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Cross-reactive monoclonal antibodies against fish parvalbumins as a tool for studying antigenic similarity of different parvalbumins and analysis of fish extracts

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ABSTRACT

Fish parvalbumins are heat-stable calcium-binding proteins that are highly cross-reactive in causing allergy symptoms in fish-sensitized patients. The reactivities of parvalbumin-specific monoclonal or polyclonal antibodies with parvalbumins of different fish species allowed their application for development of various immunoassays for allergen identification in fish samples. In this study, monoclonal antibodies (MAbs) were generated against two parvalbumins – natural Atlantic cod parvalbumin and recombinant common carp β-parvalbumin expressed in *E. coli*. Large collections of recombinant parvalbumins and natural allergen extracts of different fish species and other animals were used to identify the specificities of these MAbs using ELISA, Western blot, and dot blot. MAbs demonstrated different patterns of cross-reactivities with recombinant parvalbumins. Their binding affinities were affected by the addition and removal of Ca^{2+} ions. Moreover, all MAbs showed a broad reactivity with the target antigens in natural fish, chicken, and pork extracts. The ability of two MAbs (clones 7B2 and 3F6) to identify and isolate native parvalbumins from allergen extracts was confirmed by Western blot. Epitope mapping using recombinant fragments of Atlantic cod parvalbumin (Gad m 1) and common carp parvalbumin (Cyp c 1) revealed that 4 out of 5 MAbs recognize parvalbumin regions that contain calcium binding sites. In conclusion, the generated broadly reactive well-characterized MAbs against fish β-parvalbumins could be applied for investigation of parvalbumins of fish and other animals and their detection in allergen extracts.

1. Introduction

Fish is considered as one of the major food allergen source, causing allergy symptoms, like diarrhea, vomiting, rhinitis, urticaria, that can affect either the gastrointestinal tract, respiratory tract or skin, and can even lead to anaphylaxis (Carvalho et al., 2020; Hilger et al., 2017). These symptoms may occur not only after eating or cooking fish, but also in fish-processing environments, where workers may develop asthma or contact dermatitis (van der Ventel et al., 2011; Sharp et al., 2014). Different fish species are consumed all over the world and their choice is related to geographic regions, where certain fish species are more common, to eating habits, to fish supply in local stores and to selected fish preparation methods (fried, smoked, canned, uncooked and others) (Klueber et al., 2019; Feketea et al., 2021). In Europe, the most popular fish species are carp, cod, salmon, tuna, Alaska pollock and herring, while in the United States – salmon, tilapia, tuna, catfish, cod and Alaska pollock (Kuehn et al., 2010; Dasanayaka et al., 2022; Hilger et al., 2017). To manage fish allergy, patients are usually advised to strictly avoid fish and its products in their diet (Sharp et al., 2014). Allergy to fish for patients can be determined either using skin prick tests (SPT) with commercial fish extracts, or performing immunoassays for detection of parvalbumin-specific IgE antibodies and analyzing patients' clinical history (Sharp et al., 2014; Fernandes et al., 2015).

Twelve different fish proteins have been identified, characterized and registered as fish allergens by the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-Committee database (http://www.allergen.org/). β-parvalbumin, β-enolase, aldolase A, tropomyosin, collagen alpha are

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the most studied and analyzed fish proteins that have been identified in different fish species like Atlantic cod, striped catfish, Atlantic salmon, common carp and others. Due to increased fish consumption and the diversity of fish species, new fish allergens are being continuously determined (like grass carp β-parvalbumin (Cten i 1), Atlantic salmon creatine kinase (Sal s 7), common carp β-enolase (Cyp c 2)), that could be used as antigens for fish allergy diagnostics (Leung et al., 2020; Ruethers et al., 2021; Sližienė et al., 2022).

Parvalbumins are found in all vertebrates and are 10–13 kDa heat-stable proteins that also demonstrate resistance to enzymatic digestion and denaturating chemicals (Kalic et al., 2019; Yuk et al., 2021). Moreover, they belong to the EF-hand protein family, which bind bivalent metal ions such as calcium or magnesium (Kuehn et al., 2014; Hilger et al., 2017). These allergens are subdivided into two distinct evolutionary lineages: α-parvalbumins and β-parvalbumins, based on their amino acid sequence similarity, both of these lineages have been identified in different fish species muscles (Fernandes et al., 2015; Chen et al., 2006; Ma et al., 2008). α-parvalbumins are mainly present in cartilaginous fishes and are considered as non-allergenic proteins (Kalic et al., 2019; Hilger et al., 2017). In contrast, β-parvalbumins are predominantly found in bony fishes and are known as the major fish allergens, since most fish-allergic patients (70–90 %) have parvalbumin-specific IgE (Carvalho et al., 2020; Kleine-Tebbe and Jakob, 2017). Fish has two types of muscle: white (or light) muscle and dark (or red) muscle, that not only differ in their functions and composition, but also in the amount of parvalbumins. It was shown, that parvalbumin concentration is much lower in dark than in white muscle (Kobayashi et al., 2006). That is why certain fishes such as tuna, skipjack and swordfish, that have more dark muscle than other bony fishes like cod, flounder or whiff, are considered to be less allergenic fish species (Griesmeier et al., 2010). So, in the most commonly consumed fish species, the content of β-parvalbumin is different. It was determined, that the amount of β-parvalbumin in the raw muscle of carp and herring was found to be 3.75 mg/g and 4.75 mg/g, respectively, while it is only 0.5 mg/g in mackerel and 0.03 mg/g in tuna (Kuehn et al., 2010). β-parvalbumins of different fish species share high amino acid sequence similarity (60–80 %), which could explain the occurrences of clinical cross-reactivity in fish-allergic patients (Carvalho et al., 2020; Kuehn et al., 2013). Besides that, IgE cross-reactivity has also been observed between parvalbumins of fish and other animals (chicken, frog, crocodile), and even between β-enolases of several different fish species (Kuehn et al., 2016, 2013). However, there are cases when fish-allergic patients are monosensitized only to salmonid fishes (Kuehn et al., 2014). Humans also contain α-parvalbumin (PVALB) and β-parvalbumin (oncomodulin (OCM)) and the analysis of sequence similarities between human, frog, pike and other animals parvalbumins revealed, that mammalian OCM is phylogenetically distinct from many β-parvalbumins of lower vertebrates (Climer et al., 2019).

Even though fish-allergic patients avoid eating fish or its containing products, allergy symptoms may occur because of hidden allergens in food, due to cross-contamination during the production process or incorrect product labelling. Detecting fish allergens in food would improve allergen risk management. That is why various methods were developed and applied for parvalbumin detection in food: real-time polymerase chain reaction (PCR), Western blot, enzyme-linked immunosorbent assay (ELISA), immunochromatographic assay (ICA), biosensors and others (van der Ventel et al., 2011; Fernandes et al., 2015; Zhang et al., 2021). In most of these methods, the presence of allergens in samples is determined using parvalbumin-specific monoclonal or polyclonal antibodies. Monoclonal antibody (MAb) PARV-19 (Merk, Germany), raised against frog skeletal muscle parvalbumin and cross-reactive with other parvalbumins, is a widely used mouse antibody for fish parvalbumin detection in fish extracts (like carp, catfish, cod, pomfret, Indian anchovy and others) (Chen et al., 2006; Lim et al., 2008). However, its specificity to certain fish species is limited (Saptarshi et al., 2014). Allergen extracts prepared from catfish, pilchard, silver carp, Atlantic salmon, Atlantic cod and other fish species, and even purified allergens were used to develop new collections of fish parvalbumin-specific monoclonal and polyclonal antibodies, that demonstrate cross-reactivity with various fish extracts or selected specificity to certain fish species. Generated antibodies could be used to develop antibody-based assays, like ELISA or ICA, for detection of fish parvalbumin in food samples (Gajewski et al., 2009; Sharp et al., 2015; van der Ventel et al., 2011; Zhang et al., 2021; Koppelman et al., 2012). Several commercial ELISA kits and rapid test kits for detecting fish proteins in food are available: „3 M™ Fish Protein ELISA Kit“ (3 M Science. Applied to Life, USA), „The AgraQuant® Fish“ (Romer Labs Division Holding GmbH, Austria), „Agitest Food Allergen Rapid Test Fish“ (Rega Biotechnology Inc, Taiwan) and others (Fernandes et al., 2015; Yuk et al., 2021). Besides that, parvalbumin-specific monoclonal and polyclonal antibodies could be used for characterization of recombinant fish parvalbumins and analysis of fish extracts (Sun et al., 2019; Lee et al., 2012).

In the current study, a panel of MAbs was raised either against natural Atlantic cod parvalbumin or recombinant common carp β-parvalbumin produced in *Escherichia coli* (*E. coli*) cells. Various immunoassays have been applied to characterize the newly developed MAbs and investigate their reactivity with a large collection of recombinant fish and chicken parvalbumins as well as natural extracts from several fish species and other organisms. This study provides new data on the antigenic similarity of different parvalbumins, which may explain the pattern of cross-reactive sensitization observed in some patients with diagnosed fish allergy.

2. Materials and methods

2.1. mRNA isolation from common carp tissue and cDNA synthesis

Fresh common carp (*Cyprinus carpio*) was purchased from a local carp farm and was sacrificed in accordance with the Law on welfare and protection of animals of the Republic of Lithuania and the European Directive 2010/63/UE on the protection of animals used for scientific purposes. mRNA was isolated from a carp muscle tissue using the Quick-RNA Miniprep Kit (Zymo Research, USA) and the first strand cDNA was synthesized using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) following the manufacturer's instructions.

2.2. Cloning of parvalbumin genes

The cDNA coding for common carp β-parvalbumin was amplified to introduce the restriction sites *Bam*HI and *Xho*I at 5‘- and 3‘-end of the polymerase chain reaction (PCR) fragment using primers 5’-CGGATC-CATGGCATTCGCTGGAATTCTGAATG and 5’-GCTCGAGTTATGCCTT-GACCAGGGCAGC. PCR was performed using Phusion Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific, USA). The amplified DNA sequence was inserted into pJET1.2 vector and the resulting plasmid was transformed into *E. coli* DH10B cells. The cloned DNA sequence of the selected positive clones was verified by sequencing.

The sequences of genes of parvalbumins from other fish species and chicken α-parvalbumin were taken from the WHO/IUIS Allergen Nomenclature Sub-Committee (http://www.allergen.org/), ALLERGOME (https://www.allergome.org/) and Uniprot (https://www.uniprot.org/) databases (Table 2). The genes were synthesized by Invitrogen (USA) and cloned into pMA-RQ vector.

2.3. Expression of recombinant fusion proteins in E. coli

The recombinant plasmid pJET1.2 bearing the DNA sequence similar to β-parvalbumin-encoding partial sequences (GenBank accession No. LHQP01000860.1 and LHQP01019434.1) was selected for further experiments. The parvalbumin-encoding DNA fragment was excised from

pJET1.2 with *Bam*HI and *Xho*I restriction endonucleases and cloned into respectively digested pET28-MBP-TEV vector (a gift from Zita Balklava & Thomas Wassmer, Addgene plasmid #69929; http://n2t.net/addgene:69929; RRID:Addgene_69929) (Currinn et al., 2016). The resulting construct pET28-MBP-TEV-Cyp c 1 included the β-parvalbumin encoding gene (abbreviation Cyp c 1) fused to the sequence coding for the maltose binding protein (MBP). Selected recombinant plasmid was transformed into *E. coli* Tuner (DE3) cells (Novagen, Merck, USA). The synthesis of β-parvalbumin was induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma Aldrich, Germany), then cells were cultivated for 4 h at 25 °C. The cells were harvested and disrupted by sonication (Bandelin Sonopuls HD 3100, Bandelin Electronic, Germany). The soluble and insoluble fractions were separated by centrifugation at 32,500g for 5 min at 4 °C and were analysed on 12 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions.

The genes of fish and chicken parvalbumins were excised with *Bam*HI and *Xho*I restriction endonucleases (from recombinant pMA-RQ plasmid) and cloned into respectively digested pET28-MBP-TEV plasmid. The synthesis of recombinant MBP-fused parvalbumins in *E. coli* Tuner (DE3), BL21 (DE3) or BL21 (DE3) Star was induced with 0.1 mM IPTG, then cells were cultivated for 4 h at 25 °C (*E. coli* Tuner (DE3) strain) or for 3 h at 37 °C (other *E. coli* strains).

E. coli strains DH10B (Thermo Fisher Scientific, USA), Tuner (DE3), BL21 (DE3) (Novagen, Merck, USA) and BL21 (DE3) Star (Novagen, Merck, USA) were grown at 37 °C in Luria-Bertani (LB) medium (Roth, Germany). For recombinant pJET1.2 and pMA-RQ plasmids LB medium was supplemented with 100 μg/mL ampicillin (Sigma Aldrich, Germany), while for recombinant pET28-MBP-TEV-based plasmids LB medium was supplemented with 30 μg/mL kanamycin (Sigma Aldrich, Germany).

2.4. Purification of recombinant MBP-fused parvalbumins

Purification of recombinant MBP-tagged parvalbumins was performed as described previously for recombinant MBP-tagged enolase (Sližienė et al., 2022).

2.5. Production of truncated MBP-fused parvalbumin variants for epitope mapping

DNA fragments encoding two overlapping fragments of Gad m 1, number 1 (#1) (aa 1–84), number 2 (#2) (aa 21–110), and two overlapping fragments of Cyp c 1, number 3 (#3) (aa 1–73) and number 4 (#4) (aa 30–104) were amplified from the respective full-length parvalbumin sequences by PCR using four pairs of primers with restriction endonuclease recognition sites, start and stop codons (Table 1). Amplified fragments were cloned into pJET1.2 vector. Recombinant plasmids were sequenced and from the selected plasmid DNA fragment was digested with *Bam*HI and *Xho*I restriction endonucleases and inserted into *Bam*HI/*Xho*I-digested pET28-MBP-TEV vector. MBP-fused fragments were produced in *E. coli* Tuner (DE3) strain. The protein synthesis was induced with 0.1 mM IPTG and after 4 h of cultivation at 25 °C, the cells were collected by centrifugation at 0.8 x g for 5 min

2.6. Generation of MAbs against fish parvalbumins

Hybridomas were generated as described previously (Sližienė et al., 2022). Spleen cells of BALB/c mice, immunized either with purified recombinant MBP-Cyp c 1 produced in this study or with natural Atlantic cod parvalbumin (nGad m 1) (DST, Germany), were fused with mouse myeloma Sp2/0 cells using polyethylene glycol solution (PEG-4000, Sigma-Aldrich, Germany). The target hybridomas were screened with growth medium supplemented with 15 % fetal bovine serum (FBS, Biochrom, UK) and hypoxanthine/aminopterin/thymidine (HAT, Sigma-Aldrich, Germany). Positive hybridoma clones were selected by an indirect ELISA for antigen-specific antibodies using recombinant MBP-Cyp c 1 or nGad m 1 and MBP protein (as a negative control) and then cloned by a limiting dilution assay. More than a week later after cloning, viable cell clones were tested by an indirect ELISA and the selected clones were propagated, then cultivated *in vitro* or frozen for a storage in liquid nitrogen.

Mouse Sp2/0 myeloma and hybridoma cells were cultured as described previously (Sližienė et al., 2022).

The maintenance of mice and experimental procedures were performed by a certified staff in accordance with FELASA guidelines and conformed to Lithuanian and European legislation in the Department of Biological Models (Institute of Biochemistry, Life Sciences Center, Vilnius University). The permission to use BALB/c mice for immunizations was obtained from the Lithuanian State Food and Veterinary Agency (permission No. G2–117, issued 11 June 2019).

2.7. Indirect ELISA

This assay was used to determine serum antibody titers of immunized mice, to analyze hybridoma supernatants for selection of positive clones and to investigate MAb specificity and was performed as described previously (Sližienė et al., 2022). Briefly, 96-well polystyrene plates (MaxiSorp, Thermo Fisher Scientific, USA) were coated either with natural or recombinant protein or allergen extract (50 μl per well) diluted in coating buffer (0.05 M sodium carbonate buffer, pH 9.5), to a concentration of 5 μg/mL. After blocking, the plates were incubated with either mouse blood samples diluted 1:200–1:145800 in PBS-T (0.1 % Tween 20 in PBS) or with the purified MAbs (prepared in concentrations ranging from 10 μg/mL to 13,7 ng/mL) or with undiluted hybridoma supernatants (50–150 μl/well) for 1 h at RT. Washing of the plates, incubation with HRP-labelled anti-mouse IgG (Bio-Rad, USA) and detection of the enzymatic reaction were performed as described previously.

To evaluate the reactivity of recombinant MBP-fused parvalbumins and allergen extracts with IgE from blood serum specimens of fish-allergic patients, 96-well plates were coated overnight at 4 °C with either the purified recombinant parvalbumin or allergen extract or MBP as a negative control (50 μl/well) diluted in coating buffer (a final concentration of 5 μg/mL). After blocking the plates, human serum specimens of patients with a confirmed fish allergy (*PlasmaLab International*, USA) diluted 1:10–1:90 in RotiBlock-T (0.1 % Tween 20 in 1x RotiBlock) were added to the wells (50 μl/well) and incubated for 2 h at RT. Washing of the plates, incubation with mouse anti-human IgE Fc-

Table 1
PCR primers used to generate overlapping fragments of Gad m 1 and Cyp c 1.

Allergen	Fragment number	Fragment	PCR primer sequences	Restriction endonuclease
Gad m 1	#1	aa 1–84	5'-GGATCCGCATTTGCAGGTATTCTG-3'	BamHI
			5'-CTCGAGTTAGGTTTCTGCATCGGT-3'	XhoI
Gad m 1	#2	aa 21–110	5'-GGATCCGAAGCAGCAGAAAGCTTTAG-3'	BamHI
			5'-CTCGAGTTAGGCTTTAACCAGAACTG-3'	XhoI
Cyp c 1	#3	aa 1–73	5'-GGATCCATGGCATTCGCTGG-3'	BamHI
			5'-CTCGAGTTAGAAGTTCTGCAGGA-3'	XhoI
Cyp c 1	#4	aa 30–104	5'-GGATCCAAGAGCTTCTTCGC-3'	BamHI
			5'-CTCGAGTTACTCATCAACTCCAATCT-3'	XhoI

HRP (SouthernBiotech, USA) and detection of the enzymatic reaction were performed as described previously.

To investigate the effect of calcium ions on MAbs binding to parvalbumins, 96-well plates were coated with purified recombinant parvalbumin and MBP proteins (50 μl/well) diluted in coating buffer (a final concentration 5 μg/mL) overnight at 4 °C and then blocked for 1 h at RT. After plates were washed, purified MAbs were diluted (a final concentration 5 μg/mL) in PBS-T buffer alone or in the presence of either 10 mM $CaCl_2$ or 10 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′, N′-tetraacetic acid (EGTA) and incubated for 1 h at RT. Washing of the plates, incubation with HRP-labelled anti-mouse IgG (Bio-Rad, USA) and detection of the enzymatic reaction were performed as described previously.

The isotypes of MAbs were determined by an indirect ELISA using the Mouse Immunoglobulin Isotyping ELISA Kit (BD Biosciences, USA) following the manufacturer's instructions.

2.8. Determination of MAb apparent dissociation constant (K_d)

The apparent dissociation constants (K_d) of the MAbs were determined by an indirect ELISA. The multiwell plates, coated with recombinant MBP-Cyp c 1 protein or with nGad m 1 (a final concentration 5 μg/mL), were incubated with purified MAbs (prepared in concentrations ranging from 3.3×10^{-8} M to 1.863×10^{-13} M) diluted in PBS-T for 1 h at RT. After incubation with HRP-labelled anti-mouse IgG (Bio-Rad, USA) and then with TMB substrate, the values of MAb K_d were calculated from titration curves as described previously (Zvirbliene et al., 2010) and defined as a molar concentration (M) of the MAbs that corresponds to the curve's midpoint between the maximum OD_{450} value and the background. Titration curves were drawn by the Origin Pro 8 program (OriginLab, USA) using indirect ELISA results.

2.9. MAb purification from hybridoma growth medium

MAbs were purified from hybridoma supernatants by affinity chromatography using AKTA purifier 100 chromatography system equipped with the sample pump P-960 and the fraction collector Frac-920 (GE Healthcare Bio-Sciences AB, Sweden) according to the protocol described previously (Sližienė et al., 2022). After the elution step at pH 3.0, 50 μl of 1 M Tris-HCl buffer (pH 8) was added to each eluted antibody sample. After dialyzing purified antibodies overnight at 4 °C against PBS, samples were sterile filtered and stored at 4 °C. The MAbs concentrations were determined using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA).

2.10. Conjugation of MAbs to horseradish peroxidase (HRP)

One mg of the purified MAb was labeled with horseradish peroxidase (HRP) according to the protocol described previously (Stravinskiene et al., 2019).

2.11. Western blot

Recombinant and natural allergens (1 μg per lane), cell lysates (10 μl per lane) and allergen extracts (10 μg per lane), were subjected to SDS-PAGE under reducing conditions.

After SDS-PAGE, proteins were transferred to a polyvinyldifluoride (PVDF) membrane (Carl Roth, Germany) (pre-wetted with methanol). Non-specific binding of the membrane was blocked with 2 % milk powder (Roth, Germany) in PBS for 1 h at RT and after washing with PBS-T, the membrane was incubated with either hybridoma supernatants diluted 1:2, or human serum specimens diluted 1:50, or with purified MAbs at a final concentration 5–10 μg/mL in 2 % milk powder in PBS-T for 1 h at RT. Following the next washing step, the membrane was incubated with either goat anti-mouse IgG antibody conjugated to HRP (Bio-Rad, USA) diluted 1:4000 or with mouse anti-human IgE Fc-HRP (SouthernBiotech, USA) diluted 1:1000 in 2 % milk powder in PBS-T for 1 h at RT. After the final washing cycle, the membrane was developed with 1-Step™ TMB-Blotting Substrate Solution (Thermo Fisher Scientific, USA) for 5–10 min and the enzymatic reaction was stopped by washing membranes in deionized water.

2.12. Dot blot

Samples of purified recombinant parvalbumins (2 μl), allergen extracts (5 μl) and MBP protein (2 μl) (a final concentration 1 μg/μl, diluted in PBS) were spotted onto the PVDF membrane (pre-wetted with methanol) in a series of small dots. Membranes were dried for 10 min at RT and then blocked with 2 % milk powder in PBS for 1 h at RT. After washing them twice with PBS-T, membranes were incubated with purified MAb (a final concentration 5 μg/mL) diluted in 2 % milk powder in PBS-T for 1 h at RT. The subsequent membrane washing, incubation with HRP-labelled anti-mouse IgG (Bio-Rad, USA) and treatment with TMB-Blotting Substrate Solution was performed as described for Western blot analysis.

2.13. Serum specimens

A collection of serum specimens of allergic patients was purchased from *PlasmaLab International* (USA). Serum specimens of patients with confirmed fish allergy were selected from this collection based on the information provided by the supplier. Twenty-one serum specimen of patients who experienced at least one of the typical clinical symptoms of allergy (allergic rhinitis, dermatitis, urticaria, diarrhea, asthma, or anaphylactic reaction) after a contact with fish, were selected for this study. As indicated by the supplier, the levels of IgE antibodies specific to natural cod extract in serum specimens were determined by ImmunoCAP (Phadia, Thermo Fisher Scientific, Uppsala, Sweden). Among the selected 21 serum specimen, the levels of cod-specific sIgE exceeded 50 kU/L in 9 serum specimens (S1, S2, S4, S5, S6, S7, S9, S10 and S15), while 12 specimens had cod-specific sIgE levels less than 50 kU/L. Serum specimens from patients that had other allergies, but no detectable fish-specific IgE by ImmunoCAP, were used as negative controls (S22, S23 and S24).

2.14. Allergen extracts

Commercial allergen extracts were purchased from two different manufacturers (DST, Germany and Stallergenes Greer, Switzerland).

In-house allergen extracts from different fish species, chicken (*Gallus domesticus*) and pork (*Sus scrofa domesticus*) were purchased from a local store and were prepared as described previously (Sližienė et al., 2022).

2.15. Immunoprecipitation

One-hundred μl of rProtein A Sepharose Fast Flow (GE Healthcare Life Sciences, USA) were washed 4 times with 0.1 M Tris-HCl solution (pH 8), each time centrifuged at 3000 x g for 3 min. The prepared rProtein A Sepharose was mixed with 0.5 mg of purified MAbs and incubated for 1 h at RT with rotation. Then the mixture was centrifuged at 3000 x g for 5 min and the MAb-Sepharose complex was washed 4 times with PBS, each time centrifuged at 3000 x g for 3 min. After that the MAb-Sepharose was equally divided to different allergen extracts (each 50 μl) and incubated overnight at 4 °C with rotation. Samples were centrifuged at 3000 x g for 5 min and then washed 4 times with PBS, each time centrifuged at 3000 x g for 3 min. After final wash, the pellets were resuspended in 100 μl PBS and analyzed by SDS-PAGE and Western blot, using parvalbumins specific HRP-labeled MAb 3F6 diluted 1:500 in 2 % milk powder in PBS-T for 1 h at RT or using diluted human serum specimens as described for Western blot analysis.

3. Results

3.1. Expression and purification of recombinant fish parvalbumins fused to maltose binding protein

Recombinant carp β-parvalbumin was used as an immunogen for MAb generation. The cDNA sequence, coding for the common carp (*Cyprinus carpio*) β-parvalbumin, was obtained from mRNA, extracted from the skeletal muscle of a common carp. When cDNA was ampified and ligated into the pJET1.2 vector, one recombinant clone was selected, containing 342 base-pair (bp) fragment, showing high similarity to the common carp β-parvalbumin sequence published in GenBank. This DNA fragment was then inserted into the pET28-MBP-TEV vector to produce recombinant common carp β-parvalbumin with the N-terminal MBP (MBP-Cyp c 1) in *E.coli*.

Parvalbumins of other fish species and chicken α-parvalbumin fused with MBP were produced according to the procedure used for MBP-Cyp c 1 protein, except that parvalbumin-coding genes were synthesised according to sequences available in the WHO/IUIS Allergen Nomenclature Sub-Committee (http://www.allergen.org/), ALLERGOME (https://www.allergome.org/) and Uniprot (https://www.uniprot.org/) databases (Table 2, SM Fig. 1).

In total, a collection of 13 recombinant parvalbumins was generated. All produced recombinant parvalbumins were mostly found in soluble fractions of *E.coli* lysates and were purified by affinity chromatography using MBPTrap HP column prepacked with Dextrin Sepharose (Fig. 1). The yields of the purified recombinant parvalbumins were about 4–6 mg/300 mL of bacterial cultures.

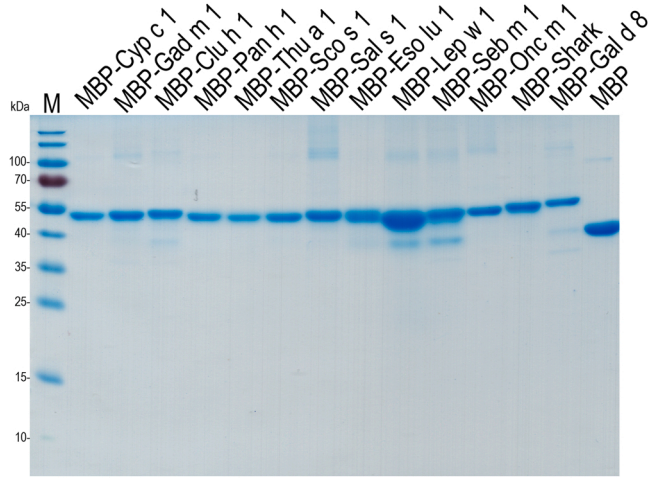

Fig. 1. SDS-PAGE analysis of purified recombinant proteins. Lane M: protein molecular weight marker (Thermo Fisher Scientific, USA); MBP protein (44.2 kDa).

3.2. The reactivities of recombinant parvalbumins with blood serum IgE

Purified recombinant fish parvalbumins were tested for IgE binding capacity by ELISA using 21 serum specimens of patients that were sensitized to fish allergens (*PlasmaLab International*, USA). At first, these serum specimens were tested with the purified MBP-Cyp c 1 protein and 14 of them were found to be reactive with this recombinant allergen (Fig. 2 A). For the characterization of the whole collection of recombinant parvalbumins, 4 serum specimens of fish-allergic patients were used. These serum specimens were reactive not only with fish β-parvalbumins, but also demonstrated a cross-reactivity with allergens of different fish species and with chicken α-parvalbumin. Besides that, these serum specimens showed no reactivity to recombinant MBP-fused *Anisakis simplex* allergen (MBP-Ani s 4), used as a control MBP-fused allergen to confirm that serum specimens specifically recognize parvalbumins (Fig. 2 B). Moreover, 4 serum specimens were tested both by ELISA and Western blot with commercial cod and carp extracts (DST, Germany) to analyze their IgE reactivity with natural allergens. All tested specimens were reactive with these fish extracts in ELISA, while 2 serum specimens were also reactive in Western blot with cod and carp extract (Fig. 2 C, SM Fig. 2). Since serum specimens from fish-allergic patients recognized natural allergens in tested fish extracts and also reacted with our purified recombinant α- and β-parvalbumins, this suggests antigenic similarities of recombinant allergens with natural allergens present in fish extracts. All tested serum specimens showed no reactivity with recombinant MBP used as a negative control (Fig. 2, SM Fig. 2).

3.3. Generation and characterization of MAbs against fish allergens

To generate hybridomas, BALB/c mice were immunized with either purified recombinant MBP-Cyp c 1 produced in this study or natural Atlantic cod parvalbumin (nGad m 1) (DST, Germany). After fusion of mouse myeloma cells with spleen cells of the immunized mice with high-titered antibody response (>1:16000), hybrid clones secreting antibodies specific either to MBP-Cyp c 1 or to nGad m 1, respectively, but non-reactive with MBP protein, were screened by an indirect ELISA. Selected hybridoma clones were cloned by limiting dilution assay and

Table 2
List of recombinant parvalbumins produced and analyzed in this study.

Recombinant parvalbumin	MW_1 (kDA)	Allergen name (IUIS)	MW_2 (kDA)	Parvalbumin isoform	Common name of allergen source	Species	Order	*E.coli* strain for protein expression
MBP-Cyp c 1	55.7	Cyp c 1	11.5	β	Common carp	*Cyprinus carpio*	Cypriniformes	Tuner (DE3)
MBP-Gad m 1	55.5	Gad m 1	11.3	β	Atlantic cod	*Gadus morhua*	Gadiformes	Tuner (DE3)
MBP-Sal s 1	56.1	Sal s 1	11.9	β	Atlantic salmon	*Salmo salar*	Salmoniformes	BL21 (DE3)
MBP-Onc m 1	56.1	Onc m 1	11.9	β	Rainbow trout	*Oncorhynchus mykiss*	Salmoniformes	BL21 (DE3) Star
MBP-Clu h 1	55.9	Clu h 1	11.7	β	Atlantic herring	*Clupea harengus*	Clupeiformes	Tuner (DE3)
MBP-Pan h 1	55.8	Pan h 1	11.6	β	Striped catfish	*Pangasianodon hypophthalmus*	Siluriformes	Tuner (DE3)
MBP-Thu a 1	55.7	Thu a 1	11.5	β	Yellowfin tuna	*Thunnus albacares*	Scombriformes	Tuner (DE3)
MBP-Sco s 1	55.7	Sco s 1	11.5	β	Atlantic mackerel	*Scomber scombrus*	Scombriformes	BL21 (DE3) Star
MBP-Eso lu 1	55.6	Eso lu 1	11.4	β	Northern pike	*Esox lucius*	Esociformes	BL21 (DE3) Star
MBP-Lep w 1	55.9	Lep w 1	11.7	β	Megrim	*Lepidorhombus whiffiagonis*	Pleuronectiformes	BL21 (DE3) Star
MBP-Seb m 1	55.6	Seb m 1	11.4	β	Ocean perch	*Sebastes norvegicus*	Scorpaeniformes	BL21 (DE3) Star
MBP-Shark	56.1	-	11.9	α	Leopard shark	*Triakis semifasciata*	Carcharhiniformes	BL21 (DE3) Star
MBP-Gal d 8	56.3	Gal d 8	12.1	α	Chicken	*Gallus domesticus*	Galliformes	BL21 (DE3)

MW_1 – calculated molecular weight (MW) of the synthesized recombinant parvalbumin.
MW_2 – molecular weight of the allergen according to WHO/IUIS Allergen Nomenclature Sub-Committee (http://www.allergen.org/), ALLERGOME (https://www.allergome.org/) and Uniprot (https://www.uniprot.org/) databases.

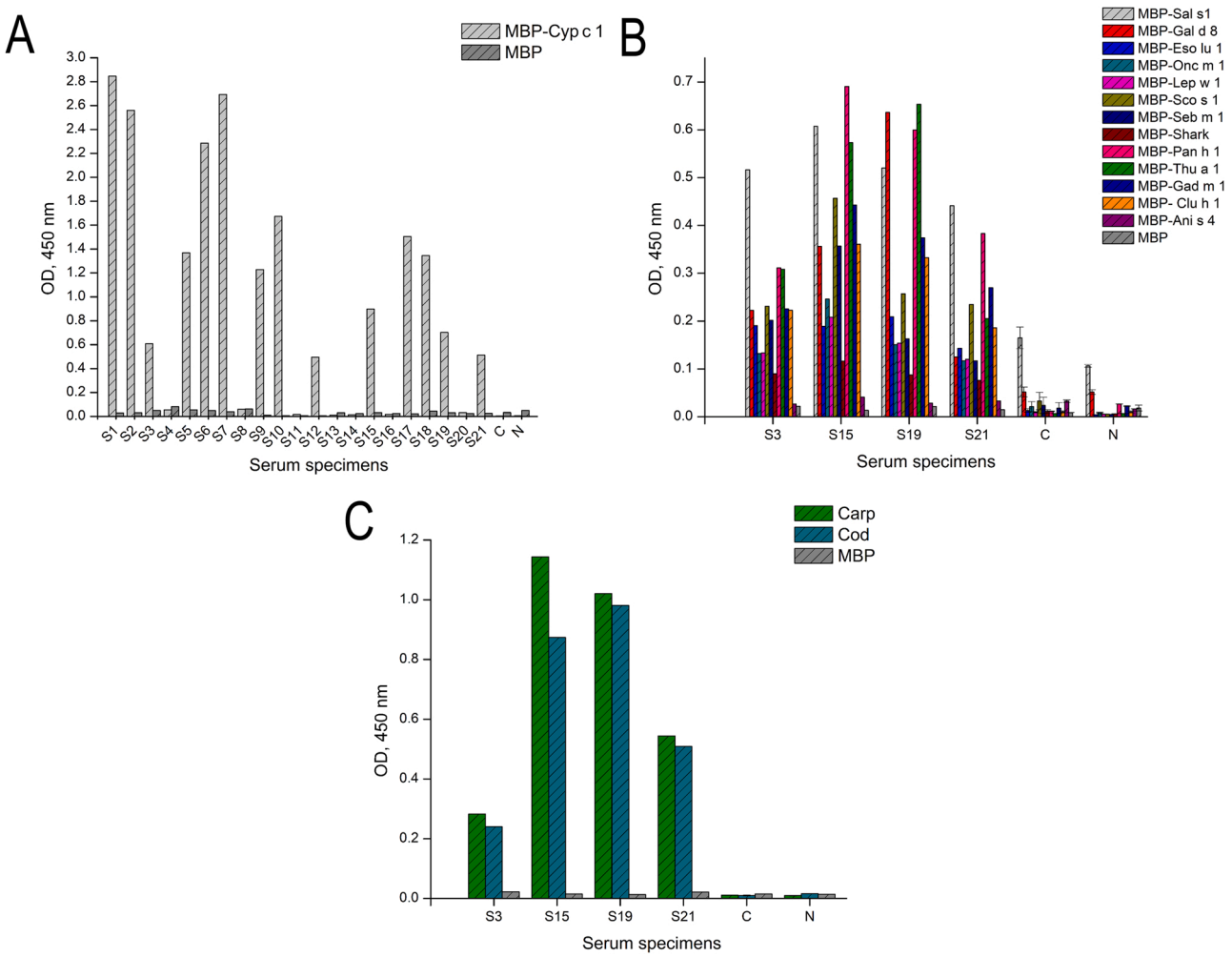

Fig. 2. The reactivities of serum IgE of fish-allergic patients with purified recombinant MBP-fused allergens and commercial allergen extracts as determined by ELISA. S1–S21: serum specimens of patients with confirmed fish allergy; C: negative control, serum specimens of patients with other allergies, S22–S24; N: incubation without serum specimen, only with anti-human IgE Fc-HRP (SouthernBiotech, USA). Serum samples were diluted 1:10. OD indicates optical density.

tested again for their specificity for the antigen. In total, five stable hybridoma cell lines of IgG1 subtype were developed: one MAb (clone 3F6) against recombinant common carp β-parvalbumin and four MAbs (clones 7B2, 2C1, 18H3 and 16B3) against nGad m 1. The supernatants of these hybridomas were collected and used for the purification of MAbs and their characterization.

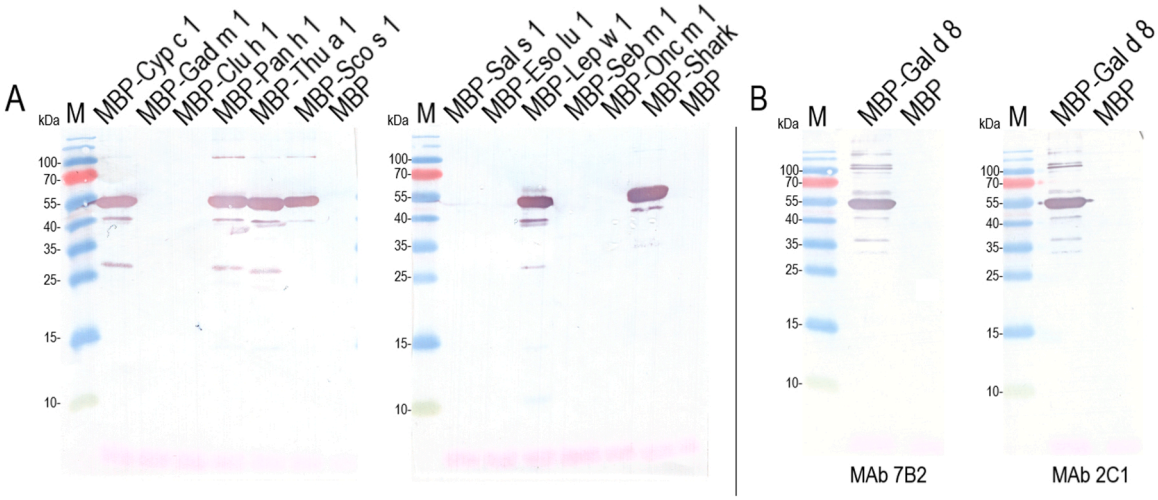

Fig. 3. The reactivity of MAb 7B2 with heated recombinant fish allergens (A) and the reactivities of MAb 7B2 and 2C1 with heated recombinant chicken α-parvalbumin (B) by Western blot. Lane M: protein molecular weight marker.

All generated MAbs were reactive with their respective antigens and showed no reactivity to recombinant MBP protein both by indirect ELISA and Western blot (SM Fig. 3). The affinity of each MAb to the antigen was determined by calculating the apparent dissociation constant (K_d) from the results of an indirect ELISA. The K_d values ranged from 2.43×10^{-10} M to 2.7×10^{-9} M, indicating high affinity of all MAbs.

3.4. The reactivities of MAbs with parvalbumins of other fish species

To determine whether MAbs are specific only to one fish species or may recognize parvalbumins from other fish species, a collection of purified recombinant fish parvalbumins was used. All MAbs were shown to be cross-reactive with recombinant allergens by ELISA, Western blot and dot blot although demonstrated slightly different pattern of reactivity (Fig. 3, Fig. 5 and Fig. 7, SM Fig. 4, SM Fig. 8 and SM Fig. 10). MAb 3F6 raised against common carp β-parvalbumin showed a strong cross-reactivity by ELISA with all tested recombinant fish β-parvalbumins, except MBP-Eso lu 1. Four MAbs raised against nGad m 1 recognized most of recombinant fish β-parvalbumins and demonstrated different strength of cross-reactivity with 9 allergens while were non-reactive with MBP-Eso lu 1, MBP-Sal s 1, MBP-Onc m 1 (only MAb 16B3 reacted by ELISA) and MBP-Seb m 1. Moreover, all MAbs reacted with recombinant Leopard shark α-parvalbumin (Table 3).

To prove the ability of MAbs to recongize not only recombinant fish parvalbumins but also natural allergens, their ability to detect β-parvalbumins in natural fish extracts was investigated using four commercial fish allergen extracts of carp, herring, salmon and cod (DST, Germany) and 12 in-house prepared fish allergen extracts.The cross-reactivity pattern was investigated by the same three immunoassays – ELISA, Western blot and dot blot (Table 4). All MAbs recognized β-parvalbumins in all fish extracts by ELISA and dot blot (Fig. 5, SM Fig. 6 and SM Fig. 8). Western blot analysis of heated fish extracts with the MAbs revealed about 10–13 kDa protein bands at positions that correspond to the molecular weight of full-length α- or β-parvalbumins (Fig. 4 and SM Fig. 7 A-D.

Only MAb 3F6 raised against common carp β-parvalbumin detected parvalbumins of all tested fish species by Western blot. MAbs 7B2 and 2C1 did not recognize any target antigen in European smelt, while MAb 18H3 and 16B3 in Eurasian ruffe extract. Neither MAb 2C1 nor 18H3 detect any target protein in Atlantic salmon and trout extracts. To exclude the potential non-specific reactivity in Western blot, we used an irrelevant MAb 19C19 against the recombinant MBP protein as a negative control to prove that all parvalbumin-specific MAb bind only parvalbumins in fish extracts. MAb 19C19 did not stain any protein band in tested fish extracts thus confirming the specificity of Western blot assay (SM Fig. 7E).

These data indicate that the newly generated MAbs were able to recognize both fish parvalbumins (α- and β-parvalbumins) of different fish species. However, their cross-reactivities with parvalbumins of other animals still remained unknown. To address this question, we investigated MAb reactivities with recombinant chicken α-parvalbumin, fused to MBP (MBP-Gal d 8) and with either commercial (Stallergenes Greer, Switzerland) or in house prepared chicken and pork extracts by ELISA, Western blot and dot blot (Tables 3 and 4). All MAbs reacted with recombinant MBP-Gal d 8 (Fig. 5 and Fig. 7, SM Fig. 8) and with non-heated chicken and pork extracts, however they were not able to detect parvalbumins in heated extracts by Western blot. These data show that the newly generated MAbs recognize epitopes common to parvalbumins not only of various fish species but also other animals.

3.5. The use of MAbs for parvalbumin immunoprecipitation from allergen extracts

For further MAb characterization, we investigated their ability to isolate parvalbumins from allergen extracts by immunoprecipitation method. For this, we used commercial and in-house prepared extracts of carp, salmon and chicken and incubated them overnight with prepared Protein A-Sepharose complex with either MAb 7B2 or 3F6. After incubation, the Sepharose-antibody-parvalbumin complex was analyzed by SDS-PAGE and Western blot. About 10–13 kDa sized protein bands corresponding to parvalbumins were identified using HRP-labeled MAb 3F6 in tested extracts. MAb 7B2 was able to immunoprecipitate fish β-parvalbumin from carp extract (Fig. 6A, lanes 2 and 3), while MAb 3F6 – from the extracts of both fish species and α-parvalbumin from the commercial chicken extract (Fig. 6 B, lanes 2, 4 and 5). As a negative control for immunopreciptation, we used MAb 19C19 specific to MBP that was pre-incubated with Protein A-Sepharose and then incubated with allergen extracts. The analysis of the immunoprecipitated samples by SDS-PAGE and Western blot did not reveal any protein band corresponding to parvalbumins (10–13 kDa) after incubation with HRP-labeled MAb 3F6, thus confirming the specificity of the immunoprecipitation assay (Fig. 6C, lanes 2–7).

Since our MAbs are able to immunoprecipitate parvalbumins from fish extracts, we used one of them – MAb 3F6 – to isolate natural parvalbumins from fish extracts and then analyze their reactivities with IgE from serum specimens of fish-allergic patients. First, the complex of MAb 3F6 and Protein A-Sepharose was incubated overnight with commercial cod and carp extracts (DST, Germany) to immunoprecipitate the parvalbumins. After incubation, the immunopreciptated material was analyzed by SDS-PAGE and Western blot, incubating the membranes with diluted serum specimens and later with mouse anti-human IgE Fc-

Table 3
Summary of MAb reactivities with recombinant parvalbumins by ELISA, Western blot and dot blot assays.

	MAb 7B2			MAb 2C1			MAb 18H3			MAb 16B3			MAb 3F6		
Recombinant allergen	E	WB	DB	E	WB	DB	E	WB	DB	E	WB	DB	E	WB	DB
MBP-Cyp c 1	+++	x	x	+++	x	x	++	x	x	++	x	x	+++	x	x
MBP-Gad m 1	+		x	+		x	+++	x	x	++	x	x	+++	x	x
MBP-Sal s 1													+++	x	x
MBP-Onc m 1										+			+++	x	x
MBP-Clu h 1	+			+			++	x	x	+		x	+++	x	x
MBP-Pan h 1	+++	x	x	+++	x	x	+	x	x	++	x	x	+++	x	x
MBP-Thu a 1	+++	x	x	+++	x	x	+++	x	x	+	x	x	++		x
MBP-Sco s 1	+++	x	x	+++	x	x	+++	x	x	++	x	x	+++	x	x
MBP-Eso lu 1															
MBP-Lep w 1	+++	x	x	+++	x	x	+	x	x	+	x	x	++	x	x
MBP-Seb m 1													++	x	x
MBP-Shark	+++	x	x	+++	x	x	+	x	x	+	x	x	++	x	x
MBP-Gal d 8	+++	x	x	+++	x	x	+	x	x	+	x	x	+	x	x

x indicate MAb reactivity with the antigen; empty space – no reactivity.
E – ELISA, WB – Western blot, DB – dot blot methods.
ELISA reactivity: (+++) $OD_{450} > 1.5$; (++) OD_{450} 0.5–1.5; (+) $OD_{450} < 0.5$.

Table 4
Summary of MAb reactivities with allergen extracts determined by ELISA, Western blot and dot blot assays.

	MAb 7B2			MAb 2C1			MAb 18H3			MAb 16B3			MAb 3F6		
Allergen extract	E	WB	DB	E	WB	DB	E	WB	DB	E	WB	DB	E	WB	DB
Carp (M)	+++	x	x	+++	x	x	+	x	x	+++	x	x	+++	x	x
Herring (M)	++	x	x	++	x	x	+	x	x	++	x	x	++	x	x
Salmon (M)	+	x	x	+		x	+		x	+	x	x	++	x	x
Cod (M)	+++	x	x	+++	x	x	+++	x	x	+++	x	x	+++	x	x
Common carp	+++	x	x	+++	x	x	+	x		+++	x		+++	x	x
Atlantic herring	+++	x	x	+++	x	x	+	x		+	x	x	++	x	x
Atlantic salmon	+	x	x	+		x	+		x	+	x	x	+	x	x
Trout	+	x	x	+		x	+		x	+	x	x	++	x	x
Saithe	+++	x	x	+++	x	x	+++	x	x	+++	x	x	+++	x	x
Alaska pollock	+++	x	x	+++	x	x	+++	x	x	+++	x	x	+++	x	x
Northern pike	+++	x	x	+++	x	x	+++	x	x	+++	x	x	+++	x	x
European smelt	+		x	+		x	+++	x	x	+++	x	x	+++	x	x
Common roach	+++	x	x	+++	x	x	+++	x	x	+++	x	x	+++	x	x
Common bream	+++	x	x	+++	x	x	+	x	x	++	x	x	+++	x	x
European perch	+++	x	x	+++	x	x	+			+		x	+++	x	x
Eurasian ruffe	+++	x	x	+++	x	x	+			+		x	+++	x	x
Chicken (M)	+			+		x	+			+			+		
Pork (M)	++		x	++		x	+		x	+		x	+		x
Chicken	+			+			+			+			+		
Pork	++		x	++			++		x	++			++		x

x indicate MAb reactivity with the antigen; empty space – no reactivity.
E – ELISA, WB – Western blot, DB – dot blot methods.
ELISA reactivity: (+++) $OD_{450} > 1.5$; (++) OD_{450} 0.5–1.5; (+) $OD_{450} < 0.5$.
M – commercial extracts of different manufacturers (M).

HRP. Two serum specimens reacted in Western blot with natural cod and carp parvalbumins isolated from fish extracts (SM Fig. 9) demonstrating that IgE from these serum specimens recognize both natural and recombinant parvalbumins.

3.6. Investigation of the effect of calcium on MAb binding to parvalbumins

To determine whether MAb binding to different parvalbumins depends on calcium ions, an indirect ELISA was performed, where recombinant parvalbumins coated on ELISA plates were incubated with MAbs in the presence of either $CaCl_2$ or EGTA reagent (Fig. 7). After adding $CaCl_2$, all MAbs against nGad m 1 showed reactivity to previously non-reactive recombinant MBP-Eso lu 1, MBP-Sal s 1, MBP-Onc m 1 or MBP-Seb m 1 proteins. No significant binding differences with other proteins, except for MAb 16B3, that demonstrated decreased reactivity signals with several recombinant parvalbumins (MBP-Cyp c 1, MBP-Gad m 1 and MBP-Pan h 1) were observed. In the presence of EGTA, MAbs 7B2 and 2C1 showed a significant decrease in ELISA signal with recombinant MBP-Cyp c 1, MBP-Gal d 8, MBP-Pan h 1, MBP-Thu a 1, MBP-Lep w 1, MBP-Sco s 1 and MBP-Shark, while MAb 18H3 – with MBP-Gad m 1. Moreover, for MAb 18H3 and 16B3 we observed a decreased signal with certain recombinant allergens in the presence of either of the reagent. After adding $CaCl_2$, MAb 3F6 binding to MBP-Sal s 1, MBP-Eso lu 1, MBP-Lep w 1 and MBP-Seb m 1 increased, while a significant decrease was observed with MBP-Gad m 1, MBP-Clu h 1, MBP-Onc m 1 and MBP-Sco s 1 after their incubation in the presence of EGTA. Besides that, MAb 3F6 showed no changes in reactivity with MBP-Cyp c 1 and MBP-Pan h 1 in the presence of $CaCl_2$, while reduced binding activity to MBP-Gal d 8, MBP-Thu a 1 and MBP-Shark parvalbumins was observed (Fig. 7).

3.7. Localization of MAb epitopes

To determine the epitopes of recombinant Gad m 1 and Cyp c 1 proteins recognized by the MAbs, two overlapping recombinant MBP-fused fragments of Gad m 1 (fragment #1, aa 1–84, fragment #2, aa 21–110) and two overlapping recombinant MBP-fused fragments of Cyp c 1 (fragment #3, aa 1–73, fragment #4, aa 30–104) were constructed (Fig. 9). All recombinant fragments were expressed in *E. coli* Tuner (DE3) cells and the reactivities of MAbs with the lysates of transformed *E. coli* cells expressing the respective fragments were investigated by Western blot (Fig. 8). MAbs 7B2 and 2C1 reacted with fragment #4 that represents the C-terminal region of Cyp c 1 (aa 30–104). MAb 18H3 was reactive with fragment #2 in the C-terminal region of Gad m 1 (aa 21–110). MAb 16B3 recognized fragment #1 the N-terminal region of Gad m 1 (aa 1–84), while MAb 3F6 reacted both with fragment #2 and fragment #4 representing the C-terminal regions of Gad m 1 (aa 21–110) and Cyp c 1 (aa 30–104), respectively. Based on the reactivities of the MAbs with parvalbumin fragments, it was concluded that the epitopes of MAbs 7B2 and 2C1 are located between aa 74–104 of Cyp c 1, the epitope of MAb 18H3 – between aa 85–110 of Gad m 1, the epitope of MAb 16B3 – between aa 1–20 of Gad m 1 and the epitope of MAb 3F6 – between aa 74–104 of Cyp c 1 (Fig. 9). Amino acid sequence alignment of the predicted epitope region of each MAb with recombinant parvalbumins revealed high sequence similiarity across parvalbumins of different fish species and chicken (Fig. 10).

4. Discussion

Fish contains valuable nutrients, however, for certain patients it can cause allergic reactions (Dasanayaka et al., 2022; Feketea et al., 2021). The prevalence of allergy to fish varies according to geographic regions, eating habits and fish preparation methods (Feketea et al., 2021; Klueber et al., 2019). Cod, carp, salmon, mackerel, tuna are one of the most popular and frequently consumed fish species around the world (Feketea et al., 2021). Fish-allergic patients could be sensitized to a single fish species or to various fish species (Kuehn et al., 2014). Parvalbumins are known as the most important fish allergens that have been identified in a broad spectrum of fish species and are abundant in the fish white muscle (Fernandes et al., 2015; Dijkema et al., 2022). Even though fish-allergic patients avoid eating fish, they are still at risk of developing allergic reactions due to cross-contamination or inhalation of fish vapour during food processing (Fernandes et al., 2017; van der Ventel et al., 2011). The need for detecting fish allergens (like parvalbumins) in foods has led to the development of various immunoassays (ELISA, ICA), where parvalbumin-specific antibodies or recombinant parvalbumins are used (Fernandes et al., 2017).

To improve food allergy diagnostics, treatment or studying the

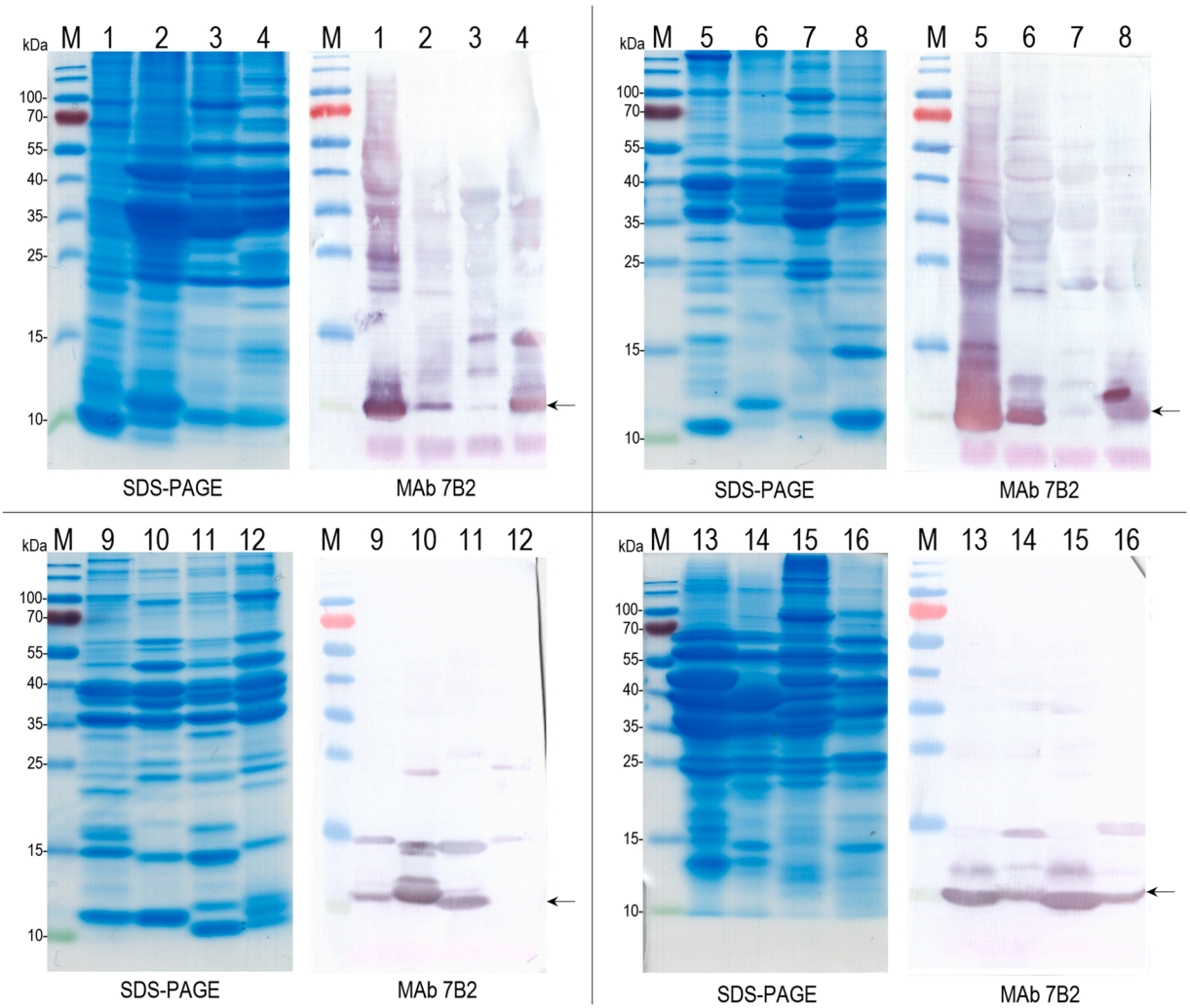

Fig. 4. The reactivity of MAb 7B2 with heated fish extracts by Western blot. Lane M: protein molecular weight marker; lane 1: Carp extract (M); lane 2: Herring extract (M); lane 3: Salmon extract (M); lane 4: Cod extract (M); lane 5: Common carp extract; lane 6: Atlantic herring extract; lane 7: Atlantic salmon extract; lane 8: Trout extract; lane 9: Saithe extract; lane 10: Alaska pollock extract; lane 11: Northern pike extract; lane 12: European smelt extract; lane 13: Common roach extract; lane 14: Common bream extract; lane 15: European perch extract; lane 16: Eurasian ruffe extract. M – commercial extracts, the others are in-house prepared extracts. The position of β-parvalbumin is indicated by an arrow.

structural characteristics of specific food allergens, recombinant food allergens are used. These recombinant proteins can be produced in unlimited amounts and at constant quality, are stable and might be immunologically identical to the native protein (Lorenz et al., 2001). Recombinant fish parvalbumins of various fish species (Atlantic salmon, common carp, Atlantic cod, Alaska pollack, wolf-herring, Japanese flounder) and recombinant β-enolase of common carp were shown to be recognized by specific human IgE, suggesting their potential application in fish allergy *in vitro* diagnostics (Van Do et al., 1999, 2003, 2005b; Swoboda et al., 2002a; Ma et al., 2008; Mohammadi et al., 2017; Sun et al., 2019; Sližienė et al., 2022). Standardization or replacement of certain fish extracts that are used in SPT by recombinant fish allergens, could improve the specificity and sensitivity of the test (Lorenz et al., 2001). Currently, only recombinant cod parvalbumin (rGad c 1) is used in the ImmunoCAP®ISAC (Thermo Fisher Scientific) assay. Moreover, production of recombinant allergens with reduced allergenic activity is considered to be effective and safe to use for allergen immunotherapy. Recombinant common carp parvalbumin, containing mutations in both calcium-binding sites showed a reduced allergenic activity and was selected by the European Union project FAST (food allergy-specific immunotherapy) to develop hypoallergenic vaccine for subcutaneous immunotherapy (SCIT) of fish allergy (Lorenz et al., 2001; Swoboda et al., 2007; Zuidmeer-Jongejan et al., 2015).

In the current study, we describe the production and characterization of 13 soluble recombinant fish allergens fused to MBP that enabled their purification on the amylose resin (Reuten et al., 2016). The reactivities of recombinant allergens with serum samples of fish-allergic patients suggest their antigenic similarities with natural allergens. This collection of recombinant proteins comprises different fish parvalbumins that have been analyzed in previous studies. In addition, we included Northern pike, Leopard shark and chicken parvalbumins to investigate MAb specificity for a broad spectrum of α- and β- parvalbumins.

Standardization of allergen extracts, development of new immunoassays for allergen detection and isolation of allergen components from analyzed samples are the major application areas for monoclonal and polyclonal antibodies raised against allergens (Jeong et al., 2017; Kiyota et al., 2016). In many studies, polyclonal antibodies produced in rabbits and mice against fish parvalbumins from Atlantic salmon, catfish, Atlantic cod, carp, barramundi, basa, pilchard were used to detect parvalbumins in various fish extracts and samples or to study their antigenic cross-reactivities (Ruethers et al., 2021; Lindstrøm et al., 1996; Koppelman et al., 2012; Faeste et al., 2008; Sharp et al., 2015). The first MAbs (clones 235, 239 and 267) were generated against carp parvalbumin and showed reactivity with carp, mouse, rat and monkey

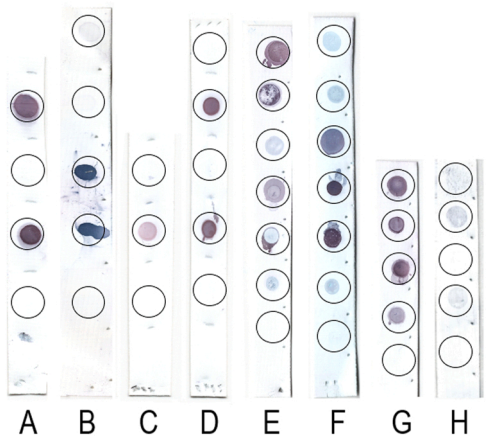

Fig. 5. The reactivity of MAb 7B2 with different recombinant proteins and allergen extracts by dot blot. Strip A: MBP-Cyp c 1 protein; MBP-Sal s 1 protein; MBP-Gal d 8 protein; MBP (negative control). Stirp B: MBP-Gad m 1 protein; MBP-Clu h 1 protein; MBP-Pan h 1 protein; MBP-Thu a 1 protein; MBP (negative control). Strip C: MBP-Eso lu 1 protein; MBP-Lep w 1 protein; MBP (negative control). Strip D: MBP-Onc m 1 protein; MBP-Sco s 1 protein; MBP-Seb m 1 protein; MBP-Shark protein; MBP (negative control). Strip E: Carp extract (M); Herring extract (M); Salmon extract (M); Cod extract (M); Common carp extract; Atlantic herring extract; MBP (negative control). Strip F: Atlantic salmon extract; Trout extract; Saithe extract; Alaska pollock extract; Northern pike extract; European smelt extract; MBP (negative control). Strip G: Common roach extract; European perch extract; Common bream extract; Eurasian ruffe extract; MBP (negative control). Strip H: chicken extract (M); pork extract (M); chicken extract; pork extract; MBP (negative control). M – commercial extracts, the others are in-house prepared extracts. Samples were spotted onto the PVDF membrane from top to bottom.

parvalbumins (Celio et al., 1988). Another MAb was developed against southern bluefin tuna, that recognized a native epitope of both tuna and common carp parvalbumins and against silver carp parvalbumin, which was used to develop the sandwich ICA strip based on Fe_3O_4/Au nanoparticles for rapid parvalbumin detection in foodstuff (Kawase et al., 2001; Zhang et al., 2020). MAb PARV-19 is a widely used antibody, raised against frog parvalbumin. It recognizes parvalbumins of various fish species (carp, catfish, cod, tilapia, Elephant shark and others). However, in one study, MAb PARV-19 showed no cross-reactivity with yelowfinn tuna extract, while in another study it did not react with swordfish, pollock and other fish species extracts (Chen et al., 2006; Gajewski et al., 2009, Saptarshi et al., 2014). This demonstrates the need for broadly-reactive MAbs that could be used as a reliable tool to evaluate parvalbumin content in different extracts. In the current study, we generated a panel of MAbs against recombinant MBP-Cyp c 1 and natural Atlantic cod parvalbumin (nGad m 1) that were strongly reactive with their target proteins. To evaluate their ability to recognize parvalbumins of different species, a comprehensive analysis of their cross-reactivies with a large collection of both recombinant and natural parvalbumins was performed.

Many studies have described that parvalbumins of different fish species share high amino acid sequence identities and fish-allergic patients' IgE antibodies can cross-react with extracts of various fish species (Kuehn et al., 2014; Van Do et al., 2005a; Ruethers et al., 2019). Therefore, we investigated the ability of MAbs to recognize parvalbumins of other fish species by different immunoassays. MAb 3F6 raised against recombinant Cyp c 1 demonstrated the broadest cross-specificity being reactive with all tested fish allergens, except MBP-Eso lu 1, suggesting its epitope localization at a highly conserved region of fish parvalbumins. Four MAbs raised against nGad m 1 reacted with the majority of tested recombinant fish allergens both in ELISA and dot blot. However, they recognized only certain antigens by Western blot, which

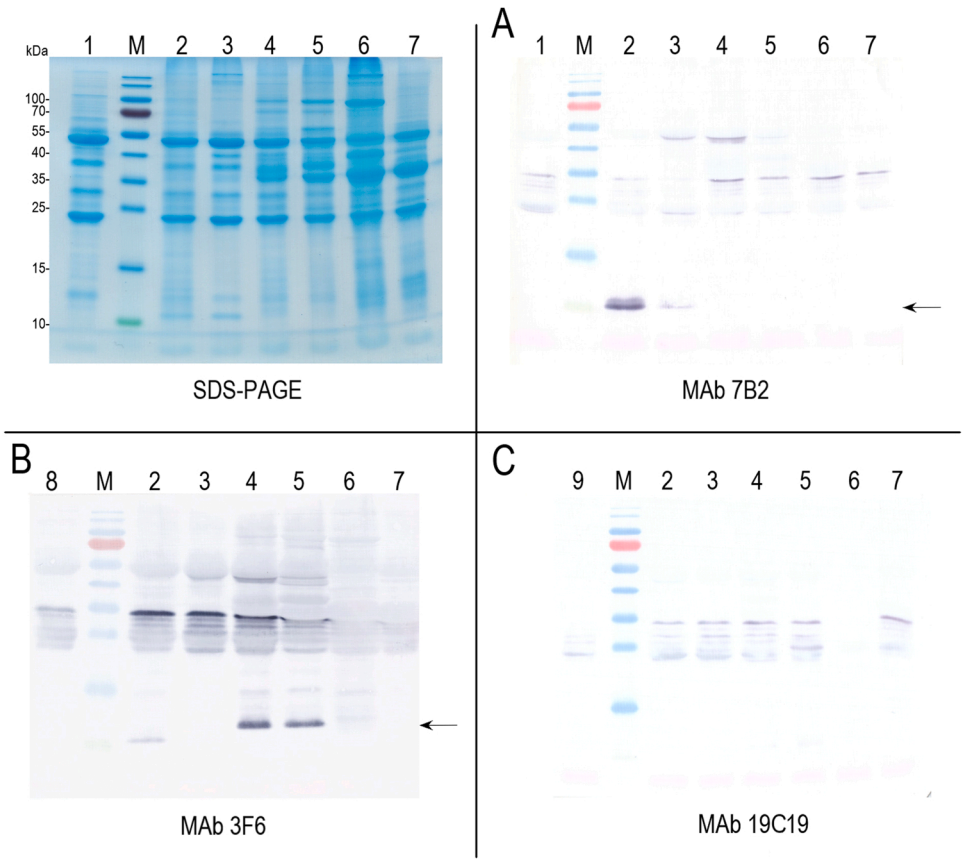

Fig. 6. Parvalbumin immunoprecipitation from allergen extracts either using MAb 7B2 (A) or MAb 3F6 (B) and detection with HRP-labeled MAb 3F6 by Western blot. Lane M: protein molecular weight marker; lane 1: Protein A-Sepharose complex with MAb 7B2; lane 2–3: carp extract; lane 4–5: salmon extract; lane 6–7: chicken extract; lane 8: Protein A-Sepharose complex with MAb 3F6; lane 9: Protein A-Sepharose complex with anti-MBP MAb 19C19 (negative control); Lanes 2, 4 and 6: commercial extracts; lane 3, 5 and 7: in-house prepared extracts. Anti-MBP MAb 19C19 was used as a negative control (C). The position of parvalbumin is indicated by an arrow.

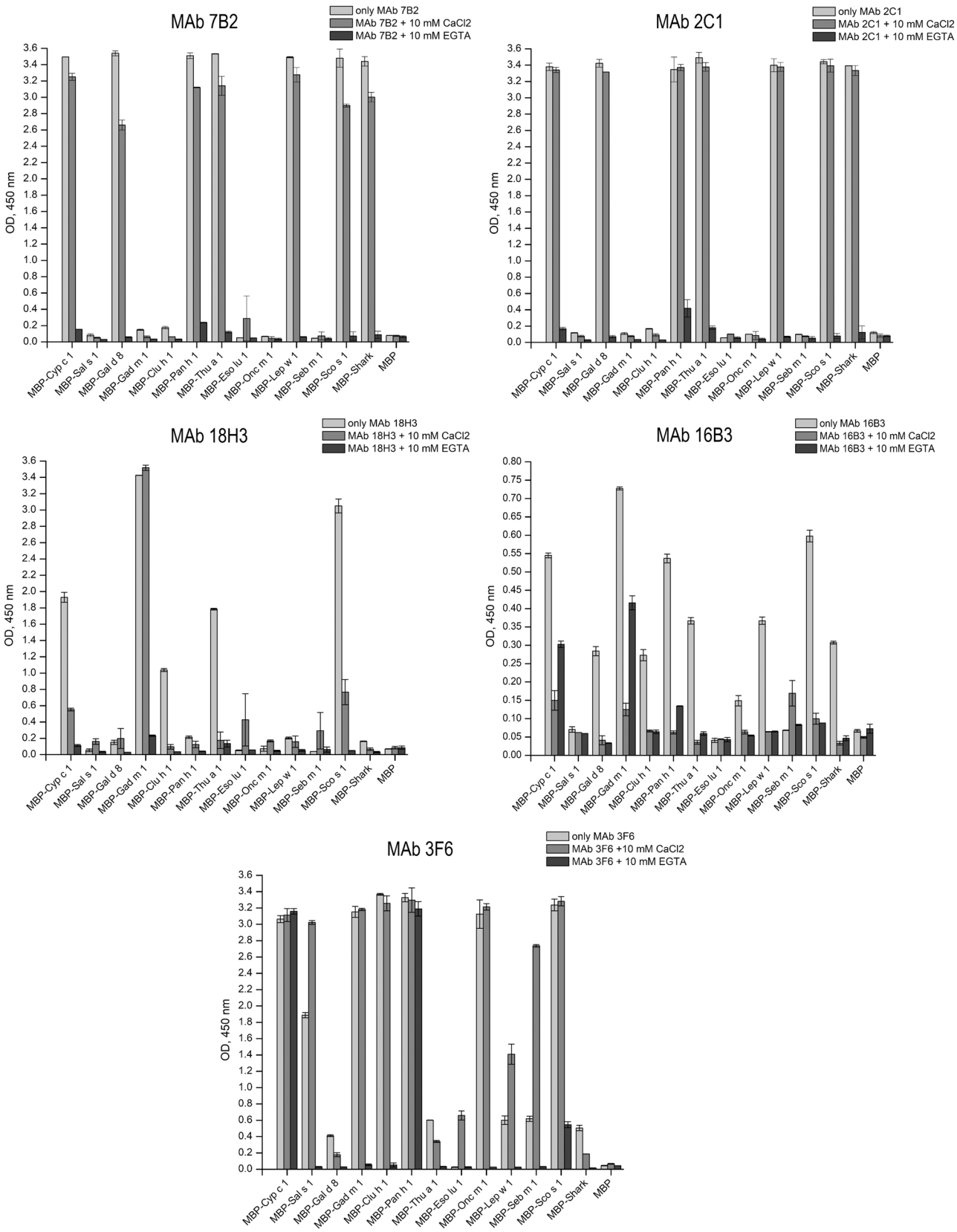

Fig. 7. The reactivities of MAbs with recombinant parvalbumins alone or in the presence of either $CaCl_2$ or EGTA by an indirect ELISA. MAb clones are indicated on the top of each picture.

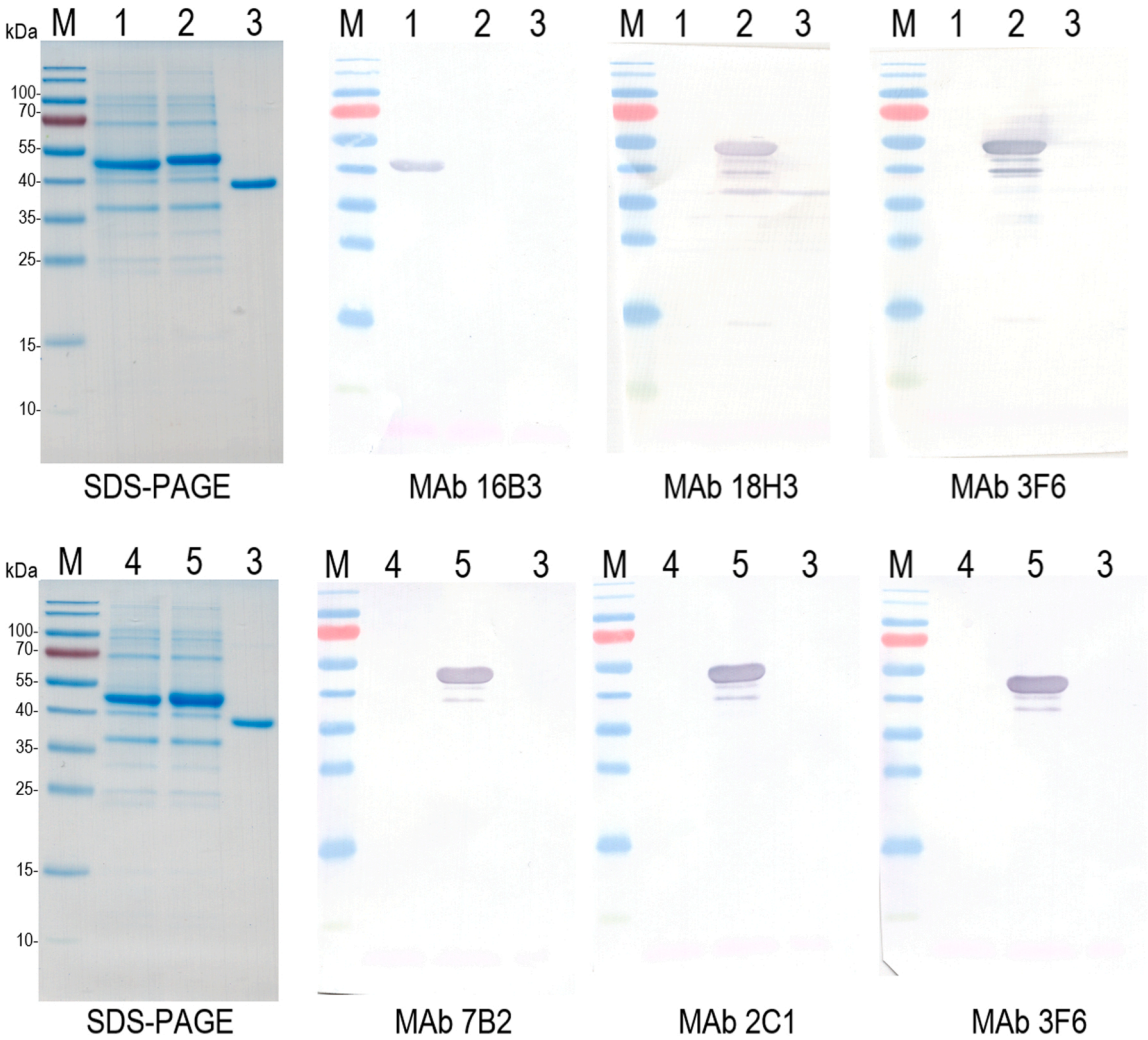

Fig. 8. The reactivity of MAbs with recombinant overlapping MBP-fused fragments of Gad m 1 and Cyp c 1 in *E. coli* cell lysates analyzed by Western blot. Lane M: protein molecular weight marker; lane 1: fragment #1 (52.9 kDa); lane 2: fragment #2 (53.9 kDa); lane 3: recombinant purified MBP as a negative control (44.2 kDa); lane 4: fragment #3 (51.9 kDa); lane 5: fragment #4 (52.2 kDa).

suggests that their epitopes are conformation-sensitive. These MAbs did not show any reactivity with recombinant Eso lu 1, Onc m 1 (except MAb 16B3), Sal s 1 and Seb m 1 parvalbumins. To evaluate the homology among these parvalbumins, the aa sequence of common carp β-parvalbumin (Cyp c 1) was aligned with Northern pike β-parvalbumin (Eso lu 1) aa sequence, while Atlantic cod β-parvalbumin (Gad m 1) aa sequence was aligned with Eso lu 1, Rainbow trout β-parvalbumin (Onc m 1), Atlantic salmon β-parvalbumin (Sal s 1) and ocean perch β-parvalbumin (Seb m 1) aa sequences, using protein Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins). The alignment revealed high identity levels (80 %, 74 %, 65 %, 66 % and 73 %, respectively) of aa sequences among these allergens. The differences in certain aa sequences may explain, why MAbs did not react with those recombinant allergens. Besides that, even though the MAbs were generated against the natural Atlantic cod parvalbumin, four isotypic variants (Gad m 1.0101, Gad m 1.0102, Gad m 1.0201 and Gad m 1.0202) of Atlantic cod β-parvalbumin are known and reported in WHO/IUIS database (http://www.allergen.org/viewallergen.php?aid=708). These isoforms have been investigated and compared in several studies (Van Do et al., 2003; Ma et al., 2008). For production of recombinant MBP-Gad m 1 protein, we selected Gad m 1.0101 parvalbumin-coding sequence, since this Atlantic cod variant shared higher aa sequence identity with Cyp c 1 (81.65 %) than other variants (Gad m 1.0102 shared 80.73 %, Gad m 1.0201 80.73%, Gad m 1.0202 79.82 %). Since MAb 7B2 and 2C1 showed a weak reactivity with recombinant MBP-Gad m 1 protein by ELISA and dot blot, while no reactivity by Western blot, we concluded that these two antibodies may recognize other isotypic variants of Atlantic cod β-parvalbumin. Isotypic variability also could explain MAbs against nGad m 1 reactivity with Atlantic salmon and trout allergen extracts and no binding to recombinant MBP-Sal s 1 and MBP-Onc m 1 proteins. It is possible that these MAbs recognize other isotypic variants of β-parvalbumins of Atlantic salmon and trout, that are present in prepared extracts.

Even though fish α-parvalbumin is considered as non-allergenic, there are patients that could be sensitized to certain cartilaginous fish (Kalic et al., 2019). In previous studies, fish α-parvalbumins have been purified from Atlantic salmon, Thornback ray and Gummy shark and have been analyzed for IgE reactivity using serum samples from patients with confirmed IgE-mediated fish allergy. The results showed low allergenicity of fish α-parvalbumins in patients sensitized to bony fish (Sharp et al., 2015). Surprisingly, we demonstrated, that not only samples of fish-allergic patients were reactive with recombinant Leopard shark α-parvalbumin, but also all our MAbs cross-reacted with this parvalbumin, although it shares low sequence similarity with Gad m 1 and Cyp c 1 proteins (48–55 % identity). This suggests that the generated MAbs are broadly-reactive and may be applied for analyzing both isoforms (α- and β-) of fish parvalbumins.

The next step in MAb characterization was investigation of their ability to detect fish β-parvalbumins in commercial and in-house

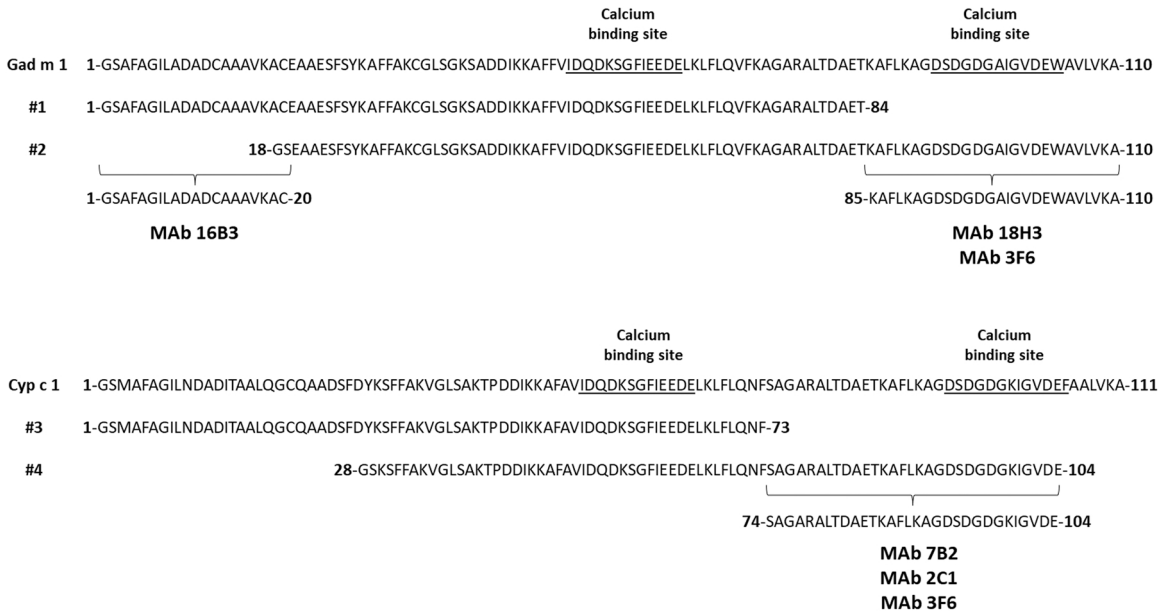

Fig. 9. Mapping of MAb epitopes using recombinant overlapping MBP-fused fragments of Gad m 1 and Cyp c 1 proteins. The sequences recognized by the respective MAbs are indicated.

prepared fish extracts by various methods. There are studies, where extracts of different cartilaginous and bony fish species have been prepared and analyzed with parvalbumin-specific antibodies (Sharp et al., 2015; Faeste et al., 2008). In our study, besides 12 previously analyzed fish extracts, we used extracts of two fish species that are more common in our country (European smelt and Eurasian ruff) and that have not been studied before. All MAbs were able to recognize β-parvalbumins in the analyzed fish extracts. The ELISA results suggest that each antibody could be applied to analyze extracts of certain fish species or fish families. For example, MAbs 7B2, 2C1 and 3F6 would be more preferred for characterization of European perch and Eurasian ruffe extracts, while MAbs 18H3, 16B3 and 3F6 – for European smelt extract. Fish extracts from various fish species have been prepared and analyzed in previous studies and many of them have demonstrated that the amount of fish allergens (parvalbumins, β-enolases or collagen) highly varies in different extracts (Kuehn et al., 2010; Saptarshi et al., 2014; Ruethers et al., 2019). In our study, the comparison of MAb reactivity with in-house prepared and commercial fish extracts of common carp, Atlantic herring and Atlantic salmon revealed different MAb reactivity patterns with the analyzed extracts. Moreover, we demonstrated that the amount of β-parvalbumin differs among different extracts prepared from the same fish species. This suggests the need for extract standardization before their use for allergy diagnostics. Standardized protocols for allergen extract preparation, selected proper storage conditions and determined amounts of certain allergen components could improve the quality of allergen extracts for allergy testing (Ruethers et al., 2019). Since the newly generated MAbs demonstrated a broad cross-reactivity with various fish extracts, we considered that they could be applied for characterization of fish allergen extracts.

Cross-reactivities of potential allergens of fish and other animals (frog, chicken and crocodile) due to parvalbumins have been reported in several studies. In one recent study, parvalbumins were detected in freshly prepared cod, crocodile, frog, and chicken extracts based on their reactivities with serum samples of fish-allergic patients (Haroun-Díaz et al., 2021). Another study described the identification of three new chicken allergens (parvalbumin, aldolase and enolase), that were recognized by serum samples of patients with conformed fish and chicken allergy. The performed competitive inhibition ELISA revealed that IgE against fish and chicken allergens are highly cross-reactive (Kuehn et al., 2016). In our study, the cross-reactivity between fish and chicken parvalbumins has been demonstrated by showing that serum samples of fish-allergic patients also reacted with recombinant Gal d 8. Moreover, the analysis of MAbs reactivities with recombinant chicken α-parvalbumin and with the in-house prepared and commercial chicken and pork extracts by different immunoassays revealed both conformation-dependent and independent common epitopes. All MAbs reacted with recombinant Gal d 8, even though this allergen shares only 56% aa sequence identity with Cyp c 1 % and 54 % aa sequence identity with Gad m 1. On the other hand, Gal d 8 has a higher protein sequence identity with Leopard shark α-parvalbumin (>68 %) that is recognized by all MAbs. Sharp et al. in 2015 demonstrated, that rabbit polyclonal antibodies raised against barramundi, basa, pilchard and Atlantic salmon showed no reactivity with prepared chicken and pork extracts that were used as negative controls in the study (Sharp et al., 2015). In contrast, in our study, all MAbs detected parvalbumins in the analyzed chicken and pork extracts by ELISA, but no reactivity was seen with heated and SDS-denatured extracts by Western blot, suggesting that extracts either contain insufficient amount of parvalbumins to be detected by MAbs or the MAb epitopes are sensitive to structural changes of heated/denatured parvalbumins.

To study the potential of MAbs for the characterization and standardization of allergen extracts, we tested their ability to immunoprecipitate parvalbumins from allergen extracts. For this procedure, we selected MAbs 7B2 and 3F6 to isolate parvalbumins from the in-house prepared and commercial extracts of common carp, Atlantic salmon and chicken. MAb 3F6 immunoprecipitated parvalbumins from the extracts of both fish species and from the commercial chicken extract, while MAb 7B2 immunoprecipitated parvalbumin only from carp extract. This demonstrates a very high potential of MAb 3F6 for extract standardization because of its broad reactivity with parvalbumins of various fish species and ability to recognize both native and recombinant parvalbumins.

Parvalbumins belong to the EF-hand protein superfamily and they bind divalent cations (Ca^{2+}, Mg^{2+}), that are important for protein structure stabilization and its functions (Matricardi et al., 2016; Swoboda et al., 2007). Several studies have been performed to investigate whether the binding of IgE antibodies from fish-allergic patients to fish parvalbumins is affected by depletion of calcium ions using ELISA and Western blot methods. The majority of analyzed serum samples demonstrated a reduced IgE binding activity to parvalbumins after

A

Gad m 1	**1**-GS-AFAGILADADCAAAVKAC-**20**	Identity (%)
Cyp c 1	**1**-GSMAFAGILNDADITAALQGC-**21**	70%
Sal s 1	**1**-GSMACAHLCKEADIKTALEAC-**21**	45%
Onc m 1	**1**-GSMACAHLCKEADIKTALEAC-**21**	45%
Clu h 1	**1**-GSMALASLLKGADIDAALKAC-**21**	60%
Pan h 1	**1**-GSMAFAGVLNDADIAAALDAC-**21**	75%
Thu a 1	**1**-GSMAFAGILTEADITAALAAC-**21**	70%
Sco s 1	**1**-GSMAFASVLKDAEITAALDGC-**21**	55%
Eso lu 1	**1**-GS-SFAG-LKDADVAAALAAC-**19**	74%
Lep w 1	**1**-GSMTFAGL-DAAEIKAALDGC-**20**	45%
Seb m 1	**1**-GSMALAASLNAADITAALAAC-**21**	55%
Shark	**1**-GSPMTKVLKADDINKAISAF-**20**	30%
Gal d 8	**1**-GSMAMTDVLSAEDIKKAVGAF-**21**	40%

B

Gad m 1	**85**-KAFLKAGDSDGDGAIGVDEWAVLVKA-**110**	Identity (%)
Cyp c 1	**86**-KAFLKAGDSDGDGKIGVDEFAALVKA-**111**	88%
Sal s 1	**86**-KAFLKAGDADGDGMIGIDEFAVLVKQ-**111**	81%
Onc m 1	**86**-KAFLKAGDADGDGMIGIDEFAVWVKQ-**111**	77%
Clu h 1	**86**-KAFLKAGDADGDGMIGVDEFAVMIKP-**111**	77%
Pan h 1	**86**-KIFLKAGDTDGDGKIGVDEFASLVKA-**111**	81%
Thu a 1	**86**-KAFLMAGDSDGDGKIGIDEFAALVKA-**111**	81%
Sco s 1	**86**-KAFLKAGDSDGDGKIGVDEFAAMIKG-**111**	77%
Eso lu 1	**84**-KAFLADGDKDGDGMIGVDEFAAMIKA-**109**	69%
Lep w 1	**85**-ANFLKAGDVDGDGKIGIEEFTDLVRS-**110**	54%
Seb m 1	**86**-TAFLKAGDSDGDGMIGMDEFAAMVKG-**111**	73%
Shark	**85**-KALLAAGDSDHDGKIGADEFAKMVAQ-**110**	62%
Gal d 8	**86**-KALLAAGDKDGDGKIGADEFATMVAE-**111**	62%

C

Cyp c 1	**74**-SAGARALTDAETKAFLKAGDSDGDGKIGVDE-**104**	Identity (%)
Gad m 1	**73**-KAGARALTDAETKAFLKAGDSDGDGAIGVDE-**103**	94%
Sal s 1	**74**-CPKARELTDAETKAFLKAGDADGDGMIGIDE-**104**	77%
Onc m 1	**74**-CPKARVLTDAETKAFLKAGDADGDGMIGIDE-**104**	77%
Clu h 1	**74**-KAGARALTDAETKAFLKAGDADGDGMIGVDE-**104**	90%
Pan h 1	**74**-KSDARALTDNETKIFLKAGDTDGDGKIGVDE-**104**	81%
Thu a 1	**74**-SAGARALTDAETKAFLMAGDSDGDGKIGIDE-**104**	94%
Sco s 1	**74**-KAGARALSDAETKAFLKAGDSDGDGKIGVDE-**104**	94%
Eso lu 1	**72**-SPSARALTDAETKAFLADGDKDGDGMIGVDE-**102**	84%
Lep w 1	**73**-SASARALTDKETANFLKAGDVDGDGKIGIEE-**103**	77%
Seb m 1	**74**-SATARALTEAETTAFLKAGDSDGDGMIGMDE-**104**	84%
Shark	**73**-SAHGRDLNDTETKALLAAGDSDHDGKIGADE-**103**	71%
Gal d 8	**74**-TPDGRDLSDKETKALLAAGDKDGDGKIGADE-**104**	65%

Fig. 10. Sequence alignment of the identified MAb epitope regions with parvalbumins of other fish species and with chicken α-parvalbumin. A – N-terminal region of Gad m 1 (aa 1–20); B – C-terminal region of Gad m 1 (aa 85–110); C – C-terminal region of Cyp c 1 (aa 74–104).

calcium depletion, that could be explained by the conformational changes of the protein structure (Bugajska-Schretter et al., 1998; Swoboda et al., 2002b). Another study showed that the immunoreactivities of anti-parvalbumin-specific MAbs 3E1 and PARV-19 with fish and meat extracts increased after adding EGTA reagent, suggesting that removal of calcium ions may have exposed the hidden MAbs epitopes (Gajewski et al., 2009). In our study, we investigated the effect of calcium ions on MAb binding to recombinant parvalbumins by ELISA. All MAbs demonstrated different patterns of their immunoreactivities with recombinant parvalbumins in the presence of either $CaCl_2$ or EGTA reagent. After adding EGTA, MAbs 7B2, 2C1 and 18H3 showed a significantly reduced reactivity to recombinant parvalbumins, which in the control samples showed high ELISA absorbance values (OD_{450} >1.5). Moreover, adding either $CaCl_2$ or EGTA reagent only reduced binding activity of MAbs 18H3, 16B3 and 3F6 to certain parvalbumins. All MAbs against nGad m 1 incubated with $CaCl_2$ showed an increase in ELISA signal with one of the recombinat parvalbumins (MBP-Eso lu 1, MBP-Sal s 1, MBP-Onc m 1, MBP-Seb m1), that previously showed no reactivity, while MAb 3F6 in the presence of $CaCl_2$ showed an increased reactivity with MBP-Sal s 1, MBP-Lep w 1 and MBP-Seb m 1 proteins. Besides that, only MAb 3F6 did not change its immunoreactivity with MBP-Pan h 1 and MBP-Cyp c 1 in the presence and absence of calcium ions. In most cases, MAbs demonstrated either increased or reduced binding reactivities with recombinant allergens after adding or removing calcium ions, that could be explained by conformational changes in parvalbumin structure.

Finally, we localized the epitopes of recombinant Gad m 1 and Cyp c 1 proteins, that are recognized by MAbs, using overlapping recombinant MBP-fused fragments of Cyp c 1 and Gad m 1. MAbs 7B2, 2C1 and 3F6 reacted with the C terminally located epitopes of Cyp c 1, MAb 16B3 identified the N terminally located epitope of Gad m 1, while MAbs 18H3 and 3F6 recognized C terminally located epitopes of Gad m 1. The predicted localization of MAb epitopes revealed that all MAbs, except for MAb 16B3, recognize aa regions of either Gad m 1 or Cyp c 1 that contain calcium binding sites. The identified aa sequences recognized by all MAbs were aligned with aa sequences of parvalbumins of different fish species and chicken, allowing to analyze their specificity and reactivity for certain parvalbumins. As for MAb 3F6, the homology between the C-terminal region of Cyp c 1 (aa 74-104) and other parvalbumins is more than 65 % and this could explain the broad cross-reactivity of the antibody with parvalbumins of different fish species and other animals. In contrast, the N-terminal region of Gad m 1 (aa 1–20), that is recognized by MAb 16B3, demonstrates low sequence similiarity with other parvalbumins, especially with α-parvalbumins (30–40 %), suggesting that this antibody could be applied only for studying certain fish β-parvalbumins.

Summarizing, this study describes four MAbs raised against natural Atlantic cod parvalbumin and one MAb against recombinant common

carp β-parvalbumin, that were comprehensively characterized by different immunoassays and tested with a broad range of potential allergens. Confirming their cross-reactivity with parvalbumins of different fish species and even chicken α-parvalbumin, all generated MAbs, in particular the broadly-reactive MAb 3F6, could become a useful tool for detection of major fish allergens and standardization of fish allergen extracts.

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CRediT authorship contribution statement

Aistė Sližienė: Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Visualization, Writing – review & editing. **Milda Plečkaitytė:** Methodology, Investigation, Resources, Writing – review & editing. **Vytautas Rudokas:** Formal analysis, Investigation. **Karolina Juškaitė:** Formal analysis, Investigation. **Gintautas Žvirblis:** Conceptualization, Resources, Writing – review & editing, Supervision. **Aurelija Žvirblienė:** Conceptualization, Resources, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

Data availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.molimm.2023.01.001.

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