids with a cyclized glutamine as the NH₂-terminal residue. The authors mention that this storage protein presents chemical and physicochemical characteristics in common with allergens and protease inhibitors of castor bean seeds. Comparing its amino acid composition to that of RC-13, some similarity is seen, principally as regards the number of glutamic/glutamines. However, differences appear in the number of residues of some other amino acids such as serine and phenylalanine. The latter is absent in RC-13 and presents three residues in the protein studied by Sharief and Li.

Thorpe *et al.*, (1988) fractionated castor bean proteins by SDS-PAGE. The 2S storage protein (Sharief and Li, 1982) so obtained were confirmed by Western blotting and radio allergen sorbent test to be the major allergenic fraction from the seeds of *Ricinus communis*.

An increasing number of atopic allergens has been found to contain carbohydrate. At present very little is known about the sugar structure of these allergenic glycoproteins. Monosaccharide composition is known only for a few, such as ryegrass pollen (Howlett *et al.*, 1981), Kentucky blue grass pollen (Ekramoddoullah *et al.*, 1980) and birch pollen (Vik *et al.*, 1982). No studies have yet been performed on the correlation between the carbohydrate moiety of the molecule and its allergenic properties.

The purpose of this paper was to initiate structural studies of allergen RC-13 through the isolation and characterization of a glycopeptide obtained by tryptic hydrolysis.

II - MATERIAL AND METHODS

ALLERGEN RC-13:

RC-13 was isolated from the group of *Ricinus communis* (Castor Bean) allergens, CB-1A, as described by Trugo *et al.*, 1985.

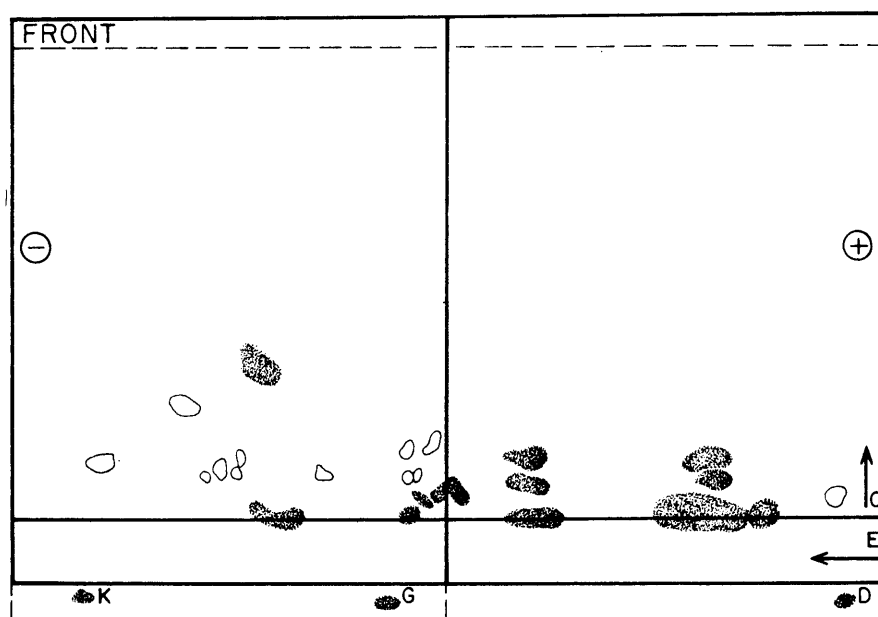


Fig. 1 — Peptide map of the 57-hours tryptic digest of reduced and S-carboxymethylated allergen RC-13. *First dimension*: High voltage electrophoresis run in 0.05 M pyridine acetate buffer, pH 6.35, 90 min. Current was maintained constant at 1 mA per centimeter of paper width (Whatman n° 3MM paper, 46.5 × 57 cm). *Second dimension*: Ascending paper chromatography using N-butanol/acetic acid/water (4:1:1 by volume), at room temperature for 15 hours. Ninhydrin stain. Dark - major peptides. K - Lysine; G - Glycine; D - Aspartic acid.

REDUCTION AND S-CARBOXYMETHYLATION:

300 mg of allergen RC-13 were reduced and S-alkylated employing essentially the same scaled-up methodology as described analytically (Silva-Júnior *et al.*, 1987).

ENZYMATIC HYDROLYSIS:

160 mg of reduced and S-carboxymethylated RC-13 (RCM-RC-13) were dissolved in 11.4 ml of distilled water and titrated to pH 8.0 using 0.1 M NH_4OH . Digestion was initiated by the addition of 0.65 ml of a 0.5% (w/v) trypsin (Worthington) solution in 10^{-3} M HCl and carried out in the cell of a Radiometer pH-Stat (Type TTT₁), under N_2 atmosphere, at 38°C. The pH 8.0 was maintained by automatic addition of 0.1 M NH_4OH . A second addition of the same enzyme solution (0.35 ml) was made 6.5 hours later. After a total of 24 hours

the hydrolyzate was lyophilized in order to have its complexity analyzed by finger-printing. The results obtained led to extension of digestion time to 57 h on a whole. The digest was lyophilized and analyzed again by analytical finger-printing (Bennett, 1967).

PEPTIDE PURIFICATION:

The 57-hour tryptic digest was fractionated by chromatography on a Dowex 50-X4-200 column (150×1.9 cm), equilibrated in 0.05 M pyridine-acetate buffer, pH 2.5. The sample was applied in 2 ml of this buffer, which initiated elution and was followed by pH and ionic strength gradients (Jones, 1970) established by passing through the column simple or gradually mixed pyridine-acetate buffers, in the following order: 0.05 M/pH 2.5 (880 ml); 0.2 M/pH 3.1 and 0.05 M/pH 2.5 (1,360 ml); 2.0 M/pH 5.0 and 0.125

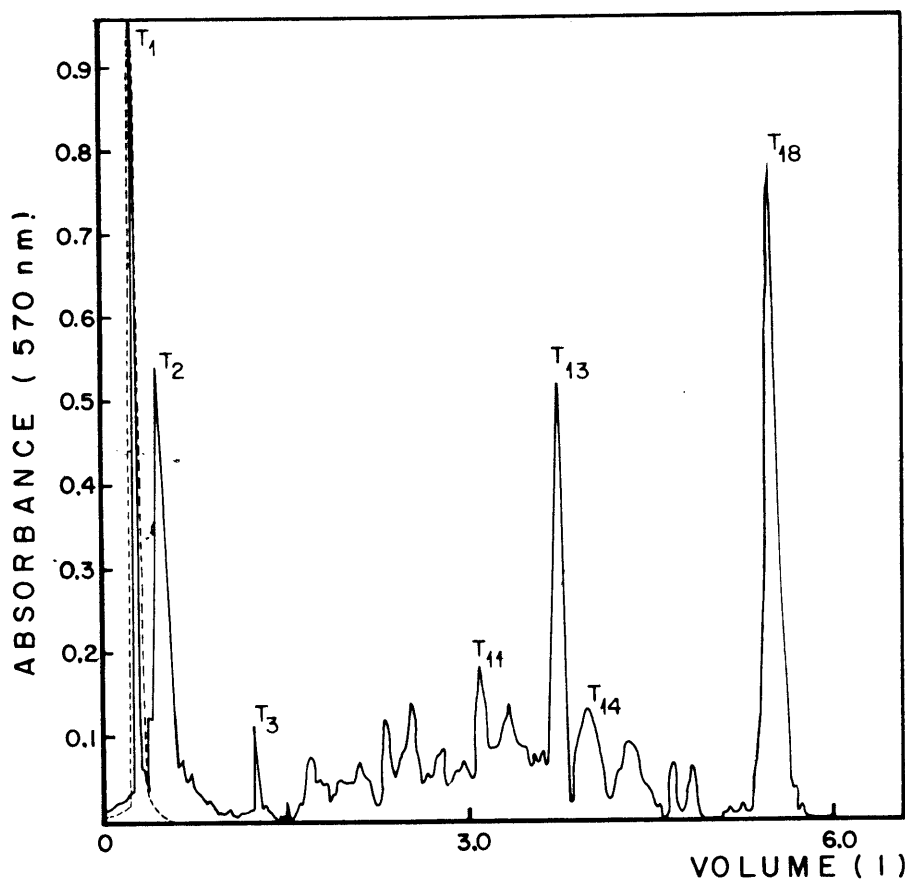


Fig. 2 — Profile of the 57 hours tryptic digest of reduced and S-carboxymethylated allergen RC-13 on Dowex 50 X-4 cation exchanger. The column (150×1.9 cm) was maintained at 40°C. Elution was performed with ionic strength and pH gradients (see Material and Methods). Flow rate was 45 ml/h. Peptide and carbohydrate identified by the ninhydrin reaction (—) and phenol - sulphuric method (----).

M/pH 2.8 (5,250 ml); 2.0 M/pH 5.0 (1,000 ml); 8.0 M/pH 5.6 and 0.2 M/pH 5.0 (1,400 ml); 8.0 M/pH 5.6 (1,090 ml) and 0.2 M NaOH (2,550 ml) were used. 3 ml were collected per tube. Ninhydrin reaction after alkaline hydrolysis (Moore and Stein, 1954) identified the tubes containing peptides. Phenol sulphuric method (Dubois *et al.*, 1956) was employed to search for glycopeptides. Fractions were pooled and lyophilized. Carbohydrate fraction T₁ peptide complexity was examined by analytical finger-printing.

T₁ was further purified by using high-voltage paper electrophoresis, run at pH 6.35 (0.05 M pyridine acetate buffer), for 180 minutes, current being kept constant at 1 mA per centimeter of paper width. Peptides were located on guide strips by ninhydrin reaction. Fractions were eluted using 0.1 M pyridine solution.

AMINO ACIDS ANALYSES:

Amino acid analyses of glycopeptide T₁E₃ was performed essentially as described for its originating allergen RC-13 (Silva-Júnior *et al.*, 1987). Times of hydrolyses were 18 and 36 hours.

CARBOHYDRATE ANALYSES:

Carbohydrate analyses of T₁E₂ and T₁E₃ were also carried out as for RC-13 (Silva-Júnior *et al.*, 1987). Phenol-sulphuric, gas-liquid chromatography (Sawardeker *et al.*, 1965), Elson-Morgan reaction (Belcher *et al.*, 1954) and hexosamine analysis using the amino acid analyser (Fanger and Smith, 1970), were the methods employed.

C-TERMINAL AMINO ACID ANALYSES:

C-terminals of reduced and alkylated allergen RC-13 (3.3 mg) and component glycopeptide (1.4 mg) were determined by hydrazinolysis (Fraenkel-Conrat and Tsung, 1967). The samples were treated with 0.4 ml of hydrazine at 100°C, for 6 hours, in vacuum-sealed tubes. The hydrazinolyzates were immediately freeze-dried, dissolved in 0.25 M NH₄OH and passed through Permutit-H 70 columns (10 × 1 cm) employing the same solution, at a flow-rate of 12 ml/h. Twenty-five ml of eluate were collected from each column and lyophilized. The materials were then redissolved in 1.4 ml (RCM-RC-13) and 0.7 ml

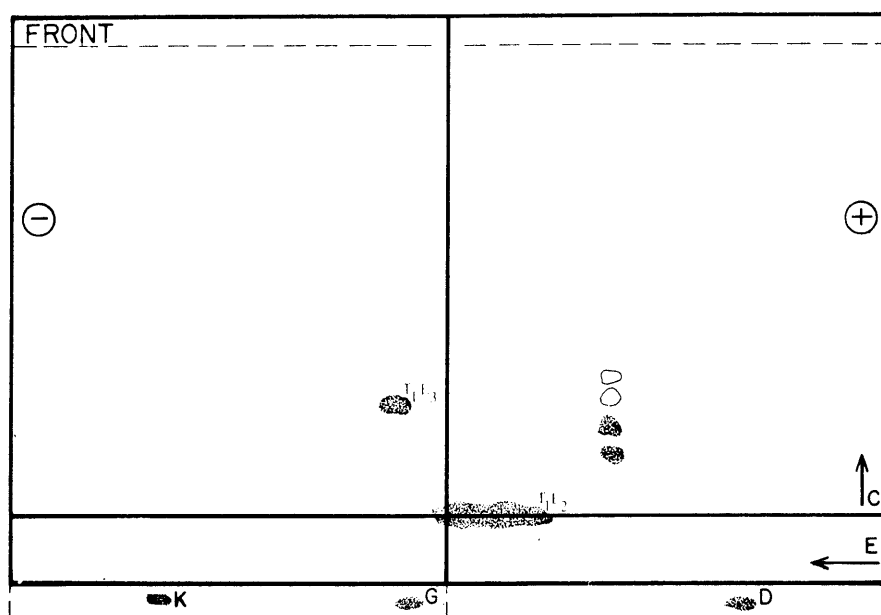


Fig. 3 — Peptide mapping of carbohydrate - containing T₁. First dimension: High voltage electrophoresis run in 0.05 M pyridine acetate buffer, pH 6.35, 90 min. Current was maintained constant at 1 mA per centimeter of paper width (Whatman n° 3MM paper, 46.5 × 57 cm). Second dimension: Ascending paper chromatography using N-butanol/acetic acid/water (4:1:1 by volume), at room temperature for 15 hours. Ninhydrin stain.

Dark - major peptides. K - Lysine; G - Glycine; D - Aspartic acid.

(glycopeptide) of 0.2 M citrate buffer pH 2.2 and submitted to analyses in an 121 Beckman amino acid analyzer.

III - RESULTS AND DISCUSSION

Trypsin was chosen for digestion as a result of experimental procedures reported previously (Silva-Júnior *et al.*, 1987). According to the amino acid composition of allergen RC-13, a maximum of 11 peptides would be expected after tryptic hydrolysis, assuming integral splitting after lysine and arginine residues and provided there existed

no restrictions for enzyme action. Since the 24 hours map exhibited, among other significant positive ninhydrin spots, a strong band which dragged to the anode, suggesting the presence for protein which had not been totally cleaved by trypsin, hence hydrolysis was continued. Analysis of this 57-hours hydrolyzate showed no dragging and the presence of 13 strong spots, as approximately expected (Fig. 1).

Cation-exchange chromatography on Dowex 50-X4, separated tryptic peptides into 17 major

TABLE I
Amino Acid and Hexosamine Compositions of T₁E₃, RC-13 and CB-1A

Amino Acid	Nanomoles		Molar ratio ⁽¹⁾	T ₁ E ₃	RC-13 ⁽³⁾	CB-1A ⁽⁴⁾
	18 h	36 h				
GlcN	9.89	4.92	6.19 ⁽²⁾	6	—	—
Lys	0.59	3.08	1.29	1	3	4
His	—	—	—	—	1	1
Arg	—	—	—	—	7	11
Asp	1.29	1.49	0.62	1	6	3
Thr	3.49	5.03	2.10	2	2	1
Ser	6.25	8.53	3.57	4	11	7
Glu	2.28	2.39	(1.00)	1	29	26
Pro	3.83	4.18	1.75	2	2	2
Gly	4.26	6.18	2.59	2	7	5
Ala	3.22	4.68	1.96	2	4	3
Cys	—	0.34	0.14	—	6	6
Val	2.51	2.77	1.16	1	5	3
Met	—	—	—	—	1	1
Ile	—	—	—	—	3	3
Leu	—	0.21	0.09	—	3	4
Tyr	—	—	—	—	1	1
Phe	—	—	—	—	—	1

⁽¹⁾ Based on results obtained after 36 hours hydrolysis assuming Glu as one residue.

⁽²⁾ Corrected for acid destruction, assuming zero time hydrolysis.

⁽³⁾ Silva-Júnior *et al.*, 1987.

⁽⁴⁾ Trugo *et al.*, 1984.

fractions. Only fraction T₁ presented positive results for the phenol-sulphuric reaction (Fig. 2).

Analytical peptide mapping of carbohydrate containing T₁ is shown in Figure 3. Purified by high voltage paper electrophoresis T₁ gave rise to three fractions, two of which - T₁E₂ and T₁E₃ -

Table I presents the results of the amino acid and hexosamine analysis of T₁E₃ together with CB-1A (Trugo *et al.*, 1984) and RC-13 (Silva-Júnior *et al.*, 1987) compositions. The first two columns represent nanomoles of amino acids and glucosamine obtained after 18 and 36-hours

TABLE II
Amino Acid Analyses of RCM-RC-13 and T₁E₃ Hydrazinolyzates

Amino Acid	Nanomoles		Molar ratio ⁽²⁾	Assumed
	Without correction	Corrected ⁽¹⁾		
RCM-RC-13				
Lys/Orn	5.87	9.77	0.34	—
His	3.84	6.78	0.24	—
Asp	1.37	3.49	0.12	—
Ser	18.39	28.69	1.00	1
Glu	2.29	5.36	0.19	—
Gly	4.62	7.22	0.25	—
T1E3				
Lys/Orn	0.36	0.60	0.20	—
Asp	0.46	1.17	0.38	—
Ser	1.74	2.71	0.89	1
Glu	0.15	0.35	0.11	—
Gly	1.95	3.05	1.00	1

⁽¹⁾ According to Zaganelli Filho, G. L., M. Sc. Thesis, Dept. Biochemistry, Institute of Chemistry, UFRJ, 1974, Rio de Janeiro, Brasil.

⁽²⁾ Assuming the highest corrected nanomole as 1.00.

were carbohydrate positive.

Analyses of T₁E₂ and T₁E₃ by the Dubois' method revealed, respectively, 31% and 47% of hexoses. Gas-liquid chromatography showed the presence of galactose and mannose, the minimum composition being Man₁ Gal_{3,30} for T₁E₂ and Man₁ Gal_{1,33} for T₁E₃. Since T₁E₃ was the major glycopeptide it was selected for proteic studies.

hydrolyses. Molar ratios calculated assuming glutamic acid as one residue are presented in the next column. Glucosamine content was corrected for acid destruction. The minimum molecular mass calculated from the summation of the masses derived from the assumed integer values is 2,708 Da.

Analysis of T₁E₃ by the Dubois' method revealed 47% of hexoses whereas the gas-liquid

chromatography showed the presence of galactose and mannose, its minimum composition being $\text{Man}_1:\text{Gal}_{1.33}$, to a calculated total hexose mass of 2,401 Da. This gives a nearest ratio of $\text{Man}_6:\text{Gal}_8$.

Since T_1E_3 contains the only two threonine residues found in RC-13, its actual chemical composition is: $\text{Lys}_1, \text{Asp}_1, \text{Thr}_2, \text{Ser}_4, \text{Glu}_1, \text{Pro}_2, \text{Gly}_2, \text{Ala}_2, \text{Val}_2, \text{GlcN}_6, \text{Man}_6, \text{Gal}_8$.

Many glycoproteins are known to have pentasaccharide core composed of $\text{GlcNAc}_2\text{Man}_3$. In their chemical structure the chytobiose residue is found in the polypeptide chain asparagine by its reductor terminal and to one of the three mannosyl residues by its non-reductor terminal. Accordingly, T_1E_3 has an Asx residue which could be an anchor point for the carbohydrate core. This Asn-GlcNAc linkage is found, for example, in glycoproteins of the vegetal kingdom, e.g., soybean agglutinin and bromelain (Kornfeld and Kornfeld, 1976).

Table II lists the analytical data obtained for the free amino acids purified from RCM-RC-13 and T_1E_3 hydrazinolyzates. In spite of lysine being present in T_1E_3 glycopeptide, this residue was not recovered in significant amounts in the hydrazinolysis experiment. Since T_1E_3 has two proline residues, a possible explanation for the fact that lysine was not experimentally defined in the C-terminal amino acid could be the presence of a Lys-Pro bond which is known to be not-susceptible to trypsin cleavage (Kasper, 1975). Serine appears to be the C-terminal residue of RC-13 whereas for the glycopeptide results point to glycine and serine. Since glycine is a common contaminator acquired during paper electrophoresis purification procedure this leaves serine as the most probable glycopeptide C-terminal residue.

According to these assumptions and considering that T_1E_3 originated from a tryptic hydrolysis it can be postulated that this glycopeptide belongs to the C-terminal region of the RC-13 allergen.

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