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Immunochemical characterization of *Glycine max* L. Merr. var Raiden, as a possible hypoallergenic substitute for cow's milk-allergic patients

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Clinical and Experimental Allergy

Summary

Background Cows' milk allergy (CMA) is the most common cause of food allergy in infancy. The only proven treatment is the complete elimination of cows' milk proteins (CMPs) from the diet by means of hypoallergenic formulas. Soybean-based formulae are widely used although intolerance to soy has been reported to occur in 15–40% of infants with CMA.

Objective The aim of this work was to analyse the *in vitro* reactivity of the soybean cultivar Raiden, which naturally lacks glycinin A₄A₅B₃, to evaluate whether this genotype could be a safe CMP substitute for CMA patients.

Methods The reactivity of conventional soybean (CS) and Raiden soybean (RS) genotypes and also recombinant glycinin A₄A₅B₃ and $\alpha\beta$ -conglycinin with casein-specific monoclonal antibodies and CMP-specific polyclonal serum was evaluated by immunoblotting and ELISA. A sequential competitive ELISA with the polyclonal antiserum and different soluble inhibitors was performed. In addition, an indirect ELISA with sera of atopic children with CMA was carried out to analyse the IgE-binding capacity of the different soybean components.

Results We have shown that CS contains four components that cross-react with CMP, while RS has only one. The remaining cross-reactive component in RS was identified as α -subunit β -conglycinin. By means of inhibitory ELISA, we demonstrated that CS, RS and the α -subunit β -conglycinin extracts inhibited the binding of CMP-specific antibodies to the CMP-coated solid phase. Finally, we showed that CS, RS and the recombinant proteins were recognized by human CMP-specific IgE antibodies.

Conclusion This work shows that although Raiden has fewer cross-reactive components than conventional soybean, it still has a residual cross-reactive component: the α -subunit β -conglycinin. This reactivity might make this genotype unsuitable to treat CMA and also explains adverse reactions to soybean in CMA infants.

Keywords caseins, cow's milk, cross-reactivity, food allergy, Raiden, soy beta-conglycinin, soy glycinin

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Introduction

Allergies are the most prevalent immune pathologies worldwide. In the last decades, their incidence and severity of symptoms have increased globally, in industrialized and developing countries [1–3]. Among them, the prevalence of adverse reactions to foods varies from 1.5% in adults compared with up to 8% in children younger than 3 years old [4–8]. Cows' milk allergy (CMA) is the most common cause of food allergy in infants and children, affecting approximately 2–3% [9, 10]. The only proven

treatment for CMA is the complete elimination of cows' milk proteins (CMP) from the infant's diet by means of hypoallergenic feeding formulas [11]. Hydrolysed CMP (HP), prepared from different fractions such as whole milk, whey proteins or caseins, extensively hydrolysed (eHP), and soy-based formulas (SF) are the most widely used substitutes for allergic patients [11–13]. Residual allergenic activity has been described in pHP, eHP [12, 14–16] and in SF [14, 16, 17]. Despite sequence and structural similarities between the main allergenic components in milk from different animal species, they have been

indicated for infant feeding. Cross-reactivity and, hence, allergenicity has been reported [18].

CMA has been classified as IgE-associated or non-IgE-associated reactions. The most problematic reactions are those mediated by IgE antibodies (immediate hypersensitivity) mainly in infancy. As quoted by Zeiger *et al.* [19], the prevalence of soy intolerance in CMA has ranged between 0% and 63%, including both IgE- and non-IgE-associated CMA patients. Nevertheless, a large cohort size study performed with IgE-associated CMA patients in the United States showed that only 14% of these patients had soy intolerance, suggesting that soy feeding may be a safe substitute [19]. A similar study, performed also for IgE-mediated CMA in Israel, showed 17% cross-sensitization to soybean [20]. However, in Korea, the prevalence of sensitization to soy in CMA infants decreased with age: 36.8% in the first year of life, 16.4% in the second year and 13.7% in the third year [17]. These results suggest that SF should be carefully used as a substitute for CMP in CMA patients, especially during infancy [17]. If there is no sensitization to soy proteins, the clinical manifestations induced by SF in CMA patients can only be explained by the immune recognition of cross-reactive allergens. Two (or more) allergens are cross-reactive if IgE antibodies or a T cell receptor react with both [21]. The identification of high-affinity IgE antibodies and the cross-reactive IgE epitopes in phylogenetically unrelated allergens may constitute the basis to understand multiple food allergies [22]. Soy belongs to the group of the eight most frequent human food allergens, and 16 allergens have been characterized [23]. Our group has identified the glycinin A₄A₅B₃ as the first soybean protein that shares IgE epitopes with bovine caseins [16]. Further studies of the soybean components recognized by CMP-specific antibodies will allow us to design adequate hypoallergenic soy formulas for CMA patients. In addition, this knowledge will constitute a useful tool to develop novel therapeutic strategies.

In this work, the soybean cultivar Raiden was immunologically studied by immunoblotting and ELISA. Despite the lack of the glycinin A₄A₅B₃, we showed that this genotype contains a residual cross-reactivity with CMP due to the α -subunit β -conglycinin, which is one of the major soybean allergens [24]. This suggests that Raiden soybean (RS) should be carefully considered as a CMP substitute in CMA patients.

Material and methods

Soybean protein extracts

Crude extracts of seed storage proteins were obtained from two *Glycine max* L. Merr. genotypes: Asgrow [named in this work as 'conventional soybean' (CS)] and Raiden. The seeds were crushed and extracted with 0.01 N NaHCO₃

at 90 °C. The extract was homogenized with an Ultraturrax UT 20 000, and centrifuged at 2500 *g* for 20 min at room temperature. Lipids were extracted with chloroform overnight at 4 °C. The extract was dialysed against distilled water and stored at -20 °C until used. Commercial soybean defatted flour (Bunge, São Paulo, Brazil) was also used to prepare CS extracts. The total protein content was quantified with the Bicinchoninic acid kit (Pierce, Rockford, IL, USA), using bovine serum albumin as a standard.

Casein monoclonal, cows' milk proteins and soy β -conglycinin-specific polyclonal antibodies

Three monoclonal antibodies with high specificity for α -casein (1D5-I), β -casein (2A1-I) and κ -casein (3B5-I), and a CMP-specific rabbit polyclonal antiserum were used in this study and were described previously [15]. Preparation and characterization of the α -subunit β -conglycinin-specific antibodies were reported previously [25]. Antibody titres were determined by indirect ELISA for each antigen.

Patient sera used for *in vitro* characterization of the allergen content

Human serum specimens obtained from 15 atopic children (ages ranking from 3 months to 10 years, mean age: 4.5 years) diagnosed as IgE-mediated CMA patients were used. Patients showed clinical pictures of immediate reactions associated with ingestion of cows' milk (CM; vomiting, diarrhoea, dermatitis, bronchospasm, urticaria and rhinorrhoea). We previously detected CMP-specific IgE antibodies by immunoblotting, ELISA and EAST, and, in some cases, skin prick tests performed with CMP and common environmental allergens were positive. Sera from 20 subjects (age- and sex-matched) with no history of food allergy and no clinical manifestations after ingestion of CM were included as controls.

cDNA cloning and constructs for expression of A₄A₅B₃ in *Escherichia coli*

The glycinin A₄A₅B₃ globulin gene (GenBank accession number X86970) was amplified from a cDNA library prepared from developing soybean (*G. max* L. Merr.) seeds [25]. We used the oligonucleotides F-A₄A₅B₃-27: cccgtagcatgaagctcaacgagtgc, *NheI* site underlined, and R-A₄A₅B₃: cctggatccttatgcgactttaacacgg, *BamHI* site underlined, designed to amplify the gene encoding to A₄A₅B₃ without the sequence encoding for the signal peptide (27 amino acids), and to fuse it to a six-histidine tag at the N terminus. The PCR product was cut with *NheI* and *BamHI* and inserted into the plasmid pET28a (Novagen, Madison, WI, USA) to obtain pETA₄A₅B₃. The construct was verified by sequencing analysis.

*Production of A₄A₅B₃ proglobulin and α -subunit β -conglycinin in *E. coli**

pETA₄A₅B₃ and pET-m α [25] were transformed into *E. coli* strain BL21(DE3)pLysS for protein expression. Production and purification of the recombinant proteins are described elsewhere [25].

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and immunoblot analysis

SDS-PAGE was performed according to Laemmli [26] on discontinuous 12.5% acrylamide gels either with or without 5 M urea (Bio-Rad Laboratories, Hercules, CA, USA). Samples were prepared in sample buffer with or without 5% β -mercaptoethanol (reduced or non-reduced conditions, respectively). Total protein load was 10–20 μ g per lane. After electrophoresis, gels were fixed and stained with Coomassie Brilliant Blue R 250 or wet-blotted onto nitrocellulose membranes (Schleicher & Schuell Bioscience, Keene, NH, USA) according to Towbin [27]. Blots were blocked in TBST (TBS-0.1% Tween 20) and incubated with the primary antibody (either mouse monoclonal antibodies or rabbit polyclonal antiserum) for 1 h at 37 °C, followed by the appropriate conjugated secondary antibody: biotinylated goat serum specific to mouse immunoglobulin G (Chemicon Int., Temecula, CA, USA) or biotin-conjugated RG-16 monoclonal antibody specific to rabbit immunoglobulins (Sigma-Aldrich, St Louis, MO, USA)/alkaline phosphatase-conjugated avidin (Sigma-Aldrich), and HRP-conjugated serum specific to mouse immunoglobulins (Bio-Rad Laboratories). Protein bands were visualized with either 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (5-BCIP/NBT) (Biokit, S.A., Barcelona, Spain) or a luminol chemiluminescent substrate and exposed to an X-ray film (Kodak, New Haven, CT, USA).

Sequential competitive enzyme-linked immunosorbent assay

Polystyrene microtitre plates (NUNC, Maxisorp, Denmark) were coated with 1 μ g/well of CMP and then blocked with blocking buffer containing 5% horse serum in PBS pH 7.4 for 2 h at 37 °C. CMP-specific rabbit polyclonal antiserum (1:200 000 diluted in 3% horse serum in PBS) was incubated separately for 2 h at 37 °C with different dilutions of several inhibitors (CMP, CS, RS, recombinant A₄A₅B₃ and α -subunit). One hundred microlitres of each pre-mixed dilution were then incubated in the CMP-coated wells for 30 min at 37 °C, followed by the secondary antibody diluted 1:4000 (HRP-conjugated anti-rabbit IgG; Santa Cruz, Santa Cruz, CA, USA) for 1 h at 37 °C. Colour was developed with *o*-phenylenediamine and 30% H₂O₂ in 0.1 M citrate-phosphate buffer pH 5.0, and OD₄₉₀

was measured in an ELISA reader (Sirio S SAECS, Radim Company, Buenos Aires, Argentina).

Indirect enzyme-linked immunosorbent assay for immunoglobulin E reactivity

Polystyrene microtitre plates (NUNC) were coated with 1.25 μ g/well of CMP or 0.05 μ g/well of each soy protein extract (CS, RS, recombinant A₄A₅B₃ or α -subunit β -conglycinin). After blocking as described before, 100 μ L/well of each patient serum was added against each antigen overnight at 4 °C. Alkaline phosphatase-conjugated monoclonal anti-human IgE (Sigma-Aldrich) was used as a secondary antibody for 2 h at 37 °C. Colour was developed with *p*-nitrophenylphosphate in buffer pH 9.6, and OD₄₀₅ was measured in an ELISA reader (Sirio S SAECS). Cut-off values were obtained statistically (SEM + 2 SD) with control sera.

Results

Expression and immunoreactivity of the recombinant A₄A₅B₃ soy glycinin

A soybean 30-kDa component was previously identified as a cross-reactive protein with α -, β - and κ -casein-specific monoclonal antibodies and a CMP-specific polyclonal antiserum. This fraction was sequenced and identified as the A₅-B₃ glycinin (11S globulin) [16]. This soybean component was then cloned and expressed in *E. coli* to obtain the recombinant protein. As *E. coli* is unable to perform the post-translational modifications that occur in developing soybean seeds, the recombinant glycinin A₄A₅B₃ was obtained in a non-processed 61.2-kDa form, known as proglobulin (Fig. 1a shows the A₄A₅B₃ structure). Pro-glycinin A₄A₅B₃ was successfully expressed in *E. coli* (Fig. 1b) and its reactivity with the three casein-specific monoclonal antibodies was confirmed by immunoblotting (Fig. 1c).

Comparison of immunoreactivity between different soybean protein extracts

CS and RS extracts were run under different electrophoretic conditions to distinguish the protein patterns (Fig. 2). In the presence of 5 M urea and under non-reduced conditions, both extracts showed almost identical profiles (Fig. 2a). In contrast, the absence of the A₄ polypeptide was observed in RS when the separation was performed with urea and under the reduced condition (Fig. 2a). The exclusion of urea and β -mercaptoethanol allowed the detection of a 31-kDa band only in CS (Fig. 2b). In contrast, under reducing conditions, an indistinguishable electrophoretic pattern was obtained for CS and RS (Fig. 2b, R).

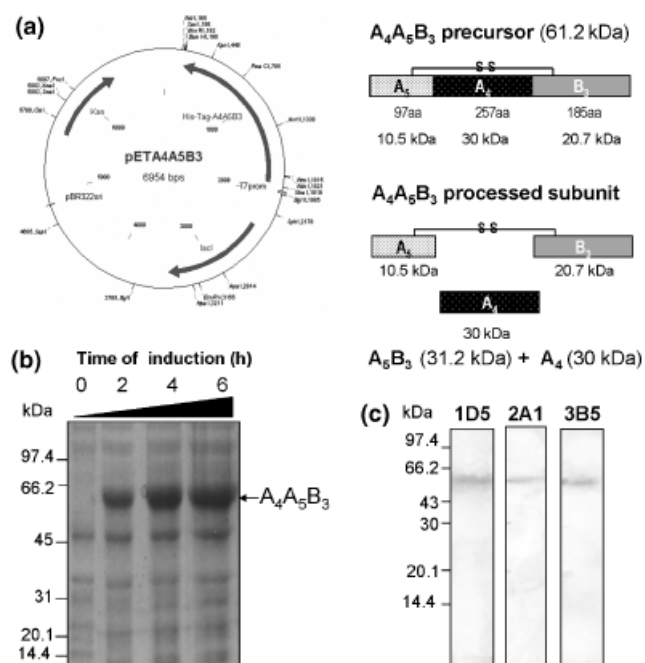


Fig. 1. Expression and immunoreactivity of A₄A₅B₃. (a) A₄A₅B₃-subunit expression vector. The cDNA without the signal peptide (27 amino acid residues) was fused to a six-histidine sequence, thrombin site and T7 tag at its 5' end. (b) SDS-PAGE, 10% under reducing conditions of total cell extracts of *E. coli* BL21 containing pET A₄A₅B₃ before (lane 0) and 2, 4 and 6 h after induction with IPTG (lanes 2, 4 and 6, respectively). (c) Immunoblotting analysis of purified A₄A₅B₃ recombinant protein with mouse monoclonal antibodies raised against α-casein (1D5), β-casein (2A1) and κ-casein (3B5). Molecular masses are given on the left in kDa. The arrow indicates A₄A₅B₃-subunit (proglobulin). Molecular masses are given on the left in kDa.

Cross-reactivity between CS and RS extracts with CMP was analysed by immunoblotting, excluding urea and β-mercaptoethanol in the electrophoretic running. Monoclonal antibodies specific to caseins and a polyclonal serum specific to CMP were used. The β-casein-specific monoclonal antibody (2A1) recognized four proteins (18, 31, 55 and 70 kDa) in the CS extract and one component of 70 kDa in the RS extract (Fig. 3). Similar results were obtained for the α-casein-specific monoclonal antibody (1D5) (Fig. 3). In contrast, the κ-casein-specific monoclonal antibody (3B5) and the CMP-specific polyclonal antiserum recognized mainly the 18-kDa component in the CS extract.

When CS and RS extracts were developed by immunoblotting using a polyclonal antiserum specific for soybean α and α' subunits of β-conglycinin [25], two bands in the region of 70 kDa were detected (Fig. 3). The 70-kDa band (lower band) corresponding to the α-subunit of β-conglycinin was identical to the one recognized by the casein-specific monoclonal antibodies.

As depicted in Fig. 4, the inhibition ELISA showed that CS, RS and alpha extracts could inhibit the binding of CMP-specific antibodies to CMP attached to the solid phase. The highest amount of soluble inhibitor to achieve 50% of binding inhibition corresponded to approximately 10 mg/mL of RS extract. The stronger inhibition of CS as compared with RS may be due to A₄A₅B₃. This experiment also showed that the α-subunit of β-conglycinin specifically inhibited the binding of the antibodies to CMP. Furthermore, a 50-fold increase in the concentration of RS was necessary to achieve the same inhibition as compared with the α-subunit of β-conglycinin concentration (0.2 mg/mL).

Finally, we characterized the IgE-binding capacity of the recombinant A₄A₅B₃ and soybean protein extracts by

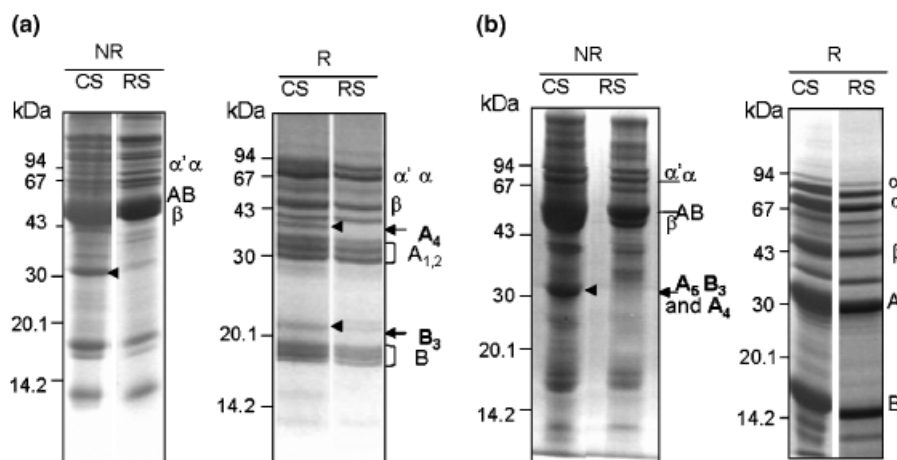


Fig. 2. SDS-PAGE analysis of conventional and Raiden soybean protein extracts. Gels were prepared and run with 5 M urea (a) or without 5 M urea (b). NR and R indicate non-reduced and reduced conditions, respectively (with 5% of β-mercaptoethanol). α', α and β indicate subunits of β-conglycinin (7S globulin). AB, A and B indicate AB-subunit, acidic and basic polypeptides of glycinin (11S globulin), respectively. Subunit composition differences between both varieties are shown with arrows. Molecular masses are given on the left in kDa.

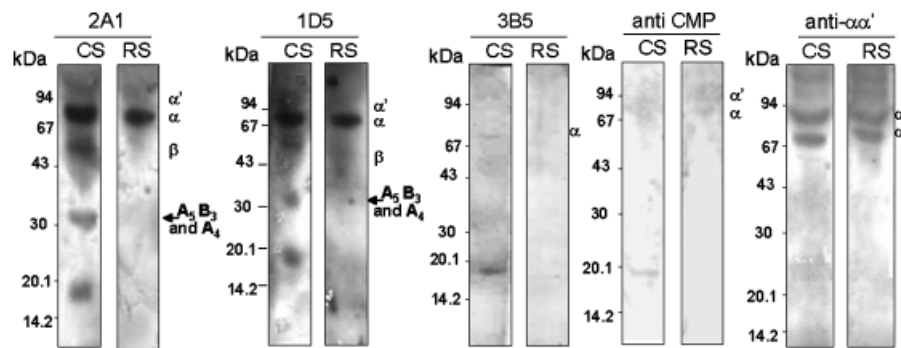


Fig. 3. Immunoblotting of conventional and Raiden soybean protein extracts. Conventional soybean (CS) and Raiden soybean (RS) extracts were developed by immunoblotting with mouse monoclonal antibodies raised against β -casein (2A1), α -casein (1D5), κ -casein (3B5), a rabbit polyclonal antiserum specific for cows' milk proteins (CMP) and with a mouse polyclonal antiserum raised against α , α' subunits of β -conglycinin. Protein load: 10–20 μ g/lane.

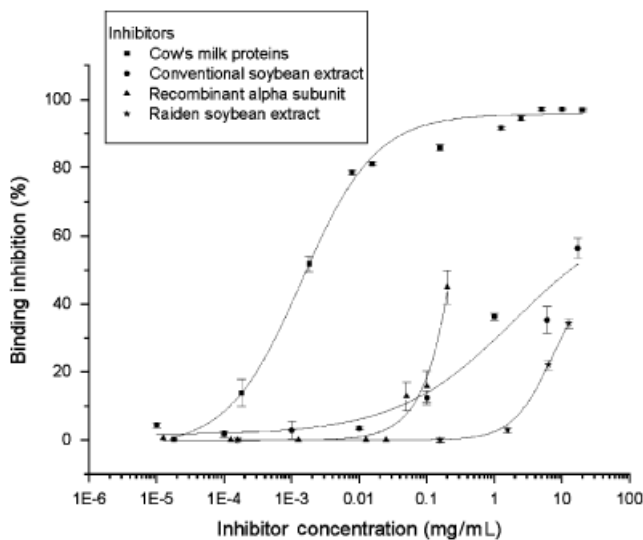


Fig. 4. Sequential competitive ELISA with rabbit polyclonal antiserum specific for cows' milk proteins (CMP). Polystyrene microtitre plates were coated with 1 μ g/well of CMP and incubated with a pre-mixed solution of 1/200 000 rabbit antiserum and different soluble inhibitors (CMP, CS, RS and α -subunit β -conglycinin) at different concentrations.

indirect ELISA (Fig. 5). CMP-specific serum IgE antibodies were bound to components present in CS, RS and to the recombinant $A_4A_5B_3$ and α -subunit of β -conglycinin. All the sera tested recognized components present in RS (15/15), while 93.3% of the sera were bound by CS and $A_4A_5B_3$ (14/15) and 86.7% (13/15) by the α -subunit of β -conglycinin. Only one serum with a low level of CMP-specific antibodies (class 1) did not recognize any of the soy components.

Discussion

In this work, we clearly show that Raiden soybean lacking the component $A_4A_5B_3$, previously described by our group as a cross-reactive component between soy and CM, has a residual immunochemical cross-reactivity due

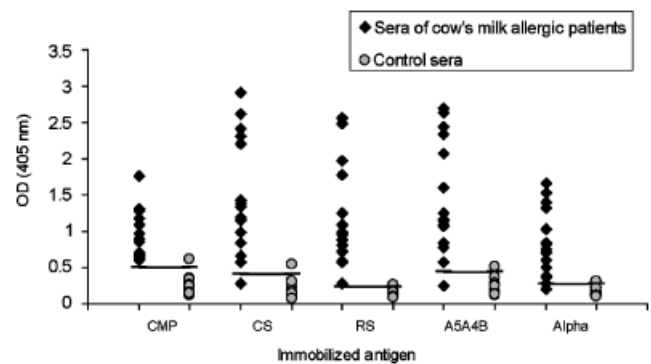


Fig. 5. Indirect ELISA with human sera containing CMP-specific IgE antibodies. Polystyrene microtitre plates were coated with cows' milk proteins (CMP; 1.25 μ g/well) or different soy extracts (CS, RS, $A_4A_5B_3$ and α -subunit β -conglycinin) (0.05 μ g/well) and incubated with undiluted human sera overnight at 4 °C. Alkaline phosphatase-conjugated monoclonal anti-human IgE 1/2000 was used as secondary antibody. One hundred percent of the positive sera recognized the Raiden soybean (RS) extract, while 93.3% (14/15) recognized CS and $A_4A_5B_3$ and 86.7% (13/15) recognized the α -subunit.

to a 7S globulin (α -subunit β -conglycinin). We showed that CS, RS and α -subunit β -conglycinin are recognized specifically by CMP-specific antibodies. As inhibition curves corresponding to CS and RS are not parallel, different populations of antibodies, with different specificities and affinities, may be involved in this cross-reactivity.

Our results indicate that although the RS extract contains fewer cross-reactive components than CS, it still has the 70-kDa protein that is strongly recognized by two casein-specific monoclonal antibodies. The finding that casein-specific monoclonal antibodies raised against different fractions recognized the same soybean component agrees with the fact that caseins have both distinct and common epitopes [28], and that different epitopes may be involved in this cross-reactive recognition. There is no information in the literature explaining such a case. In addition, a major number of soybean cross-reactive proteins were recognized by the α -casein- and

β -casein-specific monoclonal antibodies. These caseins are the most abundant fractions in CM and the main target of human-specific IgE, as they are not secreted by human mammary glands [28]. On the other hand, the 70-kDa soy component, presumably the α -subunit β -conglycinin, is one of the soybean major allergens [23, 24]. The α' -subunit of β -conglycinin, which shares a 90% homology with the α -subunit, was not recognized by the casein-specific monoclonal antibodies.

Soy formulas are one of the most widely used formulas for feeding infants with CMA as they are palatable and less expensive than CMP-based formula. The risk for concomitant soy allergy is between 15% and 40% of infants depending on age, genetic and/or environmental factors [17, 19, 29, 30]. Although soybean is also an important food allergen, the protein components responsible for adverse reactions in CMA patients have not yet been studied. The fact that components of the 7S and 11S globulin family and caseins are recognized by different immunoglobulins is somehow unexpected. Caseins and soybean storage proteins are not evolutionary relatives and this is the first report to demonstrate cross-reactivity between 7S (vicilins) and 11S (legumins) globulin families. These two families do not have more than five identical contiguous amino acid residues, although their three-dimensional structure is very similar [31, 32]. Based on this three-dimensional structure they are commonly called cupins. The cupin superfamily includes allergenic seed storage components belonging to the vicilin- and legumin-type proteins that are present in soybeans, peanuts and tree nuts. Cross-reactivity among cupins has been reported between legumins, for instance glycinins G1 ($A_{1a}B_{1r}$) and G2 (A_2B_{1a}), peanut Ara h3 and Ara h4, which share sequence similarities in some of the identified epitopes [33–36]. Similarly, it has been described that vicillins, such as β -conglycinin, and peanut (Ara h1), lentil (Len c1) and pea (Pis s1), can cross-react [37–39]. Cross-reactivity within the group of legumins or vicilins is sustained by a high degree of sequence identity [40]. In contrast, and because of the low sequence identity between legumins and vicilins, the observed cross-reactivity between glycinin $A_4A_5B_3$ and the α -subunit β -conglycinin can only be explained by the conservation of three-dimensional epitopes. Cross-reactivity between allergens with an overall sequence identity below 40% has been reported previously for the Bet v1 family, unlike for the cupin superfamily [40]. Allergens of the Bet v1 family are present in pollen, vegetables and fruits that are eaten without thermal treatment. In contrast, soybean IgE epitopes involved in the reactions described in this work are resistant to the thermal treatment used to prepare the extracts and also to the denaturing electrophoretic conditions. The resistance to proteolytic digestion and denaturation, anti-proteolytic and protease inhibitory properties are some of the features that contribute to

oral sensitization and predisposition to intestinal allergic inflammation [41].

This work also shows that although soybean 7S globulin family is mainly composed of highly homologous members (α , α' and β subunits), only the α -subunit was recognized by the β -casein- and α -casein-specific monoclonal antibodies and by the IgE serum of CMA patients. Similar results have been reported in studies of soy-sensitive patients [24]. Therefore, some special structural feature of the α -subunit may be important to confer its immunogenicity and allergenicity. We could demonstrate by a competitive ELISA that the observed cross-reactivity between different soy proteins and bovine casein is specific, and that the purified soy cross-reactive proteins are able to bind human IgE antibodies. In a previous work, we found that CM and soy are immunochemically cross-reactive. The component $A_4A_5B_3$ was identified as being responsible for this reactivity [16]. In this study, we detected an additional component, the α -subunit of β -conglycinin, that may be involved in this cross-reactivity.

Soy proteins and their derivatives are increasingly used in a wide range of processed foods and they constitute one of the major food allergens both in eastern and in western countries [23]. Therefore, their use as a CMP substitute should be carefully evaluated. These *in vitro* cross-reactions should be further studied to determine their clinical significance. The challenge is to find new alternative tools for novel therapies such as tolerance induction.

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