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Molecular and Immunological Characterization of Grass Carp (*Ctenopharyngodon idella*) Parvalbumin *Cten i 1*: A Major Fish Allergen in Hong Kong

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Abstract

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Background: Grass carp is the most commonly consumed fish species in Hong Kong. The allergenicity of grass carp and its allergen content are yet to be reported. This study characterized the major allergen in grass carp and investigated its allergenicity.

Methods: 69 subjects with history of IgE-mediated allergic reaction to grass carp were recruited. The protein content in steamed grass carp extract was resolved by SDS-PAGE and the major allergen was identified by immunoblotting with serum from subjects allergic to grass carp. The identity of allergen was elucidated by mass spectrometry and amino acid sequence obtained by amplifying the specific gene from cDNA library of grass carp. The cross-reactivity between parvalbumins from grass carp and other phylogenetically close (common carp) or commercially important (cod and salmon) species were investigated by competitive inhibition ELISA.

Results: A major IgE binding protein was found at approximately 9 kDa and identified as parvalbumin by immunoblotting and mass spectrometry. Grass carp parvalbumin was more allergenic than common carp, salmon and cod parvalbumins despite sharing high sequence homology. This newly identified major allergenic parvalbumin isoform from grass carp was registered as *Cten i 1* in World Health Organization and International Union of Immunological Societies allergen database.

Conclusions: Grass carp parvalbumin is identified as the major fish allergen in Hong Kong. The strong allergenicity of *Cten i 1* contributes to the high IgE reactivity of grass carp. Grass carp, among other fish species, should be considered when managing fish-allergic patients.

Keywords: Cross-inhibition ELISA, cross-reactivity, fish allergy, food allergen, grass carp parvalbumin, immunoblot

Impact Statement

Grass carp parvalbumin is more allergenic than common carp, salmon and cod parvalbumins despite its high sequence homology. The strong allergenicity of 9 kDa parvalbumin isoform contributes to high IgE reactivity of grass carp. This newly identified allergen was registered as *Cten i 1* in the World Health Organization/International Union of Immunological Societies allergen database. Fish allergy diagnosis based on cod-specific IgE is suboptimal in Chinese patients. Grass carp or *Cten i 1* specific IgE tests should be included to improve the accuracy of fish allergy diagnosis in carp-consuming populations.

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INTRODUCTION

Food allergy is a common health problem affecting up to 10% of the general population and its prevalence has increased over the past 3 decades.¹ Allergic reactions can be life-threatening and even fatal, leading to heavy socioeconomic burden ² and negative impact on quality of life of food-allergic patients and families ³. While many foods can cause an allergic reaction, fish alongside cow's milk, hen's egg, peanut, tree nut and shellfish are responsible for more than half of all food allergic reactions.⁴ Fish is a leading cause of parent-reported, doctor-diagnosed adverse food reactions in Hong Kong, affecting 0.25% of its pre-school childhood population.⁵

Fish allergy has an early onset and persists throughout life.^{6,7} Allergic reactions to fish range from mild skin or gastrointestinal symptoms to life-threatening anaphylaxis.⁸ The major fish allergen is the heat-stable muscle protein parvalbumin. It is highly conserved in fish and leads to clinical and serological cross-reactivity among different fish species.⁹⁻¹¹ Collagen, aldolase and enolase are other fish allergens registered in the World Health Organization and International Union of Immunological Societies (WHO/IUIS) allergen database. However, these proteins are heat-labile in nature and their clinical relevance might be limited to populations that commonly consume raw fish.^{12,13}

Codfish has been traditionally used as a model in fish allergy research, and its allergen as a representative marker for fish allergy diagnosis.¹⁴ Nevertheless, variable allergenicity was found among different fish species due to the differential expression levels of parvalbumin in muscle^{15,16}, as well as the varying allergenic property of parvalbumin.¹⁷ Some fish allergic patients can tolerate selective fish species even if they had specific IgE (sIgE) against another fish species.¹⁸ In addition, the IgE reactivity to different fish species is determined by the fish consumption pattern.¹⁹ Testing with diet-relevant instead of generic fish species can improve diagnostic accuracy of fish allergy.

Grass carp (*Ctenopharyngodon idella*) is the most commonly consumed fish species in Hong Kong.²⁰ Unlike cod or salmon in which the allergens and clinical relevance are well characterized, the allergenicity of grass carp remains largely unknown. The physiochemical properties of parvalbumin from grass carp was previously characterized²¹, but the allergenicity, clinical relevance and cross-reactivity with other parvalbumins have not been reported.

This study aimed to characterize the molecular and immunological properties of grass carp parvalbumin among subjects allergic to grass carp in Hong Kong. Since grass carp would only be consumed in a cooked form, this study focused on allergens found in cooked grass carp extract. We also compared IgE reactivity of grass carp against that of cod, salmon and common carp (*Cyprinus carpio*) at both extract and component levels. Cod and salmon were chosen for comparison due to their commercial importance, and common carp was chosen due to its close phylogenetic relationship with grass carp.²² Lastly, we identified and sequenced the major allergenic parvalbumin isoform from grass carp and registered it as *Cten i 1* in WHO/IUIS allergen database (<http://www.allergen.org/viewallergen.php?aid=951>).

METHODS

Patient characteristics

Sixty-nine subjects were recruited from all four paediatric allergy referral centres in Hong Kong in 2016-2019. Patients were included if they had physician-diagnosed IgE-mediated allergy against grass carp, which was defined as allergic reactions occurring within 2 hours of grass carp intake within two years before recruitment, and a positive sIgE (≥ 0.35 kU_A/L) against grass carp by ImmunoCAP (Thermo Fisher, Uppsala, Sweden). Sixteen of the subjects were confirmed to be

129 grass carp allergic by double-blind placebo-controlled food challenge.²³ Patients were excluded if
130 they reported allergic reactions to fish but failed to identify grass carp as the causative species. All
131 subjects had blood taken for sIgE measurement against cod, salmon, grass carp, r*Gad c 1* and r*Cyp*
132 *c 1* by the ImmunoCAP assay on a Phadia 250. Our team collaborated with Thermo Fisher to
133 develop the experimental ImmunoCAP assay for grass carp according to published method.²⁴ Five
134 subjects with no history of IgE-mediated food allergy were included as control. This study was
135 approved by institutional review boards of all hospitals, and subjects and/or their parents gave
136 informed written consent to participate.

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138 **SDS-PAGE and immunoblotting of grass carp extracts**

139 20 µg of grass carp extracts were separated in a 13.5% SDS-polyacrylamide gel and the protein
140 bands were stained with SimplyBlue SafeStain (Thermo Fisher). The proteins were then
141 transferred to a PVDF membrane (Bio-rad) with the Trans-Blot Turbo (Bio-rad) after separation
142 on the SDS-PAGE gel. Non-specific binding of the membrane was blocked with 5% non-fat dry
143 milk in TBST (blocking solution) for 1 h at room temperature. The presence of parvalbumins were
144 detected by anti-parvalbumin antibody (Sigma-Aldrich). Twenty-three serum samples representing
145 different range of IgE levels, symptoms, cross-reactivity and age were chosen for probing IgE-
146 binding proteins. The antibody (1:10000) and serum samples (1:5, 1:10 or 1:20) were diluted in
147 blocking solution and incubated with the membrane overnight at 4°C. The membrane was then
148 washed with TBST and incubated with HRP-conjugated anti-mouse (1:20000) or anti-human IgE
149 (1:1000) (Southern-Biotech) secondary antibodies. Parvalbumins and IgE-binding proteins were
150 visualized by incubating the membrane with SuperSignal West Pico Plus chemiluminescent
151 substrate (Thermo Fisher) and the chemiluminescent signal was detected with a ChemiDoc MP
152 imaging system (Bio-rad).

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154 **Enzyme-linked immunosorbent assay (ELISA)**

155 Purified parvalbumins (5 µg/ml) diluted in coating buffer (100 mM Na₂CO₃, 100 mM NaHCO₃,
156 pH 9.6) were coated onto Maxisorp microtiter plates (Nunc) and incubated at 37°C for 3 h. The
157 plates were washed with 0.05% Tween 20/PBS (PBST) and blocked with 8% fetal bovine serum
158 (Gibco) diluted in PBS at room temperature for 2 h. Serum samples were diluted with blocking
159 buffer at 1:10 or 1:20 and incubated at 4°C overnight. The plates were then washed with PBST
160 and incubated with biotinylated anti-human IgE antibodies at 1:1000 dilution (Vector Labs) at

room temperature for 1 h. After washing with PBST, the plates were incubated with HRP avidin D at 1:1000 dilution (Vector Labs) at room temperature for 30 min, washed with PBST for five times and incubated with 100 µl/well of 1-step Ultra TMB-ELISA Substrate Solution (Thermo Fisher) for colour development. The reaction was terminated by adding 0.1 M sulfuric acid and the optical density (O.D.) at 450 nm was measured using a microplate reader (BioTek). Results were considered positive only if the O.D. is two-fold higher than those of negative controls. For cross-inhibition ELISA, the inhibitor was co-incubated with pooled serum samples at various concentrations for 2 h at room temperature before adding to ELISA plates coated with grass carp parvalbumin. Each assay was performed with 4 different pooled serum samples, each containing serum from four randomly selected patients diluted at 1:10.

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172 **Statistical analysis**

Statistical analyses were performed by GraphPad Prism 8. Differences in sIgE among different fishes or components were compared using Friedman's ANOVA test and Dunn's multiple comparisons post-hoc test. The correlations between total IgE and sIgE against cod, salmon and grass carp measured by ImmunoCAP were compared by Spearman test. A p-value <0.05 was considered statistically significant.

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180 **RESULTS**

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182 **Patient characteristics**

The median age of fish-allergic subjects at evaluation was 6 years old (range 2-16), and 62.3% were male. The most common clinical features associated with fish allergy were angioedema and urticaria. Grass carp, followed by salmon and grouper were the most common fish species reported to have caused allergic reactions (Supplementary Fig. 1). Twenty-two subjects (35.5%) were able to tolerate at least one fish, with salmon being the most commonly tolerated fish species (n=16, 72.7%). Table 1 summarizes their clinical features and sIgE values.

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190 **IgE reactivity to fish extracts and recombinant parvalbumins**

Specific IgE levels against cod, salmon, grass carp, and recombinant parvalbumins r*Gad c 1* and r*Cyp c 1* were measured by ImmunoCAP test (Fig. 1a). Specific IgE against cod (median: 1.75

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193 kU_A/L; IQR: 0.44-4.84 kU_A/L) and salmon (median: 1.34 kU_A/L; IQR: 0.24-4.04 kU_A/L) were
194 lower than to grass carp (median: 4.85 kU_A/L; IQR: 1.97-18.7 kU_A/L). Fifteen (21.7%) and 17
195 (24.6%) subjects had negative ImmunoCAP (<0.35 kU_A/L) against cod and salmon, respectively.
196 Alternatively, all 69 subjects had a positive ImmunoCAP value against grass carp. For the two
197 recombinant parvalbumins, sIgE against rCyp *c 1* (median: 6.40 kU_A/L; IQR: 2.19-21.4 kU_A/L)
198 was also higher than that to rGad *c 1* (median: 3.83 kU_A/L; IQR: 1.62-11.6 kU_A/L). Specific IgE
199 against grass carp was strongly correlated with both rCyp *c 1* (Fig. 1b, $r = 0.87$) and rGad *c 1* (Fig.
200 1c, $r = 0.85$). When comparing IgE reactivity against cod extract and cod parvalbumin, sIgE
201 against rGad *c 1* was higher than that of cod extract ($p < 0.0001$). Strong correlations were also
202 found among fish sIgEs by ImmunoCAP ($p < 0.0001$; $r = 0.77-0.95$). All five non-allergic controls
203 were negative (<0.35 kU_A/L) against all five ImmunoCAP assays.

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205 **Identification of a major IgE binding protein in grass carp extract**

206 The identity of grass carp purchased from wet market was confirmed by DNA barcoding of the
207 COI gene (Supplementary Table 1). SDS-PAGE of raw and steamed grass carp extract showed
208 that a number of proteins were denatured after steaming, but several heat-resistant proteins
209 remained (Fig. 2a). Immunoblotting of the steamed grass carp extract with anti-parvalbumin
210 antibody identified two parvalbumin isoforms with a molecular weight of 11 kDa and 9 kDa,
211 respectively (Fig. 2b, lane "PV"). The two parvalbumin isoforms were subsequently isolated for
212 mass spectrometric analysis. Serum samples from 23 selected subjects revealed the 9 kDa
213 parvalbumin, but not the 11 kDa isoform, to be the major IgE binding protein. In addition, most
214 subjects were solely reactive to the 9 kDa parvalbumin, with only a few subjects (P37, P40, P53,
215 P65) exhibited additional IgE reactive bands of higher molecular weights (Fig. 2b).

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217 **Characterization of two parvalbumin isoforms in grass carp**

218 The two parvalbumin isoforms were excised from SDS gels and the peptide fingerprints were
219 analyzed by mass spectrometry. The identity of the isoforms was revealed by aligning the
220 identified peptides with the reported grass carp transcriptome, and the full-length amino acid
221 sequence of the isoforms were confirmed by PCR with specific primers (Table 2). The 9 kDa
222 parvalbumin shared a high sequence homology with Cyp *c 1* (91.7%) and moderate similarity with
223 the cod parvalbumin Gad *m 1* (79.8%), but less so to Gad *c 1* (68.8%) and Sal *s 1* (68.8%).
224 Alternatively, the 11 kDa parvalbumin was more similar to the alpha-parvalbumins from salmon

(77.1%) and chicken (67.0%), rather than the other beta-parvalbumins (49.5-59.6%) (Supplementary Table 2).

IgE reactivity and cross-reactivity of parvalbumins from different species

Native parvalbumins (*Cten i 1*, *Gad m 1*, *Sal s 1* and *Cyp c 1*) were purified to homogeneity by anion-exchange and size-exclusion chromatography (Supplementary Fig 2). IgE reactivity against *Cten i 1* was found to be highest among all, followed by *Cyp c 1* and *Gad m 1* (Fig. 3). On the contrary, reactivity to salmon parvalbumin *Sal s 1* was lowest, where 25 subjects (36.2%) had IgE levels lower than the threshold value. In accordance with the sequence homology, *Cyp c 1* was able to inhibit 80% of IgE binding to grass carp parvalbumin at the highest concentration, closely followed by the cod parvalbumin *Gad m 1* (Fig. 4). Alternatively, *Sal s 1* was only able to inhibit around 50% of IgE binding to grass carp parvalbumin even at the highest concentration.

DISCUSSION

Diagnosis of fish allergy is often hampered by the huge variety of consumable fish species and the varying allergenicity among different fish species. To date, most studies on fish allergy focused on commercially important species consumed in the West such as cod, salmon, mackerel, hake and pilchard, but the allergenicity and molecular nature of allergens in other fish species remain largely unknown. Except for salmon, the aforementioned fish species have low consumption in Hong Kong and their taxonomies are distantly related to grass carp (Cyprinidae), the fish species most commonly consumed in this region. In the absence of suitable assays, sIgE against cod and salmon are often chosen as the substitute test when evaluating patients with suspected fish allergy.⁸ Yet, the diagnostic value and clinical relevance of sIgE to cod and salmon are poor in Hong Kong population and we would like to explore whether a diet-relevant species (i.e. grass carp) could serve as a better diagnostic tool. More importantly, grass carp is the top aquaculture species globally, counting up to 5.5 million tonnes produced a year and being farmed in 38 different countries.²⁵ We believe our findings could be of great significance to other countries (e.g. China, Bangladesh, etc.) where grass carp serve as the staple food fish rather than exotic marine species.

In the present study, we investigated the allergenicity of the grass carp as well as the molecular aspects of its parvalbumin *Cten i 1*. In addition, we determined the diagnostic value of

257 cod and salmon sIgE in our grass carp allergic patients. Our data showed that grass carp had a
258 higher IgE reactivity when compared to cod and salmon, such that a substitute test using cod or
259 salmon would lead to lower sensitivity and falsely negative results. The strong IgE reactivity of
260 grass carp was likely contributed by both the high IgE binding capacity of *Cten i 1* (Fig. 3) and the
261 high parvalbumin content in grass carp muscle.¹⁵ In our study, parvalbumin was the only clinically
262 relevant allergen in grass carp. Immunoblotting of 23 individual patients showed that 20 of them
263 were monosensitized to parvalbumin. In addition, all 69 subjects tested were IgE positive to native
264 *Cten i 1* by ELISA and 68 of them were positive against *Cyp c 1* by ImmunoCAP. Last but not
265 least, the use of grass carp ImmunoCAP, which was prepared with raw tissues, did not render a
266 higher IgE reactivity when compared to using *Cyp c 1* or *Gad c 1* alone (Fig. 1a). A strong
267 correlation was also observed between grass carp-specific IgE and r*Cyp c 1*- (Fig. 1b) or r*Gad c 1*-
268 (Fig. 1c) specific IgE. Taken together, our data suggested that parvalbumin was the predominant
269 allergen in most patients, although other minor allergens could be present in a few subjects. This is
270 not surprising as parvalbumin is the only heat-stable allergen, and that grass carp is usually cooked
271 thoroughly for consumption to avoid *Clonorchis sinensis* infection. Other allergens like aldolase
272 and enolase are heat-sensitive proteins and likely to be degraded during the cooking process.

273 The complete sequences of the two parvalbumin isoforms in grass carp were reported for the
274 first time in this study, and their sequences were nearly identical to the theoretical sequences
275 suggested in a previous study.²¹ Interestingly, only one of the parvalbumin isoforms, namely *Cten*
276 *i 1*, was allergenic as shown by immunoblotting. This difference in allergenicity could be
277 explained by the sequence homology, as the non-allergenic isoform at 11 kDa was more similar to
278 parvalbumins of the alpha lineage and shared less than 60% of sequence identity with *Cten i 1*.
279 The high allergenicity of *Cten i 1* was not unexpected as it shared high sequence homology with
280 *Cyp c 1* (91.7%), which was previously reported to contain most of the fish-specific IgE
281 epitopes.²² Our ELISA data also supported this finding where IgE reactivities against *Cten i 1* and
282 *Cyp c 1* were significantly higher than *Gad m 1* and *Sal s 1*. In addition, we demonstrated that *Gad*
283 *m 1* and *Sal s 1* were only able to inhibit 60% to 70% of IgE binding against *Cten i 1*. These
284 findings suggested the presence of additional or unique IgE binding epitopes in *Cten i 1*. One
285 limitation of this study is that we were not able to test the allergenicity of *Cten i 1* in other cohorts,
286 and our findings might be applicable only to those populations where carps serve as important
287 food fish.

288 In conclusion, this study reported the identification and characterization of the major
289 allergen *Cten i 1* in grass carp (*Ctenopharyngodon idella*). Grass carp had a higher IgE reactivity
290 than cod and salmon, which was contributed by the high allergenicity of its parvalbumin *Cten i 1*.
291 Our results showed that fish allergy diagnosis based on cod ImmunoCAP, the most commonly
292 chosen surrogate test, was suboptimal in Chinese due to higher likelihood of a falsely negative
293 result. Grass carp or *Cten i 1* specific IgE tests should be included to improve the accuracy of fish
294 allergy diagnosis in carp-consuming populations.

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304 **CONFLICT OF INTEREST**

305 The authors declared that they have no conflict of interest in relation to this manuscript.

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Table 1. Summary of clinical histories and laboratory data of study subjects

	Sex	Age	SPT	SPT	Fish-related symptoms	Total IgE (kU/L)	Specific IgE by ImmunoCAP (kUA/L)				Specific IgE by ELISA (OD450nm)				
			fish mix (mm)	salmon (mm)			Cod	Salmon	Grass carp	rCyp c 1	rGad c 1	Cten i 1	Gad m 1	Sal s 1	Cyp c 1
[§] P1	F	16	6	5	Ana, Ae, Gl, O, U	946	1.45	0.78	4.85	4.61	2.32	0.35	neg	neg	0.23
P2	M	16	7	2	Ery, U	1674	0.39	neg	1.75	1.51	0.53	0.26	neg	neg	0.18
P3	M	16	9	5	Ae, Ery, O	3193	neg	neg	0.74	1.21	0.68	0.24	neg	neg	0.19
[§] P4	F	5	9	7	Ae, U	285	2.54	2.22	9.66	9.79	5.36	0.91	0.70	0.47	0.73
[§] P5	M	11	7	9	Ae, O, U	343	1.15	1.09	3.28	2.22	2.03	0.65	0.52	neg	0.54
[§] P6	M	9	5	2	Ana, Ae, Ery, Gl, O	720	neg	neg	1.90	1.89	1.23	0.67	0.35	neg	0.48
[§] P7	F	7	5	8	Ana, Ae, Cu, Gl, O, U	668	2.56	1.41	7.34	8.70	5.14	0.51	neg	neg	0.34
[§] P8	M	14	4	4	Ae, Ery, O, U	614	3.09	neg	6.41	3.62	4.66	0.65	0.52	neg	0.54
P9	F	14	9	2	Ana, Ae, Cu, Res	2283	neg	neg	3.64	0.77	3.07	0.28	neg	neg	neg
[§] P10	F	11	11	5	Ery, O, U	69.7	neg	neg	0.62	0.81	neg	0.29	0.21	neg	0.19
[§] P11	M	6	9	5	Ery, U	1913	2.58	1.34	11.10	7.90	6.02	0.55	0.32	0.31	0.43
[§] P12	M	4	0	0	Ae, Ery, U	28.4	neg	neg	2.01	2.95	1.60	0.57	0.29	0.20	0.49
[§] P13	F	8	5	7	Ery, U	1195	5.06	4.50	8.89	11.10	7.51	1.08	1.00	0.95	0.75
P14	M	6	6	4	Ae, Ery	2318	21.40	59.00	53.20	72.00	42.20	1.34	1.11	1.17	1.16
[§] P15	F	9	N.D	N.D.	Ae, O, U	762	6.73	7.33	18.60	21.70	12.10	1.59	1.32	0.95	0.88
P16	M	3	N.D.	N.D.	Ery	592	0.52	1.61	2.05	2.43	0.44	1.04	0.85	0.55	0.73
P17	F	2	N.D.	N.D.	Ery	90.7	1.12	neg	4.61	5.83	3.17	0.82	0.44	neg	0.39
P18	F	4	5	2	Ae	173	neg	neg	1.22	1.66	0.69	0.31	neg	neg	neg
P19	M	8	N.D.	N.D.	Ae, Ery, O, U	691	1.40	1.74	2.10	2.55	2.26	0.52	0.29	0.34	0.46
[§] P20	M	3	3	0	Ae, Ery, U	4481	6.45	5.59	25.20	38.10	27.60	1.26	0.64	0.30	1.28
[§] P21	M	9	6	8	Gl	4359	40.50	71.80	83.10	214.00	179.00	1.84	1.74	1.52	1.60
P22	M	8	7	5	Ery, Gl, O, U	647	0.82	2.47	2.87	5.50	2.93	0.53	0.30	0.19	0.33
P23	M	2	N.D.	N.D.	Ae, Neu, U	740	1.14	2.67	2.65	6.50	2.65	0.39	0.32	0.23	0.30
P24	F	2	4	7	Ana, Ae, Gl, O, U	266	2.80	1.34	16.50	7.89	5.74	0.61	0.48	0.44	0.56
[§] P25	F	16	7	7	Ae, O, U	343	1.39	0.64	2.68	1.32	2.01	0.45	0.27	0.22	0.32
P26	M	13	10	6	Ana, Ae, Gl, O, U	285	0.97	0.42	3.36	6.40	3.90	0.36	0.24	0.18	0.20
[§] P27	F	11	7	N.D.	Ae, Ery, U	3167	10.40	2.69	31.80	36.80	29.60	0.88	neg	neg	0.40
P28	F	6	N.D.	N.D.	Ae, Ery, U	1311	25.10	6.73	39.10	33.30	29.00	1.23	1.43	1.20	1.60
P29	M	8	3	0	Ery, O, U	4084	1.92	1.91	3.13	7.80	7.26	0.24	0.18	neg	0.20
[§] P30	M	6	11	10	Ae, Ery, Neu, O, U	145	0.53	1.60	4.40	7.44	3.57	1.69	0.58	0.63	0.92
[§] P31	M	7	5	9	Ery, O	4400	1.37	4.78	4.95	6.05	3.89	0.49	0.42	0.33	0.31
[§] P32	F	8	7	5	Ery, O, U	617	3.94	1.81	19.60	27.00	10.30	0.51	0.28	neg	0.46
[§] P33	M	3	5	4	Ery, U	2934	30.80	27.40	71.00	174.00	72.00	1.72	0.97	0.26	1.44
[§] P34	M	6	7	4	Ae, Neu, O, U	2409	16.30	19.40	68.70	38.90	43.00	1.86	1.76	1.59	1.79
[§] P35	M	4	7	4	Ae, Ery, O, U	664	4.54	1.06	12.20	14.90	11.10	0.87	0.41	0.59	0.61
P36	M	4	0	5	Ae, Ery, O, U	409	0.70	4.58	1.92	5.01	3.45	0.45	0.26	0.23	neg
[§] P37	M	5	0	0	Ae, Ery, O	>5000	14.40	63.10	22.40	40.20	36.90	1.79	1.69	1.40	1.19
P38	F	3	10	3	Ae, O, U	359	2.54	1.99	10.50	14.60	5.64	1.09	1.14	1.10	1.18
[§] P39	M	2	13	6	Ae, O, U	1033	4.80	3.37	10.30	9.80	8.71	1.55	0.64	0.37	0.69
[§] P40	M	16	5	6	Ae, Gl, O, U	329	0.93	1.02	4.80	5.91	2.85	1.31	1.16	0.68	0.94
P41	M	11	7	8	Ae, Gl, O, Res	449	1.75	1.18	3.76	3.43	2.48	0.79	0.87	0.40	0.85
P42	M	4	5	4	Ae, O, U	65.8	neg	neg	1.16	0.82	neg	0.36	0.19	0.28	0.30
P43	F	4	N.D.	N.D.	Ery	175	0.48	neg	5.09	5.27	3.77	0.51	0.33	neg	0.19
[§] P44	M	3	N.D.	N.D.	Ery	654	neg	0.37	1.32	4.30	1.22	0.97	0.38	neg	0.92
[§] P45	M	8	7	N.D.	Ery, O	348	1.84	0.70	7.98	9.85	5.78	0.52	0.27	neg	0.34
P46	F	4	N.D.	N.D.	Ae, Ery, U	616	2.13	1.33	13.90	21.00	7.10	0.45	0.27	neg	0.26
P47	M	4	N.D.	N.D.	Ae, Ery, U	2227	7.87	3.05	24.60	27.70	17.50	0.91	0.46	0.25	0.38
P48	M	5	6	4	Ana, Ae, Ery, O, Res, U	157	1.89	0.41	4.23	4.69	3.19	0.62	0.51	neg	0.41
P49	F	2	0	0	Ery, U	204	0.56	0.86	1.70	3.21	2.28	0.34	0.42	0.36	0.33
[§] P50	M	2	9	3	Ae, Ery	659	4.99	3.58	23.40	32.50	20.00	0.89	0.59	0.49	0.62
P51	F	2	0	0	Ery, O, U	1173	neg	neg	0.51	0.73	neg	0.52	0.31	neg	0.34
[§] P52	F	4	7	5	Ae, Ery, O, U	872	12.40	8.92	43.20	29.80	20.70	1.44	1.29	1.05	1.59
[§] P53	M	2	2	2	Ae, Ery, O, U	566	2.11	10.30	9.13	12.80	4.68	1.36	0.83	0.61	0.71
[§] P54	M	6	7	8	Ae, Ery, U	2190	4.68	2.65	29.50	38.80	23.00	1.67	0.85	0.72	0.93
P55	M	4	0	0	Ae, Neu, U	336	neg	1.33	0.47	0.93	0.62	0.40	neg	neg	0.26
[§] P56	M	2	7	2	Ae, Ery, O, U	205	4.88	2.65	18.80	32.30	19.00	1.24	1.08	0.80	1.19
P57	M	16	5	2	Ae, Cu, Ery, Gl, O	1098	24.30	168.00	22.80	24.30	14.40	1.21	0.37	0.27	0.53
P58	F	15	8	6	Ae, Ery, O, U	226	0.36	0.41	2.08	2.15	1.69	1.29	0.83	0.32	0.73
[§] P59	F	16	7	7	Ae, O, Res, U	865	3.55	6.08	9.61	11.60	10.30	1.03	0.94	1.04	0.71
[§] P60	M	7	5	4	Ae, Ery, O, U	353	0.76	0.82	1.49	1.83	1.29	0.54	0.44	0.43	0.49
P61	F	16	5	0	Ae, O	237	neg	neg	0.63	0.44	0.51	0.19	0.19	0.22	neg
P62	M	4	2	0	Ery, O	103	neg	neg	0.53	0.86	0.55	0.28	0.29	neg	0.24
P63	M	4	2	0	Ery, O	198	neg	neg	1.35	2.52	1.63	0.76	0.81	0.19	0.74
P64	F	8	4	0	Ae, Ery, Gl, U	2206	4.56	1.72	13.40	15.50	10.20	1.10	0.79	0.56	0.87
[§] P65	M	8	3	6	Ae, Gl, O	628	7.79	3.08	19.80	31.80	22.50	1.48	1.45	0.86	1.60
P66	F	4	2	2	O, U	45.7	neg	neg	0.76	0.53	neg	0.29	neg	0.29	0.23
P67	M	4	0	2	Ae, Ery, O	592	8.05	8.82	23.00	16.10	14.50	0.97	0.84	0.43	0.82
P68	F	7	N.D.	N.D.	Ae, O, U	174	0.66	1.15	1.80	1.14	neg	0.28	0.24	0.24	0.26
P69	M	8	N.D.	N.D.	Ae, Ery	38.5	neg	neg	2.15	neg	neg	0.36	0.21	neg	neg

† Abbreviations: N.D. = not done; neg = negative (<0.35 kU_A/L for ImmunoCAP & OD450nm <0.17 in ELISA)

‡ Abbreviations for symptoms: Ana = Anaphylaxis; Ae = Angioedema; Cu = Contact urticaria; Ery = Erythema; GI = Gastrointestinal; Neu = Neurological; O = Oral; Res = Respiratory; U = Urticaria

§ Subjects who underwent grass carp DBPCFC

¶ Subjects' serum were used for immunoblotting

Table 2. Multiple sequence alignment of parvalbumins

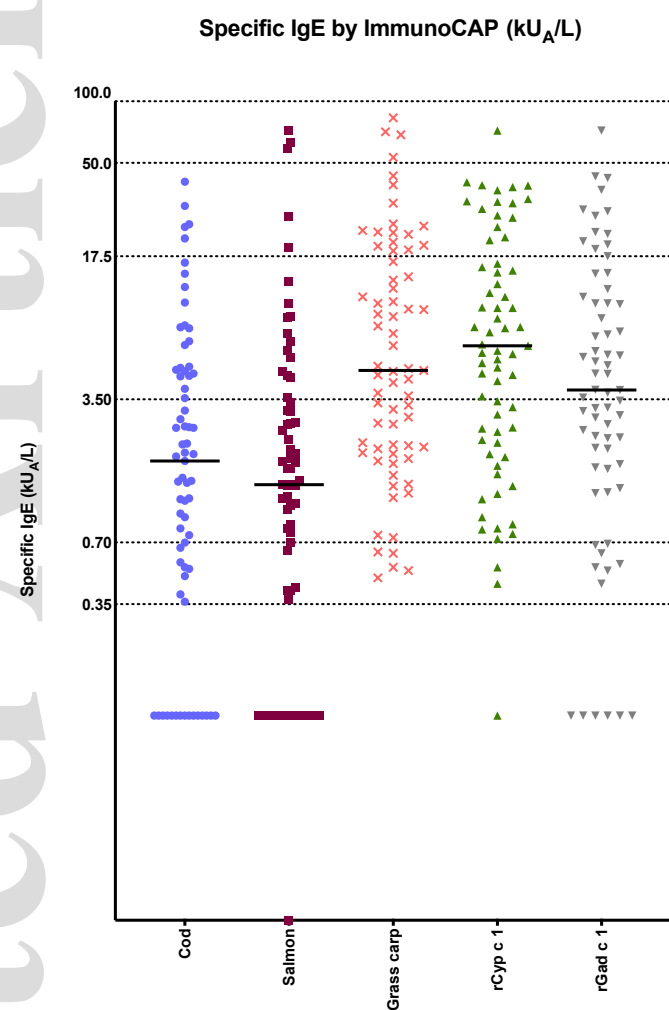
	1	5	10	15	20	25	30	35
Cten i 1	M	A	F	A	G	V	L	N
Cyp c 1	M	A	F	A	G	V	L	N
Gad m 1	M	A	F	A	G	I	L	A
Gad c 1	M	A	F	K	G	I	L	S
Sal s 1	M	A	C	A	H	L	C	K
Grass carp PV (11kDa)	M	A	M	K	N	L	L	K
Salmon alpha PV	M	A	M	N	S	I	L	N
Chicken alpha PV	M	A	M	T	D	V	L	S
	36	40	45	50	55	60	65	70
Cten i 1	L	S	A	K	S	G	D	D
Cyp c 1	L	T	S	K	S	A	D	D
Gad m 1	L	S	G	K	S	A	D	D
Gad c 1	L	D	A	F	S	A	D	E
Sal s 1	F	A	S	K	S	A	D	D
Grass carp PV (11kDa)	L	K	A	L	S	A	D	N
Salmon alpha PV	L	K	A	K	S	A	E	D
Chicken alpha PV	L	K	K	K	S	P	E	D
	71	75	80	85	90	95	100	105
Cten i 1	F	K	A	D	A	R	A	L
Cyp c 1	F	K	A	G	A	R	A	L
Gad m 1	F	K	A	G	A	R	A	L
Gad c 1	F	A	A	D	L	R	A	L
Sal s 1	F	C	P	K	A	R	E	L
Grass carp PV (11kDa)	F	S	A	D	G	R	D	L
Salmon alpha PV	F	A	S	D	G	R	D	L
Chicken alpha PV	F	T	P	D	G	R	D	L
	109							
Cten i 1	L	V	K	A				
Cyp c 1	L	V	K	A				
Gad m 1	L	V	K	A				
Gad c 1	L	V	D	K	W	G	A	K
Sal s 1	L	V	K	Q				
Grass carp PV (11kDa)	L	V	H	E				
Salmon alpha PV	L	V	H	E				
Chicken alpha PV	M	V	A	E	S			

† Shaded boxes indicate identical amino acid with *Cten i 1*

‡ Underlined boxes indicate peptide fragment identified by mass spectrometry

Figure 1. Specific IgE measured by ImmunoCAP assay and correlation of grass carp-specific IgE with parvalbumin-specific IgE

a)



b)

c)

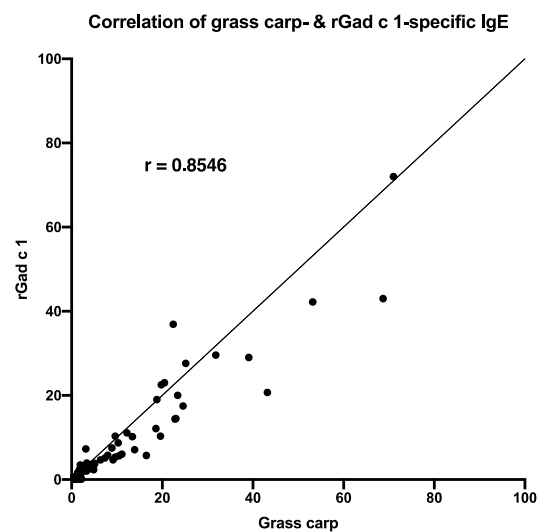
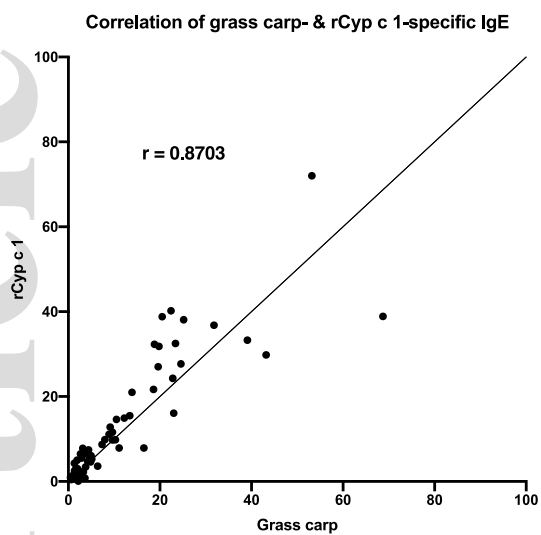


Figure 2. SDS-PAGE and immunoblot of grass carp extract

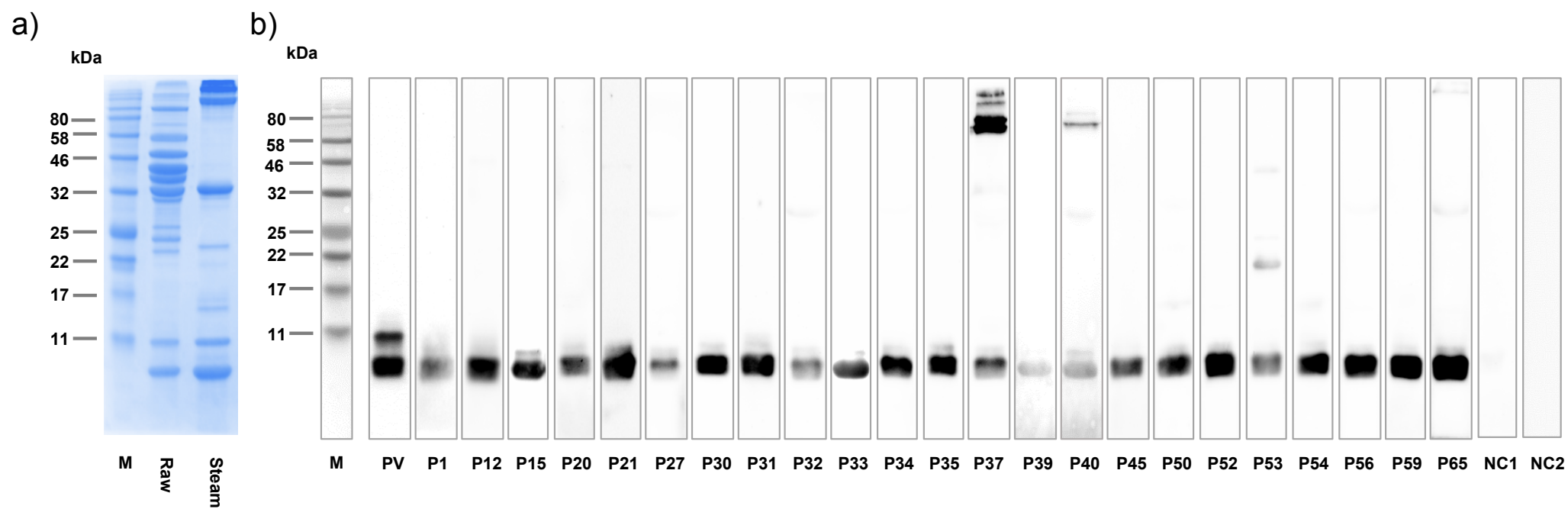


Figure 3. Specific IgE to different fish allergens as measured by ELISA

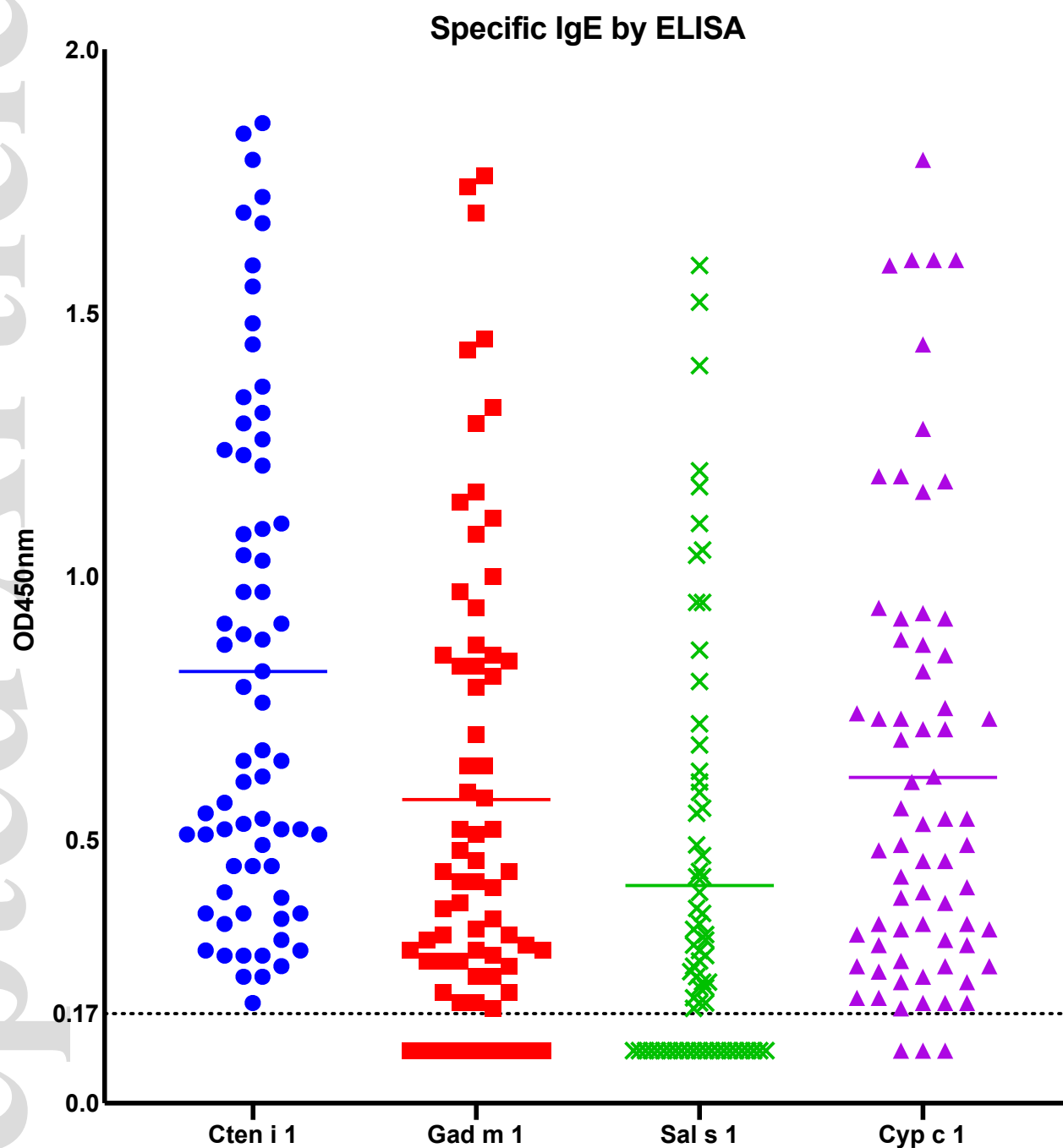


Figure 4. Cross-inhibition ELISA of parvalbumins

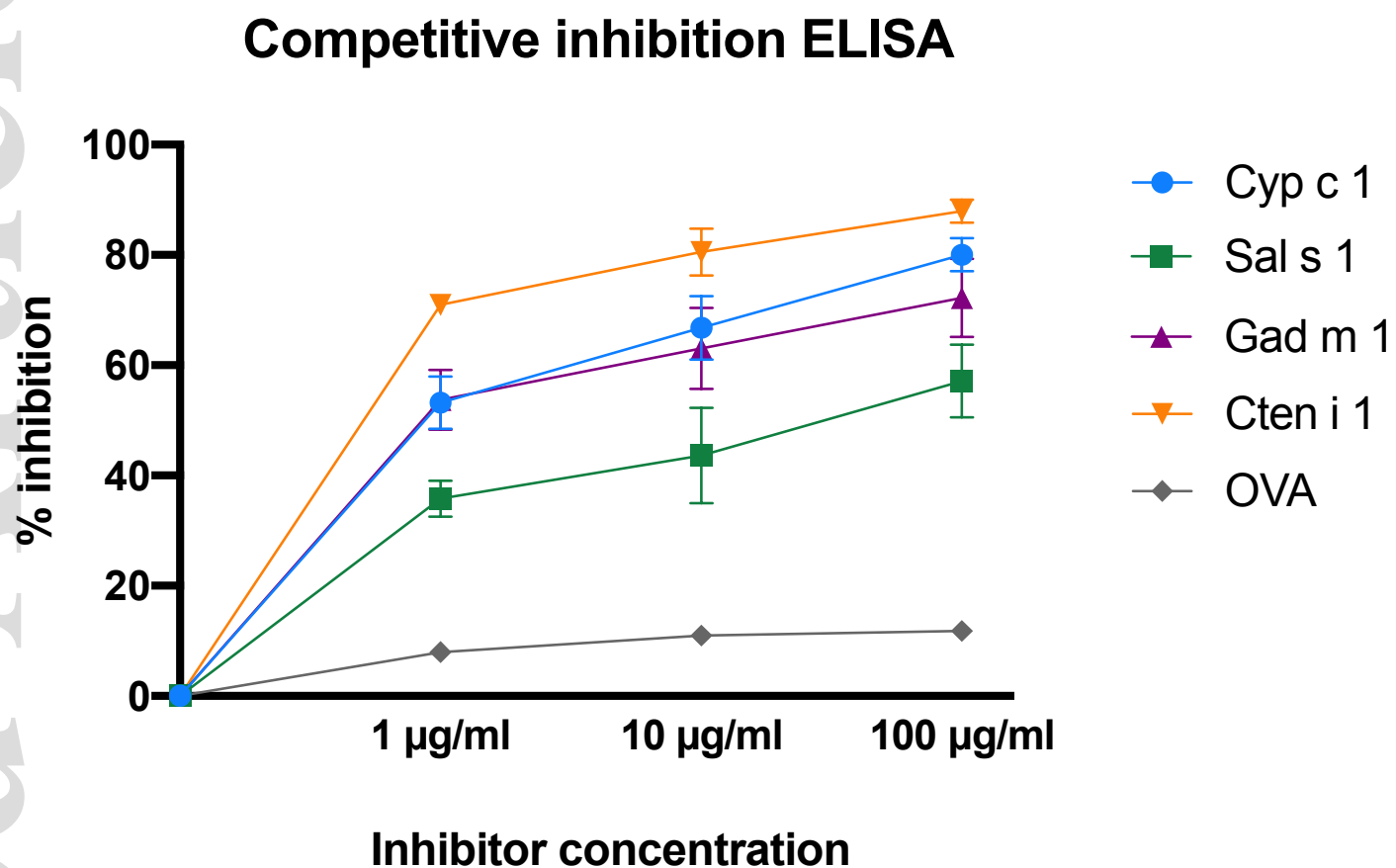


Figure Legends

Figure 1

a) Specific IgE against cod, salmon, grass carp, rCyp *c 1* and rGad *c 1* measured by the ImmunoCAP assay. The line indicated the median value for each ImmunoCAP and the dotted lines indicated the classification of IgE titers set by the ImmunoCAP system. All ImmunoCAP values < 0.35 kU_A/L were presented as 0.1 kU_A/L. Specific IgE of grass carp was higher than cod and salmon ($p < 0.0001$), as well as to rGad *c 1* ($p = 0.02$) but not rCyp *c 1* ($p = 0.22$). Similarly, the ImmunoCAP value of rCyp *c 1* was also higher than rGad *c 1* ($p < 0.0001$). b) & c) Correlation of specific IgE against grass carp extract and recombinant parvalbumins rCyp *c 1* and rGad *c 1*. Pearson's correlation test showed that the grass carp extract had a strong correlation with rCyp *c 1* ($r = 0.87$) and rGad *c 1* ($r = 0.85$), suggesting parvalbumin as the predominant allergen in the grass carp extract.

Figure 2

a) SDS-PAGE and b) immunoblotting (right panel) of grass carp proteins resolved on a 13.5% gel. The Color Prestained Protein Standard, Broad Range (New England Biolabs) was used as a molecular weight marker. 20 µg of raw and steamed grass carp extract were applied to each lane for SDS-PAGE while only steamed extract was used for immunoblotting with patients' serum. For immunoblotting, anti-parvalbumin antibody (lane PV) as well as serum from non-allergic controls (lanes NC1 & NC2) were included as controls.

Figure 3

Specific IgE levels against purified native *Cten i 1*, *Gad m 1*, *Sal s 1* and *Cyp c 1* as measured by ELISA. Dotted line indicated the positive threshold value ($OD_{450nm} = 0.17$) that was two-fold higher than the average background value obtained from the non-allergic controls. All values below 0.17 were presented as 0.10. The line in each column indicated the mean value of IgE reactivity for each parvalbumin. IgE reactivity was different for all pairwise comparisons ($p < 0.001$), except for *Gad m 1* versus *Cyp c 1*.

Figure 4

Cross-inhibition ELISA with *Cten i 1* immobilized on a solid phase. Different concentrations of inhibitors were co-incubated with serum IgE for 2 h before incubating with the solid phase. The egg allergen ovalbumin (OVA) was included as an irrelevant allergen control. The cross-inhibition ELISA was performed with 4 serum pools and the data was presented as the mean and SEM of the 4 pools. Serum pools were prepared by equal mixture of samples diluted 1:10 in blocking buffer (Pool 1: P1 + P13 + P25 + P30; Pool 2: P34 + P35 + P37 + P39; Pool 3: P47 + P50 + P52 + P53; and Pool 4: P31 + P44 + P59 + P60).