TABLE S1 Clinical characteristics of 77 fish-allergic pediatric patients recruited for this study.

Patients			sIgE (kU/L)			Skin prick test results (mm)					
#	Sex	Age (yrs)	Tuna	Salmon	Parvalbumin†	Tuna	Salmon	Seabass	Crocodile	Implicated fish‡	Symptoms
1	M	14	1.53	3.96	y	0.0	0.0	13.0	5	Snapper	AE, OAS
2	M	5	-	-	y	5.0	8.5	15.0	-	Salmon, sardine	AN, U
3	M	9	-	-	y	0.0	6.5	15.0	-	White bait	AE, C, U
4	M	1	-	-	y	3.0	6.0	24.5	-	Asian seabass, salmon, white fish	AE, OAS, U
5	M	5	-	-	y	4.5	4.5	10.0	-	Murray cod	AN, GIS, RD
6	F	13	-	-	n	4.5	0.0	12.0	-	Asian seabass, catfish	GIS, RD, U
7	F	10	-	-	n	0.0	0.0	3.0	-	Salmon, white fish	AE, GIS, U
8	M	11	4.83	25.30	y	4.0	12.0	11.5	-	Tuna, white fish	AE, GIS, OAS, U
9	F	5	-	-	y	0.0	5.5	7.0	-	Salmon	GIS, U
10	M	9	-	-	n	2.0	7.5	5.0	-	Salmon	U
11	F	7	-	-	y	6.5	9.0	16.0	5.5	Salmon, tilapia, tuna, yellowtail kingfish	AE, OAS, U
12	M	10	-	-	n	3.0	4.0	8.0	-	White bait	AN, RD, U
13	M	10	0.01	<0.01	n	2.5	0.0	3.5	-	Asian seabass	OAS, U
14	M	7	0.10	0.64	n	0.0	5.0	18.5	-	White fish	OAS, U
15	M	13	1.99	1.24	y	2.5	4.5	21.0	-	White fish	OAS, U
16	M	14	0.28	0.21	n	2.5	0.0	10.5	-	Salmon	AE
17	M	3	0.03	0.03	n	4.0	0.0	5.0	-	Catfish	AE, U
18	F	12	1.41	3.43	y	4.0	11.0	19.0	-	White fish	OAS, U
19	F	13	0.02	0.01	n	2.5	0.0	9.5	-	Murray cod or tuna	U
20	M	4	<0.01	<0.01	n	0.0	0.0	3.0	-	White fish	U
21	F	2	0.78	0.60	n	0.0	3.5	0.0	-	Salmon	AE, U
22	M	1	-	-	y	0.0	4.5	3.5	-	Catfish	AE
23	M	12	5.91	9.01	y	9.0	8.0	16.5	-	Asian seabass	U
24	M	9	2.15	5.33	y	5.5	3.0	15.5	-	Trevally	AN, RD, U
25	M	11	0.14	0.11	n	0.0	0.0	3.5	-	White fish	AE, AN, RD, U
26	F	1	5.14	5.58	y	0.0	3.0	15.5	14.5	Asian seabass	AE, RD, U
27	M	8	4.67	9.80	y	4.5	2.0	7.0	-	Salmon	OAS
28	F	2	31.00	73.40	y	5.5	13.5	18.5	-	Cod	AE, U
29	M	8	21.00	70.50	y	6.5	12.0	19.0	18	White fish	GIS, RD, U
30	F	6	-	-	y	3.0	4.5	19.0	-	White fish	AE
31	M	9	0.06	0.46	n	2.0	6.5	6.5	-	Croaker	AE
32	F	10	0.15	0.14	n	0.0	0.0	17.0	-	Catfish	GIS, RD, OAS
33	M	5	6.78	11.50	y	5.0	6.0	12.5	-	Leather jacket	AE
34	M	7	0.70	7.63	y	0.0	6.0	7.5	-	Trout	AE, OAS, U
35	F	7	1.19	3.08	y	2.5	0.0	16.0	-	White fish	U
36	M	4	17.30	21.00	y	0.0	7.0	10.0	-	White fish	AP, U
37	M	14	0.71	1.60	y	1.0	10.5	9.5	-	Salmon	OAS, U
38	M	7	0.95	0.54	y	2.0	2.0	17.5	-	Tilapia	C, GIS

39	F	4	0.71	0.47	n	0.0	0.0	17.0	-	Catfish	AE, E, U
40	F	15	0.86	1.39	n	2.5	5.0	16.5	3.5	Asian seabass	AE, U
41	M	18	1.06	3.21	y	4.0	6.5	13.0	-	Tuna	RD, U
42	M	12	0.18	0.24	n	3.0	3.0	14.0	-	Unknown	RD, U
43	M	9	2.38	3.74	y	4.0	6.5	14.0	-	Silver perch	AE
44	F	2	-	-	y	4.5	7.0	17.0	12.0	Salmon, yellowtail kingfish	U
45	F	12	14.90	38.90	y	3.0	4.5	14.0	-	Flathead	AE
46	M	1	<0.01	<0.01	n	0.0	0.0	3.5	-	Salmon	AE, U
47	F	3	-	-	n	0.0	0.0	0.0	-	Hake	AN, U
48	F	11	0.02	0.04	y	5.5	0.0	4.0	-	Silver bream	AN, AE, OAS, RD
49	F	17	-	-	y	4.0	0.0	7.0	-	Catfish	GIS
50	M	8	-	-	y	4.5	4.5	10.0	-	Cod, white fish	AN, R, RD, U
51	M	20	-	-	n	0.0	3.0	14.5	-	White fish	AE, U
52	F	13	9.43	20.60	n	5.0	9.5	15.5	-	Salmon	U
53	M	1	-	-	y	1.0	6.0	12.0	-	Tilapia, yellowtail kingfish	AE, U
54	M	5	-	-	n	0.0	3.0	13.5	-	White fish	AE, OAS, U
55	M	15	-	-	y	4.0	12.0	19.0	-	Milkfish	U
56	M	10	-	-	y	0.0	4.5	14.0	-	Perch	U
57	M	8	-	-	n	0.0	6.5	20.5	-	Bream	AN, AE, RD
58	M	16	-	-	n	5.5	4.5	17.0	-	Snapper	U
59	M	11	-	-	n	3.5	3.0	3.5	1.0	Salmon, white fish	AE, C, RD
60	M	15	-	-	n	2.0	2.5	8.5	0.0	Asian seabass, salmon	AP, GIS, RD
61	M	18	-	-	y	0.0	4.5	5.0	2.5	Catfish, white fish	GIS
62	F	17	3.18	2.88	n	1.0	2.5	11.5	-	Asian seabass, croaker	OAS, R
63	M	14	1.17	8.09	y	4.5	11.0	6.5	5.0	Catfish	RD, U
64	F	13	-	-	n	0.0	0.0	5.0	0.0	White fish	OAS, U
65	M	14	-	-	n	6.0	0.0	9.5	-	Asian seabass	AN
66	M	11	39.50	66.10	y	5.0	4.5	21.0	-	Salmon	AE
67	M	15	12.10	17.60	y	5.5	8.5	20.0	-	Ling	AE, OAS
68	F	10	7.33	9.08	y	2.0	3.5	9.0	-	White fish	AE, OAS, RD
69	M	2	-	-	y	4.0	7.5	4.5	-	Salmon	U
70	M	12	3.90	31.80	y	0.0	7.0	3.0	-	Salmon, tuna	E, U
71	M	7	-	-	y	4.0	7.5	13.5	-	Ling	AE, U
72	F	21	1.27	0.58	n	0.0	0.0	0.0	-	Snapper, tuna	AE, GIS, U
73	F	17	-	-	n	3.5	0.0	0.0	-	White fish	AE, AN, GIS, RD
74	M	18	-	-	n	3.0	6.0	9.5	-	Salmon, silver perch, shark	AE, AN, C, RD
75	M	10	13.50	54.20	y	5.5	10.5	17.5	-	Asian seabass, tuna	AE
76	F	15	-	-	y	5.5	5.0	16.5	-	Salmon	E, U
77	F	13	-	-	n	0.0	4.5	16.0	3.0	Milk fish, tilapia, white fish	C, RD, U

Note: M, male; F, female; yrs, years; '-', not determined; AE, angioedema (lip/face swelling); AN, anaphylaxis; AP, abdominal pain; C, conjunctivitis; E, eczema; GIS, gastrointestinal syndrome (vomiting); OAS, oral allergy syndrome (itchy mouth or swelling throat/tongue); R, rhinitis (iriitation inside nose, sneezing); RD, respiratory distress; U, urticaria (erythema, hives, rash). †IgE-binding to salmon or catfish parvalbumin as determined by immunoblotting in [S2]; y, yes; n, no. ‡ Fish species which was associated with clinical reaction upon ingestion.

SUPPORTING INFORMATION

METHODS

Gel-electrophoresis and immunoblotting analyses

Proteins were separated according to their molecular weights using a Criterion™ SDS-PAGE system (Bio-Rad) or Dual Double Wide Mini Vertical System (C.B.S. Scientific). Crocodile parvalbumin (PV) isoforms were separated by their isoelectric point using ReadyStripTM IPG Strips (11 cm, pH 3-6) in a PROTEAN IEF cell (Bio-Rad) prior to SDS-PAGE as described previously.[S1] Proteins were visualized by Coomassie Brilliant Blue R-250 staining.

For subsequent immunoblotting, proteins were transferred onto a nitrocellulose membrane and incubated with antibodies or serum as described previously.[S2,3] PV isoforms were detected using the commercial monoclonal PARV-19 antibody raised against frog α-PV and a polyclonal in-house anti-fish β-PV antibody raised against seabass PV.[S4] Monoclonal mouse anti-human IgE antibody (sc-53346; Santa Cruz) was used for all IgE-immunoblots. The Surf-Blot Antibody Screening System (Idea Scientific) was used to investigate serum IgE-binding from all patients to the same extract, including grid immunoblotting, as described previously.[S1,2,5–9] All immunoblots were developed with a corresponding infra-red-labeled antibody (DyLight anti-mouse/rabbit 4xPEG; ThermoFisher Scientific), followed by visualization using an Odyssey CLx infra-red imaging system (LI-COR®) and densitometric analyses with Image Studio Version 5.2 (LI-COR®), allowing sensitive and semi-quantitative evaluation of signals.

Recombinant protein expression

The sequences for crocodile β- and α-PV (XP_019397705 and XP_019400389, respectively, in the NCBI database (www.ncbi.nlm.nih.gov/protein)) and thornback ray (*Raja clavata*) α-PV

(P02630) were optimized for *E. coli* expression and cloned into the pET-28b(+) vector at BamHI and EcoRI restriction sites with cleavable polyhistidine-tag to permit purification of the native protein. Recombinant PVs were expressed in BL-21(DE3) *E. coli* strain,[S10] and purified by immobilized metal affinity chromatography (NiNTA) with Tris-HCl buffer and an imidazole gradient at pH 7.5. Following size-exclusion chromatography, polyhistidine-tags were cleaved with tobacco etch virus (TEV) protease according to a previously established protocol.[S11] Effectiveness of purification and TEV cleavage was verified with SDS-PAGE. Proteins were dialyzed into 50 mM Tris-HCl with 150 mM NaCl buffer (pH 7.5) and stored at -80°C prior to immunological analyses.

Mass spectrometry analysis

Whole crocodile protein extracts, purified parvalbumin and IgE-binding bands were digested with trypsin and analyzed by mass spectrometry as described previously.[S2,12] Results were analyzed using both Mascot (version 2.4) search engine and MaxQuant (version 1.6.10.43) against an NCBI database containing amino acid sequences of all proteins from the corresponding species or closest higher classification with at least three annotated genomes/transcriptomes (May 2021). The protein abundance was expressed in relative intensity-based absolute quantification (iBAQ%) value.[S13] Identified protein groups with at least one unique peptide and a minimum of two razor/unique peptides were included in the analyses.

LAD2 cell degranulation assay

Crocodile β- and α-PV were evaluated for cellular reactivity using the LAD2 cell line as described previously.[S14,15] Seabass β-PV served as reference and positive control. Briefly,

human mast cells were cultured in complete StemPro™-34 media (ThermoFisher) containing stem cell factor (Peprotech). Cells were incubated overnight with serum from a fish-allergic subject or control individual, recombinant IgE or buffer, and treated with PV or polyclonal anti-IgE (Vector Laboratories) at 37°C for 45 min. To measure degranulation, β-hexosaminidase was quantified by incubation with p-nitrophenyl-N-acetyl-β-D-glucosaminide (Glycosynth) followed by addition of glycine. β-hexosaminidase release was expressed as a percentage of total available cellular enzyme, determined by Triton X-100 (0.1%)-induced cell lysis. Spontaneous release of β-hexosaminidase (typically <5%) was subtracted from all values.

***In silico* analyses and predictions**

Amino acid sequence alignment was prepared using Jalview and MUSCLE.[S16,17] Sequence identities and similarities were calculated using the Sequence Manipulation Suite.[S18] Linear epitopes with a minimal length of five amino acids were predicted using BcePred, Bepipred, and BepiPred-2.0.[S19–21] Peptides with one mismatch or two mismatches but a higher similarity than identity were considered to be likely recognized by the same IgE antibody, based on Ayuso et al.[S22] A model for crocodile β-PV was generated with Swiss Model (based on the crystal structure of chicken β-PV (PDB code: 3FS7)), and visualized using PyMOL (version 2.0).[S23]

EPITOPE PREDICTIONS

Sequence identity of the investigated PV isoforms was 27-85% with three as well as two IgE-binding regions reported for seabass and salmon PV, respectively (**Table S4**).[S9,24] The highest sequence identity among all PVs was in the seabass PV's IgE-binding region 2. As this could

not explain all observed IgE-binding patterns, 20 linear epitopes for crocodile β-PV were predicted *in silico*. Their sequences were compared with corresponding peptides from above-mention PVs, identifying three possible epitopes (residues 59-65 (IEEDELQL), 79-85 (TDAETKA), and 76-81 (RALTDA)), which could explain observed frequent IgE-binding of multiple β-PV but not α-PV (**Table S5**).

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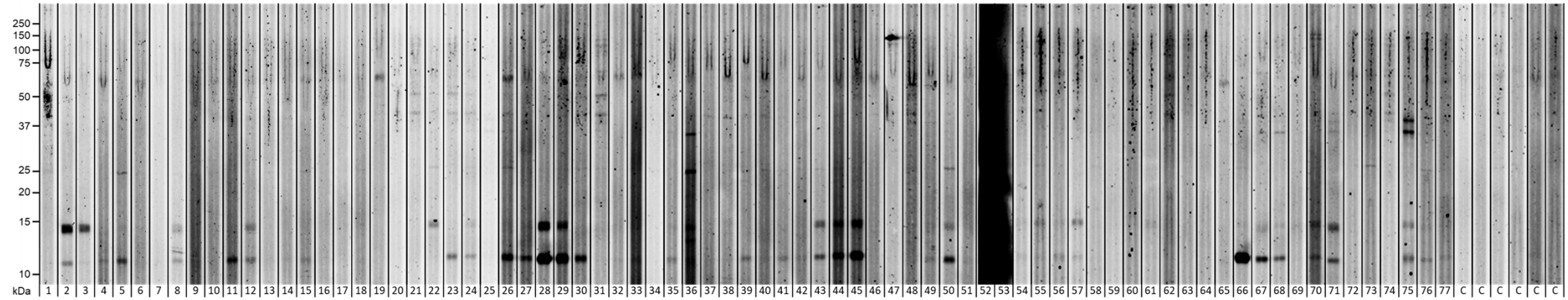
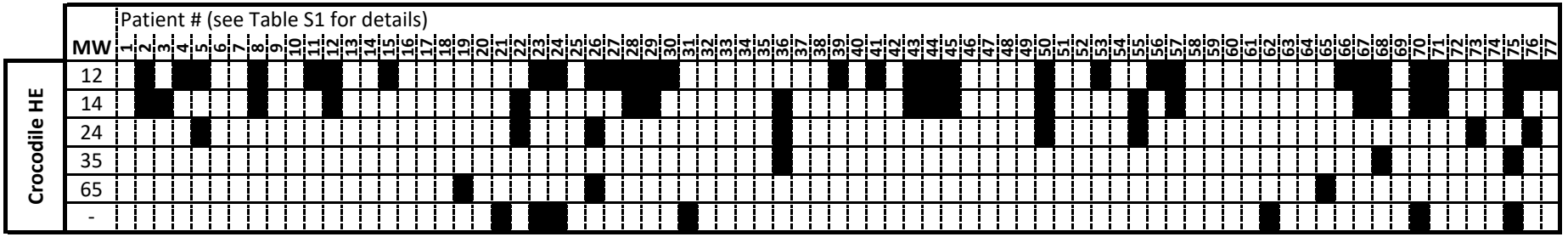


FIGURE S1 IgE-surfblots with heated crocodile extracts. See Table S1 for patient details and Table S2 for corresponding evaluation by densitometric analyses. Note: C, controls.

Table S2 Individual IgE recognition of proteins in heated extract (HE) from crocodile.



Note: A black box indicates IgE-binding, which was evaluated by densitometric analyses of IgE-surfblots shown in Figure S1. MW, molecular weight of the IgE-binding band in the corresponding extract. '-', one or multiple other IgE-binding bands.

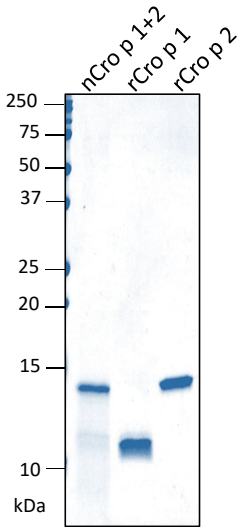


FIGURE S2 SDS-PAGE protein profiles of purified parvalbumin (PV) from crocodile: Natural purified (nCro p 1+2), recombinant β-PV (rCro p 1) and α-PV isoform (rCro p 2). Note: Same protein content in each lane.

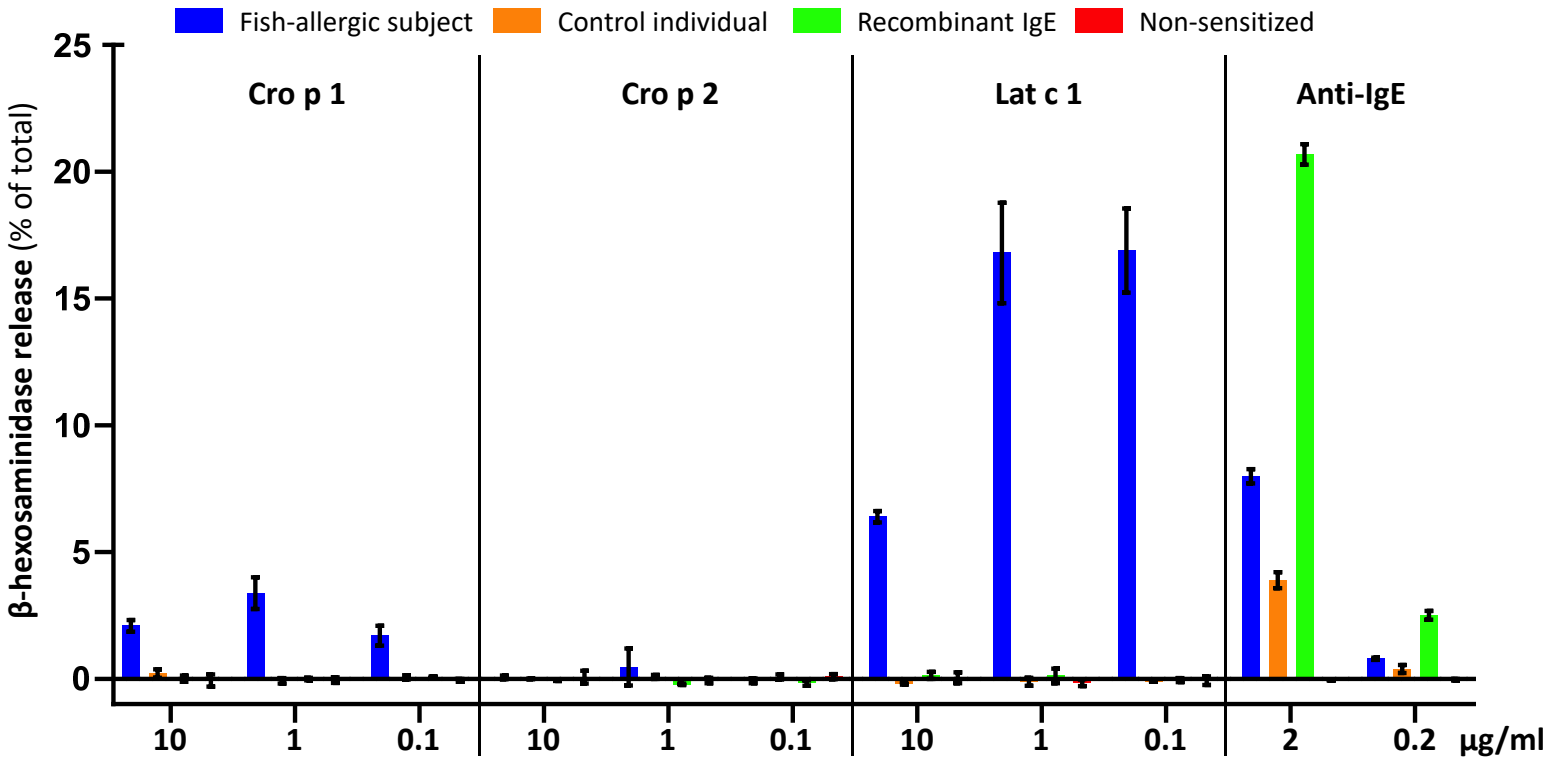


FIGURE S3 Degranulation activity of crocodile β-parvalbumin (Cro p 1) and α-parvalbumin (Cro p 2), seabass β-parvalbumin (Lat c 1), and anti-IgE in LAD2 cell assay.

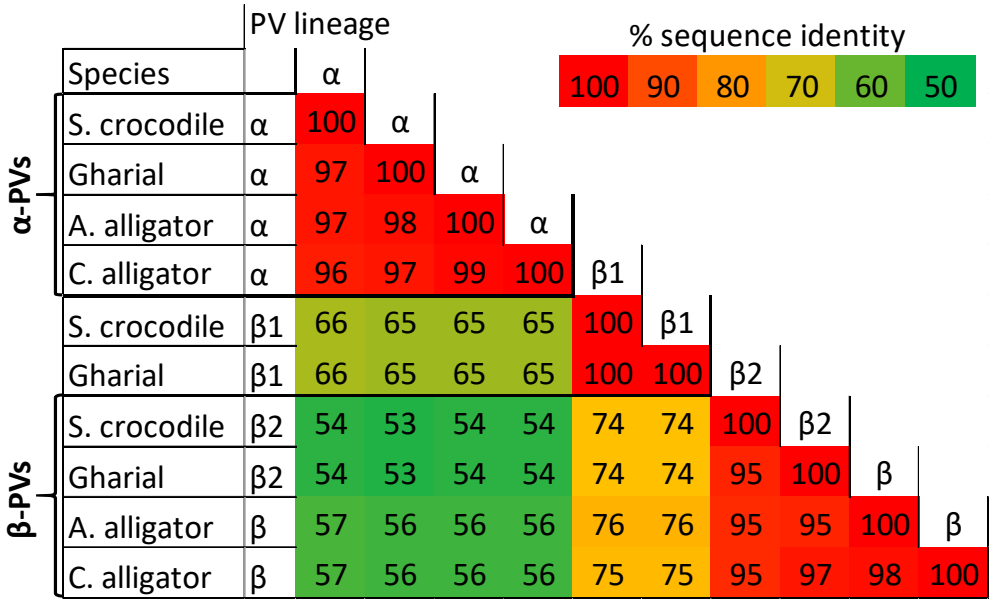


FIGURE S4 Sequence identity matrix for amino acid sequences from Crocodilian α- and β-parvalbumin (PV) isoforms available in the NCBI database (www.ncbi.nlm.nih.gov/protein). Note: S., Saltwater; A., American; C., Chinese.

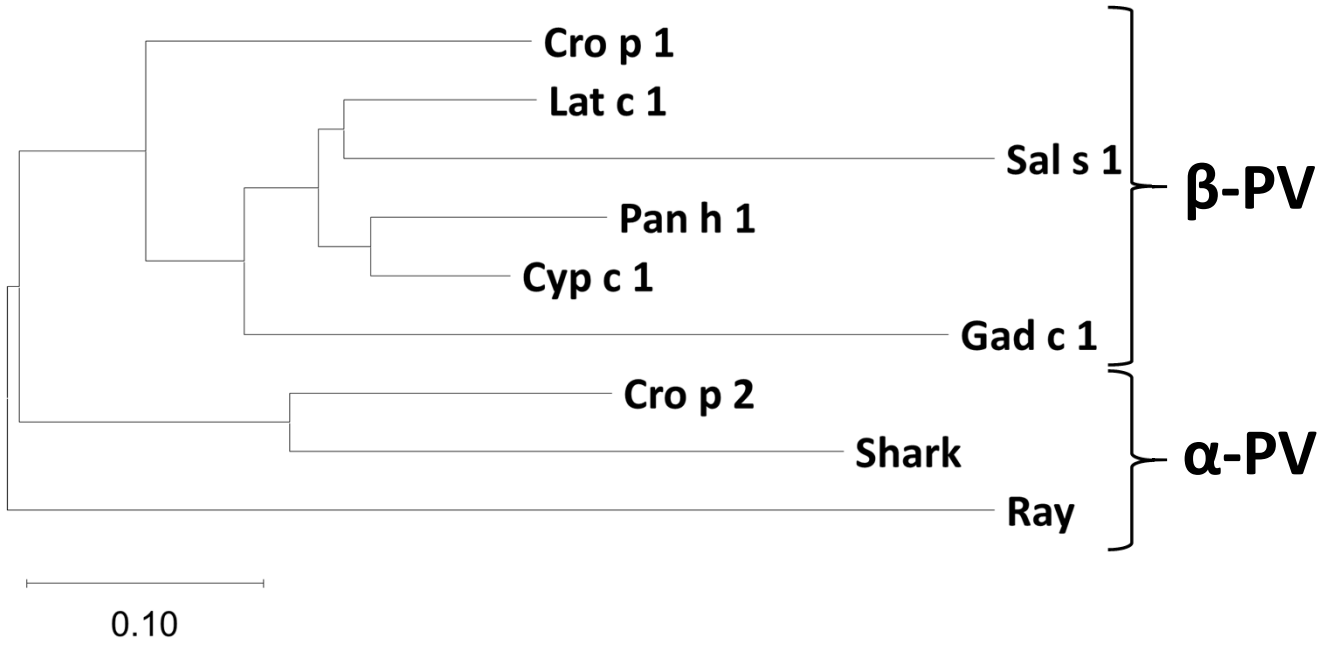


FIGURE S5 Molecular phylogenetic tree of six β- and three α-parvalbumin (PV) isoforms, which were investigated in the study. The optimal tree using the Neighbor-Joining method is shown.[1] The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site.[2]

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TABLE S3 Sequence identity and similarity of crocodile β-parvalbumin (PV, Cro p 1) and α-PV (Cro p 2) with fish derived β- and α-PV isoforms. See Figure 5 for the corresponding sequence alignment. Accession I.D. refers to NCBI database (www.ncbi.nlm.nih.gov/protein).

Accession I.D.	PV	Name	Identity with Cro p		Similarity with Cro p	
			1	2	1	2
XP_019397705.1	β	Cro p 1	100%	65%	100%	71%
XP_019400389.1	α	Cro p 2	65%	100%	71%	100%
5AHW83198.1	β	Lat c 1	72%	59%	79%	69%
CAA66403.1	β	Sal s 1	59%	53%	71%	65%
P02630.1	α	Ray	49%	49%	58%	61%
AFP11872.1	α	Shark	56%	69%	64%	80%
BAF98924.1	β	Pan h 1	71%	60%	77%	66%
P02622	β	Gad c 1	58%	50%	67%	61%
Q8UUS2	β	Cyp c 1	71%	61%	79%	67%

Table S4 Sequence identity of investigated parvalbumin (PV) isoforms in the IgE-binding regions reported for seabass and salmon PV, Lat c 1 and Sal s 1, respectively. The highest identity for each PV is highlighted in bold.

			Amino acid sequence identity (%) with corresponding peptide from PV						
PV	**IgE-binding region**		**β Cro p 1**	**α Cro p 2**	**β Lat c 1**	**β Sal s 1**	**α Shark**	**α Ray**	**β Pan h 1**
Lat c 1	1	**MAFAGILNEADITAALAACQAADSFKHKDFFVKVGLAGKSD**	59	49	100	54	49	27	71
	2	**GDSDGDGKIGVDEFAALVKV**	**85**	**75**	100	**75**	**70**	**70**	**85**
Sal s 1	1	**MACAHLCKEADIKTALEA**	39	44	56	100	39	39	50
	2	**KTFFHTIGFASKSADDVK**	61	56	61	100	56	28	61
	3	**VEELKLFLQNFCPKARELTDA**	62	48	71	100	38	57	67

Table S5 Epitopes predicted for Cro p 1 and their comparison with corresponding peptides from three β- (Lat c 1, Sal s 1, Pan h 1) and three α-parvalbumin isoforms (Cro p 2, ray, shark).

	Predicted **Cro p 1** epitope using **BcePred**	Length	Position	Number of identical amino acids/length of peptide						Similarity (%)						Identity (%)					
				Cro p 2	Lat c 1	Sal s 1	Ray	Shark	Pan h 1	Cro p 2	Lat c 1	Sal s 1	Ray	Shark	Pan h 1	Cro p 2	Lat c 1	Sal s 1	Ray	Shark	Pan h 1
1	KGKSADQVKK	10	37-46	7/10	7/10	7/10	2/10	4/10	8/10	80	80	80	40	50	90	70	70	70	20	40	80
2	LDQDKSGF	8	51-58	6/8	7/8	6/8	5/8	7/8	7/8	88	100	88	75	88	100	75	88	75	63	88	88
3	**IEEDELQL**	**8**	**59-65**	6/8	7/8	5/8	4/8	6/8	7/8	75	88	75	63	75	88	75	88	63	50	75	88
4	**TDAETKA**	**7**	**79-85**	4/7	6/7	7/7	3/7	3/7	5/7	71	86	100	43	71	86	57	86	100	43	43	71
5	DTDGDGK	7	91-97	6/7	6/7	5/7	5/7	6/7	7/7	86	100	71	86	86	100	86	86	71	71	86	100

	Predicted **Cro p 1** epitope using **Bepipred**	Length	Position	Number of identical amino acids/length of peptide						Similarity (%)						Identity (%)					
				Cro p 2	Lat c 1	Sal s 1	Ray	Shark	Pan h 1	Cro p 2	Lat c 1	Sal s 1	Ray	Shark	Pan h 1	Cro p 2	Lat c 1	Sal s 1	Ray	Shark	Pan h 1
1	LKGKSADQ	8	36-43	5/8	5/8	4/8	3/8	4/8	6/8	75	75	63	50	63	88	63	63	50	38	50	75
2	KSGFI	5	55-59	4/5	5/5	4/5	3/5	5/5	5/5	100	100	80	80	100	100	80	100	80	60	100	100
3	**RALTDA**	**6**	**76-81**	3/6	6/6	5/6	3/6	2/6	5/6	67	100	83	67	67	83	**50**	**100**	**83**	**50**	**33**	**83**
4	AGDTDGDGKIGV	12	89-100	11/12	11/12	9/12	10/12	11/12	12/12	92	100	93	92	92	100	92	92	75	83	92	100

	Predicted **Cro p 1** epitope using **Bepipred-2.0**	Length	Position	Number of identical amino acids/length of peptide						Similarity (%)						Identity (%)					
				Cro p 2	Lat c 1	Sal s 1	Ray	Shark	Pan h 1	Cro p 2	Lat c 1	Sal s 1	Ray	Shark	Pan h 1	Cro p 2	Lat c 1	Sal s 1	Ray	Shark	Pan h 1
1	DILSAK	6	5-10	4/6	3/6	3/6	3/6	4/6	3/6	83	50	67	33	67	67	67	50	50	50	67	50
2	QAAESFN	7	20-26	6/7	5/7	3/7	4/7	3/7	3/7	86	86	71	43	86	57	86	71	43	57	43	43
3	GLKGKSADQ	9	35-44	6/9	6/9	5/9	4/9	5/9	7/9	80	80	70	60	70	89	67	67	56	44	56	78
4	DQDKSGFIEED	11	52-62	9/11	11/11	8/11	6/11	10/11	11/11	91	100	82	73	91	100	82	100	73	55	91	100
5	NFSSSARALTD	11	70-80	5/11	5/11	5/11	5/11	5/11	5/11	64	91	64	64	55	82	45	45	45	45	45	45
6	TDGDGKIGVD	10	92-101	9/10	9/10	7/10	8/10	8/10	10/10	90	100	80	90	80	100	90	90	70	80	80	100

Note: Both similarity and identity refer to Cro p 1. Peptides with only one mismatch or two mismatches but a higher similarity than identity are shaded, suggesting cross-binding by IgE antibodies.
Peptides which can explain the observed cross-binding to β- but not α-parvalbumin isoforms are in bold.