

Cloning and immunobiochemical analyses on recombinant chymopapain allergen Cari p 2 showing pollen-fruit cross-reaction

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ABSTRACT

Papaya is reported to trigger food and respiratory allergy. Here, we identified chymopapain Cari p 2 as an allergen that can sensitize atopic individuals through fruit consumption followed by respiratory hazards through pollen exposure. Recombinant Cari p 2 displayed IgE-reactivity with 78% of papaya allergic sera. rCari p 2 also displayed allergenic activity through basophil degranulation. rCari p 2 is correctly folded and showed irreversible denaturation in the melting curve. rCari p 2 displayed IgE-cross-reactivity with homologous cysteine proteases from kiwi and pineapple. Cari p 2 transcript was also detected in papaya pulps. rCari p 2 was resistant to pepsin digestion and retained IgE-reactivity after 60 minutes of pepsin digestion. In mouse model, rCari p 2 was found to elicit inflammatory responses in the lung and gastrointestinal epithelium. Hence, Cari p 2 is a newly characterized allergen with diagnostic and immunotherapeutic potential for managing allergic disorders in papaya sensitized individuals.

1. Introduction

Allergy is a prevalent type of immunological hypersensitivity mediated by IgE antibodies and triggered by environmental allergens derived from airborne bioparticulates or food. The worldwide incidence of allergic diseases is continuing to rise over the past few decades affecting more than 300 million of the world population (Pawankar et al., 2011; Pawankar, 2014; Valenta et al., 2018). Among the aeroallergens, pollen grains are major allergy elicitors affecting 10–30% of the world population (Su et al., 2017). Pollinosis is a type of allergy elicited by pollen allergens and is often associated with the subsequent development of food allergy (Bartra et al., 2009). Patients with pollinosis experience adverse reactions after consuming plant-derived food more frequently than the non-pollen allergic individuals. The magnitude of this problem is aggravated by the increasing prevalence of pollen allergy. Approximately 20–70% of patients sensitive to pollen allergens experience pollen-food allergy syndrome (PFAS) after having raw fruits, vegetables, nuts, or certain spices (Bircher et al., 1994; Osterballe et al., 2005; Flores et al., 2012; Carlson & Coop, 2019). In PFAS, patients are first sensitized

by inhalant pollen allergen, and symptoms appear after consuming food that contains homologous antigens. The most common example of such a reaction is Oral allergy syndrome (OAS) which is characterized by oral-pharyngeal pruritus. However, symptoms may be more severe involving districts other than the oral cavity leading to systemic reactions including even anaphylaxis (Asero et al., 2020; Webber and England, 2010). Geographical differences in pollen distribution and different food habits influence the prevalence and implicated foods in PFAS. Hence, identification and characterization of the local dominant pollen and food allergens are essential for proper diagnosis and immunotherapy of pollen allergy and associated PFAS. With the advent of molecular biology technologies, the discovery of allergens has been paced up in the last two decades. Use of recombinant allergens in singleplex ImmunoCAP and multiplex ISAC chip platforms have revolutionized the component resolved allergy diagnosis (Ansotegui et al., 2020). Besides diagnosis, recombinant allergens are also being used in therapeutic approaches such as allergen specific immunotherapy (AIT) (Dorofeeva et al., 2021).

Papaya (*Carica papaya* L., of family Caricaceae) is a major tropical

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fruit yielding plant. Native to Mexico and northern South America, papaya is now cultivated in most tropical countries led by India with 45% of the global production. It flowers almost round the year and pollen grains are significant constituents of aeropollen load (Chakraborty et al., 2005). Papaya fruit is consumed in both unripe (as vegetable) and ripe (as fruit) form. Previous studies have shown that both pollen and fruit of papaya are sources of allergens, and allergic reactions have been reported among atopic individuals (Blanco et al., 1998; Mandal et al., 2009). In a RAST inhibition study, significant cross-reactivity was observed among papaya pollen and fruit (Blanco et al., 1998). Few allergic components were detected from papaya pollen and fruit pulp (Chakraborty et al., 2005; Sarkar et al., 2018). However, only one allergen named Cari p 1 has been studied in detail and enlisted in the IUIS allergen database (Sarkar et al., 2018). In the present study, we are reporting another major allergen from papaya designated as Cari p 2 by IUIS, which has been found to be present in both papaya pollen and fruit.

2. Materials and Methods

2.1. Sample collection

Pollen grains with less than 1% non-pollen contaminants were collected from the anthers of *Carica papaya* L. flower following the method as described in (Mandal et al., 2012).

2.2. Human subjects

A questionnaire survey was performed on allergy patients visiting a tertiary care hospital located in Kolkata, India and skin prick test was performed on 51 atopic individuals using commercial papaya extract (Evexia Life Sciences, Mumbai, India). As revealed in the questionnaire, those patients had typical outdoor respiratory symptoms as confirmed by spirometry and additionally some of them also experienced typical food allergic reactions after consuming papaya. Blood samples from 14 out of 18 such individuals with positive skin reactions for papaya were collected after obtaining written consent from them. Demographic and clinical details of these patients are given in Table 1. Blood samples from 4 healthy individuals were also collected as negative controls. The entire study was approved by the institutional human ethics committee [Reference number, BIHEC/2014-15/4].

Table 1

Clinical and demographic details of human subjects participated in the study

Subjects	Sex	Age	tIgE (kU _A /ml)	sIgE for PE (OD _{405nm})	FEV ₁ (%)	Respiratory Symptoms	Food allergy Symptoms
P1	F	22	112	0.874	59	AR + BA	H
P2	F	36	210	0.756	56	U + BA	U
P3	M	34	187	0.678	48	AR + BA	-
P4	F	45	452	1.008	61	SOB	SOB
P5	F	21	342	0.884	48	BA	-
P6	M	28	361	1.689	52	BA	MR
P7	M	27	188	0.788	53	AR + BA	-
P8	F	44	204	0.877	52	SR	A + MR
P9	M	26	234	0.743	54	BA	-
P10	F	23	241	0.788	48	AR + BA	A + U
P11	M	58	244	0.841	64	SOB	H
P12	F	44	302	0.795	52	AR + BA	-
P13	M	26	178	0.884	41	AR + BA	-
P14	M	28	321	1.008	43	SR	A
H1	M	61	59	0.061	89	ns	ns
H2	M	45	32	0.009	103	ns	ns
H3	M	43	37	0.018	102	ns	ns
DMS	F	33	352	0.078	52	BA	-

*Abbreviations: A - Angioedema, AR- Allergic Rhinitis, BA- Bronchial Asthma, H - Hives, MR - Maculopapular Rash, U- Urticaria, SOB- Shortness of Breath, SR- Seasonal Rhinitis, ns- No Symptom, H1 –H3 –Non-atopic healthy subjects, DMS- Dust Mite allergic Serum.

2.3. Specific IgE estimation

Further confirmation of seroreactivity of the collected samples was obtained by measuring specific IgE-level against papaya pollen extract (Sircar et al., 2012). Wells of microtiter ELISA plates (Nunc, Thermo Fisher, Waltham, Massachusetts, USA) were coated with crude antigenic extract of papaya pollen (250 ng / well) and incubated overnight at 4 °C. Next day, wells were washed thrice with 0.1 M PBS (pH 7.2) containing 0.05% Tween-20 (PBS-T) and blocking was done with 3% Bovine Serum Albumin (BSA) (Sigma-Aldrich, St Louis, MO, USA) for 2 h at 37 °C. After blocking, primary antibody incubation was done with 50 µL of 1:10 diluted individual patient's sera at 4 °C for 16 h and secondary antibody incubation was done with monoclonal anti-human IgE- alkaline phosphatase conjugate produced in mouse (Sigma-Aldrich) for 3 h at room temperature and para -Nitro phenylphosphate (pNPP) (Sigma-Aldrich) was used as substrate. The reaction was stopped by adding 3 N NaOH and absorbance was measured at 405 nm in ELISA reader (Multiskan, Thermo Fisher).

2.4. Protein extraction and profiling

Approximately 100 mg of papaya pollen was crushed with liquid nitrogen and incubated overnight in 4 ml of chilled acetone containing 10% TCA, 1% dithiothreitol (DTT), and 10 µL/ml plant-specific protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA) at 4 °C. Following centrifugation, the pellet was washed thrice with chilled acetone containing 1% DTT and finally resuspended in urea buffer (7 M Urea, 4% CHAPS, 5 mM EDTA) and incubated at –20 °C for 16 h. The supernatant was then collected and concentration was measured by Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA).

About 50 µg of total protein was resolved in 12% SDS-PAGE using mini vertical gel electrophoresis apparatus (GE-Healthcare, Chicago, Illinois) along with protein molecular weight marker (Bio-Rad Laboratories, Hercules, CA, USA). The protein profile was visualized by CBB – R 250 staining.

2.5. 2D Gel Electrophoresis

Approximately 200 µg of protein was processed using Focus Perfect 2D Clean up Kit (G Biosciences, St. Louis, MO, USA) following manufacturer's protocol and reconstituted in 125 µL rehydration buffer (IEF) containing 1% immobilized pH gradient (IPG) buffer for pH 3-10 (v/v) (GE-Healthcare), 25 mM DTT and traces of bromophenol blue.

Rehydration loading was done in 7 cm Immobiline Dry Strip (IPG strip, pH 3–10 L) (GE-Healthcare) in a re-swelling tray and left overnight at room temperature. Next day, isoelectric focusing was performed using Ettan IPG phor-3 isoelectric focusing system (GE-Healthcare) as per manufacturer's protocol. Briefly, the strip was focused at 0–200 V for 1 min, 200–3500 V for 1.30 h and 3500 V for 1.15 h, with a total of 8 kV accumulated. On the following day, the strip was equilibrated with equilibration buffer – I (6 M Urea, 75 mM Tris-Cl pH 8.8, 30% glycerol, 2% SDS and 1% w/v DTT) for 15 min followed by equilibration buffer-II (same as equilibration buffer-I with 2.5% w/v iodoacetamide instead of DTT). Finally, the strip was run in 12% SDS-PAGE with mini vertical Electrophoresis System (GE-Healthcare).

2.6. IgE specific western blot

Proteins from 1D and 2D gel were electrotransferred to PVDF membrane (Merck, Darmstadt, Germany) and membranes were blocked with 3% BSA in gold buffer (52 mM Na₂HPO₄, 7.2 mM NaH₂PO₄, 0.5% Tween-20, 0.5% BSA) for 1 h at 37 °C. Primary antibody incubation was done with patient sera at 1:10 v/v dilution for overnight at 4 °C. In 1D immunoblots six individual patients' sera and in 2D immunoblot pooled sera of four papaya allergic patient were used as primary antibody. After washing with TBST, membranes were exposed to alkaline phosphatase-conjugated monoclonal anti-human IgE produced in mice (Sigma-Aldrich) at 1:1000 v/v dilutions for 3 h at room temperature. IgE-reactive bands/spots were developed with NBT-BCIP (Abcam, Cambridge, UK) substrate.

2.7. LC MS/MS

The desired protein spot was subjected to in-gel trypsin digestion (Ghosh et al., 2015). Extracted peptides were then subjected to the Xevo® G2-XS QT of mass spectrometry (Waters Corporation, Milford MA, USA) in ESI (+ve) platform at 2.5 kV. Raw MS/MS spectra were analyzed in the Progenesis QI search engine (Waters Corporation, Milford MA, USA) against UniProt database.

2.8. cDNA cloning

Total RNA was extracted from freshly procured papaya pollen using TRIzol™ Reagent (Thermo Fisher, MA, USA) following the manufacturer's protocol. The quality of the RNA was checked in Nanodrop™ spectrophotometer at 260 nm and in 2% formaldehyde agarose gel. Residual genomic DNA contamination was removed by DNase-I (New England Biolabs, UK) digestion. The first-strand cDNA was synthesized from 2 µg of total RNA extract using iScript™ kit (Bio-Rad). The full-length cDNA of Cari p 2 (Accession no. X97789.1) was PCR amplified using gene-specific primers such 5'-GAAGGATCCATGGCTACGATGAGTTCAAT-3' as forward primer and 5'-AAGCTCGAGTCAAGCAAAACCTTTAAAAG-3' as reverse primer. The amplified cDNA was cloned into pET21d-TEV vector (BioBharati LifeScience Pvt. Ltd., Kolkata, India) and transformed into *E. coli* DH5α cells. Positive transformants were selected on LB agar plates with 100 µg/ml ampicillin. The integrity and orientation of the Cari p 2 reading frame were confirmed by DNA sequencing.

2.9. Purification of recombinant Cari p 2

The *E. coli* BL21 (DE3) Rosetta cells (BioBharati LifeScience Pvt. Ltd., Kolkata, India) were transformed with a Cari p 2 clone, and the expression of recombinant protein was induced in 1 L of LB broth with 0.5 mM IPTG for 16 h at 20 °C. Cells were lysed by sonication in 7 M Urea, 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM imidazole. The N-terminal 6xHis tagged recombinant protein was purified using Ni-NTA resin (Qiagen, Hilden, Germany) column under denaturing condition following the manufacturer's protocol. The denatured protein was then

refolded by rapid dilution method using glutathione buffer as described in (Sarkar et al., 2018). The purity of the recombinant protein was checked by 12% SDS-PAGE and identification was reconfirmed by MALDI TOF/TOF. IgE-reactivity was verified by IgE-specific ELISA and IgE-immunoblot.

2.10. Circular Dichroism Spectroscopy

About 5 µM of rCari p 2 protein was buffer exchanged with 10 mM NaH₂PO₄ (pH 7.5) and 1% glycerol. The CD spectra were recorded at 25 °C and 50 nm min⁻¹ scan speed in a Jasco J-810 spectropolarimeter (Jasco, Tokyo, Japan) and analyzed in the CAPITO server (Wiedemann et al., 2013). The system's temperature was then increased stepwise up to 90 °C, and CD spectra were recorded at an interval of 10 °C. After denaturation, the system was cooled down to 20 °C, and the spectra were recorded once again.

2.11. Gelatin zymography

About 2 µg of Refolded rCari p 2 was run in 12% SDS-PAGE mixed with 0.1% gelatin. Following electrophoresis, the gel was incubated at 37 °C for 30 min with 5 mM β-ME in 50 mM Tris-Cl, pH 8.0 and 1 mM EDTA for activation and then incubated overnight at room temperature for proteolysis in the same buffer without the β-ME. The zone of proteolysis was visualized by CBB-R250 staining.

2.12. Rat Basophil Leukemic (RBL) cell assay

RBL-2H3 cell line (ATCC CRL-2256) expressing human FCεR1 receptors was sensitized with sera from 5 papaya allergic patients at 1:10 v/v dilution followed by stimulation with refolded rCari p 2 within a concentration range from 1–10000 ng/ml following the protocol as described in Sircar et al., 2016.

2.13. Animal experiments

Mouse model of Cari p 2 induced papaya allergy was established following the original protocol of our earlier reported Cari p 1 (Sarkar et al., 2018). After final challenge, lung and intestinal tissues were harvested from the sacrificed animals. The histopathological studies were outsourced from Dr LalPath Labs Diagnostic centre, Kolkata, India for tissue microtome, staining, and slide preparation. Cell counting was done microscopically by a clinical pathologist in a single blind fashion. Microscopic images were obtained in a CX43 upright light microscope (Olympus, Japan).

2.14. Bioinformatics study

Multiple sequence alignment of Cari p 2 with other cysteine protease allergens reported IUIS database was done using Clustal Omega server (Sievers et al., 2011). A homology-based model for Cari p 2 and Ana c 2 were built in SwissModel server (Waterhouse et al., 2018) followed by stereochemical quality checking in Procheck server (Laskowski et al., 1996). The crystal structures of 2 other related allergens such as Act d 1 (PDB; 4BCT) and Amb a 11 (PDB; 5EGW) (Groeme et al., 2016) were obtained from PDB database. The similarity in surface structure among the 4 models was analysed in Pymol.

2.15. Immunoblot inhibition

Crude protein extract of pineapple, kiwi, soybean, papaya pollen, and papaya fruit was prepared using phosphate buffer extraction protocol. Briefly, 1 g tissue was mixed with 5 ml of sterile 0.1 M Phosphate buffer (PB), pH 7.2 and incubated overnight with constant stirring at 4 °C. The supernatant was collected after centrifugation at 12,000 × g for 20 min. PVDF membranes containing around 15 µg of the crude protein

extracted from pineapple, kiwi, soybean, papaya fruit, and papaya pollen then exposed to either pooled sera from 3 papaya allergic patients (1:10 v/v) or the same pooled sera overnight incubated with 0.3 µg/ml of rCari p 2. Bound IgE-antibodies were detected by immunoblot.

2.16. Simulated gastric fluid (SGF) digestion assay

4 µg of purified rCari p 2 was digested with SGF containing 3.4 mg/ml swine pancreatic pepsin following the protocol described in (Sarkar et al., 2017). The digestion was carried out at 37 °C for 4 different periods such as 0, 15, 30 and 60 min. The reaction was stopped with 5 N NaOH. The digested samples were then run in 12% SDS-PAGE, and IgE-reactivity was checked by immunoblotting with pooled sera from 4 papaya allergic patients.

2.17. Detection of Cari p 2 in papaya fruit

First-strand cDNA was prepared from the pulp and seeds of raw and ripe papaya fruits as described earlier for pollen. The presence of Cari p 2 transcript was detected by PCR using gene-specific primers.

3. Results

3.1. Chymopapain - an IgE-sensitizer from papaya

35% (18 out of 51) of the enrolled allergy patients suspected to have papaya allergy were diagnosed as skin test positive for papaya extract. Sera from 14 out of these 18 patients were collected. Among these 14 papaya pollen allergy subjects, 8 were reported to experience food allergy symptoms after consuming papaya. Elevated specific IgE-level in the sera of 14 such patients was further verified by ELISA against papaya pollen extract. For the detection of individual allergenic components in the papaya pollen extract, immunoblot was performed with patient serum. As shown in Fig. 1A, a prominent IgE-reactive band of 28 kDa was observed in all the 1D immunoblots, which was further selected for detailed characterization. For better resolution, papaya pollen extract was separated in a 2D gel followed by IgE-immunoblot with pooled patient sera. The spot in the 2D gel corresponding to the most prominent spot in the blot with visually highest IgE-binding signal at 28 kDa MW zone (Fig. 1B) was excised and identified by mass spectrometry. Searching MS/MS spectra of this 28 kDa protein against the NCBI nr database revealed 11 unique peptides, all of which displayed significant match with chymopapain (a cysteine protease) from *Carica papaya* L. (Table 2).

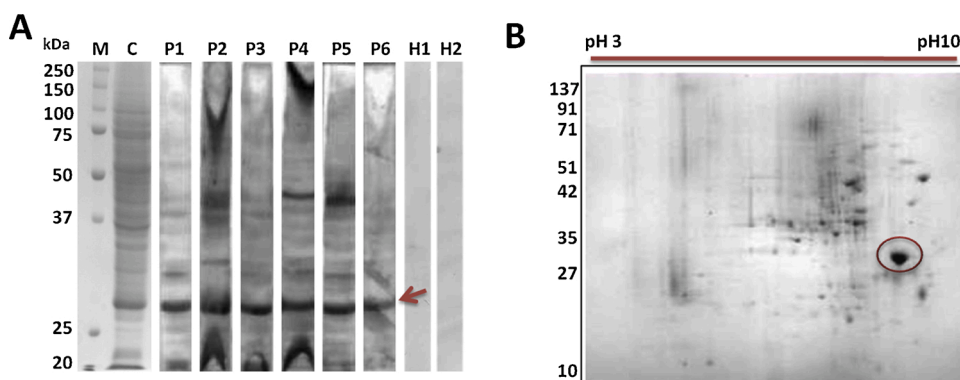


Fig. 1. Immunodetection of papaya pollen allergens. (A) The crude extract of papaya pollen (lane C) was resolved in 12% reducing SDS-PAGE and then exposed to 6 sera from papaya allergic patients (lanes P1–P6). An IgE-reactive band of 28 kDa MW (marked in red arrow) was detected in all the blots with high signal. Negative control blots were performed with 2 non-atopic healthy sera (lane H1–H2). Left margin shows MW marker. (B) Total proteome of papaya pollen was resolved in a 15% 2D gel with pH gradient from 3 to 10. The proteome was then exposed to a pool of 4 papaya positive sera and the IgE-reactive components were detected. The 28 kDa MW allergen is labeled within a red circle. The spot on 2D gel corresponding to this IgE-reactive spot on 2D blot was excised

Table 2

Identification of the 28 kDa IgE reactive protein by LC-MS/MS

Unique Peptide	Peptide mass (Da)	Sequence coverage (%)	Score	Protein (UniProt ID)
AKGAVTPVK	869.533	2.5	5.385	Chymopapain (P14080)
CRATDKPGPK	1128.587	2.8	5.256	
DNLMYIDETNKK	1482.688	3.4	4.620	
GGYQTSLQYVANNGVHTSK	2124.003	5.6	5.568	
ITGYKR	736.430	1.7	4.998	
IYRFEIFR	1142.612	2.27	4.720	
LDHAVTAVGYGTSDDGK	1589.762	4.54	4.991	
LIQLFDSWMLKHNK	1787.923	3.97	4.86	
NSWGPNWGEKGYMR	1696.746	3.97	4.729	
RQSGNSQGTCTGVYK	1540.692	3.97	5.116	
VYPYQAK	867.443	1.98	5.263	

3.2. Recombinant Cari p 2 reacts with serum IgE from papaya allergic patients

Using gene-specific primers, a 1086 bp long full-length ORF of Cari p 2 was PCR amplified, which was then cloned in a bacterial expression vector. Recombinant Cari p 2 was expressed in inclusion bodies of *E. coli*, so it was purified under denaturing conditions. The steps of affinity purification of rCari p 2 are shown in Fig. 2A. The yield of rCari p 2 was about 4 mg L⁻¹. The denatured purified protein was then refolded into its soluble form by rapid dilution method. The second round of purification using Superdex S-75 gel filtration column was performed to obtain >90% purity of rCari p 2. The purity of the rCari p 2 was checked by SDS-PAGE as shown in Fig. 2B. The IgE-reactivity of rCari p 2 was quantitatively determined by ELISA. 11 out of 14 papaya allergic patients displayed elevated specific IgE-level against rCari p 2 as shown in Fig. 3A. As expected, the IgE-levels against crude papaya extract were significantly higher than that of rCari p 2 ($p < 0.001$) as determined by the Mann–Whitney U test. However, there was a positive correlation ($r = 0.75$; $p < 0.0001$) between the specific IgE-levels for crude extract and rCari p 2 in these 14 patient sera as estimated by Spearman's correlation analysis (Fig. 3A). This observation suggests that the in-vitro IgE-binding capacity of purified rCari p 2 is as strong as papaya's crude pollen extract. For further confirmation, IgE-immunoblot was performed with 6 selected sera, and all of them showed IgE-reactivity with rCari p 2 (Fig. 3B). Both these results suggest that Cari p 2 is an important allergen of papaya, and the recombinant form retains IgE-reactivity.

3.3. rCari p 2 displayed allergenic activity

Stimulation of humanized RBL cells with rCari p 2 resulted in the release of β -hexosaminidase at various extents depending upon the

and identified by mass spectrometry.

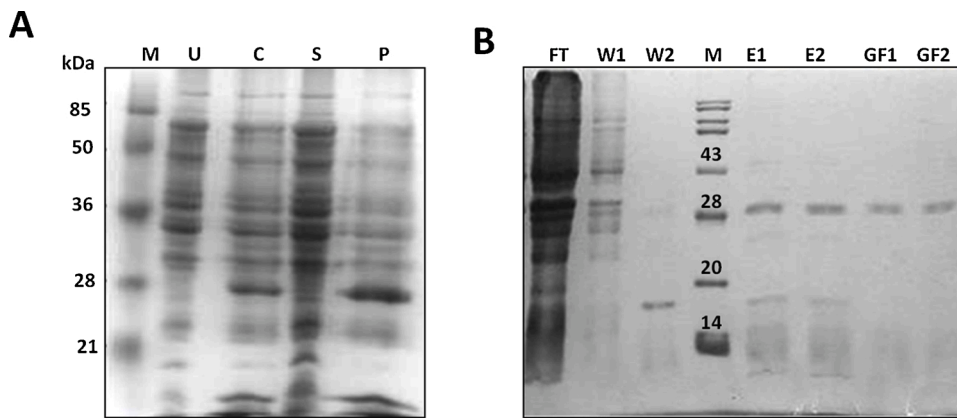


Fig. 2. Expression optimization and purification of rCari p 2. (A) Expression of Recombinant version of Cari p 2 was induced in *E. coli* Rosetta cells with 0.5 mM IPTG at 20 °C for 16 h. The 28 kDa rCari p 2 was expressed in crude *E. coli* extract (lane C) as compared to uninduced extract (lane U). Furthermore, rCari p 2 was found to be expressed only in the inclusion bodies of the pellet fraction (lane P) soluble in 7 M urea buffer but not in the supernatant fraction (lane S) soluble in phosphate buffer. (B) N-terminal 6xHis tagged rCari p 2 was purified in denaturing form by metal affinity chromatography using Ni-NTA resins. After removing the flow through (lane FT), the column bound proteins were washed twice (lanes W1 and W2) with the same buffer containing 40 mM imidazole followed by elution of the rCari p 2 with 250 mM imidazole (lanes E1 and E2). The rCari

p 2 was refolded by rapid dilution method using glutathione buffer followed by a second round of purification in gel filtration column to remove non-specific *E. coli* proteins (lanes GF1 and GF2). Lanes of MW markers are labeled as M.

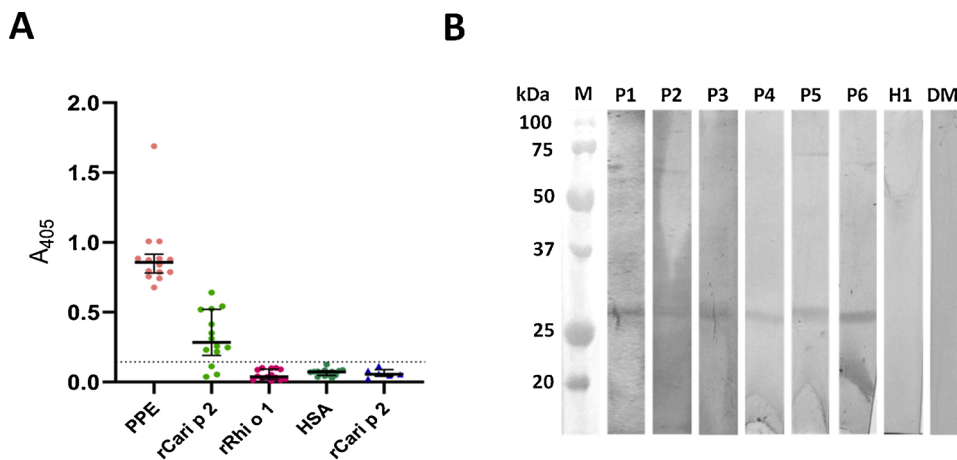


Fig. 3. IgE-reactivity of rCari p 2. (A) Scatter plot showing IgE-binding capacity of rCari p 2 was checked in ELISA using sera from 14 papaya allergic subjects (colored circles) and compared with papaya pollen extract (PPE as positive control), 1 unrelated allergen rRhi o 1 and Human Serum Albumin as negative controls. A third set of negative control was performed in which plate-bound rCari p 2 was exposed to 6 non-atopic sera (blue triangles). Horizontal lines and bars indicate median and SD respectively. Mean of all negative controls was set as cut-off value (dotted horizontal line) above which any value is considered as positive. Absorbance at 405 nm for each serum was plotted on Y-axis against each plate-bound antigen in X-axis. (B) For reconfirmation, rCari p 2 was immunoblotted separately with 6 representative papaya allergic sera (lanes P1 to P6). Negative control blots were performed with 1 non-atopic serum (lane H1) and one house dust

mite reactive serum (lane DMS).

concentration of the allergen (Fig. 4). RBL cells sensitized with IgE-antibodies of papaya allergic patients started releasing the mediators at 100 ng/ μ l of rCari p 2, and optimum release (31–33%) was observed at 10 μ g/ μ l of rCari p 2 concentration after which the dose-dependent curve reached a plateau. No mediator release was observed in the negative control sets.

3.4. rCari p 2 is properly folded with functional activity

The CD spectra of rCari p 2 showed that the protein was folded correctly and composed of a mixture of different secondary structural elements with high contents of beta sheeted elements. The absorbance minimum (λ_m) was obtained at 213 nm (Fig. 5A). The refolding pattern of rCari p 2 was studied by generating a melting curve as shown in Fig. 5B and C in which a temperature-dependent unfolding was observed. The unfolding was initiated at 48 °C, and the protein became entirely unfolded at 85 °C. Transition temperature (T_m) was obtained at 55 °C. An irreversible pattern of denaturation was observed for rCari p 2 as the unfolded protein didn't come back to its native conformation after cooling down the system to 25 °C. The presence of functional cysteine protease activity of rCari p 2 after refolding was verified by gelatin zymography. In this in-gel assay Fig. 5D, the rCari p 2 band appeared as a hyaline zone due to degradation of gelatin by the proteolytic activity

of rCari p 2, whereas rest of areas on SDS-PAGE turned blue upon CBB-R250 staining.

3.5. Cari p 2 can act as a food allergen

As shown in Fig. 6A, we were able to amplify the full-length Cari p 2 transcripts from the cDNA prepared from the pulp of raw as well as ripe papaya fruit. However, the presence of Cari p 2 transcripts couldn't be detected from the seeds of papaya fruit at any stage of maturity. The potential role of Cari p 2 as a food allergen was verified by digesting the protein in simulated gastric fluid followed by checking the IgE-reactivity of the digested products. As shown in Fig. 6B, treatment of rCari p 2 in SGF buffer containing pepsin didn't result in the release of any polypeptide fragment even after 60 min of digestion. When the digested products were probed with serum IgE from papaya allergic patients, the intact rCari p 2 of 28 kDa MW was found to retain IgE-reactivity throughout the digestion periods.

3.6. Cari p 2 showed IgE-cross reactivity with other cysteine proteases

Multiple sequence alignment of Cari p 2 sequence with 3 other cysteine proteases reported as allergens showed >30% identity within them as shown in supplementary Fig. S1. However, alignment of the Cari

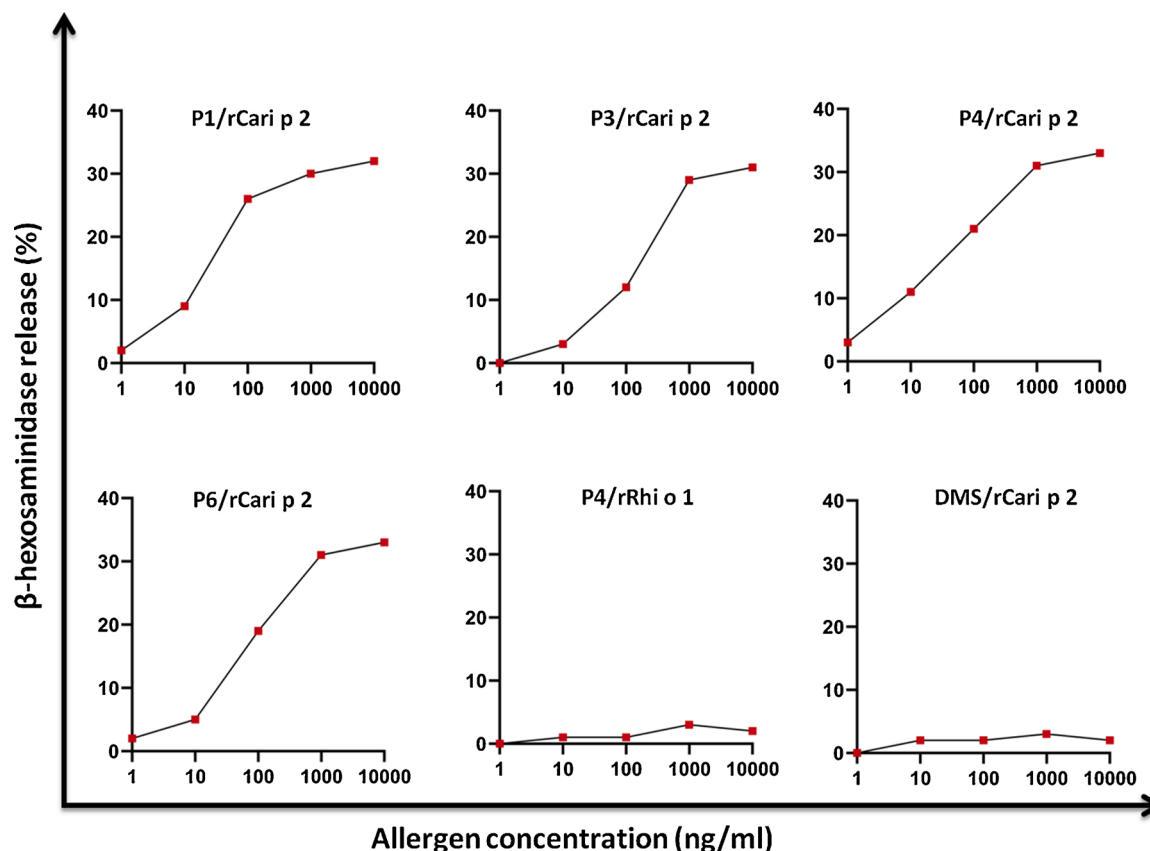


Fig. 4. Allergic activity of rCari p 2. RBL cell lines displayed dose-dependent degranulations (in X-axis) when stimulated with increasing concentrations (in Y-axis) of rCari p 2. Individual patient serum used to sensitize RBL cells with IgE-antibody and corresponding antigen used for stimulation is written on each plot. 2 sets of negative controls were prepared in which RBL cells were either sensitized with papaya allergic serum P4 but stimulated with unrelated allergen rRhi o 1 or sensitized with a dust mite allergic serum but stimulated with rCari p 2. No degranulation (i.e. < 5% β-hexosaminidase release) was observed in either case.

p 2 homology model separately with either the crystal structures of Amb a 11 (ragweed) and Act d 1 (kiwi) or with the homology model of Ana c 2 (pineapple) revealed significant similarities in certain solvent-accessible surfaces, suggesting a possibility of cross-reactivity among them (Fig. 7A). Hence, we performed IgE-immunoblot inhibition to check the presence of Cari p 2 cross-reactivity with the corresponding cysteine proteases present in the extracts of kiwi and pineapple. Another cysteine protease is reported from soybean in AllFam database (Radauer et al., 2008), which has not yet been given any IUIS nomenclature. Hence, in the immunoblot inhibition experiment, we also included soybean extract. As shown in Fig. 7B, Cari p 2 partially inhibited the IgE-binding to allergenic components of MW 22 kDa and 30 kDa present in the extracts of pineapple, and kiwi respectively, which correspond to the previously reported cysteine proteases from these fruits. As expected, visible inhibition of IgE-binding was observed in papaya fruit and pollen extract at a region corresponding to the molecular weight of Cari p 2. However, no visible inhibition of IgE-binding was observed in soybean extract.

3.7. rCari p 2 displayed in-vivo allergic inflammation in a mouse model

Allergic potency of the recombinantly produced Cari p 2 was assessed in a mouse model of respiratory and food allergy. The BALB/c mice sensitized with rCari p 2 displayed inflammatory changes in the lung and GI tissue after being challenged with papaya pollen (intranasal) and fruit (oral) extracts, respectively (Fig. 8A). As shown in Fig. 8 B-C, eosinophil infiltration was observed in the lung and GI epithelium after H/E staining. Similarly, as shown in Fig. 8D-E goblet cell hyperplasia and enhanced mucus secretion were observed in lung and GI histology. Quantitative measurements of these 2 parameters revealed a

comparable level of inflammatory changes between rCari p 2 and ovalbumin (positive control) challenged mice histology.

4. DISCUSSION

The role of papaya in triggering allergic hypersensitivity has been reported in various earlier literatures (Chakraborty et al., 2005; Mandal et al., 2009; Blanco et al., 2015). Papaya pollen grain and fruit are the sources of inhalant and food allergens, respectively (Chakraborty et al., 2005; Sarkar et al., 2018). Interestingly, certain allergenic proteins are commonly present in the pollen and fruit (Sarkar et al., 2018; Irañeta et al., 2005). Hence, these proteins, in a way similar to PFAS, can lead to food allergy in individuals who are originally sensitized by pollen inhalation in their respiratory mucosa and vice versa (Valenta & Kraft, 1996; Price et al., 2015; Carlson & Coop, 2019). One such papaya allergen is Cari p 1 that has earlier been reported by us (Sarkar et al., 2018). In the present study, we report a second papaya allergen designated as Cari p 2. Cari p 2 is a cysteine protease popularly known as chymopapain. A serological investigation combined with mass spectrometry identified the presence of IgE-reactive chymopapain in the pollen grains and fruits of papaya. Sequence analysis of this chymopapain revealed a conserved cysteine protease domain (P¹³⁶-S³⁴³) with a conserved C¹⁵⁹ residue in the catalytic domain. Availability of genome information of papaya facilitated to amplify the full-length cDNA of Cari p 2. The recombinant version of Cari p 2 showed IgE-reactivity almost similar to its native counterpart. rCari p 2 reacted positively with IgE-antibodies in the sera from 11 out of 14 (78%) papaya allergic patients. The data indicates sufficient frequency in IgE-binding; however, a cross-sectional study, including a larger patient cohort, is warranted to claim Cari p 2 as a major papaya allergen. In addition to frequency, rCari

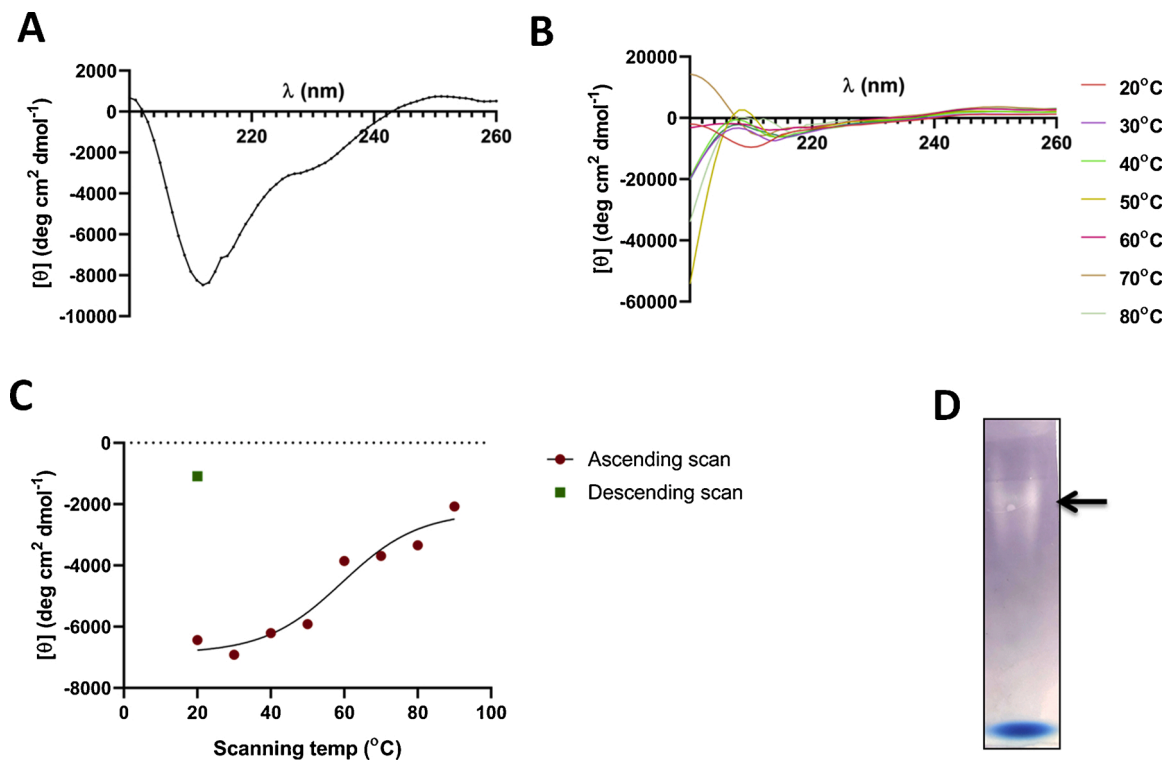


Fig. 5. Folding pattern of rCari p 2. (A) Far UV CD spectra of rCari p 2 showing molar ellipticities (Y-axis) plotted against wavelengths (X-axis) at 25 °C. (B) CD spectra of rCari p 2 were also recorded at increasing temperature (20 °C to 80 °C) to study the denaturation pattern of the protein. (C) Sigmoid melting curve of rCari p 2 in which molar ellipticities at 213 nm (λ_{max} in Y-axis) were plotted against temperature (in X-axis). rCari p 2 was unfolded by gradually increasing the temperature (ascending scan as red dots) and no refolding of the fully denatured rCari p 2 was observed by lowering the temperature up to 20 °C (descending scan as green square). (D) Gelatin zymography showing proteolytic activity of rCari p 2.

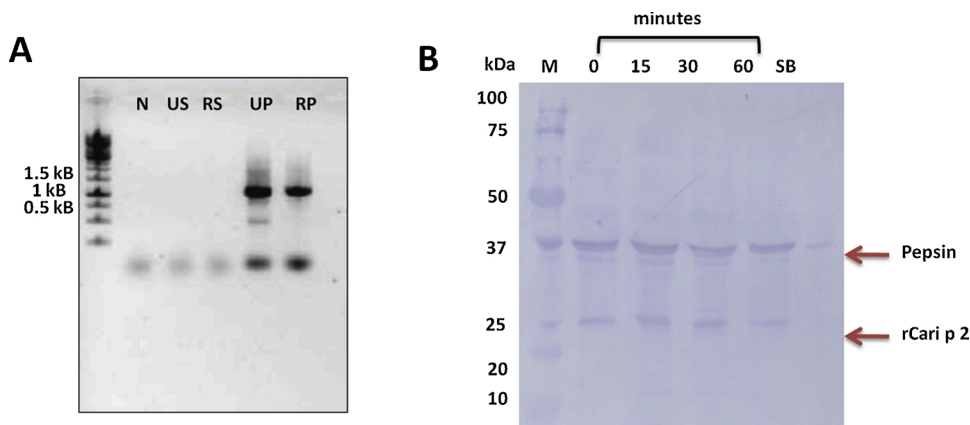


Fig. 6. Cari p 2 as a food allergen. (A) Cari p 2 transcript was detected by PCR-amplification from the cDNA of ripe pulp (RP) and unripe pulp (UP) of papaya but not from ripe seed (RS) and unripe seeds (US). (B) SGF assay in which rCari p 2 was digested with pepsin for increasing time period (lanes 0 to 60 minutes) and then immunoblotted with a pool of 4 papaya allergic sera. 28 kDa rCari p 2 remained intact possibly due to resistance to pepsin digestion and displayed IgE-reactivity even after 60 minutes of SGF treatment. For comparison, a substrate blank (SB) without rCari p 2 was run on the same gel.

p 2 displayed strong IgE-binding, which is almost comparable to the crude papaya extract. rCari p 2 was properly folded and retained its cysteine protease activity. However, after cooling down from heat denatured state, rCari p 2 remained unfolded and was not found to retain any of its native secondary structures. This observation led us to infer that no partial refolding of denatured rCari p 2 (performed under denaturing SDS-PAGE condition) on PVDF membrane was possible in immunoblots. Hence, IgE-binding in immunoblot was mediated by linear epitopes, which is a characteristic of food allergens. Unlike many other cysteine proteases, rCari p 2 was not found to form any oligomer in our study. However, certain earlier reports suggested the presence of naturally occurring dimeric forms of chymopapain (Maes et al., 1996). However, the bacterially expressed recombinant version of Cari p 2, after refolding from a denatured version, might have lost some surface

potential that hindered the protein aggregation. However, this monomeric form of rCari p 2 was found to show allergenic activity in RBL assay. Hence, recombinant Cari p 2 is a suitable candidate for skin test-based diagnosis as well as more advanced basophil activation tests. Sequence alignment revealed the presence of specific peptide stretches in Cari p 2, which are homologous to some other reported cysteine protease allergens. Further, *in silico* comparative structural analyses of these allergens have shown the presence of a high degree of similarities in various structural folds within these allergens. This observation led us to anticipate the presence of cross-reactive epitopes in these allergens. The immunoblot inhibition experiment confirmed certain degree of cross-reactivity between Cari p 2 and the corresponding cysteine proteases present in pineapple (Ana c 2) and kiwi (Act d 2). In the blots, only partial inhibition was observed which might be explained as either Cari

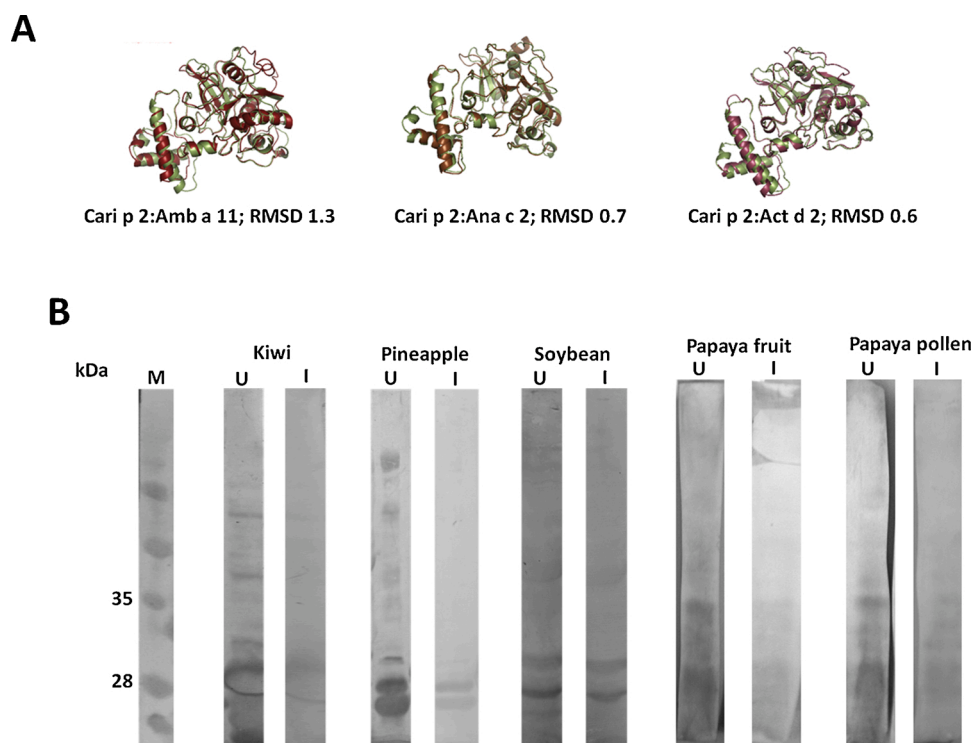


Fig. 7. IgE-cross reactivity of Cari p 2. (A) Homology model of Cari p 2 (green ribbon) was aligned with either the homology model of Ana c 2 or the crystal structures of Act d 2 and Amb a 11 (in 3 different shades of red) to locate the highly conserved structural elements on the surface of these allergens which might be responsible for cross-reactivity. (B) IgE-immunoblot inhibition in which the fruit extracts of pineapple, kiwi, and soybean were exposed either to a pool of 3 papaya allergic sera overnight incubated with 0.3 g/ml of rCari p 2 (inhibited; I) or the sera pool only (uninhibited control; U). rCari p 2 mediated partial inhibition of IgE-binding to the corresponding cysteine proteases (at a region similar to reported MW) present in pineapple and kiwi extracts were observed. However, no inhibition was observed for soybean extract. In auto-inhibition control blots, rCari p 2 displayed significant IgE-inhibition of protein present in papaya pollen and fruit.

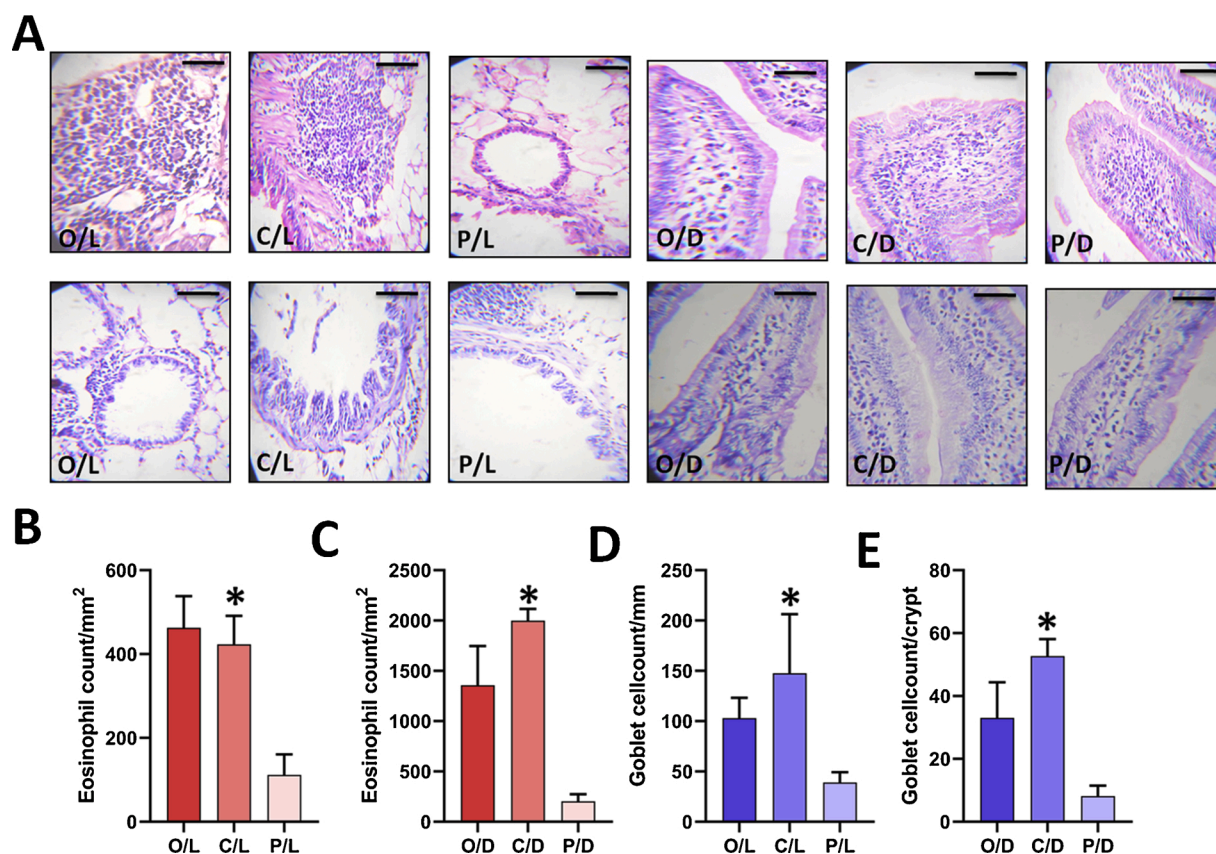


Fig. 8. In-vivo allergenic activity of rCari p 2 in mouse model. BLAB/c mice (n = 6) were challenged with either rCari p 2 (C) or ovalbumin as positive control (O) or PBS as negative control (P). (A) Representative histopathology of mouse lung (L) and duodenum (D) after H/E (upper panel) and PAS staining (lower panel) to observe eosinophil infiltration and goblet cell hyperplasia respectively. Bar graphs with mean $\pm$ SD (n = 6 mice) showing quantification of eosinophils (B and C) and goblet cells (D and E). Asterisk represent significant ($p < 0.005$) difference in rCari p 2 treatment group versus PBS treated group.

p 2 having less potent IgE-epitopes than those cysteine proteases or the presence of a different IgE-reactive protein with similar molecular weight. In our study, we couldn't check cross-reactivity with Amb a 11 due to the unavailability of ragweed pollen. On the contrary, no cross-reaction was observed for soybean, which has been reported to contain an uncharacterized and putative allergenic cysteine protease. In addition to pollen, chymopapain is reported to be present in papaya latex (Azarkan et al., 2003). We observed Cari p 2 transcripts amplified from the fruit pulps but not from the seeds. Also, in the cross-inhibition experiment, it was observed that rCari p inhibited IgE-binding to a papaya fruit protein having the same MW as Cari p 2. Hence, Cari p 2 can be thought of being present in papaya fruit and having the potential to act as a food allergen sensitizing the gastric mucosa. SGF assay revealed the presence of IgE-reactivity in intact Cari p 2 after prolonged digestion of the protein with pepsin. Hence, Cari p 2 was found to be somewhat resistant to pepsin digestion at a pH of 1.2. A similar pattern was observed in various other food allergens of the LTP family (Asero et al., 2001; Duffort et al., 2002; Vassilopoulou et al., 2006), which can be correlated with the occurrence of typical food allergic symptoms in papaya sensitized patients seropositive for Cari p 2. The allergenic potential of Cari p 2 was further evaluated and strengthened in a mouse model of papaya allergy in which the recombinant allergen was found to elicit inflammatory responses both in the lung as well as in gut epithelium in a way similar to Cari p 1 (Sarkar et al., 2018).

5. CONCLUSION

Taken together, Cari p 2 is a new addition to the increasing list of cross-reactive plant allergens. This allergen is unique as it is capable of triggering respiratory symptoms as well as food allergy symptoms in sensitized patients. Hence, the purified rCari p 2 can be an ideal candidate for component-resolved diagnosis of patients suffering from cyteine protease group of allergens. It is tempting to speculate that further antigenic analyses of Cari p 2 will help to locate the antigenic regions, which in turn facilitate to design epitope-based immunotherapeutic for the management of papaya and related allergy.

Author's contributions

Conceptualization; SGB, GS
Data curation; MB, MBS, RKS, ADG, KJ, SS
Formal analysis; GS, SGB, MBS, SS
Funding acquisition; SGB
Investigation; SGB, GS, MB
Methodology; MB, MBS, RKS, ADG, KJ, SS
Project administration; SGB
Resources; SGB
Software; SS
Supervision; SGB, GS
Validation; All the authors
Visualization; SGB, GS, MB, ADG, SS, KJ
Writing - original draft; SGB, GS, MB
Writing - review & editing; All the authors

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Declaration of Competing Interest

The authors declare that they have no conflicts of interest with the contents of this article.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.molimm.2021.06.010>.

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Supplementary Fig. S1: Sequence analysis of Cari p 2. Multiple sequence alignment in which Cari p 2 was used as template and amino acid sequences of 3 reported cysteine protease allergens were aligned to locate the conserved regions. Gaps are denoted as dashes and identical residues in the aligned sequences are denoted as asterisks.

Carip2	MA--TMSSISKIIFLATCLIIHMGSLSSADFYTVGYSDDLTSIERLIQLFDSWMLKHNKI	58
Amba11	-----ME*N*LVCFSFS*VLIL**VE----SFH*HERE*E*E*GFMGMY*R*REQ**IE	50
Anac2	-----A*KVQLV**FLF*CA-----MWASPSAASRDEPNPMMKR*EE**AEYGRV	47
Actd1	*GLPKSFVSMILL*FS*L**LSLAFNAKNL-----TQRTNDEVKAMY*LI*YG*S	52
Carip2	YESIDEKIYRFEIFRDNLMIYIDETNKK-NNSYWLGLNGFADLSNDEFKKKYVGFVAEDFT	117
Amba11	MR*----PE**NV*KY*VRR*H*S**M-DKP*K*KV*E***MT*L**VNT*ANSKISH*Q	105
Anac2	*KDD**MR**Q**KN*VKH*ETF*SRNE***T*I*Q*T*MTKS**VAQ*T*VSLPLNI	107
Actd1	*N*LG*WER*****KET*RF***H*ADT*R**KV***Q****TDE**RST*L**TSGSNK	112
Carip2	GL-----EHFDNEDFTYKHVTINYPQSIDWRAKGAVTPVKNGACGSCWAFSTIATVE	169
Amba11	A*RGSAFGSIDTDP*K*I*AN**KI*DKV***E*N***D**G**G*****AAVVAL*	165
Anac2	ER-----*P--VVS*DDVNISAV*****DY***NE*****NP*****S*AA*****	157
Actd1	TK-----V---SNQYEPRVGQVL*SYV***SA***VDI*S**E**G*****A*****	161
Carip2	GINKIVTGNLLELSEQLVDCDKH--SYGCKGGYQTTSLQYVANN-GVHTSKVYPYQAKQ	226
Amba11	***A*R**K*VKF***Q*****MT--NA**D**LMEPAFT**IKHG*IAPEAS***VG*R	223
Anac2	**Y**K**Y*VS*****VL**AV-----*****WVNKAYDFIIS*N**T*EEN***L*Y*	214
Actd1	*****V*IS*****I**GRTQNTN**NV**I*DG*FII**G*IN*EEN***T*QD	221
Carip2	YKCRATDKPGPKVKITGYKRVPSNCETSFLGALANQPLSVLVEAGGKPFQLYKSGVFDGP	286
Amba11	ET*DKAKIKD-VL**D*RQN**GLD*EALRK*V*H**VATGIQLS*HGL*F*SE**YT*D	282
Anac2	GT*N*NSF*NS-AY****SY*RR*D*R*MMY*VS***IAA*ID*-SEN**Y*NG***S**	272
Actd1	GE*NVDLQNEKY*T*DT*EN**Y*N*WALQT*VTY**V**ALD*A*DA*KH*S**I*I**	281
Carip2	CGTKLDHAVTAVGYGTS-DGKNYII IKNSWGPWGEKGYMRLKRQSGNSQGTGCVYKSSY	345
Amba11	***EPN*G*GI****ENEK*IKFWTV*****T*****IH*Q*GARK-E*L***AMH*S	341
Anac2	***S*N**I*II***QDSS*TK*W*VR*****SS***G**V*MA*GVSS*S*V**IAMAPL	332
Actd1	***AI*****I*****E-G*ID*W*V***DTT**E***IL*NV*G-A****IATMPS	339
Carip2	YPFKGFA-----	352
Amba11	F*IMNDPNPPKDD-----PNGPKDDPDAPKDPKFKTTQRLQGIRTKLLEL	386
Anac2	F*TLQSGANAEVI--KMVSET-----	351
Actd1	**V*YNNQNHPKSYSSSLINPPAFSMSNDGPVGVDDG-----QRYSA-----	380