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Profiling C-Phycocyanin: From Extraction to Structure, IgE Recognition and Digestive Transformation of Spirulina's Major Pigment-Protein

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Microalgae have emerged as promising sources of sustainable nutrients and bioactive compounds, attracting growing interest for their potential use in future food systems. *Arthrospira platensis* (commonly known as Spirulina) is particularly valued for its high protein content and functional pigments, among which C-phycocyanin (C-PC) is the most abundant. Although Spirulina is generally recognized as safe by regulatory agencies, recent reports have described allergic reactions in sensitized individuals, raising concerns about the allergenic potential of C-PC and related proteins. In this study, C-PC was extracted and purified from *A. platensis,* and its structural integrity and oligomeric state were characterized. Immunoreactivity was assessed using sera from Spirulina-sensitized patients and allergic to crustaceans, mites, and fish to explore potential cross-reactivity. Simulated gastrointestinal digestion was then performed to evaluate the stability and allergenic behavior of the protein. C-PC displayed clear IgE-binding activity in its intact form, confirming its relevance as a potential allergen. However, simulated gastrointestinal digestion reduced IgE-binding activity and caused extensive fragmentation and high-resolution LC–MS analysis. This work provides new insights into the digestive profile and immunoreactivity of Spirulina proteins, supporting a more accurate assessment of their safety and informing their responsible use in food and nutraceutical applications.

In recent years, microalgae have garnered considerable attention due to their ecological importance, nutritional value, and wide range of potential applications in biotechnology. This interest has become increasingly relevant as the continuous growth of the world's population poses a global public health challenge by exacerbating food shortages and increasing the demand for sustainable nutrient sources[1,2].

Arthrospira platensis, commonly known as Spirulina, is one of the most extensively studied microalgae. Spirulina is a filamentous, spiral-shaped, multicellular blue–green microalga belonging to the cyanobacteria group, which thrives in warm, alkaline aquatic environments. It is rich in natural pigments that are widely used in the food industry as colorants, including phycocyanin, chlorophyll, and carotenoids, which play crucial roles in photosynthesis[1,3].

Among these pigments, C-phycocyanin (C-PC) is the most abundant and functionally significant pigment-protein complex. C-PC is a blue-colored phycobiliprotein (PBP) that functions as an accessory light-harvesting pigment in the photosynthetic apparatus of cyanobacteria. It is a water-soluble biliprotein located within phycobilisomes supramolecular complexes responsible for capturing and transferring light energy to chlorophyll a[3–6]. Structurally, C-PC consists of two distinct subunits, α (12–19 kDa) and β (14–21 kDa), each comprising approximately 160–180 amino acids. These subunits associate to form (αβ) monomers, which further aggregate into trimeric [$(\alpha\beta)_3$] or hexameric [$(\alpha\beta)_6$] complexes that constitute the functional light-harvesting units[1,7].

The intense blue coloration of C-PC is due to its chromophores, known as phycocyanobilin. These are linear tetrapyrrole prosthetic groups that are covalently bound to cysteine residues of the apoprotein via thioether

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linkages. The interaction between the chromophore and the protein matrix determines the characteristic pigment absorption spectrum, typically peaking around 620 nm[8,9].

Beyond its photobiological role, Spirulina is also recognized for its exceptional nutritional composition and bioactive properties, which explain its widespread use as a dietary supplement. It is particularly valued for its high protein content, constituting 60%–70% of its dry weight, and its complete amino acid profile[9–11]. Moreover, Spirulina provides essential vitamins (B-complex, C, E, and K), minerals (iron and magnesium), essential fatty acids, and potent antioxidants. These compounds collectively contribute to its functional properties, including antioxidant, anti-inflammatory, and immunomodulatory effects, supporting immune system function and overall well-being[4,12,13].

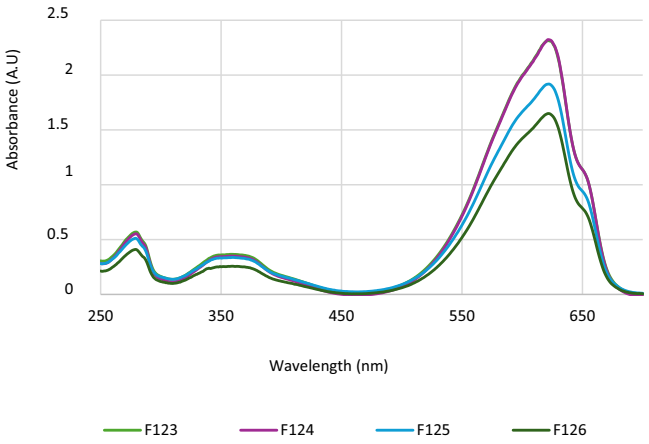
In terms of regulatory aspects, Spirulina has been extensively evaluated by food safety authorities. In the United States, the Food and Drug Administration (FDA) has classified several microalgal species, including *Arthrospira platensis* and *Arthrospira maxima*, as Generally Recognized as Safe (GRAS). Similarly, the European Food Safety Authority (EFSA) has reported no safety concerns regarding the toxicity or use of Spirulina-based products in food applications[12,14].

Nevertheless, emerging evidence indicates that Spirulina may elicit allergic reactions in susceptible individuals. Reported symptoms range from mild manifestations, such as skin irritation and gastrointestinal discomfort, to severe, life-threatening reactions, including anaphylaxis[15,16]. The C-PC beta subunit of spirulina is already known as a food allergen, as reported on the Allergome database (https://www.allergome.org/script/tools.php, protein code: 8833), in which it was inserted after the case of anaphylaxis reported by Petrus et al.[16]. More recently, additional proteins, including thioredoxins, superoxide dismutase, glyceraldehyde-3-phosphate dehydrogenase, and triosephosphate isomerase (TPI) have been implicated in its allergenic potential[17]. Among them, TPI is particularly noteworthy, as it is a well-recognized pan-allergen also present in house dust mites and therefore represents a plausible molecular basis for the suspected cross-reactivity[18]. Thioredoxin, another conserved protein with documented allergenic potential in various species, may further contribute to this theoretical cross-reactivity, although its role is less clearly defined. These proteins are recognized allergens in other biological systems and may contribute to sensitization in Spirulina consumers. Thus, potential cross-reactivity between Spirulina proteins and other known food allergens remains an area of ongoing investigation. In silico analyses have revealed sequence similarities with allergens from diverse biological sources[17,19]. In particular, several suspected proteins occurring in extracts of Spirulina microalgae—showing significant sequence homology with known food allergens, most commonly those associated with fish and crustaceans—have been annotated[17,19]. However, clinically validated cross-reactions have not yet been demonstrated. Thus, while theoretical risks exist, further research is required to determine the clinical relevance of these findings and to ensure the safe use of Spirulina-derived ingredients in future food applications. In this study, C-PC was extracted from Spirulina, and its oligomeric state was characterized. Additionally, the immunoreactivity of the purified protein was assessed using IgE-binding assays to evaluate its recognition by allergic patients and its potential for cross-reactivity (considering, in particular, patients allergic to dust mites, fish, and crustaceans). These analyses provide new insights into the allergenic properties of C-PC and its implications for Spirulina use in food and health-related applications.

Results

Extraction and purification of C-PC from Spirulina

C-PC was successfully extracted from Spirulina biomass and subjected to a two-step purification protocol. SDS–PAGE analysis of the crude extract revealed multiple protein bands of various molecular weights, reflecting the complex protein mixture present in the algal extract (Fig. S1a, Supplementary Information). Following anion-exchange chromatography, fractions collected from the column showed a marked enrichment in proteins of ~15–20 kDa, corresponding to the expected α and β subunits of C-PC.



	Fractions SEC			
	123	124	125	126
280nm	0.57	0.55	0.50	0.41
620nm	2.31	2.31	1.94	1.64
Ratio 620/280	4.1	4.2	3.7	4.0

Fig. 1 | Purity of C-PC from *Spirulina platensis*. UV–vis spectra and A_{620}/A_{280} ratios of different C-PC fractions obtained after size-exclusion chromatography.

To further improve homogeneity, the pooled fractions, with an A_{620}/A_{280} ratio greater than 1.8, were subjected to size-exclusion chromatography. The resulting fractions displayed a characteristic blue color, and SDS-PAGE confirmed the presence of two predominant protein bands at ~15–20 kDa, with minimal contaminating proteins (Fig. S1b, Supplementary Information). The electrophoretic pattern is consistent with the α and β subunits of C-PC, demonstrating that the two-step purification strategy was effective in isolating highly pure C-PC from Spirulina. Spectroscopic analysis confirmed the successful enrichment of C-PC following size-exclusion chromatography. UV–vis analysis of the fractions collected by size-exclusion chromatography revealed a characteristic absorption maximum at 620 nm, corresponding to C-PC. All spectra displayed a pronounced peak at 620 nm and minor absorption at 280 nm, consistent with the presence of aromatic amino acids and trace protein impurities (Fig. 1). Fractions chosen for further experiments exhibited A_{620}/A_{280} ratio above 4.0, indicating high purity and minimal contamination from other proteins (Fig. 1). Protein concentrations of the main FPLC fractions ranged from 4.35 to 5.76 mg/mL. These results indicate that the purification procedure effectively isolated intact C-PC suitable for subsequent biochemical and biophysical analyses.

Characterization of the structure and oligomeric state of extracted C-PC

The structural identity of C-PC was first examined by LC–MS. Chromatographic separation condition allows to separate three major peaks, corresponding to the expected protein components of the complex, and fully consistent with the well-established structures reported in the literature[20]. Peak A (12.7 min) was assigned to the apo form of the β-subunit, peak B (16.0 min) to the β-subunit carrying phycocyanobilin, and peak C (16.4 min) to the α-subunit (Fig. S2, Supplementary Information). This separation pattern is fully consistent with the known composition of C-PC, in which the β-subunit can occur both with and without chromophore, while the α-subunit typically carries a single chromophore.

Mass spectrometry analysis (Fig. S2, Supplementary Information) of peak A showed a dominant deconvoluted mass at approximately 18,188 Da, in agreement with the theoretical mass of the β-subunit without chromophore (18,094 Da). This confirms that the first chromatographic fraction

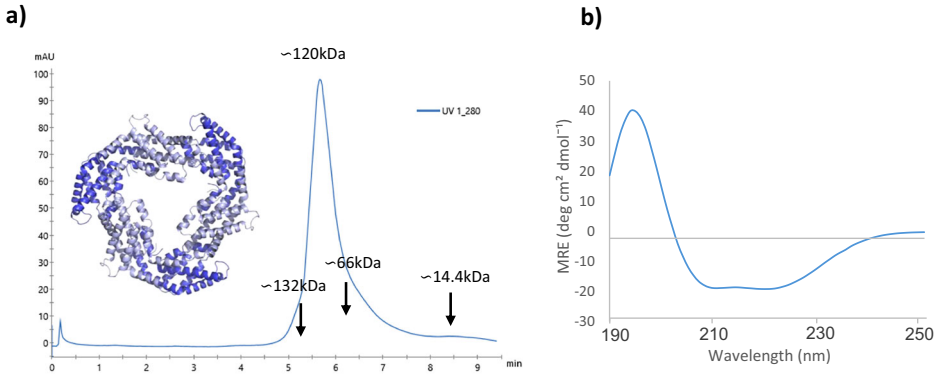


Fig. 2 | Structural characterization of C-PC from *Spirulina platensis*. **a** Analytical size-exclusion chromatography (Superdex 200 Increase 5/150 column) elution profile of C-PC. Elution times of the protein standards (bovine serum albumin dimer (132 kDa), bovine serum albumin monomer (66 kDa), and lysozyme (14.4 kDa) are indicated by arrows. The inset shows the AlphaFold 3D model of the trimeric form [$(\alpha\beta)_3$] of C-PC, where each monomer (αβ) consists of an α-subunit (dark blue) and a β-subunit (light blue). **b** CD spectroscopy analysis of purified C-PC.

predominantly contains the apo-form of the β chain, as expected for the partial loss of the chromophore that can arise during extraction or purification[21]. Peak B displayed two major deconvoluted masses at 19,279 and 19,587 Da, corresponding to β-subunit species carrying one or two phycocyanobilin chromophores (theoretical mass 19,266.95 Da). The presence of both mono- and di-chromophorylated forms is consistent with the known heterogeneity of C-PC chromophore loading and reflects the natural distribution of bilin attachment states reported in the literature[22]. Finally, peak C yielded a dominant mass at 17,846 Da, consistent with the α-subunit (17,602 Da), confirming that this fraction corresponds to the expected α chain. Overall, the LC–MS profile not only validates the chromatographic separation of the three major protein components of the C-PC complex, but also aligns with the structural organization and chromophore composition widely documented for Spirulina-derived phycocyanins[22].

Analytical size-exclusion chromatography confirmed the oligomeric state of C-PC in solution. A major peak eluting at 5.67 min corresponded to an apparent molecular mass of approximately 120 kDa, consistent with the expected hydrodynamic size of the trimeric $(\alpha\beta)_3$ assembly reported for C-phycocyanin (Fig. 2a). These results indicate that C-PC predominantly assembles as a trimer of αβ monomers, in agreement with its well-established ring-shaped structure reported in the literature[23] and with previous reports showing that at neutral pH, the protein mainly exists in a trimeric form[24]. Moreover, the far-UV CD spectrum collected at pH 7 and 20 °C showed two minima at ~210 and 220 nm (Fig. 2b), consistent with a well-ordered secondary structure dominated by α-helical elements[25,26], in agreement with the structural organization previously reported for C-phycocyanin, and a maximum at 194 nm, indicative of β-sheet contributions. In line with previous reports, these features reflect an intact polypeptide backbone and confirm the structural stability of C-PC[25,26].

Limited proteolysis experiment

To gain preliminary insight into the structural organization of the two C-PC subunits and to verify structural features previously reported in the literature, a limited proteolysis (LiP) experiment with trypsin was performed. This assay was performed to provide an analytical snapshot of which regions of the native complex are more accessible to enzymatic attack under controlled conditions. Aliquots were collected at 0 min (before enzyme addition) and at 5, 10, 20, and 40 min, and analyzed by 16% SDS–PAGE (Fig. S3a, Supplementary Information) as well as by LC–MS (Fig. S3b, Supplementary Information) to monitor the generation of proteolytic fragments. As shown by SDS–PAGE, a distinct low-molecular-weight band (approximately 5–10 kDa) appeared as early as 5 min, suggesting rapid cleavage of a specific region of the protein. LC–MS analysis further confirmed the presence of a major peak at 6135 Da (Fig. S3, Supplementary Information), corresponding to the ExPASy-predicted (ExPASy—Compute pI/Mw Tool. Swiss Institute of Bioinformatics) fragment generated by cleavage at position 57 of the β-subunit (6132.91 Da). No predicted fragments originating from the α-subunit matched this mass.

Together, these observations indicate that the β-subunit is more readily accessible to proteolysis, in line with structural models and experimental evidence previously reported for Spirulina phycocyanin[20,21,27]. This targeted LiP analysis, therefore, served to confirm the higher solvent exposure of the β-chain within the native oligomeric assembly, providing a structural reference point for interpreting subsequent biochemical investigations.

Immunoreactivity of C-PC

The immunoreactivity of the extracted protein was first assessed using commercial polyclonal IgG antibodies raised against C-PC. Dot blot analysis confirmed clear antibody recognition, indicating that the protein retained structural features relevant for antibody binding (Fig. S4a, Supplementary Information). Following this validation, IgE reactivity was assessed using sera from allergic patients with distinct sensitization profiles. Control dot blots performed without patient serum confirmed that direct binding of the secondary antibody was negligible, indicating that the signals observed in the main assay reflect specific IgE–antigen interactions (Fig. S4b, Supplementary Information).

For clarity's sake, the IgE recognition of C-PC observed in some patients sensitized to mites, crustaceans, or fish should not be interpreted as direct evidence of allergenic cross-reactivity. Although the data may suggest the presence of shared IgE-binding determinants, definitive demonstration of cross-reactivity would require dedicated inhibition or competitive binding experiments, which were beyond the scope of the present study.

The patient sera included in this assay were selected based on their suspected cross-reactivity profiles toward house dust mites, crustaceans, and fish (as outlined in the introduction), given the presence in Spirulina of conserved proteins that are recognized pan-allergens across these species. The same patients were subjected to an SPT with commercially available Spirulina in order to assess whether the suspected cross-reactivity—previously indicated by their sensitization profiles toward mites, crustaceans, and fish—would translate into a measurable in vivo response.

With regard to the skin prick test, only two patients (3 and 7) tested positive, and both were sensitized to mites and crustaceans. However, the same pattern was not observed in other patients with similar sensitization profiles (such as patients 6 and 8).

Dot blot analysis in triplicate against C-PC and BSA as a negative control. As shown in Fig. 3b, c, BSA, used as a negative control, showed consistently low background reactivity across all samples, confirming the specificity of the assay. In contrast, several patients displayed a measurable signal against C-PC, although with substantial interindividual variability.

Notably, patients who tested negative to all prick tests (patients 23 and 24) showed no detectable reactivity toward C-PC. This strongly suggests that the binding observed in other individuals is not due to nonspecific IgE interactions. Among allergic patients, the intensity of the C-PC signal varied widely: some individuals, such as patients 1, 2, 7, 10, 12, 16, exhibited an elevated antigen/negative-control ratio, whereas others showed only weak or borderline reactivity. When comparing C-PC reactivity with the allergen

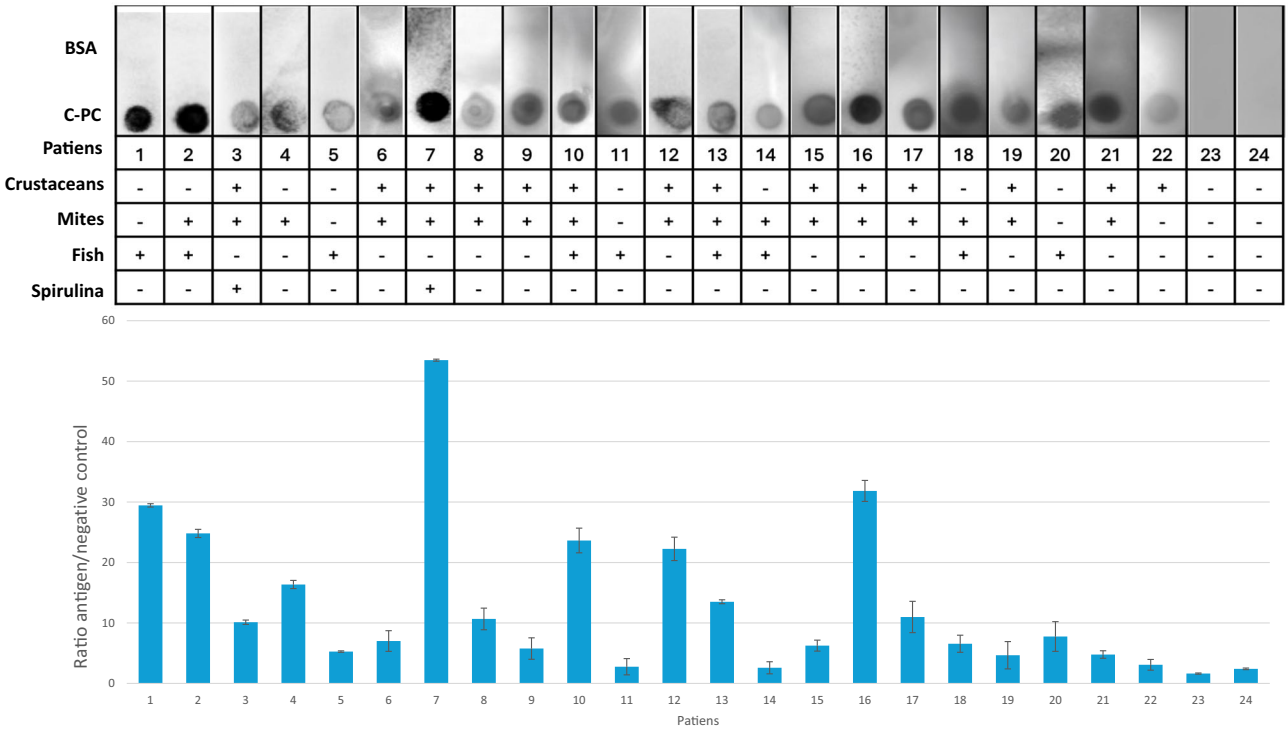


Fig. 3 | IgE reactivity of Spirulina C-PC. a Dot blot IgE immunoassay of C-PC using sera from patients with allergic sensitization to crustaceans, fish, mites, and Spirulina, as confirmed by skin prick test (SPT), and from two nonallergic individuals with negative SPT results. BSA was used as a negative control. The table reporting "+/–" indicates the SPT outcome for crustaceans, mites, fish, and Spirulina, respectively. **b** Bar chart of the quantification of dot blot signals expressed as mean integrated density ± standard deviation (SD), calculated from three independent replicates using ImageLab. For each patient, the bar represents the average C-PC signal normalized to the corresponding BSA spot (negative control), while error bars indicate the standard deviation.

profiles obtained from prick tests, no single sensitization category emerged as clearly associated with stronger IgE binding. Importantly, patient 7, who tested positive for the Spirulina prick test, showed the strongest response in the dot-blot assay. However, this pattern was not consistent across all Spirulina-sensitized individuals, as patient 3 did not display a comparable level of reactivity. Patients sensitized to mites, crustaceans, fish, or Spirulina all showed variable responses, with both reactive and nonreactive individuals within each group. This pattern indicates that C-PC recognition cannot be attributed to a single sensitization profile and may instead reflect broader cross-reactivity mechanisms.

The fact that many patients sensitized to unrelated allergen families still reacted to C-PC supports the possibility of an IgE-mediated cross-reactive response. At the same time, the observed heterogeneity among Spirulina-sensitized subjects suggests a more complex sensitization pattern. Notably, patients 3 and 7, although both tested positive in the Spirulina Skin Prick Test, displayed markedly different reactivity toward C-PC in the dot blot assay. Furthermore, not all Spirulina-sensitized patients showed strong reactivity to C-PC, indicating that this protein is unlikely to be the sole allergenic component of Spirulina. These findings suggest that additional Spirulina proteins may contribute to sensitization and allergic responses, or that distinct sensitization profiles may exist among Spirulina-allergic individuals. Further studies involving larger cohorts and a broader characterization of Spirulina proteins will be required to clarify the specific role of C-PC and to identify other potentially relevant allergens. Overall, these findings provide initial evidence that C-PC can be recognized by IgE from allergic patients, while remaining completely unrecognized by nonallergic controls. This supports the hypothesis that C-PC may contribute to allergic responses in sensitized individuals, although the molecular basis of this interaction remains to be clarified. Future studies involving larger cohorts and other Spirulina proteins will be essential to determine the prevalence of C-PC sensitization and to identify potential cross-reactive allergen families.

Simulated digestion

To assess the susceptibility of C-PC to proteolysis, the protein was subjected to a simplified in vitro digestion using the major gastrointestinal proteases[28]. The standard INFOGEST protocol was originally developed for complex food matrices and, when applied to purified proteins, may result in extensive hydrolysis that reduces the recovery of intermediate digestion products and complicates sequence-based analyses. Despite the fact that the adopted simplified digestion protocol has limitations in mimicking physiological gastrointestinal conditions (e.g., glycation reactions or other peptide modifications resulting from interactions with the matrix), milder digestion conditions used in this study were intended to preserve intermediate digestion products, allowing the exploratory evaluation of whether C-PC digestion could generate peptide sequences corresponding to known allergenic epitopes. To evaluate possible nonspecific proteolytic phenomena, in addition to the digested C-PC samples, control samples containing only buffer and digestive enzymes were also prepared (GB, gastric blank; IB, intestinal blank).

To evaluate the effect of digestion on IgE reactivity, SDS–PAGE and immunoblotting assays were performed. A dot blot was carried out using the serum of patient 7, who had demonstrated the strongest IgE response to C-PC in previous assays. As shown in Fig. 4a, in the SDS–PAGE, the band corresponding to the molecular weight of C-PC already displays a marked decrease in intensity after the gastric digestion (G) compared with the undigested sample (ND), and it disappears completely following intestinal digestion (I). No bands are detected even when samples are subjected to TCA precipitation (G-TCA, I-TCA). As can be seen in Fig. 4b, strong IgE binding was only detected for the undigested C-PC sample, whereas no signal was observed for the gastric- and intestinal-digested fractions, including those subjected to TCA precipitation. These results suggest that digestion reduces IgE recognition. This indicates that the linear or conformational epitopes responsible for allergenicity may be completely

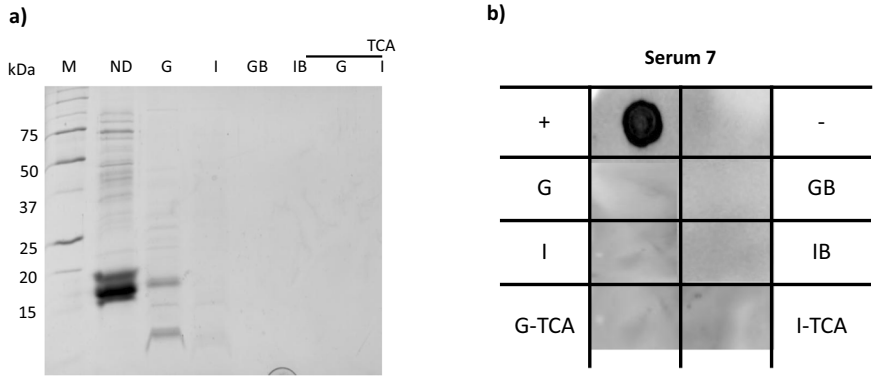


Fig. 4 | SDS-PAGE of simulated digestion of C-PC and dot blot assay. a 16% SDS–PAGE of C-PC subjected to simulated gastric and intestinal digestion. **b** Dot blot IgE immunoassay of C-PC after simulated gastric (G) and intestinal (I) digestion, performed using serum from patient No. 7. Undigested C-PC was used as a positive control (+), and BSA as a negative control (−). M protein standards, ND non-digested C-PC sample, G gastric-digested C-PC, I intestinal-digested C-PC, G-TCA TCA-precipitated gastric-digested C-PC, I-TCA TCA-precipitated intestinal-digested C-PC, BG and BI control samples containing saline solutions and digestive enzymes from the gastric and intestinal phases, respectively.

Table 1 | Patient sera used in IgE-immunoassays

ID patient	Age	Sex	Skin Prick test (SPT)				Total IgE	RAST	ISAC
			Crustaceans	Mites	Fish	Spirulina			
1	28	F	−	−	+	−	19.2	Shrimp 0,15, fish 1,32	cyp c1 1.52
2	43	F	−	+	+	−	165	Shrimp 0.33, fish 0.19	cyp c1 0.17
3	30	M	+	+	−	+	48.9		Ani s3, Bla g7, der p10, pen m1
4	30	M	−	+	−	−	33		
5	39	M	−	+	+	−	482	Shrimp 3.77	
6	30	F	+	+	−	−	683	Shrimp 1.81	pen a 1 1.76
7	54	M	+	+	−	+	106	Shrimp 0.90	
8	37	M	+	+	−	−	126		pen a1 17.5
9	24	F	+	+	−	−	306	Shrimp 1.46	
10	24	F	+	+	+	−	536		
11	36	M	−	−	+	−	1830	Shrimp 0.35	
12	44	F	+	+	−	−	105		
13	34	F	+	+	+	−	718	*D. pteronissius* >100, Shrimp 2.47	
14	30	M	−	+	+	−	311		
15	24	M	+	+	−	−	186		
16	34	F	+	+	−	−	48	Shrimp 2.15	
17	22	F	+	+	−	−	4300	Shrimp 18.4	
18	25	F	−	+	+	−	175		
19	58	F	+	−	−	−	50.3		
20	68	F	−	−	+	−	33.7	Fish 0.31	
21	49	M	+	+	−	−	199		
22	73	F	+	−	−	−	126	Shrimp 0.29	
23	56	M	−	−	−	−	63		
24	75	M	−	−	−	−	132		

+ = positive SPT result; − = negative SPT result.

degraded during gastrointestinal processing, or they are present at a very low concentration. It should also be noted that dot blot analysis has intrinsic sensitivity limitations. In particular, the concentration of individual digestion-derived peptides in the analyzed samples is unknown and may vary considerably, potentially affecting their detectability and apparent IgE reactivity. Therefore, the absence of a detectable signal should be interpreted with caution and not considered definitive proof of the complete loss of IgE-binding capacity. Future studies employing purified peptides or quantitative targeted analyses will be necessary to more accurately evaluate the persistence of IgE-binding sequences following gastrointestinal digestion.

Peptide fraction released after digestion

The complete list of identified peptides in the sample subjected to the digestion experiment is provided in Table S1 (Supplementary Information), whereas Table 1 reports only peptides with a length ≥8 amino acids, which is generally considered the minimal requirement for defining a linear epitope[29,30]. In terms of relative abundance, peptides <8 amino acids accounted for approximately 32% of the detected peptide pool, whereas peptides ≥8 amino acids represented approximately 68%.

The resulting data refer only to the intestinal digestion phase, as no peptides were detected by LC–MS during the gastric step. This is likely due to incomplete protein hydrolysis, as also suggested by the SDS–PAGE

profile (Fig. 4a), where a band corresponding to C-PC is still clearly visible at approximately 18 kDa, together with a peptide fragment of about 8 kDa. These bands are no longer observed after the intestinal digestion phase, indicating extensive protein hydrolysis. The absence of immunoreactivity following gastric digestion, despite only partial proteolysis, may suggest the involvement of conformational epitopes that are disrupted during the digestive process. Peptides derived from allophycocyanin subunits were also detected; their presence was expected, given the similar molecular weight of C-PC and allophycocyanin and the tendency of these phycobiliproteins to co-elute during purification[22,31], which can result in trace amounts of allophycocyanin remaining in the final preparation. Notably, such peptides had not been observed in earlier analyses, because those measurements were performed at lower resolution, which limited the ability to discriminate between the two highly similar protein species. For completeness, their sequences and corresponding peptide lists are reported in Table S1 (Supplementary Information). In addition, the relative abundance of C-PC and allophycocyanin is reported in Table S2 (Supplementary Information). However, as these proteins fall outside the scope of the present study, they are not discussed further here.

At this stage, none of the detected peptides showed features consistent with potential epitope activity. This observation aligns with the in silico predictions obtained using AlgPred 2.0 and BepiPred-3, to which the full amino-acid sequences of both the α- and β-subunits of C-PC were submitted. The analyses were performed using the default settings recommended for linear epitope prediction, which evaluate residue-level propensity scores and sequence patterns typically associated with IgE-binding regions. These analyses did not reveal any relevant linear epitopes, and the peptides identified in the digesta did not overlap with regions predicted to display epitope-like features. Consistently, dot blot assays performed on the digesta yielded negative results. This observation suggests that the loss of immunoreactivity following digestion may be due, at least in part, to the disruption of conformational epitopes associated with the native oligomeric structure of C-phycocyanin. Although no known allergenic epitopes were identified among the detected digestion products, the immunological relevance of the resulting peptides cannot be completely excluded and would require dedicated peptide-level studies to determine whether residual linear IgE-binding epitopes are retained after digestion. Additionally, sequence coverage of the β-subunit reached 81.9%, whereas the α-subunit showed a coverage of 35.8%. These data, together with the absence of any intact α-subunit detected by LC–MS analysis, indicate that the α-subunit is more susceptible to digestion and undergoes more extensive fragmentation, generating smaller peptides (two to three amino acids) which cannot be identified with accuracy. In any case, peptides of such short length are unlikely to be immunologically relevant.

Moreover, according to the results obtained by LiP experiments, the β-subunit has a well-defined exposed structure which can be attacked by enzymes. Given the high sequence coverage obtained after digestion, the enzymatic cleavage does not appear to proceed too rapidly. Furthermore, a possible explanation is that the initial cleavage of the more accessible β-subunit may destabilize the native C-phycocyanin complex, thereby increasing the susceptibility of the α-subunit to further proteolysis. Under these conditions, the α-subunit could be degraded into shorter peptides that are less readily detected, resulting in the lower sequence coverage observed at the digestion endpoint. Future studies will be needed to better elucidate how the amino-acid sequence of this fragment influences its interaction with proteolytic enzymes and its recognition by antibodies, particularly because the β-subunit of C-PC is a reported allergen, as discussed in the Introduction[16]. Altogether, the results seem to indicate a marked reduction in the IgE-binding capacity of C-PC following digestion. Despite that, sequence homology with known pan-allergens may suggest the presence of shared IgE-binding epitopes, but sequence similarity alone is not sufficient to predict clinically relevant cross-reactivity. The heterogeneous IgE responses observed among sensitized patients (Fig. 3) indicate that additional factors, including protein structure, epitope accessibility, and individual sensitization patterns, likely contribute to the recognition of C-PC.

Discussion

Microalgae have been consumed as food for centuries, particularly in Asia and South America, where they have long served as nutrient-rich dietary components. In recent years, their popularity has expanded to Europe as well, driven by their valuable nutritional properties. In the context of a rapidly growing global population and increasing pressure on conventional food systems, microalgae represent a promising alternative source of sustainable, high-quality proteins[32].

Within this framework, we selected Spirulina as our model species, given its status as one of the most widely consumed and commercially relevant microalgae worldwide. Its history of safe use as a food ingredient, together with its particularly attractive nutritional profile, underpins its expanding presence in European and global markets. Among the bioactive components of Spirulina, C-PC is the most abundant and functionally significant pigment–protein complex. Its inclusion in this study was further motivated by emerging evidence suggesting that C-PC—and particularly its β-subunit—may be involved in allergenic responses.

In this study, C-PC was successfully extracted and purified from Spirulina biomass using a two-step chromatographic workflow. The high A_{620}/A_{280} ratios obtained after purification confirmed minimal contamination by non-target proteins and the preservation of intact chromophore–protein complexes, ensuring suitability for downstream biochemical and immunological analyses. Structural characterization further demonstrated that the purified protein retained its native oligomeric state and secondary structure.

The immunoreactivity of C-PC was evaluated by dot blot analysis using sera from a small cohort of patients selected on the basis of sensitization profiles potentially associated with cross-reactivity toward dust mites, crustaceans, and fish. The results showed that C-PC can be recognized by IgE in some individuals, supporting the need for further investigation of its immunological relevance.

However, it should be noted that the sample contains a small amount of allophycocyanin. Therefore, a potential contribution of allophycocyanin to the observed IgE reactivity cannot be completely excluded. While some of the sequence homologies between the respective subunits are relatively low and may be considered of limited relevance, the sequence similarity between the β-subunits of C-phycocyanin and allophycocyanin is not negligible. Consequently, potential IgE cross-reactivity associated with allophycocyanin-derived peptides cannot be excluded. This, together with the exploratory nature of the study, the limited number of sera analyzed, and the fact that only two subjects showed a positive Skin Prick Test to Spirulina, these findings should be considered preliminary and not representative of the prevalence of C-PC sensitization in the allergic population. Rather, the data provide initial evidence supporting the need for further investigation of C-PC as a potential allergen. Future studies involving larger, clinically well-characterized and stratified cohorts, together with the assessment of additional Spirulina proteins, will be necessary to validate these observations and to further elucidate patterns of sensitization and potential allergenic cross-reactivity. Moreover, the discrepancy observed between patients 3 and 7, who were both positive in the Spirulina Skin Prick Test but exhibited different levels of reactivity toward C-PC in the dot blot assay, highlights the possibility that the immune response to Spirulina may also involve other allergenic proteins. This aspect requires further investigation to better characterize the spectrum of Spirulina allergens and the associated sensitization patterns.

Simulated gastrointestinal digestion provided further insight into the digestibility and stability of C-PC. Sequence coverage analysis revealed extensive fragmentation of the β-subunit (81.9%) compared with the α-subunit (35.8%), indicating that the α chain is more accessible and more readily degraded under digestive conditions. High-resolution LC–MS analysis of the digesta identified numerous peptides; however, none met the criteria for potential linear epitope activity. These results are consistent with in silico predictions, which did not identify significant epitopes, and with IgE dot blot assays performed on the digesta, which yielded negative results. Although none of the detected C-PC-derived peptides matched the criteria

adopted for the identification of known linear epitopes, their potential immunological relevance cannot be completely excluded. In particular, given the proposed allergenic role of the β-subunit, further studies are needed to evaluate whether digestion-derived fragments retain IgE-binding regions not currently represented in available epitope databases.

Taken together, the biochemical, proteomic, and immunochemical evidence suggests a reduction in the IgE-binding capacity of C-PC following digestion. While IgE binding was observed for the intact protein in sera from Spirulina-sensitized patients, the loss of IgE reactivity after digestion highlights its limited stability in the gastrointestinal environment.

Overall, these findings underscore the importance of comprehensive immunological evaluation of microalgal proteins to assess potential allergenic risks and ensure compliance with safety requirements for their use in food applications. Further studies will be needed to characterize additional Spirulina proteins capable of eliciting IgE responses, as suggested by the serum screening performed here. Collectively, this work provides a clearer understanding of the digestive profile and immunoreactivity of C-PC and contributes to a more informed assessment of the safety of Spirulina-derived ingredients in food and nutraceutical products.

Methods

Extraction of C-PC from Spirulina

Spirulina was purchased from the SPIREAT company (Spireat Spirulina Pura, Organic farm, Caselle, BS, Italy), and C-PC was extracted from flakes of microalgae. First, the flakes were ground into a fine powder using a mortar and pestle. One gram of the dried Spirulina powder was suspended in 14 mL of 100 mM sodium phosphate buffer (NaP), pH 7, and incubated overnight at 4 °C on a rotary shaker. After incubation, the mixture was centrifuged at 20,000 × *g* for 30 min at 4 °C to separate the biomass from the supernatant. The resulting supernatant was collected into a Falcon tube and filtered using 0.20 µm filters to remove any remaining particles. The blue-colored supernatant, containing the crude C-PC extract, was stored at 4 °C for further steps.

Purification of C-PC from crude extract

The crude extract was diluted fivefold with milli-Q water and subjected to a two-step purification process. First, anion-exchange chromatography was performed. The sample was loaded at a flow rate of 2 mL/min onto a HiTrap Q HP column (GE Healthcare), pre-equilibrated with 25 mM sodium phosphate buffer, pH 7.5, and connected to an ÄKTA Pure FPLC system (GE Healthcare, Chicago, IL, USA). The column was washed with the same buffer, and bound proteins were eluted using a two-step NaCl gradient (0–1 M followed by 1–2 M). Samples were collected at various stages of the purification and analyzed spectroscopically to assess the purity of C-PC using the A_{620}/A_{280} ratio. Fractions showing the highest purity ($A_{620}/A_{280} > 1.8$) were pooled and concentrated using a 10 kDa centrifugal concentrator (Merck-Millipore, Burlington, MA, USA). These purified fractions were further analyzed by 16% SDS-PAGE and visualized using Coomassie Brilliant Blue staining.

The concentrated protein was then subjected to a second purification step by size-exclusion chromatography. The sample was loaded at a flow rate of 0.15 mL/min onto a Ultrogel® AcA 44 (Sigma Aldrich), pre-equilibrated with 50 mM sodium phosphate buffer pH 7.5, containing 150 mM NaCl. Peak protein fractions were identified based on the characteristic blue color of C-PC, collected, and further analyzed both spectroscopically and by 16% SDS–PAGE (see below), followed by Coomassie staining. The purified protein was stored at −20 °C.

Electrophoretic profile

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method described by Cabanillas et al.[33] with slight modifications. In general, gels of 15% of polyacrylamide were used to evaluate the protein profile of digested samples. The samples were run with a Mini-PROTEAN® Tetra System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) with 1× Tris/Glycine/SDS buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA), in nonreducing and reducing conditions, by adding 2× Laemmli Sample Buffer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and 2× Laemmli sample buffer with 5% solution of β-mercaptoethanol (following 5 min at 95 °C) in 1:1 ratio, respectively. The electrophoresis was set to 130 V for 15 min and then increased to 160 or 170 V for up to 45 min. The gels were stained with Coomassie Blue staining solution (G-250), further stained, following image acquisition with Gel Doc™ EZ Imager (Bio-Rad Laboratories, Inc., Hercules, CA, USA) or Bio-Rad ChemiDoc™ Imagers, and processed with Image Lab 5.2.1 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

CD-spectroscopy analysis

Far-UV CD spectra (195–250 nm) were recorded on a Jasco J-1500 spectropolarimeter (Applied Photophysics Ltd., Surrey, UK) equipped with a JASCO Peltier temperature controller. Measurements were performed using a 0.1 cm pathlength cuvette, a scan rate of 50 nm/min, and a bandwidth of 1.0 nm. C-PC was analyzed at a concentration of 0.3 µg/µL in 50 mM sodium phosphate buffer (pH 7.0) at 20 °C. Three individual scans were acquired, averaged, and converted to mean residue ellipticity (θ, deg cm^2 dmol^{-1}).

Analytical size exclusion chromatography

The size-exclusion chromatography (SEC) was performed using a Superdex 200 Increase 5/150 column connected to an ÄKTA Pure FPLC system (GE Healthcare, Chicago, IL, USA). A total of 100 µg of C-PC was loaded onto the column, which had been previously equilibrated with 50 mM sodium phosphate buffer containing 150 mM NaCl, pH 7.5. The molecular mass of C-PC was estimated based on a calibration curve constructed using protein standards of known molecular mass: bovine serum albumin dimer (132 kDa), bovine serum albumin monomer (66 kDa), and lysozyme (14.4 kDa).

Determination of C-PC concentration and purity

The concentration of C-PC was determined spectrophotometrically using a Jasco V-750 UV–visible spectrophotometer by measuring absorbance at 620 nm. The C-PC concentration was calculated using an extinction coefficient of $\varepsilon_{620} = 7.3 \times 10^5$ M^{-1} cm^{-1}, as reported by Boussiba and Richmond[34]. The purity of C-PC was evaluated from the absorbance ratio A_{620}/A_{280}, where A_{620} represents the maximum absorbance of C-PC and A_{280} corresponds to the absorbance of total proteins. According to established purity standards, C-PC with a ratio of 0.7 is classified as food grade, 3.9 as reactive grade, and values exceeding 4.0 as analytical grade[25,35].

Limited proteolysis experiments

Limited proteolysis of C-PC was performed by setting up a reaction containing 30 µg of protein in 90 µL of buffer (100 mM Tris–HCl, pH 8.5), yielding a final concentration of 0.33 µg/µL. A 15 µL aliquot was withdrawn and mixed with 5 µL of reducing SDS buffer. Subsequently, 5.3 µL of trypsin (1.5 µg/µL) was added to the reaction batch, and the reaction was left to gently shake at room temperature. At 5, 10, 20, and 40 min, 15 µL aliquots were collected, and each was immediately mixed with 5 µL of SDS buffer. All samples were heated at 100 °C for 4 min and analyzed on 15% acrylamide gels as described before. For samples intended for mass spectrometry analysis, limited proteolysis was performed under the same conditions, except that ultrapure water was used instead of buffer, and each aliquot was quenched by adding 5 µL of water only, without reducing the SDS buffer.

Simulated gastrointestinal digestion

Simulated gastrointestinal digestion of extracted C-PC was carried out as described by Delfino et al.[28]: Forty microliters of protein solution (corresponding to 80 µg of protein) were subjected to simulated gastrointestinal digestion. The sample was brought to 50 µL in HCl (final conc 1 mM, pH 2) and 1 µL of pepsin solution (1 mg/mL, 727 U/mg) was added; the simulated gastric phase was conducted at 37 °C for 3 h. Then, 30 µL of 100 mM NH_4HCO_3 (pH 7.8), 1 µL of trypsin solution (1 mg/mL, 1749 BAEE units/

mg solid), and 1 µL of chymotrypsin solution (1 mg/mL, 55 units/mg of protein) were added; the simulated intestinal phase was conducted at 37 °C for 4 h. At the end of the digestion, the sample was stored at −20 °C until analysis. The samples were digested in duplicate, and two control samples were subjected to the same procedure, but without the addition of digestive enzymes, to check for the presence of peptides deriving from autolytic cleavage.

To improve peptide sequence recognition after digestion, a fractional precipitation approach was applied. An aliquot of the digested sample was analyzed by mass spectrometry without further processing (final concentration 1 mg/mL), while a second aliquot was subjected to trichloroacetic acid (TCA) precipitation, as reported in literature[36], yielding a final concentration of 0.5 mg/mL. Following digestion, the samples were analyzed using 16% SDS–PAGE, mass spectrometry, and IgE dot-blot assay as described previously. Gel images were acquired using a ChemiDoc™ MP Imaging System (Bio-Rad, USA). All reagents and consumables were supplied by Bio-Rad (Hercules, CA, USA).

Liquid chromatography coupled to low-resolution mass spectrometry analysis

The intact protein was identified by performing liquid chromatography mass spectrometry (LC–MS) on the treated samples by using a UHPLC H-CLASS/TUV/SQD2 system (Waters, Milford, MA, USA), equipped with an ACQUITY UPLC Peptide BEH C18 (300 Å, 1.7 µm, 2.1 mm 170 × 150 mm) column (Waters, Milford, MA, USA) and an ACQUITY UPLC Peptide BEH C18 VanGuard™ (300 Å, 1.7 µm, 2.1 mm × 5 mm) pre-column (Waters, Milford, MA, USA). The analysis was performed as reported by Delfino et al.[28]. Data were acquired and processed using MassLynx™ software V4.0 (Waters, Milford, MA, USA). The mass spectra of proteins were processed using the MaxEnt function with the following parameters: ranges (5000:15,000), resolution (1.00 Da/channel), damage model (uniform gaussian, width at half height 0.750 Da), minimum intensity ratios (left 33%, right 33%), iterations (iterate to convergence).

Sequence coverage calculation

Sequence coverage for each C-PC subunit was determined by mapping all peptides identified by LC–MS/MS onto the corresponding full-length amino acid sequence. For each protein, all residues included in at least one experimentally detected peptide were marked as "covered." The percentage of sequence coverage was calculated as the ratio between the number of covered residues and the total number of residues in the theoretical protein sequence. This analysis was used to assess the extent and distribution of gastrointestinal fragmentation of purified protein.

Patient Sera

A total of 24 human serum samples were included in the study, obtained from individuals aged 28–75 years with a clinical history suggestive of allergic sensitization. Both sexes were represented. For each patient, data were collected on skin prick test (SPT) reactivity to common allergens (crustaceans, mites, fish, and Spirulina), total serum IgE levels (kUA/L), and, when available, RAST or ISAC assay results. SPTs for Spirulina were performed using commercial batches purchased from SPIREAT Company (Spireat Spirulina Pura, Organic Farm, Caselle, BS, Italy). Sera from patients 23 and 24 were used as negative controls, based on negative SPT results to all tested allergens. All clinical characteristics are summarized in Table 2.

All serum samples were stored at −20 °C until use. The study protocol was approved by the Ethical Committee of the University of Parma (Approval No. 893, study 289/2024/TESS/UNIPR), and written informed consent was obtained from all participants. Sample handling complied with institutional ethical guidelines for the use of anonymized human biological material.

IgG and IgE-immunoreactivity assays

The immunoreactivity of C-PC was analyzed using IgG and IgE Dot Blot assays. Dot Blot assays were performed as previously described[34]. Briefly, 0.5 µg of C-PC was spotted onto a nitrocellulose membrane (GE Healthcare, Chicago, IL, USA) and air-dried completely at room temperature. The membrane was blocked with 5% (w/v) skim milk in TBS-T buffer (20 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.5) for 45 min at room temperature, followed by washing (three times) with TBS-T.

Table 2 | List of the identified peptides analyzed by LC–HRMS in gastrointestinal digesta (intestinal endpoint)

Subunit	Fragment label	Peptide	Sequence length
α-subunit	F3–17	TPLTEAVSIADSQGR	15
	F3–18	TPLTEAVSIADSQGRF	16
	F5–17	LTEAVSIADSQGR	13
	F5–18	LTEAVSIADSQGRF	14
	F8–18	AVSIADSQGRF	11
	F9–17	VSIADSQGR	9
	F19–26	LSSTEIQV	8
	F19–28	LSSTEIQVAF	10
	F20–27	SSTEIQVA	8
	F20–28	SSTEIQVAF	9
	F21–28	STEIQVAF	8
	F45–57	TSKADSLISGAAQ	13
	F48–58	ADSLISGAAQA	11
	F48–60	ADSLISGAAQAVY	13
	F52–60	ISGAAQAVY	9
	F53–60	SGAAQAVY	8
	F95–104	VTYCLIAGGT	10
	F100–108	IAGGTGPMD	9
	F102–110	GGTGPMDEY	9
	F111–120	LIAGIDEINR	10
	F112–120	IAGIDEINR	9
β-subunit	F8–15	VVSQADTR	8
	F6–15	TKVVSQADTR	10
	F20–27	STAQIDAL	8
	F20–30	STAQIDALSQM	11
	F23–30	QIDALSQM	8
	F43–57	RITSNASTIVSNAAR	15
	F44–57	ITSNASTIVSNAAR	14
	F58–65	SLFAEQPQ	8
	F58–69	SLFAEQPQLIAP	12
	F61–69	AEQPQLIAP	9
	F62–74	EQPQLIAPGGNAY	13
	F62–69	EQPQLIAP	8
	F96–108	AVFAGDASVLEDR	13
	F96–105	AVFAGDASVL	10
	F99–108	AGDASVLEDR	10
	F120–133	LGTPGSSVAVGVGK	14
	F124–133	GSSVAVGVGK	10
	F118–133	LALGTPGSSVAVGVGK	16
	F140–147	AIVNDPAG	8
	F140–150	AIVNDPAGITP	11
	F142–150	VNDPAGITP	9
	F157–164	ASEIASYF	8

For IgG immunodetection, the membrane was incubated with a rabbit polyclonal anti-C-PC antibody (Bioss, Thermo Fisher Scientific, USA) diluted 1:200 as the primary antibody. Subsequently, a goat anti-rabbit IgG–HRP conjugated secondary antibody (Bio-Rad, USA) diluted 1:10,000 was applied.

For IgE immunodetection, the membrane was incubated for 1 h at room temperature with patient sera diluted 1:15. The sera were obtained from individuals with positive SPT to spirulina, crustaceans, fish, and/or mites, as well as from nonallergic control individuals. After washing, a goat anti-human IgE (ε-chain specific) peroxidase-conjugated secondary antibody (Sigma-Aldrich, USA) diluted 1:3000 was applied for 1 h at room temperature.

An internal negative control was included by spotting 0.5 µg of bovine serum albumin (BSA) onto the membrane, followed by incubation with the same diluted IgG antibody or patient serum used for C-PC detection. The experiments were conducted in triplicate.

Dot Blot signals were visualized by chemiluminescence. This was done using Clarity Max Western ECL Substrate and ChemiDoc™ MP Imaging System (Bio-Rad, USA). Signal intensities were analyzed and quantified with Image Lab™ software (Bio-Rad, USA) and then normalized to the corresponding negative control on the same membrane. The data were expressed as the ratio of serum to the negative control, where a value of two represented background intensity and higher values indicated increased reactivity. The results were displayed as bar charts, with the negative serum used as the reference. Control dot blots to assess potential direct binding of the secondary antibody were carried out following the same procedure, except that the patient serum incubation step was omitted.

Liquid chromatography coupled to high-resolution mass spectrometry analysis

Liquid chromatography high resolution mass spectrometry (LC–HRMS) on the treated samples was performed by using a UHPLC H-CLASS/TUV/SQD2 system (Waters, Milford, MA, USA), equipped with an ACQUITY UPLC Peptide BEH C18 (300 Å, 1.7 µm, 2.1 mm 170 × 150 mm) column (Waters, Milford, MA, USA), following the reported procedure[37]. Detection was performed using a Vion IMS QTof Mass Spectrometer (Waters, Milford, Massachusetts) under the following conditions: experiment type, peptide map (IMS); acquisition mode, HDMSE; ionization source, ESI; polarity, positive; analyzer mode, sensitivity; transmission mode, standard; capillary voltage, 3.00 kV; sample cone voltage, 40 V; source offset, 80 V; source temperature, 120 °C; desolvation temperature, 450 °C; cone gas flow, 50 L/h; desolvation gas flow, 800 L/h. HDMSE acquisition was performed from 3.5 to 29.1 min, with a scan range of 100–2000 m/z, scan time of 0.4 s, low collision energy of 6 V, and a high-energy collision ramp of 20–45 V. Leucine enkephalin was used for automatic lock-mass correction.

Data processing was carried out using UNIFI software (Waters, Milford, Massachusetts) with the following parameters: high-energy threshold, 250 counts; low-energy threshold, 1500 counts; maximum number of peaks per channel, 20000; digestion mode, nonspecific; minimum peptide length, 2 amino acids; minimum number of matched fragments, 2; minimum percentage of matched fragments, 20%. Protein identification was performed using the UniProt entries corresponding to C-PC subunits (P72508; P72509; A0A9P1KBV0; P72505).

Data availability

All data supporting the findings of this study are available within the paper and its Supplementary Information.

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Author contributions

D.D.: Investigation, Formal analysis, Writing—original draft. K.B.: Investigation, Formal analysis, Writing—original draft. S.C.: Investigation, Formal analysis, Writing—review & editing. L.C.: Investigation, Formal analysis, Writing—review & editing. E.R.: Collecting serum samples. D.C.: Investigation. T.T.: Supervision, Conceptualization, Writing—review & editing. C.F.: Supervision, Conceptualization, Writing—review & editing.

Competing interests

The authors declare no competing interests.

Additional information

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