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# Chapter I

# General Introduction

# General introduction

## 1. Allergy

Allergy was defined as „*an altered capacity of the body to react to a foreign substance*“ by Clemens Von Pirquet in 1906 [1]. Today the definition is more restricted to immune-mediated reactions and is defined as *“a disease following a response by the immune system to an otherwise innocuous antigen”* [2].

Allergic or hypersensitivity reactions were classified into four types by Coombs and Gell in 1963 [2, 3]:

**Type I** or immediate hypersensitivity is caused by IgE antibodies and a sub-population of immune cells, the mast cells and basophils. During the sensitization phase, the atopic immune system encounters an allergen for the first time. This results in a Th-2 directed IgE synthesis of B-cells. This IgE then binds to the Fcε receptors present on the surface of mast cells and basophils. When a second exposure to the same allergen occurs, the allergen binds to at least two molecules of IgE thus cross-linking the antibodies and Fcε receptors. After cross-linking a degranulation of mast cells and basophils occurs during which histamine and other inflammatory mediators are released. Some examples of symptoms are anaphylaxis, atopic asthma, atopic eczema, oral allergy syndrome (OAS), pruritis or urticaria. An example for an inhalative allergen is Bet v 1, the major birch pollen allergen, the first recombinant pollen allergen [4], and the food allergen Gad m 1, the major allergen from cod [5].

In a **Type II** hypersensitivity reaction, IgG or IgM antibodies are directed against antigens on an individual’s own or on foreign cells, such as those acquired through a blood transfusion. These antibodies can cause tissue injuries mediated by the

cytotoxic action of T killer cells or by lysis mediated by the complement system. Examples are autoimmune haemolytic anemia, Goodpasture's syndrome or penicillin allergy [2].

In the **Type III** reaction immune complexes of circulating antigens and IgM or IgG antibodies are formed in the tissue that cannot be cleared by macrophages. A complement or a cell mediated local reaction can provoke tissue injuries and inflammation. Type III hypersensitivity reactions include serum sickness and the Arthus reaction [2].

**Type IV** or cell-mediated reactions are those in which T cells are sensitized to an antigen and release lymphokines following secondary contact with the antigen. Cytokines induce an inflammatory response, and they also activate and attract macrophages, which release inflammatory mediators. Examples are contact dermatitis, the tuberculin reaction and granulomatous hypersensitivity [2].

**Atopy** has a substantial genetic contribution, which lead to a higher susceptibility to allergic disease [2]. It is defined as *"a personal and/or familial tendency, usually in childhood or adolescence, to become sensitized and produce IgE antibodies in response to ordinary exposures to allergens, usually proteins." As a consequence, these persons can develop typical symptoms of asthma, rhinoconjunctivitis, or eczema"* [6].

Genetic predisposition but also environmental factors (changes in exposure to infectious diseases in early childhood, environmental pollution, allergen levels, and dietary changes) are responsible for an increasing prevalence of allergy [2].

## 2. Food allergy

Only few foods are responsible for the majority of allergic reactions: milk, egg, peanuts, tree nuts, fish, wheat, soy and shellfish [7, 8]. Food allergy can resolve or persist. Usually allergies that are outgrown include milk, egg, soy and wheat. In contrast, peanut, tree nuts, fish and shellfish allergy are not restricted to childhood and mainly persist. Yet, in some cases the development of tolerance was reported [9].

Although *"Food is essential for life, a major source of pleasure, and often intrinsic to our cultural identity"* [10], some people react to it with an adverse immune response [11]. Over 2000 years ago Hippocrates, and in the first and second centuries other Greek scholars already reported adverse reactions to food. Food adverse reactions are defined as *"any abnormal response upon ingestion of food"* [10].

The European Academy of Allergy and Clinical Immunology suggests a classification of adverse reactions to food [12, 13]: Food can cause dose-dependent *toxic* reactions in everyone (eg. histamine in scombroid fish poisoning), but for *non-toxic* reactions the individual susceptibility is responsible [10]. Non-toxic reactions are divided into *non-immune mediated* and *immune-mediated* (food hypersensitivity [14]). *Non-immune mediated* adverse reactions may result from enzyme deficiencies (eg. lactose intolerance) [9]. *Immune-mediated* reactions can be classified into *non-IgE mediated* and *IgE mediated* (type I) and are defined as food allergy.

### 2.1. Symptoms of food allergy

One of the most common symptoms are *cutaneous reactions*, like acute urticaria with or without angioedema, often seen in combination with manifestations of other target organs by ingestion of meat, vegetables and fruits [13, 14]. Food-induced contact dermatitis often occurs among persons who handle raw fish, shellfish, meats and eggs [14]. Further, a direct correlation between the severity of atopic dermatitis and food

allergy was reported. Around one third of infants with atopic dermatitis was found to be allergic to food [13, 15].

*Oral allergy syndrome* (OAS) is a type of contact urticaria and the most frequent allergic reaction in adults. Within 5 to 15 minutes after food ingestion the lips, tongue, palate, ears and throat start to itch and also mild-angio-oedema at the same site are observed. Beside spontaneous resolution within minutes in most cases, gastrointestinal symptoms such as vomiting, abdominal pain and diarrhoea, and very rarely urticaria and anaphylaxis may occur. OAS is predominantly observed in patients suffering from pollinosis with an associated allergy to homologous, cross-reactive allergens of vegetable foods [13]. *"The restriction of symptoms to the oral cavity seems to be related to the ability of the allergens involved (eg. Bet v 1 homologues, profilins) to the digestion process"* [16].

*Anaphylaxis* occurs when a massive release of mast cell mediators affects multiple organ systems [16]. This systemic reaction can be elicited by the ingestion of foods like cow's milk, hen's egg, peanut, fish, and crustaceans [14]. The patients may develop acute nausea, colicky abdominal pain and vomiting within minutes of food ingestion [13].

Allergic eosinophilic oesophagitis or gastroenteritis are IgE-mediated and/or non IgE-mediated *gastrointestinal reactions* characterized by infiltration of the oesophagus, stomach or intestinal walls with eosinophils. Symptoms are nausea, dysphagia, vomiting, abdominal pain and diarrhoea mostly by ingestion of milk, egg, wheat, rye and beef [10, 13, 16].

Food allergens can induce *respiratory reactions*, like allergic rhinoconjunctivitis or acute bronchospasm in association with other food allergy symptoms. Also a worsening

of asthma in sensitized individuals may occur after ingestion of food allergens or inhalation of vapours or steam from cooking food, like fish [14].

## 2.2. Prevalence of food allergy

In the United States approximately 4% of the adult population suffer from food allergy. More than 2% were estimated to be allergic to seafood in a prevalence study by random telephone survey [17]. In France, Kanny et al. [18] found a prevalence of food allergy of approximately 3.24%, and Osterballe et al. [19] published a prevalence of IgE-mediated food hypersensitivity for adults (median age 33.7 years) of 10.8% in Denmark. In a German survey the same age-group (20-39 years) had the highest frequency of 4.3% of IgE-mediated reactions to foods [20]. In all three European studies the most common elicitors of food allergy were pollen-related fruits and vegetables, whereupon the primary sensitisation came from pollen [12]. In contrast, in Spain 4-5 % of the general population are mostly allergic to animal allergens. Milk, egg or fish are mainly the inducers of food allergy in children [21].

Nevertheless, these prevalence data should be treated with care. In a meta-analysis a marked heterogeneity in the prevalence of food allergy was observed depending on study design or differences between populations. The prevalence is often overestimated in studies with self-reported symptoms in comparison to reports using objective diagnostic tools like the double-blind, placebo-controlled food challenge (DBPCFC) [22].

## 2.3. Diagnosis of food allergy

First, the patients' history, compatible with IgE-mediated symptoms, should be established [9]. Subsequently, food-specific IgE can be measured by an inexpensive and simple skin-prick test (SPT) puncturing the skin with food extract or by puncturing the native food and thereafter the skin (prick-to-prick). Another similar but more expensive diagnostic method is the *in vitro* determination of specific IgE by the CAP-FEIA system

(Pharmacia Diagnostics, Uppsale, Sweden). With these methods a food allergy can be excluded, but not confirmed [9, 13]. The most reliable diagnostic tool is the double blind, placebo controlled food challenge (DBPCFC). With this "gold standard" of food allergy adverse reactions to food can be confirmed or ruled out [9]. In the case of negative results an open and supervised challenge must confirm the outcome [11]. Further, a promising approach is the component resolved diagnosis, one of the future trends of allergy diagnosis. Purified recombinant food allergens represent new tools for diagnostic applications *in vivo* and *in vitro* and are believed to be able to replace non-standardized extracts [13].

## 2.4. Prevention and treatment of food allergy

Few data are available on food allergy prevention in the early childhood. Hydrolyzed formulas of cow milk had a long-term preventive effect on allergic manifestations and atopic eczema in children with high risk for atopy until the age of six years [23]. Novel therapeutic concepts (e.g. oral or sublingual immunotherapy) for allergy treatment are being developed, but are not commercially available yet [24]. Currently, only a strict elimination diet is the treatment of choice [9]. New immunotherapeutic tools for food allergy are under development. Swoboda et al. [25] produced a genetically engineered carp parvalbumin with an impaired IgE binding activity for specific immunotherapy. Also novel therapies for life-threatening peanut allergy are under investigation using animal models [13]: The administration of a high dose of heat-killed Escherichia coli producing mutated Ara h1, 2, and 3 can downregulate the hypersensitivity reaction to peanut in mice by a shift from Th-2 responses to Th-1 responses[26]. Another example is a Chinese herbal medicine formula, which reduced Th-2 cytokines and enhanced the production of IFN-gamma in peanut allergic mice, and could block anaphylaxis [27]. To prevent side effects of peanut immunotherapy peanut allergic mice were treated with

soybean extract. Using homologue seed storage proteins from soybean to treat peanut allergy can skew toward a Th-1 response [28].

## 2.5. Cross-reactivity

"*Structural similarity among proteins from diverse sources is the molecular basis of allergic cross-reactivity*" [29]. "*Two (or more) allergens are cross-reactive, if IgE antibodies or a T cell receptor reacts with both"* [30]. Cross-reactivity can be caused by allergens whose sequences are at least 50% to 70% identical [30, 31]. For example, IgE antibodies originally directed to pollen allergen can cross-react with homologous plant food proteins from a variety of sources (birch pollen-fruit syndrome) [32, 33]. One prominent cross-reactive animal food allergen is the major fish allergen, parvalbumin [34]. Cross-reactivity was observed among various fish species and also with edible frog species [34-36].

# 3. Fish allergy

Fish and their products are common in human nutrition, especially in coastal countries. However, their consumption may result in mild to severe allergic reactions including anaphylaxis. The first anaphylaxis to fish was reported by Phillip Sachs in the seventeenth century [10].

In Spain, where a cross-sectional study was carried out, fish allergy was diagnosed in 36 of 4991 patients (0,72%) referred to for allergy evaluation [37]. Sensitization occurs in the early childhood (> 1 year) and allergy often persists. In children under two years of age fish is the third most frequent allergen source, as fat-poor white fish (like whiff/megrim) is often introduced into the diet at this time. Ingestion, contact, or inhaling cooking vapours elicit mainly IgE mediated reactions [38].

The major allergen of fish is parvalbumin beside minor allergens such as aldehyde phosphate dehydrogenase (APDH) [39] or collagen [40]. Parvalbumins are highly cross-reactive allergens. Most patients are allergic to multiple fish species, but in some cases sensitization to only one fish was reported [41]. Due to the low parvalbumin level in dark muscles, fish species containing more dark muscles (tuna and swordfish) may be tolerated [38, 42].

Fish allergy should not be confused with other adverse reactions, such as allergic reactions to the allergens of the *Anisakis simplex* larva. This nematode infects consumers of raw or less cooked fish [43]. Hypersensitivity reactions often occur to Anisakis tropomyosin in individuals suffering from allergy to its homolog in crustaceans or mites [44, 45].

Finally, fish ingestion may also cause a not so common syndrome known as food protein induced enterocolitis syndrome (FPIES). This non-IgE mediated allergy syndrome is usually reported following the ingestion of cow’s milk or soy proteins, but

also of fish, and may lead to acidosis and shock. Only food avoidance is an appropriate method for patients' treatment [38]. Fish allergy played also a historically important role, as IgE was first found in a fish allergic individual [46].

# 4. Food allergens

*"Allergens possess special features and not every protein can become an allergen: the finding that (1) the small number of protein families in which allergens were found and (2) the frequent occurrence of certain biochemical functions among allergens support this view"* [31].

Certain biochemical features characterize food allergens. These proteins have to be abundant in the food source and should be resistant to digestion and processing [47]. Additionally, structural features enhance their thermal stability and resistance to proteolysis. Ligand binding capacity, intramolecular disulfide bonds, the ability to aggregate , the presence of glycosylation, or the interaction with cell membranes or lipid structures [33] preserve the allergens structure from thermal denaturing and degradation by digestive enzymes, low pH and surfactants, such as bile salts that are present in the gastrointestinal tract (GIT) [33].

The major food allergens identified as **class 1 food allergens** are water-soluble 10 to 70 kDa proteins, stable to heat, acid, and protease treatment. Some prominent members are beta-lactoglobulin from milk (Bos d 5), peanut vicilin (Ara h 1), or cod parvalbumin (Gad c 1) [7]. These allergens sensitize via the gastro-intestinal tract (GIT). Most of the class 1 plant food allergens belong to the prolamin or cupin protein superfamilies. Members of these superfamilies are remarkably stable to proteolysis due to the presence of structural motifs such as the conserved skeleton of cysteine residues forming four disulphide bonds in the members of the prolamins or the presence of a beta-barrel in the case of the cupins [33]. But there are also proteins that sensitize through the GIT and are sensitive to gastric digestion by pepsine, like the animal food allergens shrimp tropomyosin or milk caseins and alpha-lactalbumin [48]. It is hypothesized that these proteins form large stable protelolytic fragments with a potential to bind IgE [48]. Huby et al. [49] stated: "an allergenic protein must possess at least two IgE binding sites, each

with a minimum of 15 amino acid residues". Also the peanut allergens Ara h 1 and Ara h 2 are easily degraded by digestion enzymes, but fragments can bind IgE antibodies to elicit an allergic reaction [48, 50, 51].

Besides food allergens sensitizing via the GIT (class 1 food allergens), allergic reactions can occur as a consequence of an allergic sensitization to inhalant allergens (class 2 food allergens) [14, 32]. **Class 2 food allergens** are labile to heat and enzymatic degradation, as most of them are presumably comprised of conformational epitopes [14]. Class 2 food allergy is the result of sensitization to labile pollen allergens via the respiratory tract [11], e.g. IgE antibodies directed against inhalent allergens, like Bet v 1, recognize IgE epitopes on Bet v 1 homologues in plant foods (birch pollen-fruit syndrome) [32].

In addition, the modulation of allergenic properties by food by processing needs to be considered. Food processing, such as thermal treatments, can impair the IgE binding ability to epitopes of food allergens, as recently shown for birch pollen allergic patients [52]. Structural unfolding of the Pru av 1, the major allergen of cherries, by cooking of cherries with high water content reduced the allergic reactions to this Bet v 1 homologue [52]. However, processing can also enhance the allergenic potential by protein aggregation (e.g. Gly m 6, soy glycinin, forms heat-set gels [52]) or chemical modification (e.g. cross-linking of the peanut allergens Ara h 1 and Ara h 2 to form IgE reactive high molecular weight aggregates by the Maillard modification [52, 53].

Whereas, most members of plant food allergens are restricted to four structural protein families [54], most animal food allergens are classified into only three main families [55]. The most important plant food allergen superfamilies are the prolamin superfamily [composed of cereal storage proteins (e.g. ω-5 gliadin allergen of wheat), nonspecific

lipid transfer proteins (e.g. Pru p 3 from peach), 2S storage albumins (e.g. Ber e 1 from Brazil nut) and inhibitors of trypsin and α-amylase], the cupin superfamily with the 7S and 11S globulin storage proteins of seeds (eg. Ara h 1, 3 and 4 from peanut), Bet v 1 homologues (e.g. Mal d 1 in apple and Api g 1 in celery), and profilins (e.g. Api g 4 from celery) [31, 54].

Most animal food allergens are members of three superfamilies: tropomyosins, caseins and EF-hand proteins. All of them show an ability to bind ligands. Animal food allergens have a high degree of similarity to human homologues, thus the evolutionary distance reflects their allergenicity [55]. Jenkins et al. [55] found: *"proteins with a sequence identity to a human homologue above approximately 62% were rarely allergenic"*.

## 4.1. Ligand-binding animal food allergens

**Tropomyosins** are *"rod-shaped coiled-coil dimers that form a head-to-tail polymer along the length of an actin filament"* [56]. They mediate the interactions between the troponin complex and actin thus regulating muscle contraction. Tropomyosins are the major allergens of shellfish and molluscs and they are very stable to thermal treatments [57]. Invertrebrates tropomyosins are highly cross-reactive. High sequence identities were found in crustaceans and molluscs [58]. Additionally, studies have shown that patients allergic to the tropomyosin of house dust mite and cockroach show IgE reactivity to shrimp Pen a 1 without previous exposure to shrimp [59].

Vertebrate tropomyosins are at least 90% identical to the closest human homologues and not allergenic [55]. Tropomyosins in invertebrates as inhalative allergens (e.g. the house dust mite allergen Der p 10) and crustacean tropomyosins as food allergens (e.g. the major allergen of shrimp Pen a 1) are only approximately 55% identical to human homologues [55].

**Caseins** are *exclusively mammalian proteins found in milk* [55]. Calcium-binding is one structural feature that enhances their thermal stability and resistance to proteolysis [33]. Furthermore, caseins' structures are disordered and dynamic (rheomorphic) and therefore possess many linear, thermostable IgE epitopes. Caseins comprise 4 structural groups, alpha$_{S1}$-, alpha$_{S2}$-, beta-, and kappa-caseins. The first three mentioned components bind calcium and assemble to casein micelles in milk [60]. Kappa-caseins are important for the first step of milk-clogging [61].

Alpha$_{S2}$-caseins are not present in humans. This appears to be linked to the IgE reactivity of caseins. The highest number of cow's milk allergic children had serum IgE against alpha$_{S2}$-casein (90%), followed by alpha$_{S1}$-casein (55%) [62]. Much fewer children had IgE against beta-casein (15%), the casein with the highest identity to a human homologue [62]. Beta-caseins from mammals show sequence identities in the range of 53 to 58% to human homologues [33, 52, 55].

## 4.2. Allergens of the EF-hand superfamily

The EF-hand motif is found in calcium-binding proteins in the cytosol. Members of this superfamily contain from two to eight copies of the EF-hand domain [63]. The classical EF-hand domains, helix-loop-helix, consists of a calcium binding loop of 12 amino acid residues flanked on both sides by α-helices of 12 residues in length [63, 64]. EF-hand proteins are subdivided in 32 subfamilies [65], whereas 42 allergens were found distributed in few of them (www.meduniwien.ac.at/allergens/allfam/; data retrieved on March 26, 2009). Two major cross-reactive allergen families are of importance, the allergenic polcalcins from pollen and the allergenic parvalbumins from fish [65]. There is also an autoallergen, Hom s 4, recognized by patients suffering atopic dermatitis [66]. Calcium-binding pollen allergens, grouped into allergens with 2, 3 or 4 EF-hands, can be found in pollen of grasses, weeds or trees [65]. So far, no significant cross-reactivity

among these groups was described [65, 66]. IgE binding ability of EF hand-containing allergens was shown to be calcium-dependent [65-69].

# 5. Parvalbumins

Parvalbumins, found in various fish species, contain 2 calcium-binding EF-hand motifs (CD- and EF-site) and one silent domain (AB-site) forming a cap covering the hydrophobic surface of the two calcium-binding domains [33, 64, 70]. At least two lineages of parvalbumin existed in the ancestors of vertebrates, **alpha** and **beta** [63]. Birds and humans express only the alpha form, though in humans oncomodulin was classified as a beta parvalbumin [63]. Moncrief et al. [63] hypothesized that the beta form in mammals may be another isoform and the beta-parvalbumin encoding gene may have been deleted or inactivated [63]. Oncomodulin is a tumor protein, but is also expressed in the fetal placenta [71-74] and in the postnatal mammalian tissue [75]. Alpha parvalbumin in humans was described to protect the neurons from calcium-mediated cell death [76].

Alpha parvalbumins are abundant in fish and amphibians and are not generally allergenic. Nevertheless, there are two reports about an allergenic alpha parvalbumin in frog [55, 77]. In contrast, allergenic beta parvalbumins are known in many fish species [55]. Fish parvalbumins of the beta-lineage are only 56% identical to the human alpha form [55]. Although the two calcium-binding motifs are highly conserved in all parvalbumins, there was no IgE cross-reactivity observed among alpha and beta parvalbumins from most vertebrate muscles, including human beings. The lack of the allergenic potential of alpha parvalbumin in fish and in humans might be explained by a deletion of the IgE binding site in the calcium binding motifs during evolution [55].

The first reported allergenic fish parvalbumin was from Baltic cod (*Gadus callarias*) designated as “allergen M” (later named Gad c 1) and was identified by Elsayed et al. [78]. Since then various allergenic fish parvalbumins were cloned and characterized (e.g. Gad m 1 from Atlantic cod [5, 79], Cyp c 1 from carp [80], and Sal s 1 from

salmon [81]). Beta-parvalbumins with a pI around 4.5 have a molecular weight of approximately 11 kDa. They regulate calcium- and magnesium-dependent muscle homoeostasis in fish muscle [82]. The calcium-bound form is responsible for the relaxation of the fast twitch muscles. Parvalbumin is abundant in fish species containing more developed white muscle, like bottom dwelling fish [42]. The protein content also varies in the different parts of a fish as Lim et al. [83] described for tuna. Multiple isoforms can be found, depending on developmental stages, muscle type and parts of the fish [84, 85]. Additionally, a higher expression level of parvalbumin was observed in younger than in older fish [86].

**Beta-parvalbumin** is described as the major allergen in many fish species eliciting mild to severe symptoms in fish allergic patients mainly in coastal countries [34]. The calcium-bound form has been shown as resistant to thermal treatment and digestive enzymes [79, 87, 88]. A loss of calcium results in structural changes and a substantial reduction of IgE binding [25, 67, 80]. Depending on the fish species, food processing may impair the IgE binding ability, as reported for canned tuna and salmon [89]. IgE reactivity may also be enhanced by formation of high molecular aggregates after cooking as shown for tuna, salmon, cod, and flounder [90].

IgE binding epitopes are distributed along the whole parvalbumin polypeptide chain [91]. The AB-site, also containing IgE reactive epitopes [91], is more conserved between frog and fish and so cross-reactivity of parvalbumin among different fish species [34, 36, 55, 77, 90] and even with frog were observed [35, 55]. However, no IgE cross-reactivity outside the fish and amphibian species have been described, as *"the homology decreases with decreasing zoological relationship"* [8]. The cross-reactivity among fish species is of clinical relevance, as patients are often allergic to several fish species [36]. The degree of IgE-binding activity might be related to the amount of fish consumption [25, 36].

Parvalbumin is resistant to gastrointestinal digestion and can be found in the serum already 10 minutes after ingestion [92]. These findings may explain the rapid allergic reaction after fish ingestion in some fish allergic patients. The most severe and frequent cause of IgE-mediated fish allergy is a lethal anaphylactic shock. However, based on the extensive characterisation of fish parvalbumins, a hypoallergenic carp parvalbumin mutant was produced, by introducing mutations into the calcium binding domains [25]. Such an engineered hypoallergen could prove to be a promising tools for treating IgE-mediated fish allergic patients [25].

# 6. Aims

Allergy to fish represents a severe health problem. Beside extensive studies of the complex interplay between the immune system and the allergen, identification and characterization of the involved allergens will enable a better understanding of the molecular pathology of allergy.

The aims of this thesis were:

- to investigate and compare the structural and immunological properties of natural and recombinant allergenic fish parvalbumins.

- to determine the impact of thermal denaturation on purified parvalbumins. Cod was selected as a sea water fish and carp as a fresh water fish species.

- to examine the IgE cross-reactivity of purified parvalbumins from cod and whiff as examples for high allergenic fish, and from swordfish as an example for a dark muscled fish.

- to determine the thermal and gastric stability of native whiff parvalbumin Lep w 1 by circular dichroism (CD) spectroscopy and *in vitro* gastrointestinal digestion experiments. Further, the digestibility of EGTA treated and untreated Lep w 1 were tested. At last, the gastric stability and IgE binding ability of proteins extracted from cooked fish were examined.

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# Chapter II

## Comparison of natural and recombinant forms of the major fish allergen parvalbumin from cod and carp

Yan Ma[1]*, Ulrike Griesmeier[1]*, Markus Susani[2], Christian Radauer[1], Peter Briza[3], Anja Erler[3], Merima Bublin[1], Stefano Alessandri[4], Martin Himly[3], Sonia Vázquez-Cortés[5], Isabel Reig Rincon de Arellano[5], Emilia Vassilopoulou[6], Photini Saxoni-Papageorgiou[6], André C. Knulst[7], Montserrat Fernández-Rivas[5], Karin Hoffmann-Sommergruber[1] and Heimo Breiteneder[1]

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Research Article

# Comparison of natural and recombinant forms of the major fish allergen parvalbumin from cod and carp

**Yan Ma[1]*, Ulrike Griesmeier[1]*, Markus Susani[2], Christian Radauer[1], Peter Briza[3], Anja Erler[3], Merima Bublin[1], Stefano Alessandri[4], Martin Himly[3], Sonia Vàzquez-Cortés[5], Isabel Reig Rincon de Arellano[5], Emilia Vassilopoulou[6], Photini Saxoni-Papageorgiou[6], André C. Knulst[7], Montserrat Fernández-Rivas[5], Karin Hoffmann-Sommergruber[1] and Heimo Breiteneder[1]**

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Allergic reaction following fish consumption can trigger life-threatening reactions in predisposed individuals. Parvalbumins from different species have been identified as the major fish allergens. There are two distinct phylogenetic lineages of parvalbumins, alpha and beta. Most allergic reactions are caused by β-parvalbumins. We cloned and expressed cDNAs encoding cod (*Gadus morhua*) and carp (*Cyprinus carpio*) β-parvalbumins and purified natural cod β-parvalbumin. CD spectra of the purified proteins showed that their overall secondary structure contents were very similar. No differences in thermal stability were monitored in the calcium-bound or calcium-depleted form of natural cod parvalbumin. IgE reactivity was assessed using 26 sera of fish allergic patients from Spain, The Netherlands, and Greece in immunoblot and ELISA experiments. Twenty-five of the 26 patients with IgE reactivity to native and recombinant cod parvalbumin also reacted to the recombinant carp parvalbumin. IgE inhibition assays were performed using cod and carp extracts and purified recombinant parvalbumin of cod and carp. High crossreactivity among cod and carp parvalbumins was observed in immunoblots as well as in fluid phase assays. Natural and recombinant parvalbumins gave comparable results when performing various *in vitro* diagnostic assays.

**Keywords:** Carp parvalbumin / Cod parvalbumin / Fish allergy / Food allergy / β-Parvalbumin

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## 1 Introduction

Fish are among the most common sources of food allergens. In many countries where seafood is an integrate part of the diet, fish represent a frequent cause of food allergy [1]. The parvalbumins of fish represent the second largest animal food allergen family, the largest being the tropomyosins of crustaceans and molluscs [2]. Parvalbumins which are abundant in the white muscle of many fish species constitute a subfamily of a large evolutionary related family of proteins with mixed type binding sites for $Ca^{2+}$/$Mg^{2+}$, the so-called EF-hand [3]. The family of EF-hand proteins is the third ranking protein family in terms of numbers of allergenic members (www.meduniwien.ac.at/allergens/allfam/). The EF-hand corresponds to a helix-loop-helix motif of 30 residues in length. Both helices E and F, with 10 residues each, are flanking a central loop that contains the metal-binding residues [4]. Parvalbumins are important for the relaxation of muscle fibers by binding free intracellular

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**Abbreviations: CD,** circular dichroism; **ESI-QTOF,** ESI-Quadrupole TOF

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calcium [5]. The binding of the calcium ligand was found to be necessary for maintaining the parvalbumin in a conformation that is able to bind IgE. Loss of calcium results in a change in conformation together with an associated loss of the protein's IgE binding capacity [6–8]. Parvalbumins with bound calcium also possess a remarkable stability to denaturation by heat, denaturing chemicals, and digestive enzymes [8–10] which influences their allergenic activity.

Parvalbumins can be subdivided into two distinct evolutionary lineages based on the comparison of their amino acid sequences [11]. α-Parvalbumins comprise 109 amino acid residues and are less acidic with p*I*s at 5.0 or higher. β-Parvalbumins consist of 108 amino acid residues and are more acidic with p*I*s at 4.5 or lower [12]. In general, α-parvalbumins are not allergenic with the exception of representatives from two frog species [13, 14]. In contrast, many allergenic β-parvalbumins are found in a variety of fish species [15, 16]. Some of them have been shown to display more than two parvalbumin isotypes [17]. Today, allergenic β-parvalbumins are considered as crossreactive pan allergens in fish [18–20]. Sufficient IgE-reactive epitopes remain after cooking to trigger allergic reactions in susceptible individuals as has been demonstrated by double blind placebo controlled food challenge [21]. CD analysis of carp parvalbumin revealed a remarkable stability and refolding capacity of the calcium-bound form [7]. However, there is one contradictory report which questions the stability of parvalbumins to pepsin [22]. The authors reported a recombinant allergenic carp parvalbumin (rCyp c 1) to be completely degraded after 30 s treatment with pepsin.

In the present study, the structural and immunological properties of natural and recombinant allergenic fish parvalbumins were investigated and compared. In addition, the impact of thermal denaturation on purified parvalbumins was studied. We have selected cod as a sea water and carp as a fresh water fish species for our studies.

## 2 Materials and methods

### 2.1 Sera and antibodies

Fish allergic patients from Spain, The Netherlands, and Greece ($n = 26$; including children and adults) were identified according to convincing case histories and positive CAP values (Phadia Diagnostics, Uppsala, Sweden; Table 1) to fish. Sera were stored at −20°C until use. The mouse monoclonal antiparvalbumin clone Parv-19 antibody from Sigma (St. Louis, MO, USA) and a rabbit polyclonal anti-Gad m 1 antibody (Tepnel BioSystems, Deeside, Flintshire) were used in this study.

### 2.2 Preparation of crude fish extract

Fresh filet of Atlantic cod (*Gadus morhua*) was purchased from a local market. Fish muscle (500 g) was homogenized with three volumes of 20 mM Bis-Tris buffer pH 6.5. Proteins were extracted by stirring the homogenate for 3 h at 4°C. After centrifugation at 17 000 × *g* for 45 min at 4°C the supernatant was collected and filtered through Miracloth® (Merck Biosciences, Nottingham, UK) and filter papers, subsequently, to remove cellular debris. Cod extract was further used for purification and immunological assays. For IgE inhibition assays 4 g of carp muscle were homogenized with five volumes of double distilled $H_2O$ containing 3 mM $NaN_3$, and extracted by stirring for 3 h at 4°C. The total extract was cleared by centrifugation at 20 000 × *g* for 15 min at 4°C. The protein concentration was determined by using the BCA Protein Assay Reagent Kit (Pierce, Rockford, Ireland), according to the manufacturer's instructions. The extracts were stored at 4°C.

### 2.3 Purification of natural cod parvalbumin

As a first purification step the cod extract was cleared by filtration then incubated with Biocryl BPA-1000 (Supelco, Bellefonte, PA) to remove nucleic acids and then centrifuged at 20 000 × *g* for 10 min at 4°C. The supernatant was applied to a DEAE Sepharose Fast Flow column (GE Healthcare, Little Chalfont, UK) and washed with 20 mM Bis-Tris buffer, pH 6.5. Bound protein was eluted with a linear salt gradient from 0 to 25% elution buffer (20 mM Bis-Tris, 1 M NaCl, pH 6.5) and parvalbumin was detected by SDS-PAGE and immunoblotting using the mouse monoclonal antiparvalbumin clone Parv-19 antibody. Fractions containing parvalbumin were then loaded onto a HiPrep 16/60 Sephacryl S-200 High Resolution column (GE Healthcare) which had been equilibrated at room temperature with 20 mM Bis-Tris, 150 mM NaCl, pH 6.5. Parvalbumin was eluted as a single peak from the column.

### 2.4 cDNA synthesis and RT-PCR amplification

Total RNA was isolated from 100 mg cod muscle tissue using the RNeasy® Protect Midi Kit following the manufacturer's instructions (Qiagen, Hilden, Germany). Five micrograms of total RNA was used for cDNA synthesis. The reverse transcription was performed with an oligo-dT primer $T_{25}$NN. Gene-specific primers (5′-ATG GCA TTC GCT GGA ATT CTC G-3′ for rGad m 1.01 and 5′-ATG GCT TTC GCC GGA ATT CTG A-3′ for rGad m 1.02) were synthesized according to the published N-terminal sequences of cod parvalbumins [23]. These primers were used in conjunction with the oligo-dT primer $T_{25}$NN to amplify Gad m 1 encoding cDNAs. The PCR reaction was performed as described elsewhere [24].

### 2.5 cDNA cloning and DNA sequencing

Amplified Gad m 1 cDNA was ligated into the pCP2.1-TOPO vector (Invitrogen, Carlsbad, USA) and competent

**Table 1.** Patients' characteristics

| No. | Country | Age | Sex | CAP | Symptoms |
|---|---|---|---|---|---|
| **1** | E | 11 | f | Cod 3.18 | OAS, AD |
| 2 | E | 11 | m | Cod 17.2 | U, V, AD |
| **3** | E | 11 | m | Haddock 32.9 | OAS, U, AD |
| **4** | E | 5 | m | Cod 8.91 | U, V, AD |
| 5 | E | 6 | m | Cod 2.74 | OAS |
| 6 | E | 27 | m | Cod 2.42 | AE, D, CU, AD |
| 7 | E | 15 | m | Cod 0.52 | OAS, AE, DY, AD |
| **8** | E | 27 | m | Cod 55.4 | U, A |
| 9 | E | 4 | f | Cod 0.72 | OAS |
| **10** | E | 23 | m | Cod 9.6 | AN |
| 11 | E | 10 | m | Sole < 0.78 | V, AD |
| 12 | E | 7 | f | Cod 6.38 | U, AD |
| 13 | E | 29 | m | Cod 7.17 | DY |
| **14** | NL | 29 | f | Cod 37.0 | R, D, AD |
| **15** | NL | 22 | m | Cod 53.0 | R, ST, AD |
| **16** | NL | 41 | m | Cod 11.0 | OAS, AD |
| 17 | NL | 39 | f | Cod 3.2 | AD |
| **18** | NL | 15 | m | Cod 6.3 | OAS, U, AD |
| 19 | NL | 17 | m | Cod 12.0 | OAS, TT, AD |
| 20 | GR | 5 | f | Cod 5.22 | AN |
| **21** | GR | 10 | m | Cod >100 | A |
| 22 | GR | 14 | m | Cod 99.3 | U, G |
| 23 | GR | ND | m | – ND | G |
| 24 | GR | 12 | f | Cod 1.99 | U |
| 25 | GR | 13 | m | Cod 9.09 | AN, A, U |
| 26 | GR | 4 | m | Cod 11.2 | AN |

Patients' sera used for immunoblotting are indicated in bold. E, Spain; NL, Netherlands; GR, Greece; f, female; m, male; CAP, capsulated hydrophobic carrier polymer (kU/mL); A, asthma; AD, atopic dermatitis; AE, angioedema; AN, anaphylaxis; C, cough; CU, contact urticaria; D, dyspnoea; DY, dysphagia; OAS, oral allergy syndrome; R, rash; ST, swelling throat; TT, tightness in the throat; U, urticaria; G, gastrointestinal; V, vomiting.

TOPO10F' *E. coli* cells were transformed with the pCP2.1-Gad m 1 plasmids. Sequencing of the inserts was performed by VBC-Biotech Service (Vienna, Austria). Sequence analysis was performed using the BLAST Program of the National Center of Biotechnology Information (Bethesda, MD) and ClustalW program of EMBL-EBI.

### 2.6 Expression and purification of rGad m 1.02 and rCyp c 1.01

The cDNA coding for Gad m 1.02 was PCR amplified from the respective pCP2.1-plasmid and subcloned into the *Hin*dIII/*Bam*HI sites of expression vector pET17b (Novagen, Madison, WI). The primers for subcloning were designed in a reading frame avoiding the expression of the T7-Tag. This was achieved using the following oligonucleotide primer for the 5′ end of the clone: 5′-AC AAG CTT ATG GCT TTC GCC GGA ATT CTG A-3′, which contained a *Hin*dIII site and a primer for the 3′ end with a *Bam*HI site: 5′-AT CGG ATC CTA TGC CTT GAT CAT GGC-3′. pET17b containing the Gad m 1.02 cDNA was expressed in *E. coli* BL21 (DE3)-RIPL cells. Single colonies were grown overnight at 37°C in LB medium containing 0.1 mg/mL ampicillin and 50 μg/mL chloramphenicol. Protein expression was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubation was continued for 5 h at 30°C. Cells were harvested and disrupted by repeated cycles of freezing in liquid nitrogen and thawing in a water bath. Cell pellets were resuspended in lysis buffer (50 mM $Na_2HPO_4$, 300 mM NaCl, 10 mM imidazole, pH 8.0). Digestion with DNase I (0.1 μg/g cell pellet) and RNase A treatment (0.01 μg/g cell pellet) were performed at room temperature for 30 min under constant stirring. The lysate was centrifuged at 13 000 × *g* for 30 min at 4°C. Recombinant Gad m 1.02 was purified from the supernatant using two anion exchange columns, a DEAE Sepharose Fast Flow (buffer A: 20 mM Bis-Tris, pH 6.5, buffer B: A + 1 M NaCl) and a MonoQ 5/50 GL Tricorn high performance column (GE Healthcare, buffer A: 20 mM Tris, pH 8.0, buffer B: A + 1 M NaCl).

Recombinant Cyp c 1.01 was expressed from the pET17b-Cyp c 1 plasmid [25, 26]. *E. coli* lysate containing rCyp c 1.01 was heated up to 75°C for 40 min and centrifuged 13 000 × *g* for 30 min at 4°C. Subsequently, ammonium sulfate (75%) was added and the protein extract was centrifuged. Recombinant carp parvalbumin was obtained from the supernatant and was applied onto a Phenyl-Cellufine column (GE Healthcare) which was equilibrated with 1 M NaCl, 200 mM $NaH_2PO_4$ pH 5.0, 1 mM β-mercaptoethanol (buffer A). Bound protein was eluted by a linear gra-

dient (0 – 100%) of buffer B (25 mM Tris/HCl pH 9.3, 8% 2-propanol). Recombinant Cyp c 1.01 enriched fractions were further purified by an anion exchange chromatography (DEAE Sepharose Fast Flow column). The column was equilibrated with buffer A (20 mM imidazol pH 7.4, 2 mM β-mercaptoethanol), the bound recombinant Cyp c 1.01 was eluted by a gradient with buffer B (buffer A + 0.4 M NaCl). A final dialysis step was performed against 5 mM $NaH_2PO_4$, 2 mM β-mercaptoethanol, pH 7.4.

### 2.7 SDS-PAGE, Western blotting, IgE immunoblotting, and IgE inhibition

Purified nGad m 1, rGad m 1.02, and rCyp c 1.01 were separated by 15% SDS-PAGE as described by Laemmli [27] under reducing conditions and either visualized by CBB R-250 staining or transferred to nitrocellulose membranes (pore size 0.2 µm, Pall Corporation, Pensacola, USA) for immunodetection.

Blots were incubated with the mouse monoclonal anti-parvalbumin clone Parv-19 antibody (1:4000 diluted in TBST [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20 v/v]) or the rabbit polyclonal anti-Gad m 1 antibody (1:12 000 diluted in TBST) under constant shaking at room temperature for 2 h, respectively. Subsequently, alkaline phosphatase (AP)-conjugated swine antimouse (DAKO, Glostrup, Denmark, 1:1000 diluted in TBST) and antirabbit Igs (Jackson ImmunoResearch Laboratories, West Grove, USA, 1:5000 diluted in TBST), respectively. Development was performed with BCIP/NBTC reagent solutions. In addition, blotted proteins were incubated with fish allergic patients' sera diluted 1:5 in buffer B (42 mM $Na_2HPO_4$, 7 mM $NaH_2PO_4$, 0.05% w/v Na-azide, pH 7.5, 0.5% Tween-20) overnight at 4°C and detected by $^{125}$I-labelled rabbit anti-human IgE (MALT Allergy System Isotope Reagent, IBL Hamburg, Germany, 1:20 diluted in buffer B). Autoradiography was performed at room temperature for 2 – 48 h with intensifying phosphor screens and imaged on the Storm 860 Imager (GE Healthcare) or with a BioMax Ms film (Kodak, Sigma – Aldrich, St. Louis, USA) at –70°C. IgE inhibition assays were performed using purified nGad m 1 (50 µg/mL), rGad m 1.02 (10, 50, and 100 µg/mL) and rCyp c 1.01 (50 µg/mL) as inhibitors respectively over night at 4°C. Incubation of the blot strips and detection were performed as above.

### 2.8 IgE ELISA and inhibition assays

Purified nGad m 1, rGad m 1.02, and rCyp c 1.01 (1 µg/well diluted in 25 mM $NaHCO_3$, pH 9.6) were coated on microtiter plates (Nunc Maxisorp, Nalge Nunc International, Roskilde, Denmark) overnight at 4°C. Nonspecific binding sites were blocked with 3% milk in TBST. The coated allergens were incubated with sera from 26 fish allergic patients (1:4 diluted in TBST containing 0.5% BSA) overnight at 4°C. Bound IgE was detected with AP-conjugated mouse antihuman IgE antibody (BD-Biosciences Pharmingen, San Diego, CA, USA) and developed with the SIGMA FAST™ *p*-nitrophenyl phosphate substrate (Sigma-Aldrich). Color development was measured using an ELISA reader (Spectra Max Plus 384; Molecular Devices, Munich, Germany) at 405 and 510 nm as reference wavelength. OD values were counted positive if they exceeded the mean OD of the negative controls by more than three SDs. IgE inhibition assay was performed using cod or carp fish extracts (10 µg/mL) diluted in coating buffer (25 mM $NaHCO_3$, pH 9.6) and purified recombinant parvalbumin of cod (10 µg/mL) and carp (10 µg/mL) as inhibitors.

### 2.9 N-terminal sequencing

Fifty picomoles of purified proteins was diluted into 100 µL of 0.1% TFA and immobilized on a polyvinylidene difluoride (PVDF) membrane (ProSorb sample preparation cartridges, Applied Biosystems, Foster City, CA, USA), which was soaked in 10 µL of methanol before. The PVDF membrane was washed with 0.1% TFA. After drying at room temperature membrane was subjected to the automated gas-phase Procise 491 sequencer (Applied Biosystems). For nGad m 1, the N-terminal acetyl group was cleaved off with TFA prior to sequencing.

### 2.10 Circular dichroism (CD) and stability studies

Far UV CD spectra of purified parvalbumins were recorded with a JASCO J-810 spectropolarimeter (Jasco, Essex, UK) at 20°C in aqueous solutions. Protein samples were concentrated to 0.1 µg/µL and measured in quartz cuvettes (Hellma, Mullheim, Baden, Germany) of 0.1 and 0.2 cm path length. All spectra were corrected for the proper baseline using the corresponding aqueous solution. Absorption between 190 and 260 nm was monitored at 0.5 nm intervals. The obtained spectra represent an average of three consecutive scans. Results of each sample were averaged, and the mean residue ellipticity (θ) was expressed as $deg \cdot cm^2 \cdot dmol^{-1}$. The mean residue molecular weight of each parvalbumin was calculated from the amino acid composition. For monitoring the stability to heating, samples were incubated for 10 min at 95°C.

### 2.11 NMR analysis

The allergens rCyp c 1.01 and nGad m 1 were analyzed by NMR, according to the following protocol. Before and after the NMR experiments, the allergens were stored at –20°C. A solution of each allergen was prepared in 0.45 mL of $H_2O$ plus 0.05 mL of $D_2O$. The concentrations were 0.52 mM for rCyp c 1.01 and 0.05 mM for nGad m 1. The solutions were placed into high-quality NMR tubes with Ar as head-space

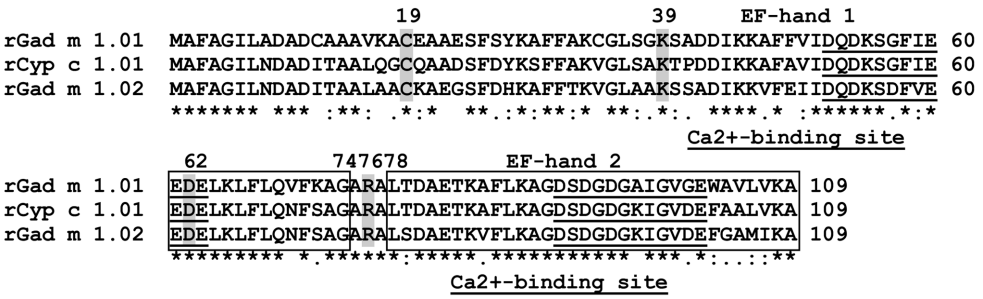


**Figure 1.** Comparison of amino acid sequences and analysis of the two cod β-parvalbumins (rGad m 1.01 and rGad m 1.02) and carp β-parvalbumin (rCyp c 1.01). The sequences are available under accession numbers AM497927 for rGad m 1.01, AM497928 for rGad m 1.02, and AJ292211 for rCyp c 1.01. Two EF-hand repeats including the calcium binding sites are boxed and the calcium binding sites are underlined. Characteristic amino acid residues for currently known parvalbumins are highlighted. Stars indicate conserved, ":" highly conserved, and "." weakly conserved amino acid residues.

gas. Two High Resolution NMR experiments were carried out, by a Bruker Avance 700 spectrometer operating at a proton resonance frequency of 700 MHz (11.7 Tesla) at 25°C. The two experiments were different in the method to manage the water signal: the zgpr experiment minimizes the water peak, while the zgesgp experiment suppresses it. For each experiment 1024 scans were programmed to analyze nGad m 1 and 256 scans for rCyp c 1.01.

### 2.12 Mass determination

For mass determination of intact Gad m 1, approximately 2 µg of protein was reduced with the reagents of the Proteoextract™ Trypsin Digestion Kit (Calbiochem, San Diego, USA). Prior to the mass analysis, salts and reagents were removed using C18 ZipTips™ (Millipore, Billerica, MA, USA), following the manufacturer's protocol, except that trifluoro acetic acid was replaced by formic acid. Proteins were eluted from the RP material with aqueous 50% v/v HPLC-grade ACN and 0.1% v/v formic acid and directly infused into an ESI-Quadrupole TOF (ESI-QTOF) mass spectrometer (Ultima Global, Micromass-Waters, Milford, MA, USA) at an infusion rate of 1 µL/min. The intact mass of rCyp c 1.01 was determined without prior Ziptip purification at a concentration of approx. 500 fmol/µL and an infusion rate of 0.5 µL/min. The Waters Nanoflow spray head was used with nitrogen as desolvation gas and a capillary voltage of 3.4 kV. The instrument was calibrated with the fragment ions of [Glu]-Fibrinopeptide B (Sigma). Spectra were recorded for 3 min in a mass/charge range from 400 to 1900. More than 200 mass scans of 1 s each were combined for optimal S/N. Multiply charged peaks were processed using the MaxEnt1™ algorithm of the MassLynx™ software package (Waters).

For nano-LC-MS/MS-based peptide mapping 50 µg aliquots of rCyp c 1.01 each were digested overnight at 37°C with 2 µg trypsin or V8 protease (Roche, Basel, Switzerland). Present cysteine residues were reduced by pretreatment of the protein with a ten-fold molar excess of DTT and a 20-fold molar excess of iodoacetamide, both incubations lasted for 30 min at room temperature. Alternatively, 5 µg of Gad m 1 was reduced, alkylated, and digested with the Proteoextract Trypsin Digestion Kit (Calbiochem). Proteolytic digests were diluted 1:20 in 0.1% formic acid and 5 fmol was injected to RP capillary HPLC (Nanoease Symmetry 300™ trap column and $0.075 \times 15$ mm$^2$ Nanoease Atlantis dC18™ separating column on CapLC, Micromass-Waters) directly coupled to ESI-QTOF in data-dependent analysis mode. Tandem mass spectra were analyzed using the ProteinLynx Global Server 2.2.5™ software (Waters) with both automatic and manual data verification. For MS/MS-based sequencing a combined Swiss-Prot/TrEMBL database was used and automatic validation was enabled. Therefore, positive identification of rCyp c 1.01- or rGad m 1.02-derived peptides by CID was based on at least four consecutive unequivocally identified *y*-ions in MS/MS mode.

## 3 Results

### 3.1 Cloning and sequence analysis

Two full-length clones of 599 and 797 bp (named Gad m 1.01 and Gad m 1.02), coding for cod β-parvalbumins were obtained. The sequences were submitted to EMBL Genbank Database (accession numbers AM497927 and AM497928). Gad m 1.01 had one amino acid exchange at position 101 (E/D) compared to cod parvalbumin with the accession number AY035584 [28]. Gad m 1.02 had four nucleotide differences with one different deduced amino acid at position 41 (S/P) compared to cod parvalbumin with the accession number AY035585 [28].

The coding regions of the two parvalbumin cDNAs each encompass 330 bp coding for 109 amino acid residue proteins both with a theoretical p*I* of 4.58 (Fig. 1). The deduced amino acid sequences of the two cod β-parvalbumin isoforms are 71% identical. Both Gad m 1 isoforms share 80–81% sequence identity with Cyp c 1.01. Like all the other

 

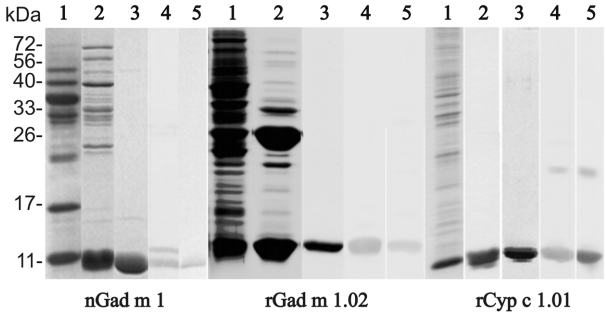


**Figure 2.** Purification of parvalbumins: nGad m 1: Lane 1: cod extract. Lane 2: protein purification by anion exchange followed by size exclusion chromatography (lane 3). Lane 4: detection of two Gad m 1 isoforms by a rabbit polyclonal anti-Gad m 1. Lane 5: detection of one Gad m 1 isoform by the mouse monoclonal antiparvalbumin Parv-19 antibody. rGad m 1.02: Lane 1: cell lysate of *E. coli* expressing rGad m1.02, lane 2: protein purification by anione exchange followed by size exclusion chromatography (lane 3), lane 4: detection of purified rGad m 1.02 by a rabbit polyclonal anti-Gad m 1 antiserum. Lane 5: detection of purified rGad m 1.02 by the mouse monoclonal antiparvalbumin Parv-19 antibody. rCyp c 1.01: cell lysate of *E. coli* expressing rCyp c 1.01. Lane 2: purification of rCyp c 1.01 by hydrophobic interaction chromatography followed by anion exchange chromatography (lane 3). Detection of purified rCyp c 1.01 by polyclonal (lane 4) and monoclonal antibodies (lane 5) showing dimers at around 20 kDa.

known fish parvalbumins, the Gad m 1 encoding sequences contain two characteristic EF-hand repeats which bind calcium and contain conserved amino acid residues (Fig. 1). An invariant aspartic acid residue is present at position 62 which is common to all currently known parvalbumins. In addition, a cysteine at position 19 and an arginine at position 76, conserved characteristics of the β lineage parvalbumins [28], were also identified.

### 3.2 Extraction and purification of nGad m 1, expression and purification of rGad m 1.02 and rCyp c 1.01

Natural cod parvalbumin was purified from cod muscle protein extract. The SDS-PAGE profile of the extract showed a prominent band at approximately 12 kDa (Fig. 2, nGad m 1, lane 1). Natural Gad m 1 was purified by a combination of anion exchange (Fig. 2, nGad m 1, lane 2) and size exclusion chromatography (Fig. 2, nGad m 1, lane 3). Purified nGad m 1 representing two isoforms was detected by immunoblotting with polyclonal and monoclonal antibodies (Fig. 2, nGad m 1, lanes 4 and 5, respectively). The total yield from 500 g fish muscle was 25 mg pure nGad m 1.

Recombinant Gad m 1.02 and rCyp c 1.01 were expressed in the pET17b expression vector and produced as nonfusion proteins in BL21 (DE3)-RIPL cells. Recombinant parvalbumin proteins were mostly found in the soluble fraction of the cell cultures (Fig. 2, rGad m 1.02 and rCyp c 1.01, lane 1). After precipitation (rCyp c 1.01), proteins were purified by several chromatographic steps (Fig. 2, rGad m 1.02 and rCyp c 1.01, lanes 2 and 3). Purified proteins were detected by immunoblotting using the mouse monoclonal antiparvalbumin clone Parv-19 antibody and the rabbit polyclonal anti-Gad m 1 antibody (Fig. 2, rGad m 1.02 and rCyp c 1.01, lanes 4 and 5, respectively).

### 3.3 N-terminal sequencing

N-terminal sequence analysis of all three purified proteins nGad m 1, rGad m 1.02, and rCyp c 1.01 revealed that the initiating methionine was cleaved off, resulting in the following first five amino acid residues AFAGI.

### 3.4 IgE binding activity and crossreactivity

Sera from 26 fish allergic patients from Spain, The Netherlands, and Greece were used for IgE ELISA. The IgE binding activities of purified natural and recombinant β-parvalbumins of cod and recombinant β-parvalbumin of carp were comparable for most sera (19/26, Fig. 3A). A serum pool from five fish allergic patients (patient no. 4, 8, 10, 18, and 21) was used for IgE inhibition experiments. The serum pool was preincubated with cod or carp muscle extract (10 μg/mL) purified rGad m 1.02 (10 μg/mL) or rCyp c 1.01 (10 μg/mL). IgE binding activity was inhibited by 91% to nGad m 1 and rGad m 1.02 and by 76% to rCyp c 1.01 (Fig. 3B). IgE binding to rGad m 1.02 was inhibited 98% by rCyp c 1.01 and IgE binding to rCyp c 1.01 was reduced 86% by rGad m 1.02 (Fig. 3C).

Sera from 10 fish allergic patients (patient no. 1, 3, 4, 8, 10, 14, 15, 16, 18, and 21) were used for IgE immunoblotting of the three parvalbumins (Fig. 3D). Serum no. 21 was used for the inhibition assay (Fig. 3D, lane 21i). The band recognized by the IgE antibodies from patients' sera was at 12 kDa. The identity of this band as parvalbumin was confirmed by immunoblots with antiparvalbumin antibodies. Most samples displayed equal IgE reactivity to all three proteins. The immunoblot inhibition experiments were performed with cod and carp muscle extract, purified nGad m 1, rGad m 1.02, and rCyp c 1.01. IgE binding to nGad m 1 and rGad m 1.02 was almost completely inhibited, and IgE binding to rCyp c 1.01 was inhibited by more than 50% with fish extracts (Fig. 3D, lane 21i). These results were in agreement with the results of ELISA inhibitions (Fig. 3B). The immunoblot inhibition experiments with purified natural and recombinant proteins showed that the IgE binding capacity of rCyp c 1.01 was completely inhibited by carp extract (Fig. 3E). The IgE binding capacity of nGad m 1 and rGad m 1.02 was inhibited more than 90 and 80% by cod extract (Fig. 3E), which was measured using ChemiImager™ 400 (Alpha Innotech Corporation). Normal human

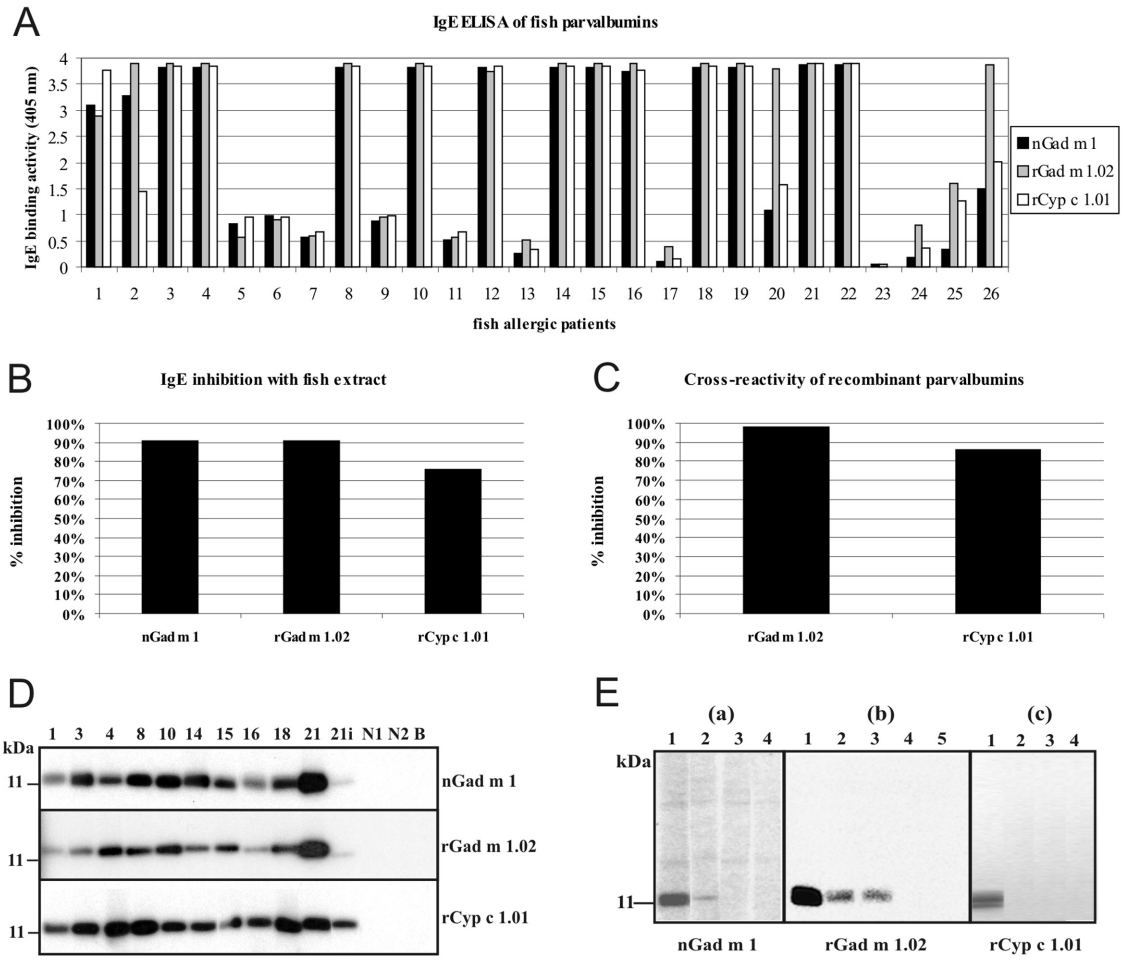


**Figure 3.** Immunological analysis of nGad m 1, rGad m 1.02 and rCyp c 1.01. (A) IgE binding to purified parvalbumins of sera from fish allergic patients from Spain, The Netherlands, and Greece. (B, C) IgE inhibition assays were performed using cod or carp extract or purified recombinant parvalbumin of cod or carp as inhibitors. OD values were counted positive if they exceeded the mean OD of the negative controls by more than three SDs. (B) Residual IgE binding to rGad m 1.02, nGad m 1, and rCyp c 1.01 after preincubation with cod and carp protein extract, respectively. (C) Crossinhibition assay: Residual IgE binding to rGad m 1.02 after preincubation with rCyp c 1.01, IgE binding to rCyp c 1.01 after preincubation with rGad m 1.02. D: IgE immunoblot and IgE inhibition analysis. 1–21, sera of fish allergic patients; 21i, serum was preincubated with cod extract for nGad m 1 (top) and rGad m 1.02 (middle), or with carp extract for rCyp c 1.01 (bottom). N1 and N2, healthy nonallergic individuals; B, buffer control. (E) Immunoblot inhibition of IgE binding to cod or carp extract by purified nGad m 1, rGad m 1.02 or rCyp c 1.01. nGad m 1: Lane 1: IgE binding to cod extract, lane 2: Immunoblot inhibition of IgE binding to cod extract by 50 μg/mL nGad m 1. rGad m 1.02: Lane 1: IgE binding to cod extract, lane 2: Immunoblot inhibition of IgE binding to cod extract by 100 μg/mL rGad m 1.02, and by 50 μg/mL rGad m 1.02 (lane 3). rCyp c 1.01: Lane 1: IgE binding to carp extract, lane 2: inhibition of IgE binding to carp extract by 50 μg/mL rCyp c 1.01. Lane 3 of (a) and (c) and lane 4 of (b): healthy nonallergic individuals. Lane 4 of (a) and (c) and lane 5 of (b): buffer controls.

sera and buffer used as controls were negative in all experiments.

### 3.5 NMR analysis

For both rCyp c 1.01 (Fig. 4A left) and nGad m 1 (Fig. 4A right) the amide region (7–9 ppm), the aromatic region (6–8 ppm), the H-α region (below and above 4.4 ppm), and the aliphatic region (0–5 ppm) were crowded with clearly separated, narrow peaks. Moreover, aromatic and amide protons showed shifts above 9 ppm and below 7 ppm, α protons showed shifts above 5 ppm. All these facts gave evidence of a complete folding of rCyp c 1.01 (Fig. 4A left). The same evaluation applied to the spectra of nGad m 1 with some difference (Fig. 4A right). The peaks were slightly broader for nGad m 1, and some part of the protein appeared not to have a rigid tertiary structure.

### 3.6 Mass determination

The identity of rCyp c 1.01 with the UniprotKB/TrEMBL entry Q8UUS3 for a parvalbumin of *Cyprinus carpio* could

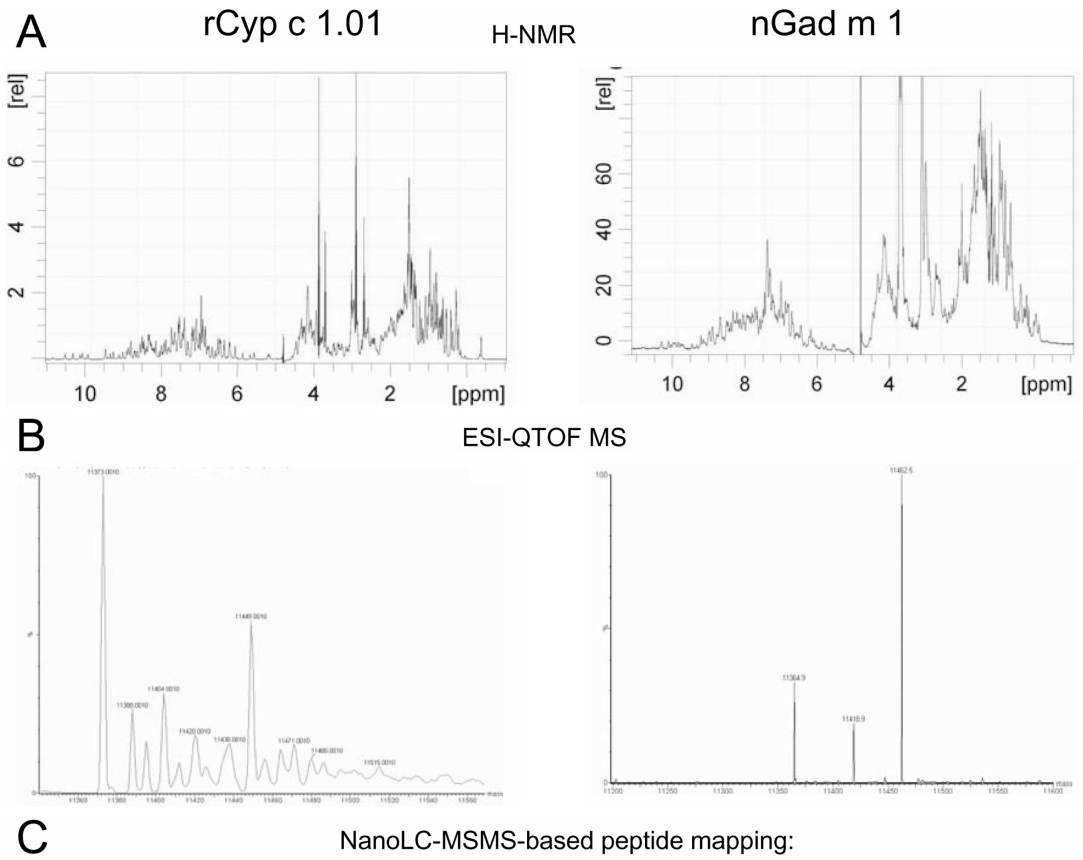


**Figure 4.** NMR and mass spectrum analysis. (A) $^{1}$H-NMR analysis: left: $^{1}$H 700 MHz spectrum of rCyp c 1.01, zgesgp experiment, 256 scans, 25°C. Right: $^{1}$H 700 MHz spectrum of nGad m 1, zgesgp experiment, 1024 scans, 25°C. (B) MS analysis: rCyp c 1.01 (left) and nGad m 1 (right). (C) Results of nano-LC-MS/MS-based peptide mapping for rCyp c 1.01 and nGad m 1, black: sequence coverage of obtained peptides, gray: no coverage.

be shown by ESI-QTOF MS (Fig. 4B left). The determined intact mass of 11 373 ± 1 Da indicated cleavage of N-terminal methionine, however, a second less prominent peak at 11 449 ± 1 Da pointed at a partial addition of β-mercaptoethanol to the free sulfhydryl groups of the single cysteine residue in position 18 (Fig. 4B). By nano-LC-MS/MS-based peptide mapping a sequence coverage of 69.4% was reached (Fig. 4C).

Mass spectroscopic analysis of the purified natural nGad m 1 revealed the presence of three peaks at 11 462.5, 11 364.9, and 11 419 Da molecular mass (Fig. 4B right). Nano-LC-MS/MS-based peptide mapping resulted in sequence fragments of two different isoforms, which showed a sequence coverage of 90% as well as 88% to previously published parvalbumin sequences from *G. morhua* (Acc No: Q90YK9, Q90YL0, [23]) (Fig. 4C). In both protein sequences, the acetylated N-terminal alanine was included. The intact mass of the least intense peak (11 419 Da) was in good agreement with the theoretical mass of the already published Gad m 1 isoform Q90YK9 (11 420 Da) [23], whereas 11 462.5 Da corresponded to the form with the deacetylated N-terminus. The mass of 11 364.9 was assigned to the N-terminal acetylated Gad m 1 isoform Q90YL0 (11 323.8). The difference of 42 Da compared to the theoretical masses are due to modification by acetylation of the N-termini. Mass analysis showed a higher amount of the isoform corresponded to the previously published Q90YK9 [23].

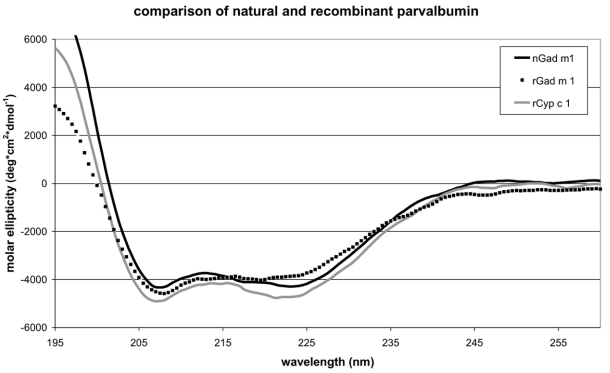


**Figure 5.** Comparison of $Ca^{2+}$-bound nGad m 1, rGad m 1.02 and rCyp c 1.01. CD spectra analysis revealed conformational differences between the natural and recombinant parvalbumins. Black: nGad m 1, black dotted: rGad m 1.02, gray: rCyp c 1.01.

### 3.7 CD and stability studies

The natural cod parvalbumin showed a far UV CD spectrum typical for α-helical proteins characterized by two broad minima at 208 and 222 nm at 20°C (Fig. 5), as previously described [29]. The recombinant carp parvalbumin was more similar to the natural than to the recombinant cod protein. The spectrum of recombinant cod parvalbumin showed a reduced minimum at 222 nm (Fig. 5).

CD spectra of nGad m 1 were recorded at pH 2.5, 7.0, and 8.5 (Fig. 6A). The spectra revealed conformational changes of the natural protein under acidic conditions. The minimum of around $-8000\ \mathrm{deg \cdot cm^2 \cdot dmol^{-1}}$ measured by 208 nm drifted to $-10\,000\ \mathrm{deg \cdot cm^2 \cdot dmol^{-1}}$. No change was caused under neutral or basic conditions. Thermal stability values were monitored at different pH values (Fig. 6B). The spectrum of nGad m 1 heated to 95°C at pH 7 showed an increase of negative dichroism. Whereas the heating of nGad m 1 to 95°C at pH 2.5 and 8.5 resulted in one broad minimum and changes in ellipticity at 208 nm to a higher negative dichroism (Fig. 6B). The spectra of unheated and heat-treated rCyp c 1.01 at pH 8.5 showed no shifts, similar to the ones of nGad m 1 (data not shown). In contrast to nGad m 1, rCyp c 1.01 changed its conformation to an irregular structure after heating at pH 2.5 (Fig. 7).

After $Ca^{2+}$-depletion by addition of 5.0 mM EGTA a decreased signal intensity was measured in the spectrum of rCyp c 1.01, as previously described [7]. In contrast, nGad m 1 displayed no change in the signal intensity, but a complete loss of the peak at 222 nm was monitored (Fig. 6C). Thermal stability of natural cod and recombinant carp parvalbumin after $Ca^{2+}$-depletion was observed after heating samples. Whereas, natural Gad m 1 treated with 5.0 mM EGTA showed a remarkable increased negative dichroism with a minimum at 208 nm after the heat treatment (Fig. 6C).

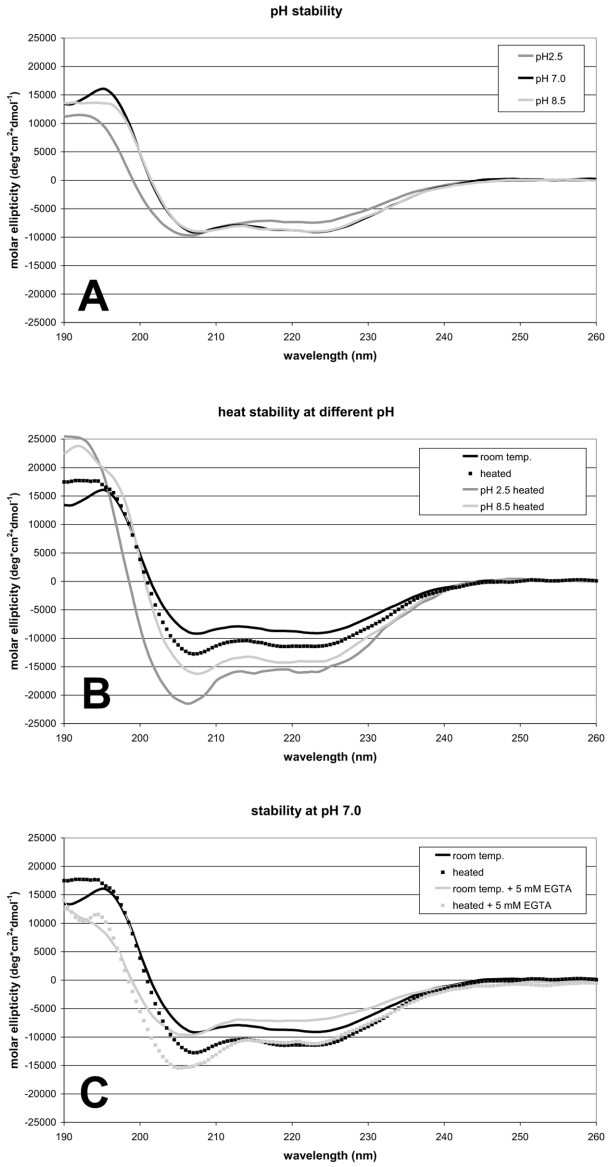


**Figure 6.** CD spectra of stability measurements of nGad m 1. (A) pH stability. No significant conformational changes at pH 7.0 and 8.5, but a complete loss of the minimum at 222 nm at pH 2.5. (B) heat stability. An increased dichroism and a loss of the minimum at 222 nm were monitored for each heated sample. (C) $Ca^{2+}$-depletion. Only one minimum at 208 nm was observed for the $Ca^{2+}$-depleted form. An increase of negative dichroism was revealed in the samples after heating.

## 4 Discussion

This work reports the comparison of natural and recombinant forms of parvalbumin of Atlantic cod and carp. Parvalbumins have previously been identified as major and cross-reactive allergens in various fish species [6, 30]. As the major cod allergen Gad m 1 is well studied and regarded as a representative for fish allergen, it is used for the develop-

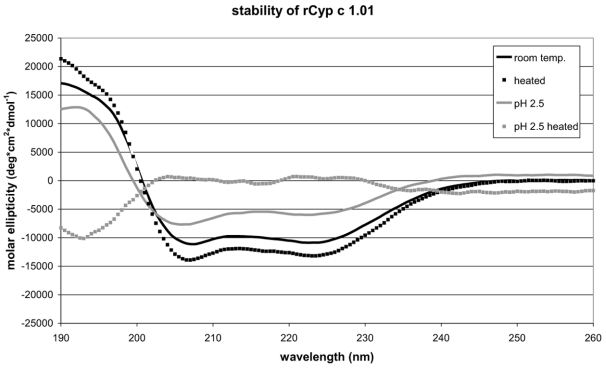


**Figure 7.** Stability of rCyp c 1.01. An increased negative dichroism after heating was monitored. Conformational change of the heated protein to an irregular structure at pH 2.5. Black line: room temperature; black dotted line: heated at 95°C; gray line: pH 2.5; gray dotted line: heated to 95°C at pH 2.5.

ment and validation of novel diagnostic tools [15, 31]. In our study, we established new purification protocols for natural and recombinant cod parvalbumin and recombinant carp parvalbumin. Cod muscle contains several IgE-reactive parvalbumin isoforms including at least two β-isoforms, which were purified and characterized by mass spectroscopy and NMR. In addition, we cloned two β-parvalbumins (rGad m 1.01 and rGad m 1.02) and produced rGad m 1.02 from cod.

IgE ELISA, IgE immunoblotting and inhibition experiments revealed a high crossreactivity between cod and carp parvalbumins (Fig. 3). Almost all sera of fish allergic patients (25/26) in this study, which had IgE reactivity to native and recombinant cod parvalbumin, also had IgE reactivity to recombinant carp parvalbumin (Fig. 3A). This result supports the assumption that cod and carp parvalbumins share at least some B-cell epitopes. The high amino acid sequence identities between the two parvalbumins of cod (71%) and between parvalbumins of cod and carp (80–81%) support this finding. The results of immunoblotting performed with the mouse monoclonal antifrog parvalbumin antibody Parv-19 and a rabbit polyclonal anti-Gad m 1 antiserum further illustrate the crossreactivity of parvalbumins (Fig. 2). The IgE binding capacity of rGad m 1.02 was comparable to nGad m 1. Interestingly, some serum samples (sera 2, 13, 17, 20, 24, 25, and 26) showed stronger IgE binding to rGad m 1.02 than to nGad m 1 (Fig. 3A). Hence, standardized batches of recombinant cod parvalbumin can replace nGad m 1 for diagnostic assays. The second band detected in immunoblot of nGad m 1 with the polyclonal anti-Gad m 1 antiserum (Fig. 2, nGad m 1, lane 4) represents a second isoform of cod parvalbumin according to the results from MS. An approximately 22 kDa band present in both lanes 4 and 5 (Fig. 2) of the rCyp c 1.01 blot could be a protein dimer according to the results of immunoblots with fish allergic patients' sera (data not shown).

CD analysis revealed that purified rGad m 1.02 and rCyp c 1.01 were present in solution as a folded protein with a predominantly α-helical secondary structure similar to that of the native cod parvalbumin, as was previously described for the recombinant carp parvalbumin rCyp c 1.01 [26]. However, we could observe a similarity between the CD spectra of rGad m 1.02 and the $Ca^{2+}$-depleted form of natural cod parvalbumin (Figs. 5 and 6C). In contrast to nGad m 1, which did not show conformational differences after adding $Ca^{2+}$ (data not shown), we could detect a conformational change of rGad m 1.02 after dialysis against a $Ca^{2+}$-containing buffer (data not shown). These data indicate that the native parvalbumin contained bound calcium ions even after undergoing the purification procedure. This may not be the case with the protein produced in *E. coli*. The $Ca^{2+}$ depletion experiments for carp parvalbumin described in the literature [7] were performed with the recombinant protein rCyp c 1.01. In contrast, our $Ca^{2+}$ depletion experiments for cod parvalbumin were performed with the natural protein. For carp parvalbumin no comparison was made between the natural and the recombinant proteins regarding their stability depending on bound $Ca^{2+}$. The conformation of natural cod parvalbumin appears to be more resistant to calcium depletion than the conformation of recombinant carp parvalbumin. Recombinant Cyp c 1.01 seems to be stable at pH 7.0 even after heating to 95°C as previously described [7]. Additionally, we could observe an effect of pH 2.5 on conformation and the stability after heating the sample (Fig. 7). As the glutamate residues of the $Ca^{2+}$-binding site [32] are uncharged at pH 2.5, the binding of the chelate ion is hindered. The similarity of the spectra of EGTA treated rCyp c 1.01 and at pH 2.5, as well as the instability of rGad m 1.02 under basic conditions (data not shown) are most likely due to the reduced amount of bound $Ca^{2+}$. In contrast, natural cod parvalbumin was stable at each pH value tested and we could observe only a loss of the broad minimum at pH 2.5, and at pH 2.5 after heating (Figs. 6A and B). These observations illustrate that the binding of the chelate ion plays an important role for the conformation and for the stability of the recombinant proteins.

The mass data obtained for rCyp c 1.01 showed a second less prominent peak at 11 449 ± 1 Da (Fig. 4B left). This pointed at a partial addition of β-mercaptoethanol to the free sulfhydryl groups of the single cysteine residue of the protein in position 18. This addition in a minor component of the recombinant protein might be a likely leftover from the purification procedure involving trace amounts of the reducing agent. The mass data for nGad m 1, in addition to the least intense peak showed two additional peaks with masses of 11 462.5 and 11 364.9, respectively (Fig. 4B right). Noticeably, both masses showed a difference of 42 Da compared to the theoretical masses of isoform Q90YK9 (11 420.9) and isoform Q90YL0 (11 323.8). We therefore speculate that the majority of nGad m 1 molecules was post-translationally modified by acetylation. This

assumption was confirmed by MS/MS analysis of the peptides obtained by tryptic digest of nGad m 1 (Fig. 4C). When 92% of the Q90YK9 sequence could be confirmed, the acetylated N-terminal alanine was included.

Our data indicate that natural and recombinant parvalbumins can be used equally well for *in vitro* diagnostic assays to detect parvalbumin-specific IgE. Nevertheless, the various batches of recombinant proteins need to be characterized by several physico-chemical methods to assure their consistent quality. However, when the natural and recombinant allergens are compared in more detail, differences do emerge.

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# Chapter III

# Expression levels of parvalbumins determine allergenicity of fish species

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**Allergy (reviewed and minor revisions required)**

## Abstract:

**Background:** Parvalbumins are the most important fish allergens. Polysensitization to various fish species is frequently reported and linked to the cross-reactivity of their parvalbumins. Studies on cross-reactivity and its association to the allergenicity of purified natural parvalbumins from different fish species are still lacking. In addition, some studies indicate that dark muscled fish such as tuna are less allergenic.

**Methods:** Total protein extracts and purified parvalbumins from cod, whiff, and swordfish, all eaten frequently in Spain, were tested for their IgE-binding capacity with 16 fish allergic patients' sera from Madrid. The extent of cross-reactivity of these parvalbumins was investigated by IgE ELISA inhibition assays. Additionally, the cDNA sequences of whiff and swordfish parvalbumins were determined.

**Results:** Extractable amounts of parvalbumins from cod were 20 times and from whiff 30 times higher than from swordfish. Parvalbumins were recognized by 94% of the patients in extracts of cod and whiff, but only by 60% in swordfish extracts. Nevertheless, a high cross-reactivity was determined for all purified parvalbumins by IgE inhibition. The amino acid sequence identities of the three parvalbumins were in a range of 62 to 74%.

**Conclusions:** The parvalbumins of cod, whiff and swordfish are highly cross-reactive. The high amino acid sequence identity among cod, whiff and swordfish parvalbumins results in the observed IgE cross-reactivity. The low allergenicity of swordfish is due to the lower expression levels of its parvalbumin.

Keywords: beta-parvalbumin, cross-reactivity, fish allergy, food allergen, whiff

## Introduction

Especially in coastal countries, fish constitute an important part of the diet. In Norway allergy to fish is found in 0.1% of the population [1,2]. In a cross-sectional study carried out in Spain, fish allergy was diagnosed in 36 of 4991 patients (0,72%) referred to for allergy evaluation [3]. Patients are often allergic to certain fish species while they tolerate others [4]. In Spain, whiff, cod and swordfish are commonly consumed [5,6]. Allergic reactions to fish can be mild to severe [5,7].

The major allergens of fish are parvalbumins besides minor allergens such as collagen and aldehyde phosphate dehydrogenase [8,9]. Parvalbumin is an acidic, calcium-binding 12 kDa protein resistant to heat and digestive enzymes [10-14]. Fish muscles express multiple parvalbumin isoforms, which can be divided into two distinct evolutionary lineages. Alpha-parvalbumins are generally not allergenic [15] with the exception of two alpha-parvalbumins reported in frog [16-19]. In contrast, various IgE-reactive beta-parvalbumins of bony fish have been described [10,20,21]. Based on the high amino acid sequence identity of beta-parvalbumins from different fish, cross-reactivity among different fish species occurs frequently [4,5,20,22-24].

Bony fish have fast twitching white muscle for rapid movements and dark muscle for continuous swimming. Active fish such as tuna, skipjack [25], and swordfish have a higher proportion of dark muscles than bottom dwelling fish, such as cod, flounder [25], or whiff. Dark muscle contains lower levels of parvalbumins, thus these fish species are expected to be of lower allergenicity [25,26].

In this study we investigated the IgE cross-reactivity of purified parvalbumins from cod and whiff as examples for allergenic fish, and from swordfish as an example for dark muscled fish.

## Material and Methods

### *Protein extracts*

Atlantic cod, swordfish, and whiff filets were purchased from local markets in Vienna, Austria, and Madrid, Spain. 500 g cod or 250 g whiff filet were homogenized and extracted in three volumes (w/v) of 20 mM Bis-Tris, pH 6.5. After centrifugation at 17,000 x g for 45 min at 4°C, the supernatants were filtered through Miracloth (Merck Biosciences, Nottingham, UK) and filter papers (Macherey-Nagel, Düren, Germany). An 80 g swordfish filet was extracted in double distilled water. The supernatant, obtained as described above, was dialysed against 20 mM Bis-Tris, pH 7.0. Protein extracts were stored at 4°C. Protein concentrations were determined by the BCA Protein Assay Reagent Kit (Pierce, Rockford, Ireland).

### *Protein purification*

All fish extracts were treated with 0.1% Biocryl BPA-1000 (Supelco, Bellefonte, PA) and centrifuged at 17,000 x g for 30 min at 4°C. The supernatants were applied to a DEAE Sepharose Fast Flow column (GE Healthcare, Little Chalfont, UK). Selected fractions were loaded onto a HiPrep 16/60 Sephacryl S-200 column (GE Healthcare). Cod or whiff parvalbumins were eluted as a single peak. For swordfish, a pool of fractions containing parvalbumin was dialyzed against 50 mM Na-acetate, pH 5.0 and purified by a SP-Sepharose Fast Flow column (GE Healthcare). The flow through was collected, dialyzed against 20 mM Tris-HCl, pH 8.0 and purified by anion exchange chromatography using a MonoQ 5/50 GL column (GE Healthcare).

### *Mass and sequence determination*

The mass of whiff beta-parvalbumin was determined as described [21]. For nano-liquid chromatography-tandem mass spectrometry (nanoLC-MSMS)-based peptide mapping

50 µg aliquots of each parvalbumin was digested overnight at 37°C with 2 µg trypsin or V8 protease (Roche Applied Science, Vienna, Austria). The N-terminal sequence of whiff parvalbumin was determined as described [21].

### *RNA isolation and cDNA synthesis*

Total RNA of 60 mg whiff or 400 mg swordfish muscle tissue was extracted according to the RNeasy kit procedure (QIAGEN, Hilden, Germany). First strand cDNA was synthesized from 2 µg total RNA with an oligo-$dT_{25}$ primer (*5′*-GGAGAAGGAT$_{25}$VN-*3′*), using MuLV reverse transcriptase (Fermentas, St. Leon-Rot, Germany).

### *PCR amplification and sequencing*

A fragment of whiff cDNA encoding beta-parvalbumin was amplified by PCR using oligo-$dT_{25}$ and the degenerate primer Wh1-fwd (*5'*-ATGACITTYGCIGGIYTIGAYGC-*3'*) designed on the basis of an internal amino acid sequence obtained by MS analysis. For swordfish, PCR amplification was carried out with the primer pair oligo-$dT_{25}$ and Sw1-fwd (*5'*-CTGAAGCTGTTCCTGCAGAAC-*3'*), which corresponded to the Chub mackerel parvalbumin sequence (EMBL: AB091470). In order to obtain the 5'ends, 5'-RNA ligase-mediated rapid amplification of cDNA ends was performed using the GeneRacer Kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. For swordfish, a modified 5'-primer was used (*5′*-GAGCACGAGGACACTGAC-*3′*). 3'-primers for whiff parvalbumin were Wh2-rev (*5'*-CCTAACAAGGTCGGTGAACTC-*3'*) and Wh3-nested: (*5'*-GCCATCAACGTCACCGGCCTTCAG-*3'*) and for swordfish Sw2-rev (*5'*-CGCAGCCGCCTTGAAGTTCT -*3'*) and Sw3-nested: (*5'*-GTTCTGCAGGAACAGCTTCAG-*3'*). PCR products were gel purified, cloned into the pCR2.1-TOPO vector (Invitrogen) and sequenced (IBL, Vienna, Austria). Sequences were aligned using AlignIR 2.0 (LI-COR Biosciences, Lincoln, NE, USA).

### *Sera and antibodies*

Sera from 16 patients with clinical histories of type 1 fish allergy were selected in the Allergy Department of the Hospital Clinico San Carlos (Table 1). Diagnosis of fish allergy was verified by determination of fish-specific IgE using ImmunoCAP (Phadia, Uppsala, Sweden) and skin prick testing to different fish species and *Anisakis simplex*, a fish parasite [27]. All patients were negative to *Anisakis*. Parvalbumins were detected by the mouse monoclonal anti-parvalbumin clone Parv-19 antibody (Sigma, St Louis, Missouri, USA) and rabbit polyclonal anti-Gad m 1 antibody (Tepnel BioSystems Ltd., Deeside, UK).

### *SDS-PAGE and immunoblotting*

Total protein extracts and purified allergens were separated by SDS-PAGE under reducing conditions and either visualized by Coomassie Brilliant Blue or transferred to nitrocellulose membranes for immunodetection of purified parvalbumins by the mouse monoclonal and rabbit polyclonal antibodies [21]. In addition, blotted proteins were incubated with individual fish allergic patients' sera and bound IgE was detected by $^{125}$I-labelled rabbit anti-human IgE (MALT Allergy System Isotope Reagent, IBL Hamburg, Germany).

### *IgE ELISA and inhibition assays*

Purified Gad m 1, whiff and swordfish parvalbumins (2 μg/ml) were coated to CovaLink NH plates (Nunc, Roskilde, Denmark). Non-specific binding sites were blocked with Tris buffered saline, 0.5% Tween-20 (TBST), 3% (w/v) milk. Plates were then incubated with sera diluted in TBST 0.5% (w/v) BSA. Bound IgE was detected with AP-conjugated mouse anti-human IgE antibody (BD-Biosciences Pharmingen, San Diego, CA, USA) and developed with p-nitrophenyl phosphate (Sigma-Aldrich, St.

Louis, MO, USA). Inhibition assays were performed by pre-incubating a serum pool with increasing concentrations of parvalbumins (1, 10, 20, 50, 100 µg/ml) or extracts (1 or 100 µg) and determining residual IgE-binding to fish extract. In order to control for IgE binding to high molecular weight (HMW) allergens of cod and whiff, chromatographic fractions containing these proteins but no parvalbumins were used as inhibitors (1 or 100 µg/ml). Cross-reactivities between parvalbumins were determined with 4 patients' sera using purified parvalbumins (50 µg/ml) as inhibitors. Inhibition values are given as percent reduction of bound IgE compared with the controls where no inhibitor protein had been added.

### *Statistic analysis*

Mean and standard deviations (sd) were calculated for the ImmunoCAP and IgE ELISA results. Comparisons of ImmunoCAP results for the 3 fish species and of the IgE ELISA for the 3 parvalbumins were carried out by non-parametric tests for paired samples (Friedman and Wilcoxon tests). Paired correlations of the IgE responses to cod, whiff and swordfish whole extracts and parvalbumins were performed with the Spearman test. P values $< 0.05$ were considered significant. Statistical analysis was carried out with SPSS (SPSS, Chicago, IL, USA).

## Results

### *cDNA cloning and sequencing of whiff and swordfish parvalbumins*

The full-length cDNA sequence of whiff parvalbumin was obtained (EMBL: AM904681, designated Lep w 1.0101 by the IUIS allergen nomenclature sub-committee). It comprised 671 base pairs (bp) with an open reading frame (ORF) of 327 bp encoding a protein of 109 amino acids including the initiating methionine. The full-length cDNA of swordfish parvalbumin consisted of 692 bp with an ORF of 327 bp encoding a protein of 109 amino acids including the initiating methionine (FM202668, designated Xip g 1.0101). Both sequences contained conserved residues characteristic of beta-parvalbumins (Fig. 1, highlighted) [21,28,29]. Theoretical isoelectric points were calculated as 4.5 for Lep w 1 and 4.43 for Xip g 1. The translated sequences were aligned with the published cod parvalbumin isoforms Gad m 1.01 (AM497927) and Gad m 1.02 (AM497928) [21] (Fig. 1A). All 4 parvalbumins shared sequence identities in the range of 62 to 74% (Fig. 1B).

### *Patients' characteristics*

Clinical data of the patients are summarized in Table 1. All had a history of type 1 allergy to more than one fish species. The patients, 10 children (age 1-3 years) and 6 adults (age 21-36), showed moderate to severe symptoms. All displayed positive skin prick test reactions to cod, whiff or swordfish. Fifteen patients (94%) had positive ($\geq$ 0.35 kU/L) ImmunoCAP results to cod and whiff, 12 (75%) to swordfish. ImmunoCAP values to swordfish were significantly lower than those to cod ($p = 0.002$) and whiff ($p = 0.003$), whereas no significant differences were observed between whiff and cod ImmunoCAP values ($p = 0.12$). A strong and significant correlation ($p < 0.001$) was observed between ImmunoCAP values to cod and whiff ($r = 0.88$), whereas the

correlations between ImmunoCAP values to cod and swordfish ($r = 0.37$) and ImmunoCAP values to whiff and swordfish ($r = 0.29$) were not significant ($p > 0.05$).

### *IgE recognition patterns to fish extracts*

All 16 patients had IgE to at least one allergen in cod or whiff protein extract (Fig. 2). Fifteen patients (94%) recognized a 12 kDa protein (Fig. 2A, B), identified as parvalbumin by a mouse monoclonal anti-parvalbumin and a rabbit polyclonal anti-Gad m 1 antibody (data not shown). In cod, two additional IgE-reactive bands were observed at 30 kDa and around 40 kDa (Fig. 2A). In whiff extract, IgE-reactive proteins were detected at 17 kDa and in the high molecular weight range (33-72 kDa) (Fig. 2B). Twelve of 16 patients showed IgE reactivity to parvalbumin in swordfish extract. One patient also had IgE to a 40 kDa protein in all fish extracts (Fig. 2C).

### *Purification and biochemical characterization of natural parvalbumins*

Protein extracts of cod and whiff displayed similar protein patterns in SDS-PAGE with a prominent band at 12 kDa. The swordfish extract showed a remarkable amount of high molecular weight proteins and two less prominent bands at 12 kDa (Fig. 3A; lanes 1). The 12 kDa proteins were identified as parvalbumins in all extracts by immunoblotting with monoclonal and polyclonal anti-parvalbumin antibodies (data not shown).

Twenty milligrams parvalbumin from cod, 30 mg from whiff and 1 mg from swordfish were purified from 100 g fish muscle (Fig. 3B). The mouse monoclonal antibody detected one band for Gad m 1, Lep w 1 and Xip g 1 (Fig. 3A; lanes 3). The polyclonal anti-parvalbumin antibody detected two isoforms of Gad m 1 and Xip g 1 (Fig. 3A; lanes 5). A purity of >98% was determined for Gad m 1 and Lep w 1 by size exclusion

chromatography (data not shown). Coomassie staining showed that Xip g 1 was >99% pure (Fig. 3A; right panel, lane 2).

The identity of Lep w 1 was confirmed by N-terminal sequencing which revealed the first five amino acid residues as TFAGL. Mass spectrometric analysis of Lep w 1 revealed a single peak at 11624 Da, which is in agreement with the predicted theoretical mass of 11581.8 Da (data not shown). The difference of 42 Da is due to the acetylation of the N-terminus as previously reported for Gad m 1 [21]. Mass spectrometric analysis confirmed the identity of the purified cod parvalbumin as Gad m 1 (data not shown) as described [21]. In swordfish, two parvalbumin isoforms were identified by nanoLC-MSMS-based peptide mapping (data not shown). The lower molecular mass isoform was consistent with the cDNA sequence.

### *IgE reactivity of purified parvalbumins*

IgE reactivity of the purified parvalbumins Gad m 1, Lep w 1 and Xip g 1 was confirmed by IgE ELISA using 14 patients' sera (Table 1). Gad m 1 and Lep w 1 were recognized by all patients in ELISA. The IgE titer to Lep w 1 was significantly higher ($p = 0.001$). Ten patients' sera recognized the swordfish parvalbumin. The IgE level to Xip g 1 was significantly lower ($p = 0.001$) than those to Gad m 1 and Lep w 1. Strong and significant paired correlations ($p < 0.001$) were observed between the ELISA values to Gad m 1 and Lep w 1 ($r = 0.99$), Gad m 1 and Xip g 1 ($r = 0.83$) and between Xip g 1 and Lep w 1 ($r = 0.85$).

Purified Gad m 1, Lep w 1 and Xip g 1 dose-dependently inhibited the IgE-binding of a serum pool (P1, 4, 8, 14) to fish protein extracts of cod, whiff and swordfish,

respectively (Fig. 4). Pre-incubation of the serum pool with 100 µg/ml Gad m 1 or Lep w 1 revealed an IgE inhibition of 89% and 92% to cod and whiff protein, respectively (Fig. 4, grey bars). IgE binding was inhibited by HMW proteins of cod (38% at 100 µg/ml) and whiff (45% at 100 µg/ml) extracts (Fig. 4, transparent bars). IgE-binding to immobilized swordfish extract was inhibited by 84% after pre-incubation with 100 µg/ml Xip g 1 (Fig. 4, grey bars). Pre-incubation of the serum pool with fish extracts as positive control showed 100% inhibition (Fig. 4, squared bars).

Gad m 1 was able to inhibit IgE-binding to immobilized Lep w 1 between 63 to 97%, and sera pre-incubated with Lep w 1 were reduced in their IgE-binding to Gad m 1 by 42 to 75% (Table 2). A reduction of IgE-binding to Lep w 1 and Gad m 1 was achieved bewtween 52 to 85% using Xip g 1 as inhibitor. The IgE reactivity to Xip g 1 was reduced between 78 to 100% by inhibition with Gad m 1 or Lep w 1. The lowest inhibition of IgE- binding of approximately 20% to whiff parvalbumin was determined for serum P3 preincubated with Gad m 1 or Xip g 1 (Table 2). Pre-incubation of serum P3 with Gad m 1 and Lep w 1 did not reduce the IgE-binding to Xip g 1.

## Discussion

Fish allergic patients are often allergic to more than one fish species due to the cross-reactivity of their beta-parvalbumins [4,30]. However, differences in IgE reactivity to dark and white muscled fish have been reported [25]. Active fish with a higher amount of dark muscles [25] were regarded as low allergenic [4].

In this study, we compared the IgE-binding of fish allergic patients' sera to cod, whiff and swordfish extracts and purified parvalbumins. We selected swordfish as an active dark muscled fish and analyzed its IgE cross-reactivity with cod and whiff, both frequent causes of fish allergy in Spain [5]. We describe for the first time the parvalbumins from whiff (Lep w 1) and swordfish (Xip g 1) as major allergens. Additionally, we cloned cDNAs of whiff and swordfish beta-parvalbumins.

We purified cod, whiff and swordfish parvalbumins under native conditions to preserve calcium-binding, which is important for the conformation of the proteins [20,21,31]. For whiff and swordfish we established new purification protocols. The low yield of swordfish parvalbumin (1 mg/100 g filet) compared to cod (20 mg/100 g) and whiff (30 mg/100 g) parvalbumins reflected the weak signals in IgE immunoblotting of the extract. We found only one parvalbumin isoform (Lep w 1) in whiff, whereas two isoforms could be identified in cod and swordfish using a polyclonal anti-parvalbumin antibody. A nearly complete inhibition of IgE-binding to cod and whiff extracts by purified Gad m 1 and Xip g 1 suggested that all IgE- reactive parvalbumin isoforms were purified. All patients' sera also recognized HMW proteins (Fig. 4, transparent bars), thus no complete inhibition could be obtained. In swordfish additional

parvalbumin isoforms may be present. Only 84% inhibition of patients' IgE to this extract was achieved after incubation with purified Xip g 1.

IgE cross-reactivity of all studied parvalbumins was shown by IgE ELISA inhibition assays. The parvalbumins may share several identical IgE-binding epitopes, consistent with their high protein sequence identities (62-74%). We detected a lower reduction of IgE-binding to Gad m 1 after pre-incubation of sera with Lep w 1 (in a range of 42 to 75%) than to Lep w 1 after sera had been pre-incubated with Gad m 1 (63-97%). These findings could indicate the presence of IgE-reactive to species-specific IgE epitopes in cod and whiff parvalbumins. Patients might not have been sensitized by swordfish parvalbumin as IgE-binding to Xip g 1 was inhibited in a range of 80 to 100% with Gad m 1 and Lep w 1, respectively. Interestingly, patient P3's serum seemed to contain mainly species-specific IgE to the parvalbumin of the frequently eaten whiff. IgE-binding to Lep w 1 could only be reduced by around 20% using Gad m 1 and Xip g 1 as inhibitor (Table 2).

In previous studies it was hypothesized that the degree of IgE-binding activity might be related to the amount of consumption [5,32]. As whiff is one of the most frequently consumed fish in Spain, it is not unlikely that this patient was initially sensitized by Lep w 1. In our present work, all fish belonged to different taxonomic orders, but the protein sequence identities of parvalbumin isoforms were similar within the species and among fish species (around 70%) as already reported for Alaska pollock, Atlantic cod and salmon [7].

In conclusion, this study demonstrates the cross-reactivity among the parvalbumins of high allergenic cod and whiff, and the "low allergenic" swordfish. High sequence

identities support this finding. Parvalbumin was the major allergen in all studied fish including swordfish. According to our results the weak reaction of only 12 sera to swordfish parvalbumin, when tested by IgE immunoblotting, was due to the low parvalbumin content of swordfish.-We therefore suggest that cod or whiff parvalbumins are responsible for sensitization of fish allergic patients. Additionally, we have demonstrated that swordfish is hypoallergenic. Clinical studies involving oral challenges are currently ongoing to confirm its lower allergenicity in fish allergic patients.

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## Figures

*Figure 1:* (A) Protein sequence alignment of Gad m 1.01 (EMBL Acc. No. AM497927), Gad m 1.02 (AM497928), Lep w 1 (AM9046811), and Xip g 1 (FM202668) generated by ClustalX, Version 1.83; highlighted: characteristic amino acid residues of beta-parvalbumins. Boxed: EF-hand 1 and 2, underlined: calcium-binding sites, indicated and highlighted: conserved amino acid residues characteristic of beta-parvalbumins, "*" indicate conserved, ":" highly conserved, "." weakly conserved amino acid residues and "-" a gap. (B) Protein sequence identities.

*Figure 2*: IgE-binding of fish allergic patients' sera to cod (A), whiff (B) and swordfish extract (C) was determined by IgE immunblotting. M, molecular weight marker (kDa); 1-16, sera of fish allergic patients; NHS, control serum; B, buffer control.

*Figure 3:* Purification and identification of parvalbumins. (A) Fish protein extracts (lane 1) and purified parvalbumins (lane 2) were detected by Coomassie staining and immunoblotting with monoclonal (lane 3) and polyclonal (lane 5) antibodies, respectively. Lanes 4 and 6 show negative controls. (B) Extraction of 100 g fish yielded different amounts of purified parvalbumin.

*Figure 4:* IgE ELISA inhibiton assay with immobilized fish extracts. Grey: Purified parvalbumins Gad m 1, Lep w 1 and Xip g 1, respectively as inhibitors; transparent: binding of IgE to HMW proteins as inhibitor; squared: fish extract were used as inhibitor.

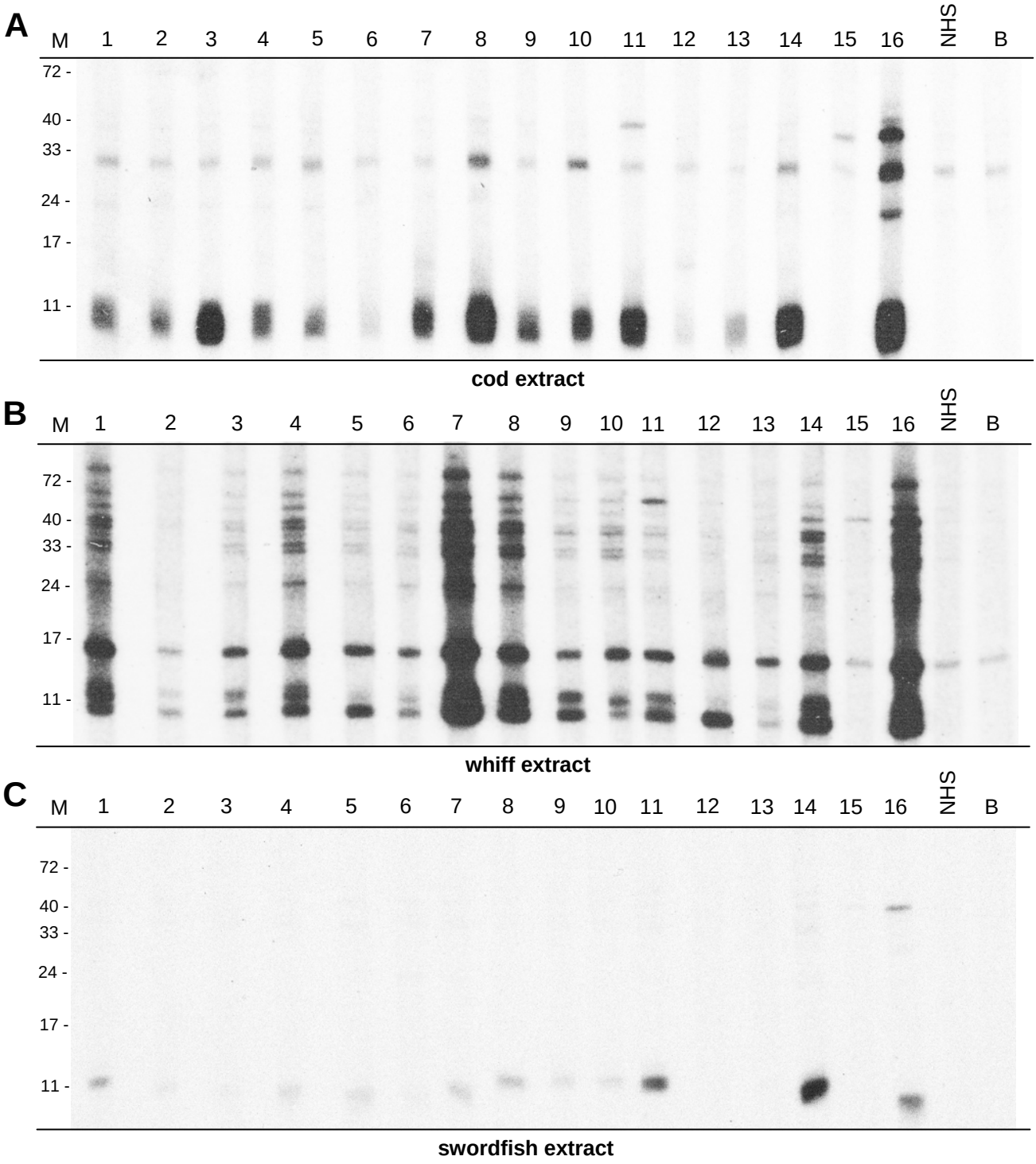
| | | | | | Cod | | Gad m 1 | | Whiff | | Lep w 1 | | Swordfish | | Xip g 1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Patient | Age (years) | Sex | Fish-related symptoms | Total IgE (kU/L) | CAP (kU/L) | ELISA (OD) | CAP (kU/L) | ELISA (OD) | CAP (kU/L) | ELISA (OD) | | | | | |
| P1 | 11 | m | AE, A | 264 | 74.5 | 3.1 | 48.5 | 3.88 | 2.33 | 0.26 | | | | | |
| P2 | 5 | m | U | 33.5 | 8.91 | 0.77 | 14.2 | 1.08 | 1.16 | 0 | | | | | |
| P3 | 12 | m | AE | 422 | 9.6 | 1.02 | 6.06 | 1.43 | 1.02 | 0 | | | | | |
| P4 | 26 | f | AN | 1272 | 12.3 | 1.79 | 10.2 | 2.43 | 4.6 | 0.049 | | | | | |
| P5 | 36 | m | OAS, U | 1633.5 | 7.37 | 1.98 | 10 | 2.64 | 0.38 | 0.2 | | | | | |
| P6 | 35 | f | U, AE | >2000 | 2.69 | 0.59 | 1.43 | 0.88 | 0.39 | 0.011 | | | | | |
| P7 | 9 | m | U, V | 56.3 | 17.2 | 2.49 | 11.3 | 3.19 | 4.32 | 0.75 | | | | | |
| P8 | 2 | f | OAS | 143 | 15.3 | 0.8 | 13.5 | 1.11 | 0.87 | 0.041 | | | | | |
| P9 | 30 | f | AE, A, D | 389 | 3.07 | 0.77 | 2.07 | 1.16 | 0 | 0.172 | | | | | |
| P10 | 34 | f | AN | 311 | 30.3 | 1.23 | 5.9 | 1.67 | 5.74 | 0.198 | | | | | |
| P11 | 21 | f | U, AE | 1724 | 45.1 | 0.98 | 19.9 | 1.32 | 9.7 | 0.039 | | | | | |
| P12 | 5 | m | OAS | 61.5 | 1.82 | 0.06 | 1.17 | 0.06 | 0 | 0 | | | | | |
| P13 | 1 | f | AN | 72.4 | 1.18 | 0.19 | 2.14 | 0.25 | 0 | 0 | | | | | |
| P14 | 3 | m | OAS | 450 | 47.5 | 3.58 | 83.8 | 3.62 | 10.9 | 0.335 | | | | | |
| P15 | 13 | m | U | 961 | 47.5 | nd | 23.2 | nd | 0 | nd | | | | | |
| P16 | 4 | m | U | 276 | 0 | nd | 0 | nd | 10.9 | nd | | | | | |
| mean | | | | | 20.27 | 1.38 | 15.83 | 1.76 | 3.29 | 0.15 | | | | | |
| sd | | | | | 22.16 | 1.06 | 21.75 | 1.20 | 4.01 | 0.20 | | | | | |

**Table 1:** Clinical and serologic characteristics of patients with type 1 fish allergy.

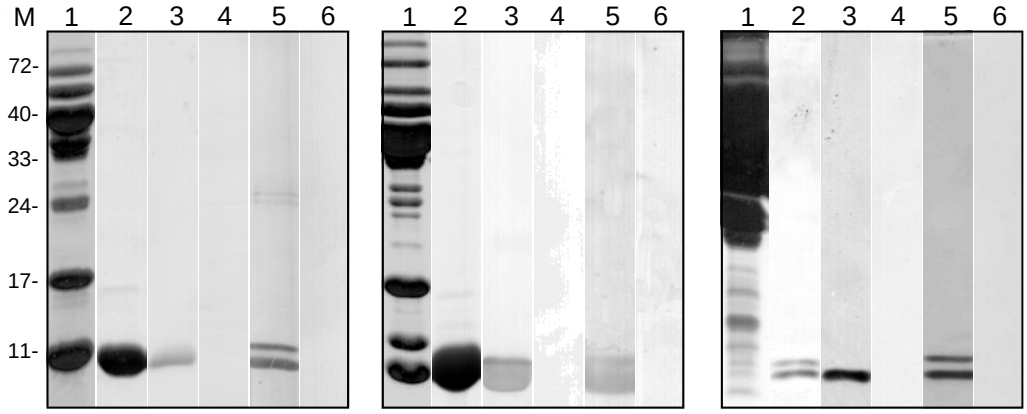
Mean, mean value; sd, standard deviation; m, male; f, female; A, asthma; AD, atopic dermatitis; AE, angioedema; AN, anaphylaxis; U, urticaria; D, dysphagia; V, vomiting; OAS, oral allergy syndrome; nd, not done.

| Coated | Gad m 1 | | Xip g 1 | | Lep w 1 | |
|---|---|---|---|---|---|---|
| Inhibitor | Xip g 1 | Lep w 1 | Gad m 1 | Lep w 1 | Gad m 1 | Xip g 1 |
| Patients | | | | | | |
| P1 | 85 | 75 | 91 | 78 | 68 | 64 |
| P2 | 57 | 42 | 99 | 86 | 63 | 52 |
| P3 | 81 | 60 | 0 | 0 | 22 | 19 |
| P4 | 75 | 59 | 100 | 88 | 97 | 80 |

**Table 2:** IgE ELISA cross-inhibition of three fish parvalbumins.


A
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 NHS B
72 -
40 -
33 -
24 -
17 -
11 -
cod extract
B
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 NHS B
72 -
40 -
33 -
24 -
17 -
11 -
whiff extract
C
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 NHS B
72 -
40 -
33 -
24 -
17 -
11 -
swordfish extract

**A**

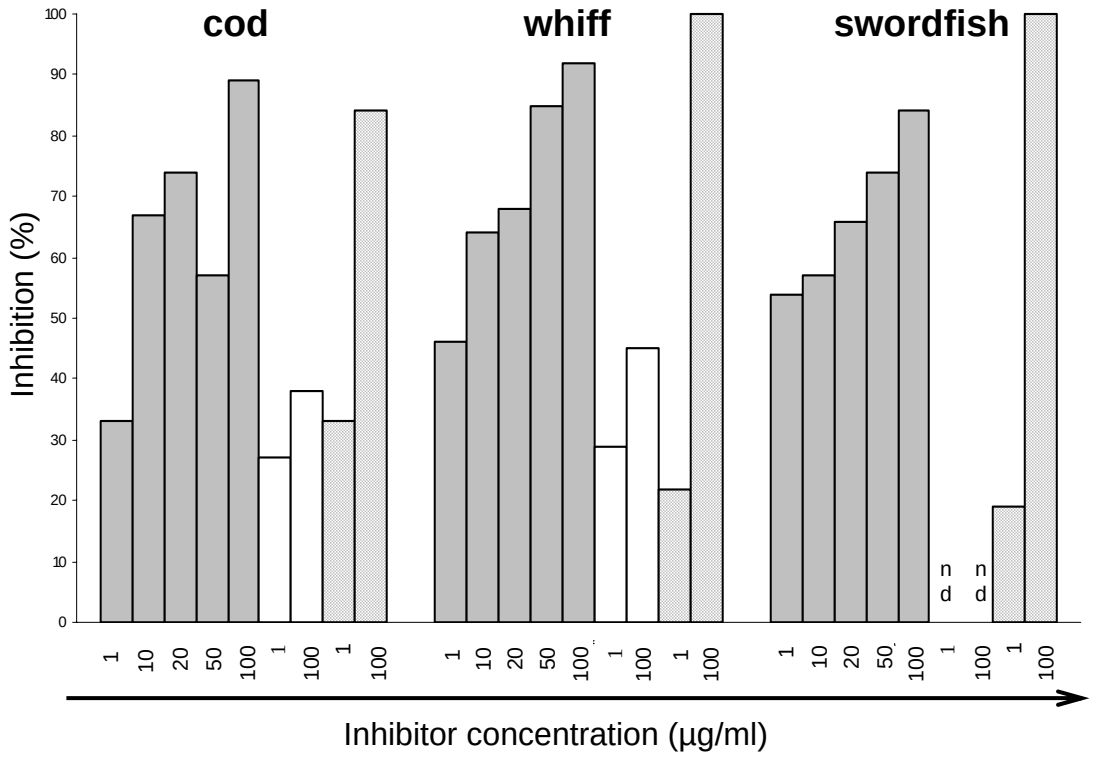



*G. morhua (cod)* *L. whiffiagonis (whiff)* *X. gladius (swordfish)*

**B**

| species | fish filet | parvalbumin |
|---|---|---|
| *G. morhua* | 100 g | 20 mg |
| *L. whiffiagonis* | 100 g | 30 mg |
| *X. gladius* | 100 g | 1 mg |

Figure 3


cod
whiff
swordfish
Inhibition (%)
100
90
80
70
60
50
40
30
20
10
0
n d
n d
1
10
20
50
100
1
100
1
100
Inhibitor concentration (µg/ml)

**A**


**B**

| | Amino acid identity (%) | | | |
|---|---|---|---|---|
| **Allergens** | Gad m 1.01 | Gad m 1.02 | Lep w 1.0101 | Xip g 1.0101 |
| Gad m 1.01 | 100 | 71 | 65 | 74 |
| Gad m 1.02 | 71 | 100 | 62 | 71 |
| Lep w 1.0101 | 65 | 62 | 100 | 69 |
| Xip g 1.0101 | 74 | 71 | 69 | 100 |

# Chapter IV

## Physicochemical properties and thermal stability of Lep w 1, the major allergen of whiff

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**Mol Nutr Food Res (reviewed and major revisions required)**

## Abstract

Whiff (*Lepidorhombus whiffiagonis*) is a fish frequently consumed in Spain. Lep w 1, its major allergen, is a calcium-binding beta-parvalbumin. The resistance of Lep w 1 to heat denaturation and to digestion were studied by circular dichroism (CD) spectroscopy and by *in vitro* gastric digestion systems. Purified Lep w 1 was thermally stable up to 65°C at neutral pH. Calcium-depletion resulted in a change of its structure as determined by CD spectroscopy. A partial loss of structure was also observed at acidic pH, however the allergen retained its full IgE binding ability. The partially denatured Lep w 1 was easily digested by pepsin within 2 minutes. Further, the IgE reactivity of proteins extracted from cooked fish and their stability to proteolysis were analysed. The extract revealed a higher number of IgE reactive bands than an extract from uncooked fish. IgE binding to these proteins could not be inhibited by an extract from uncooked fish. In contrast to a raw fish extract the cooked extract showed higher resistance to pepsinolysis. The stability of Lep w 1 to thermal denaturation and digestion, explain the high allergenicity of whiff.

## 1. Introduction

Fish is an important source of dietary protein but also a common elicitor of food allergies in coastal countries. One of the most allergenic fish species in Spain is the frequently consumed whiff (*Lepidorhombus whiffiagonis)* [1]. Allergic reactions to fish often manifest already in small children with a tendency to persist [2]. In Spain, 18% of food allergic children suffer from fish allergy [3]. Fish consumption may lead to severe clinical symptoms and even to lethal anaphylaxis [1]. The major fish allergen is parvalbumin, a 12 kDa small and soluble, acidic protein. It belongs to the second largest animal food allergen family, the EF-hand family [4]. Parvalbumins comprise 3 EF-hand motifs, two of which are able to bind calcium with high affinity [5]. The N-terminal region may regulate the binding affinity of the active calcium-binding motifs. Parvalbumins are subdivided into two different phylogenetic lineages, alpha and beta. Many allergenic cross-reactive beta-parvalbumins are found in various fish species [6], including whiff.

Beta-parvalbumins with bound calcium are remarkably stable [6-8]. Many studies report a significant resistant to heat, chemical denaturation and proteolytic enzymes [7, 9, 10]. Calcium binding is essential for the conformational stability but also for the allergen's immunoreactivity [6, 11].

It has been described that food allergens are usually abundant in the food and structurally stable [12, 13]. Additionally, food allergens must preserve their structure from degradation by digestive enzymes to be taken up by the gut and to be presented to the immune system [14]. Calcium-bound parvalbumins are known as remarkably stable food allergens [6, 11]. However, one study questions the proteolytic stability of fish

proteins. Untersmayer et al. reported the pepsinolysis of raw codfish proteins to small fragments after incubation with simulated gastric fluid, pH 2 for one minute [15]. Physiochemical changes can impair proteolysis during gastrointestinal digestion and alter the way of presentation of the protein to the immune system [16]. Modifications, like heat denaturation, during food processing are sometimes responsible for the higher allergenic potency of proteins [16]. Processing may cause interactions between proteins and other food matrix proteins [14]. Heat-denatured proteins may rearrange their disulphide bonds and form homo- and heteromeric aggregates [17]. The cooking of fish can change the protein pattern observed for raw fish extracts and as well as its allergenicity. Cooked fish extracts formed immunoreactive high molecular weight aggregates of denatured proteins [18]. At present only little data about physicochemical properties of parvalbumins has been published.

In our present study we aimed to investigate the thermal and gastric stability of native whiff parvalbumin Lep w 1 by CD spectroscopy and *in vitro* gastrointestinal digestion experiments, respectively. $Ca^{2+}$-depletion experiments were performed in order to examine the relevance of bound $Ca^{2+}$ for the protein's conformational stability and IgE reactivity. The thermal stability was studied at two different pH values, acidic pH 2.5 to simulate the conditions of gastric digestion, and pH 7.0 as control. Further, the digestibility of EGTA treated and untreated Lep w 1 was tested. Finally, we examined the gastric stability and IgE binding ability of proteins extracted from cooked fish.

## 2. Material and Methods

### 2.1. Patients' sera

Five patients'sera were selected based on the presence of a positive clinical history of type I fish allergy and positive skin-prick test to different fish species (table 1). Fish-specific IgE antibodies were determined using the Pharmacia CAP system (Pharmacia, Uppsala, Sweden). All sera contained IgE specific for Lep w 1 as tested by IgE immunoblotting.

### 2.2. Native protein extraction

Fresh whiff filets were purchased from a local market in Madrid, Spain. Twohundredfifty grams of raw fish muscle were homogenized by grinding in three volumes (w/v) of 20 mM Bis-Tris-HCl, pH 6.5. Proteins were extracted by stirring for 3 h at 4°C. Subsequently, the homogenate was centrifuged (17,000 x g, 45 min, 4°C) and the pellet discarded. After removing cellular debris by filtration through Miracloth (Merck Biosciences, Nottingham, UK) and filter papers the extract of soluble whiff proteins was freeze-dried.

For the extraction of proteins from cooked fish, a 60 g raw fish filet was heated to 100° C in 600 ml double distilled $H_20$ for 10 minutes. Subsequently, the cooked fish was homogenized in 1.6 volumes (w/v) of double distilled $H_2O$, and extracted as described above. The extract was stored at 4°C for further use.

### 2.3. Purification of Lep w 1, the whiff parvalbumin

The freeze-dried protein extract was suspended in 20 mM Bis-Tris-HCl, pH 6.5, and its protein content was determined by using the BCA Protein Assay Reagent Kit (PIERCE, Rockford, Ireland). The extract was treated with 0.1% Biocryl BP-100 (Supelco,

Bellefonte, PA) for 10 min at room temperature with stirring. Precipitates were pelleted at 17,000 x g, 30 min at 4°C and subjected to a DEAE Sepharose Fast Flow column (GE Healthcare, Chalfont St. Gilles, Great Britain). The column material was pre-equilibrated in 20 mM Bis-Tris-HCl, pH 6.5. Bound proteins were eluted by a linear gradient from 0 to 25%, 120 ml length with 20 mM Bis-Tris-HCl, pH 6.5, 1 M NaCl. The elution process was monitored at 280 nm, fractions were collected, and analyzed by 15% SDS-PAGE and immunoblotting using a rabbit polyclonal anti-Gad m 1 antibody (Tepnel Biosystems Ltd., Deeside, Flintshire, UK). Fractions containing parvalbumin, eluting at approximately 150 mM NaCl, were pooled. Gel filtration chromatography was carried out on a HiPrep 16/60 Sephacryl S-200 High Resolution column (GE Healthcare) using 20 mM Bis-Tris-HCl, 150 mM NaCl, pH 6.5. The whiff parvalbumin, designated Lep w 1 by the IUIS allergen nomenclature sub-committee, was separated from higher molecular weight proteins and eluted from the column as one peak. The fractions were analyzed by SDS-PAGE and the purified parvalbumin detected by a polyclonal anti-Gad m 1 antibody (Tepnel BioSystems Ltd.).

### 2.4. N-terminal sequencing

The N-terminus of Lep w 1 was obtained as described for Gad m 1 [6].

### 2.5. Structure modelling

Structures of parvalbumins from *Cyprinus carpio* (Cyp c 1) and *Lepidorhombus whiffiagonis* (Lep w 1) were modeled using the Swiss-Modelserver (http://swissmodel.expasy.org, [19]) The structure of carp parvalbumin (PDB code 5cpv [20]) was used as template. Visualisation, building and manipulation of the structure were carried out using DeepView/Swiss-PdbViewer 4.0.

## 2.6. Circular dichroism (CD) spectroscopy

Far ultraviolet CD spectra of native and unfolded Lep w 1 (unfolding induced by heat, $Ca^{2+}$-depletion and at pH values 7.0 and 2.5), were recorded with a JASCO J-810 spectropolarimeter (Jasco, Essex, UK) at 20°C in aqueous solutions. Protein samples dissolved in 10 mM $KPO_4$, pH 7.0, $KH_2PO_4$, pH 2.5, 4.5 or $K_2HPO_4$, pH 8.5 were concentrated to 0.1 µg/µl and measured in quartz cuvettes (Hellma, Müllheim, Baden, Germany) of 0.1 cm path length. The temperature dependence of the CD at 222 nm was measured at heating rates of 2°C/min and spectra were recorded from 25 to 95°C at intervals of 10°C, and the reversibility of the heat-induced unfolding transition was assayed by measuring the spectra of the sample cooled down in a single step to 25°C. Absorption between 190 and 260 nm was monitored at 0.5 nm intervals. Each spectrum was obtained by averaging three individual runs, and corrected by subtraction of the solvent spectrum obtained under identical conditions. The results were expressed as mean residue ellipticity ($\theta$) = $deg.cm^2.dmol^{-1}$.

## 2.7. IgE enzyme-linked immunosorbent assay (ELISA) inhibition assay

In order to evaluate the recognition of the treated proteins by patients' sera IgE Covalink NH plates (Nunc A/S, Kamstrupvej, Roskilde, Denmark) were used. Purified Lep w 1 (2 µg/ml diluted in 50 mM $KPO_4$, pH 8.2) was covalently bound to activated (1,25% (w/v) glutaraldehyde in 50 mM $KPO_4$, pH 8.5, overnight, 37°C) microtiter plates overnight at 4°C. Plates were saturated with 1 M ethanolamine, pH 8.0, and subsequently, non-specific binding sites blocked by Tris buffer saline, 0.5% Tween-20 (TBST), 3% (w/v) bovine serum albumin (BSA). Four individual patients' sera (diluted in TBST, 0.25% (w/v) BSA) were pre-incubated with 50 µg/ml untreated and treated proteins for 3 hours and further transferred to ELISA plates. Buffer and sera of three non allergic subjects were used as negative controls. OD values at 405 nm were

regarded positive when they exceeded the mean OD of the negative controls. Bound IgE was detected with alkaline phosphatase (AP)-conjugated mouse anti-human IgE antibody (BD-Biosciences Pharmingen, San Diego, Calif., USA) and developed with the SIGMA FAST$^{TM}$ p-nitrophenyl phosphate substrate (SIGMA-Aldrich, Inc., St. Louis, USA). Colour development was measured using an ELISA reader (Spectra Max Plus 384; Molecular Devices GmbH, Munich, Germany) at 405 nm and 510 nm as reference wavelength. The assay was analysed in duplicates. Samples were heat-treated by incubation for 10 min at 95°C. For calcium-depletion 5 mM EGTA were added to the protein solution and subsequently incubated for 1 hour.

### 2.8. SDS-PAGE and immunoblotting

Whiff protein extract and Lep w 1 were separated by SDS-PAGE under reducing conditions (2.5% beta-mercaptoethanol). Purity of Lep w 1 was assessed by Coomassie staining. For immunoblotting proteins were transferred to a nitrocellulose membrane and whiff parvalbumin was detected by the rabbit polyclonal anti-Gad m 1 antibody as previously described for Gad m 1 [6]. In order to determine the IgE reactivity of patients' sera to undigested and digested proteins, blotted proteins were incubated with a serum pool of 4 fish allergic patients and bound IgE was detected with an AP-conjugated mouse anti-human IgE antibody (BD-Biosciences Pharmingen, San Diego, Calif., USA) and developed with a BCIP/NBTC reagent solution.

### 2.9. In vitro gastric and duodenal digestion

*In vitro* gastric (phase I) and duodenal (phase II) digestions were performed as described by Moreno *et al.* [21]. In brief, digestions of purified Lep w 1 with or without bound $Ca^{2+}$ were performed. For calcium depletion 5 mM EGTA in $KPO_4$ buffer, pH 7.5 were added and the protein solution incubated for at least one hour. Half a milligram of Lep w 1 (2 mg/ml) with or without EGTA treatment was dialyzed against simulated

gastric fluid (SGF; 0.15 M NaCl, pH 2.5) for 3 hours at room temperature, and further dissolved in SGF (Lep w 1: 0.15 mg/ml). The pH was adjusted to 2.5 with 1 M HCl. A solution of pepsin (3.2 mg/ml in SGF, pH 2.5; porcine pepsin, Sigma, Dorset, UK; product no. P6887, activity: 4,230 U/mg) was added at an approximately physiological ratio of enzyme/substrate (1:20, w/w). The digestion was carried out at 37°C and aliquots were taken from the single digestion mixture at 0, 2, 5, 15, 30, 60 and 120 min for further analysis. The reaction was stopped by raising the pH to 7.5 adding 1 M NaOH.

*In vitro* duodenal digestion was performed as previously described [21] using 120 min *in vitro* gastric digesta as starting material. Intestinal digestion was carried out at 37°C with shaking for 120 min using 125 mM bile salt mixture (sodium taurocholate (Sigma; product no. T4009), gylcodeoxycholic acid (Sigma; product no. G9910), 1 M $CaCl_2$ and 0.25 M Bis-Tris-HCl, pH 6.5) and a solution of trypsin (Sigma; product no. T-1426, activity: 12,400 U/mg) and alpha-chymotrypsin (Sigma; product no. C-7762, activity: 52 U/mg) 1.400:100 (w/w/w). Aliquots were taken at 2, 5, 15, 30, 60, 120 min for analysis. The digestion was stopped by adding a solution of a Bowman-Birk trypsin-chymotrypsin inhibitor from soybean (0.25 mg/ml in water; Sigma; product no. T-9777). For control of gastric and duodenal enzymes, alpha-lactalbumin was digested under identical conditions. Samples without added enzymes were used as negative controls.

## 3. Results

### 3.1. Extraction and purification

Whiff parvalbumin was abundant in the protein extract as visualised by Coomassie staining (Fig. 1A, lane 1). Thirty milligram Lep w 1 were obtained from approximately 100 g fish filet. Coomassie staining of the purified parvalbumin showed only one band at 11 kDa (Fig. 1A, lane 2). The identity was confirmed by immunoblotting using a polyclonal anti-Gad m 1 antibody (Fig. 1A, lane 3) and N-terminal sequencing. The initiating methionine was cleaved off, resulting in the first five amino acid residues TFAGL.

### 3.2. Structure

On the basis of the conserved EF-hand motifs of parvalbumins, a model of Lep w 1 (Acc. No. AM904681) was constructed using the structure of the calcium-bound carp parvalbumin (PDB Acc. No. 5cpv) as template (Fig. 1B). The sequence alignment used for building the Lep w 1 model is shown (Fig. 1C). This alignment exhibits approximately 70% amino acid sequence identities. The N-terminal calcium-binding loop (CD loop) showed 4 conserved amino acid residues (Asp 51, 53, Ser 55 and Glu59) and the backbone oxygen of phenylalanine residue as binding partners. Four conserved interaction partners of the molecule with calcium were found at the C-terminal loop (EF loop) (Asp 90, 92, 94 and the backbone oxygen of the lysine residue) (Fig. 1B).

### 3.3. Stability studies by circular dichroism

The extent of secondary structural changes induced in Lep w 1 at different pH values and temperatures was observed by far-UV CD. The CD spectra of the native whiff parvalbumin unheated and heated were nearly superimposable and showed

characteristic double minima at 208 and 222 nm, and a maximum at 190 nm (Fig. 2A, black). However, at pH 2.5, the protein lost the broad minimum at 222 nm. An increase of negative ellipticity, monitored at 208 and 222 nm, indicated a partial loss of structure before and after heating (Fig. 2A, grey).

In order to compare the thermal stability at different pH values, the thermal unfolding of whiff parvalbumin at pH 7.0 and 2.5 was investigated. The secondary structure of the native protein was highly resistant to thermal unfolding. Temperatures higher than 65°C were reached before Lep w 1 started to unfold (Fig. 2B, continuous line). Upon cooling, the denaturation was reversible (Fig. 2A, dotted). As mentioned above, the protein at pH 7.0 incubated with EGTA lost its structure at room temperature (Fig. 2B, dotted). No thermal induced conformational change of the partial unfolded Lep w 1 at pH 2.5 was observed (Fig. 2B, grey).

Conformational changes and structural stability were further tested by $Ca^{2+}$ depletion at pH 7.0 and 2.5 using EGTA. The calcium-depleted Lep w 1 showed no minimum at 222 nm. Two nearly coincident spectra were obtained of the untreated and heat-treated protein at pH 7.0 without calcium (Fig. 2C, dotted black and grey). The negative ellipticity at 222 nm and the maximum at 190 nm were remarkably diminished. At pH 2.5, the spectrum obtained of Lep w 1 after EGTA treatment (data not shown) was superimposable to this of the undepleted form under acidic conditions (Fig. 2A, grey).

### 3.4. IgE reactivity of EGTA and heat treated Lep w 1

Pretreated proteins were further tested by IgE ELISA inhibition assays. Four individual fish allergic patients' sera containing IgE specific for Lep w 1 were used. Thermal and

EGTA treated proteins showed similar inhibitions of IgE binding (approximately 90 - 100%) to immobilized native and uncooked Lep w 1 (Tab. 1).

### 3.5. In vitro gastric digestion of purified parvalbumin

Calcium-bound and unbound parvalbumins were evaluated individually for gastric digestibility in SGF (Fig. 3). Immediately after adding pepsin, around 90% of Lep w 1 were degraded with or without adding EGTA (Fig. 3, lane 2 or 4). Two fragments of lower molecular weight of approximately 4.5 kDa were still visible at the earliest time point of the digestion in Coomassie staining (Fig. 3, lane 2 and 4). Both fragments were completely digested by pepsin within 2 minutes (Fig. 3, lane 3 and 5). Alpha-lactalbumin, a positive control, was degraded within 120 minutes as published previously [22] (data not shown).

### 3.6. IgE binding to uncooked and cooked fish

The protein pattern of uncooked and cooked fish extracts were compared by IgE immunoblotting (serum pool of #1, 2 and 4). The protein extract of the thermally treated fish revealed a higher number of IgE reactive bands than the extract of uncooked fish (Fig. 4A, B lane 1) as described previously for Indian fish species [23]. Several high molecular weight (HMW) bands ranging from 55 kDa to around 130 kDa were observed in the cooked fish extract (Fig. 4B, lane 1). Whereas, a double band of around 40 kDa appeared only in the uncooked fish extract (Fig. 4A, lane 1). An 11 kDa protein band, corresponding to Lep w 1, was detected in both extracts. The immunoreactivity of the extracts was analyzed by IgE inhibition experiments. IgE binding to the HMW proteins, formed after cooking, could not be inhibited by the extract of uncooked fish (Fig. 4B, lane 2). Whereas, cooked extract showed 100 % inhibition of specific IgE to uncooked fish extract proteins (Fig. 4A, lane 2).

### 3.7. In vitro gastric digestibility of uncooked and cooked fish

To assess whether the heating of fish proteins affected the digestion rate, the degradation of proteins from uncooked and cooked fish by pepsinolyis was evaluated. By *in vitro* gastric digestion, uncooked fish extract (EN) (Fig. 5A, lane 1) was degraded to small fragments evident on Coomassie stained SDS-PAGE, even after 15 minutes (Fig. 5A), which in accordance with a digestion study of raw cod protein extract, using a gastric tablet from Fédération International Pharmaceutique (FIP). [15]. In contrast to raw fish extract, the cooked extract (EC) (Fig. 5B, lane 2) showed higher resistance to gastric proteolysis. EC precipitated in SGF at pH 2.5, and subsequent *in vitro* gastric digestion gave rise to a complex mixture of peptides immediately after adding pepsin (Fig. 5B, lane 0'). Some of them were still evident at 120 minutes of the digestion reaction (Fig. 5B). In detail, the Lep w 1 (11 kDa band) started degrading immediately, after adding the enzyme. As digestion proceeded, a prominent band with a molecular weight of 130 kDa, showed a partial breakdown and disappeared after 60 minutes (Fig. 5B). Fragments of another abundant protein, at approximately 34 kDa, were still detected by Coomassie staining after two hours pepsinolysis. Additionally, one protein band at approximately 24 kDa (Fig. 5B, lane 2), absent in uncooked fish (fig. 5B, lane 1), was only degraded at the time point of 60 minutes. A turbidity of the sample was observed during all 120 minutes of incubation time. Whereas, after raising the pH to 6.5, mimicking the transfer to duodenum, precipitates were dissolved and completely degraded as visualized by Coomassie staining (data not shown).

The digested extracts EN (Fig. 6A, lane 1) and EC (Fig. 6A, lane 2) were tested for IgE reactivity by IgE immunoblotting. A serum pool of patients' sera no.1, 2, 3 and 5 was used. The immunoblot detecting EN proteins showed that the immunoreactivity was lost after 15 minutes (data not shown). In contrast, patients' serum IgE could bind to the

fragmented proteins of EC even at 120 minutes. The IgE binding decreased as the proteins, inclusiding the 34 kDa protein, were degraded (Fig. 6A). IgE from sera also bound the approximately 24 kDa protein and its digestion products. No IgE bound to the abundant 130 molecular weight protein after adding pepsin. To evaluate the time of complete digestion of Lep w 1, the anti-Gad m 1 antibody was used by immunoblotting. A double band at 11 kDa, corresponding to whiff parvalbumin, was detected in EN (Fig. 6B, lane 1) and EC (Fig. 6B, lane 2). Additionally, the anti-Gad m 1 antibody could also recognize a protein with the molecular weight of approximately 24 kDa in EC (Fig. 6B, lane 2). Although visible in the Coomassie stain and the IgE immunoblot, the polyclonal antibody failed to detect the proteolytic fragments of two minutes digestion of whiff parvalbumin in EC (Fig. 6B) and EN (data not shown).

## 4. Discussion

Complete food allergens are characterized as structurally stable to thermal treatments and gastrointestinal digestion. Thus the intact allergen can be presented to the immune system. Besides the stability, interactions with the food matrix and food processing are also responsible for the allergenicity of proteins [14].

The beta-parvalbumin Lep w 1 of whiff was stable to thermal treatment up to 65° C, but only when calcium was bound as a ligand and the tests were performed at neutral pH. In contrast to natural Gad m 1 [6], Lep w 1 lost its structure completely when calcium was depleted from the protein. In line with an earlier study of Gad m1, Lep w 1 underwent a structural change at pH 2.5 resulting in a loss of the broad minimum observed at 222 nm, (Fig. 2A). Only a partial denaturation was recorded as the glutamate residues were uncharged at acidic conditions [6]. Hence, the calcium ion of the EF-loop closer to the surface [20] was lost, only the calcium of the CD-site persisted due to the stronger binding of five binding partners. Interestingly, the structural change at pH 2.5 did not influence the IgE binding ability of the protein tested by IgE ELISA inhibition assay (Tab. 1). All treated proteins showed similar percentages of inhibition of approximately 90 to 100%. This is in contrast to one report that indicated a much stronger IgE binding to the calcium-bound form of carp parvalbumin as opposed to the calcium-depleted protein [11]. It was suggested that the binding sites of parvalbumin-specific IgE were distant from the calcium binding loop, and only the conformational changes after calcium depletion impaired the IgE binding ability [24].

The CD measurements showed a partial denaturation of the protein at pH 2.5, which could explain the efficient pepsinolyis of Lep w 1 within seconds in *in vitro* gastric

digestion assays. No difference of gastric stability was observed between the EGTA treated and untreated parvalbumin as the chelator was inactive in the acidic SGF, pH 2.5 (Fig. 3).

It is interesting to note that whiff parvalbumin, although a major allergen, is completely degraded by pepsin within seconds. Therefore, we examined the role of processing. As only thermally treated whiff is consumed in Spain, we extracted proteins from boiled and raw whiff filet and determined their IgE reactivity. IgE binding to various proteins within the high molecular weight range was only observed in the cooked fish extract (Fig. 4). The IgE reactivity to these proteins could not be inhibited by raw fish extract (Fig. 4B). These findings were in accordance with an already published report of Bernhisel-Broadbent et al., where immunoreactive high molecular weight protein conglomerates were formed from cooked protein extracts of tuna, salmon, cod, and flounder [18]. Subsequently, we examined the digestibility of the whole fish protein extract as compared to uncooked fish and determined the IgE binding capacity of the resulting digestion fragments by IgE immunoblotting. Fish allergic patients' sera could detect fragments after than 15 minutes of the digestion process of raw fish (data not shown), but after more than 120 minutes of digestion of the cooked fish extract (Fig. 6A) after digestion by pepsin. As one report detected aggregates or polymers of parvalbumins in raw cod extracts depending on storage duration by an anti-parvalbumin antibody [25], we tested our cooked protein extract for parvalbumin oligomers with an anti-Gad m 1 antibody. The antibody failed to recognize high molecular protein bands in cooked fish, but detected a protein at 24 kDa (Fig. 6B). Such a molecular weight is characteristic for a parvalbumin dimer [6, 26]. Thus, we speculate that the additional proteins in cooked fish were protein aggregations of Lep w 1 or Lep w 1 with interacting extract proteins [17].

We observed that despite the remarkable stability to heating, Lep w 1 was easily digested using physiological gastric conditions. Additionally, food processing, like cooking, could generate allergenic aggregates that were partially stable in gastric digestion. It is likely that these observations explain the high allergenicity of this fish. However, to explain the role of food allergens the importance of the food matrix should also be considered.

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The authors declare that they have no conflicts of interest.

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## Figure legends

**Figure 1. A:** Purification and characterization of whiff parvalbumin. Lane 1: protein extract of whiff; lane 2: Coomassie staining of the purified Lep w 1; lane 3: detection of Lep w 1 by anti-Gad m 1 antibody. **B:** Secondary structure of modelled Lep w 1 (Acc. No. AM904681). Binding partners of calcium are indicated. Four bidentate (Asp 51, Asp 53, Ser 55 and Glu 59) and one monodentate (Phe 57) binding partners of calcium at the CD-loop. Three bidentate (Asp 90, 92 and 94) and one monodentate (Lys 96) contacts to the cation at the EF-site. Bold: bidentate ligation; italic: monodentate ligation; spheres: calcium. **C:** Sequence alignment for building the Lep w 1 model. $Ca^{2+}$-binding sites are underlined; bold: bidentate cation-binding partners, italic: monodentate binding partners.

**Figure 2.** Stability studies of Lep w 1 by CD: **A:** Thermal stability of thermally untreated (solid line) and treated (dotted line) protein at neutral (black) and acidic (grey) pH. **B:** Thermal unfolding of native Lep w 1 at 222 nm wavelength. Black: Lep w 1, pH 7.0; dotted: calcium-depleted (+EGTA); grey: Lep w 1, pH 2.5. **C:** CD of calcium-depleted protein (+EGTA) before and after thermal treatment; black: calcium-bound, grey: calcium-depleted, dotted: calcium-depleted and cooked protein. RT: room temperature, nE: thermally treated.

**Figure 3.** *In vitro* gastric digestion of whiff parvalbumin with (+EGTA) or without (-EGTA) bound calcium. Lane 1: undigested Lep w 1 in SGF, pH 2.5; lanes 2 and 4: immediately after adding pepsin; lanes 3 and 5: 2 minutes gastric digestion.

**Figure 4.** IgE inhibition assay using cooked or uncooked whiff protein extract. **A:** Residual IgE binding to uncooked extract after incubation with cooked fish proteins (lane 2); lane 1: uncooked extact; lane 3: normal human sera. **B:** Inhibition of the IgE binding to cooked extract (lane 1) with uncooked extract. Lane 2: inhibition with uncooked extract; lane 3: normal human sera; Lep w 1 is indicated by an arrow

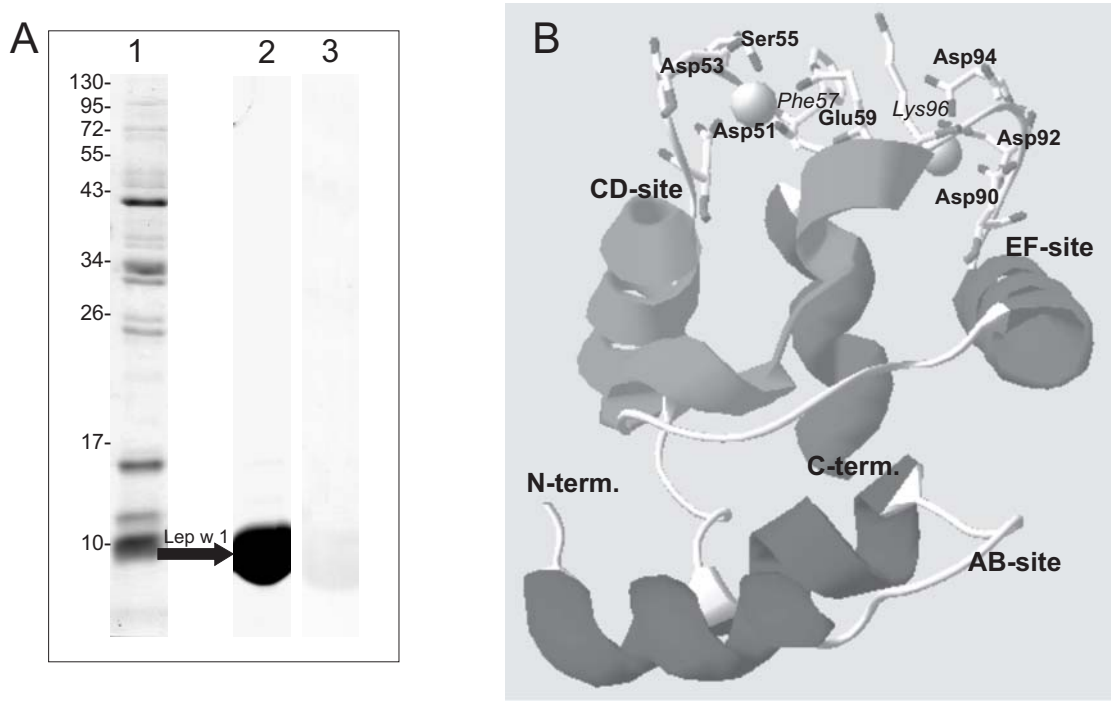
**Figure 5.** *In vitro* gastric digestibility of uncooked and cooked fish protein extract. **A:** Digestion of uncooked extract. Lane 1: raw fish extract; lanes 0', 2', 5' and 15': minutes of digestion. **B:** Gastric degradation of cooked fish extract. Lane 1: uncooked extract; lane 2: cooked extract; lanes 0', 2', 5', 15', 30', 60' and 120': minutes digestion; Lep w 1 dimer is indicated by an arrow.

**Figure 6.** Immunoblotting of *in vitro* gastric digested cooked fish extract. **A:** IgE immunoblotting using a serum pool (patients' serum no.1, 2, 3 and 5) and **B:** Detection of whiff parvalbumin by anti-Gad m 1 antibody. Lane 1 unheated and lane 2 heated fish proteins. Lanes 0', 2', 5', 15', 30', 60' and 120': minutes digestion; dimeric Lep w 1 is indicated by an arrow.

**Table 1.** Patients' characteristics and results of IgE ELISA inhibition assay.

CAP: capsulated hydrophobic carrier polymer (kU/L), A: asthma, AE: angioedema, AN: anaphylaxis, U: urticaria, nd: not done

| | | | | **Results of IgE ELISA inhibition assay** | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | **pH 7.0** | | | **pH 2.5** | | |
| **Patients** | **Symptoms** | **CAP (kU/L)** | **IgE-blot** | **Untreated** | **Cooked** | **EGTA** | **Untreated** | **Cooked** | **EGTA** |
| #1 | AE, A | 48.5 | + | 100% | 100% | 100% | 96% | 98% | 99% |
| #2 | AN | 10.2 | + | 100% | 100% | 100% | 91% | 89% | 99% |
| #3 | AN | 5.9 | + | 100% | 100% | 100% | 100% | 100% | 100% |
| #4 | U, AE | 19.9 | + | 97% | 100% | 100% | 87% | 87% | 90% |
| #5 | AE | 6.06 | + | nd | nd | nd | nd | nd | nd |

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5cpv                  AFAGVLNDADIAAALEACKAADSFNHKAFFAKVGLTSKSADDVKKAFAIIDQDKSGFIEE  60
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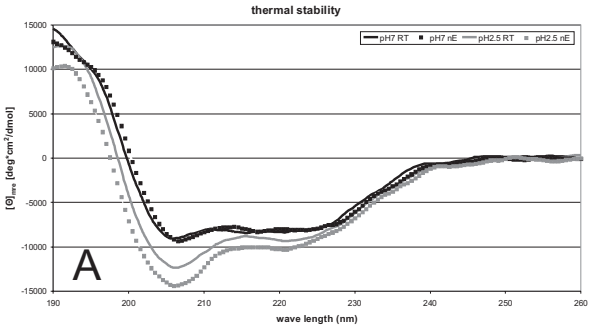
Figure 2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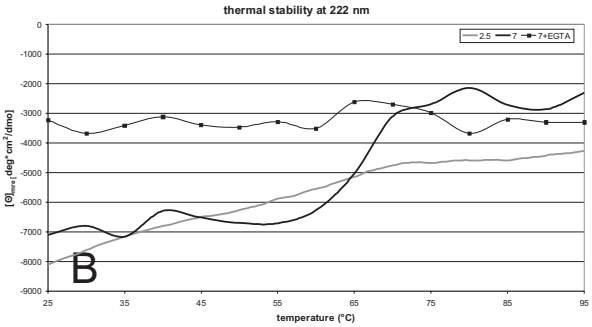
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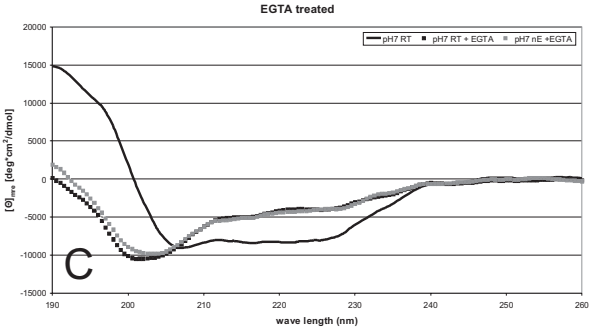
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wave length (nm)
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Figure 3

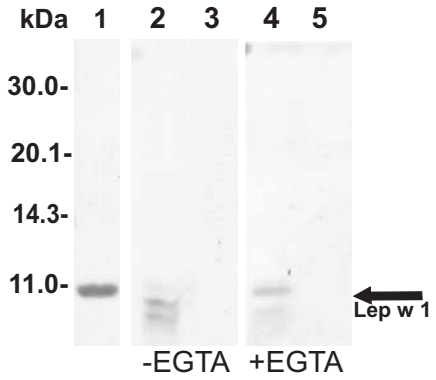
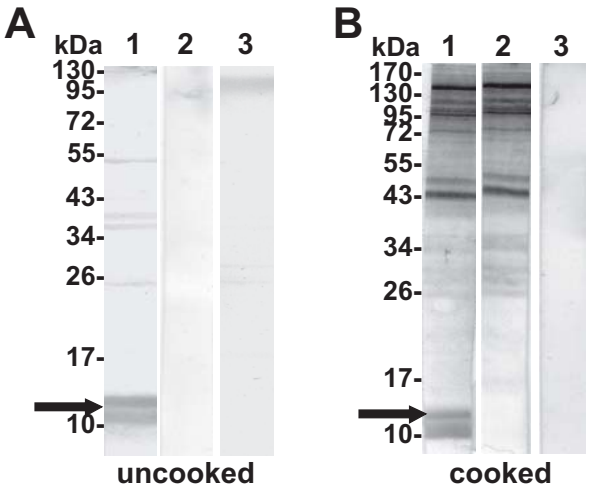


Figure 4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kDa
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uncooked
B
kDa
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170-
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cooked

Figure 5

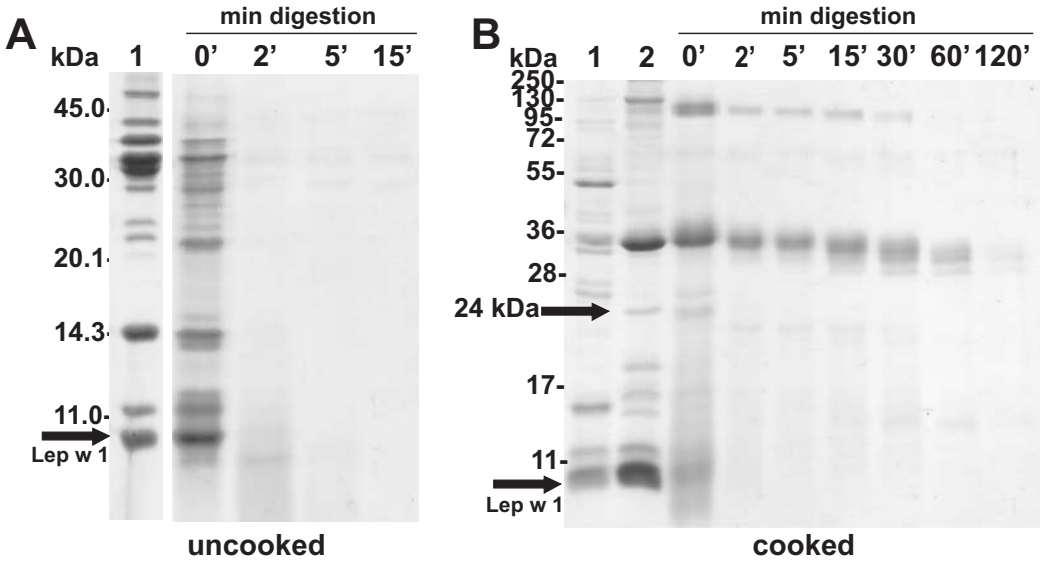
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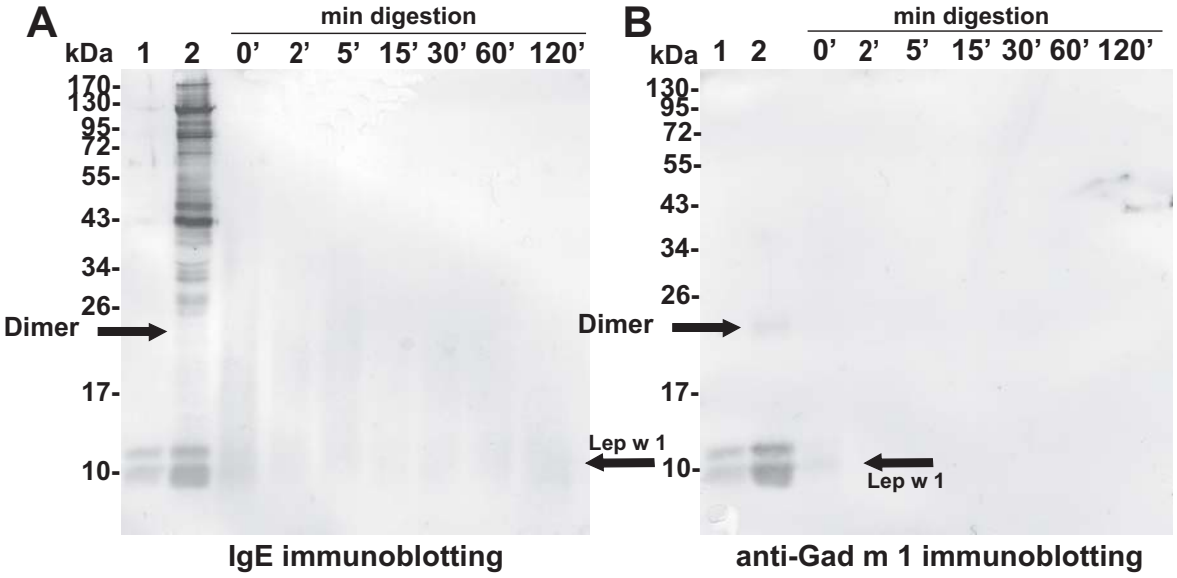
Figure 6


A
min digestion
kDa 1 2 0' 2' 5' 15' 30' 60' 120'
170-
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min digestion
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Lep w 1
anti-Gad m 1 immunoblotting

# Chapter V

# General Discussion

# Final Discussion

Only three protein families contain the majority of clinically relevant animal food allergens. These are the tropomyosins, the caseins and the parvalbumins [1]. Physico-chemical characteristics like the resistance to proteolysis and thermal denaturation, and certain biochemical features including ligand-binding as well as the abundance of the allergen in its source are some of the factors that contribute to the allergenicity of these proteins [2, 3]. Beta-parvalbumins, the major allergens of various fish species, possess a remarkable stability to thermal treatments and denaturation by digestive enzymes in their calcium bound form due to the presence of a highly conserved calcium-binding EF-hand motif [1, 4-7]. These proteins are the main elicitors of fish allergic reactions in coastal countries [8, 9]. Due to the high amino acid sequence identities among parvalbumins, fish allergic patients are often allergic to parvalbumins of various fish species [9, 10]. Interestingly, some fish species, like tuna and swordfish can be tolerated [9, 10] and may be designated as low allergenic.

In the following, the physiochemical and immunological characterization of parvalbumins of the highly allergenic cod and whiff and from low allergenic swordfish are discussed. The cDNAs of the parvalbumins from these fish species were cloned and the recombinant proteins can be used for *in vitro* single allergen based tests. Additionally, recombinant and native parvalbumins from cod and carp were compared for their application in clinical diagnosis.

Based on extensive studies of their structural and immunological properties [5, 11-14], fish beta-parvalbumins can be used for the establishment of novel diagnostic tools for fish allergy. For this purpose, we compared recombinant and natural beta-parvalbumins

regarding their IgE reactivity and structural stability. IgE ELISA, IgE immunoblotting and IgE ELISA inhibition experiments, the recombinant and natural allergens gave comparable results (Chapter II, Fig. 3). Almost all sera (25/26) of fish allergic patients displayed IgE reactivity to the tested parvalbumins (II, Fig. 3A). In addition, the results of the inhibition experiments (II, Fig. 3C) and the high amino acid sequence identities between the two parvalbumin isoforms of cod (71%) and between parvalbumins of cod and carp (80-81%) illustrated the high cross-reactivity among sea water (cod) and fresh water (carp) fish species.

Stability studies by CD spectroscopy revealed folded recombinant and natural parvalbumins with predominantly alpha-helical secondary structures (II, Fig. 5). We then subjected the proteins to various pH and temperature conditions and checked the influence on the protein structure. We observed a denaturation of the recombinant carp parvalbumin at pH 2.5 after heating (II, Fig. 7). Further, CD spectroscopy results of recombinant cod parvalbumin revealed a similarity to the untreated, calcium-depleted natural cod parvalbumin at pH 7.0 (II, Fig. 5, 6C). In addition, the protein was unstable at basic pH conditions (data not shown). These findings suggest that calcium-binding is more important for the stability of recombinant proteins than for their natural counterparts. Nevertheless, the recombinantly produced parvalbumins showed comparable IgE reactivity and thus are useful tools for *in vitro* single allergen-based tests, like component resolved diagnosis (CRD).

Despite lack of standardization and heterogeneity among different batches, total protein extracts from food have been used for routine clinical diagnosis [15, 16]. CRD is a concept utilising well defined and highly purified individual allergens instead of total protein extracts [17, 18]. This approach can improve allergy diagnosis using single

natural or recombinant allergens either on a protein CHIP or for ImmunoCAP analysis [19]. Individual allergens could serve as markers for IgE cross-reactivity elicited by homologous allergens, e.g. Bet v 1 homologues in birch pollen-related food allergy [20, 21] or highly cross-reactive parvalbumins in fish allergy. Based on the results of oral challenges on adults, cod parvalbumin was designated as a marker for fish allergy in Norway [9]. Most patients were allergic to cod parvalbumin, but could tolerate other species. Due to the high protein identity among the parvalbumins, cod parvalbumin may be used for fish allergy diagnosis in the CRD approach [9]. Whereas in Norway allergy to cod parvalbumin is very common, most Spanish patients are allergic to whiff [9, 10]. These geogragphic differences in sensitization could be ruled out by the CRD approach [16]. We suggest recombinant swordfish and whiff parvalbumins as additional diagnostic tools, especially for Spain. Swordfish parvalbumin as an example for low allergenic and whiff parvalbumin as an example for high allergenic fish may contribute to improve conventional diagnosis. Pascual et al. [22] have already used swordfish as low allergenic fish for challenges, to conclude that patients who reacted to this species "were very unlikely to tolerate any of the other tested species" [22]. Furthermore, well characterized allergens allow the development of hypoallergenic derivatives for specific immunotherapy [23].

The success of allergen isolation from its natural source or the production of the recombinant protein in a heterologous expression system very much depends on the biochemical characteristics of each individual allergen [16]. The purification from natural sources might be preferred, if the allergen is stable and abundant in the food, or if post-translationally modified [16]. Additionally, when various isoforms contribute to the overall allergenicity, the natural protein isoform mixture should be used in order to reproduce the original food composition [16]. For proteins that are either of low

abundance or degraded through the extraction from the natural source, the recombinant production is an important alternative [19, 24]. The protein abundance of fish parvalbumins depend on their different expression levels (III, Fig. 3). In our study we found that the parvalbumin of swordfish was of low abundance in the protein extract and that the purification procedure required several chromatographical steps. Thus swordfish extracts may be replaced by the individual recombinant allergen for *in vitro* diagnosis using the parvalbumin clone produced in this thesis.

Another aspect of this thesis was to determine the allergenicity of some fish species that are consumed in Spain. In countries, where fish is frequently consumed and processed, fish allergy represents a severe health problem causing mild to severe symptoms, and in some cases anaphylaxis [8-10, 22, 25]. Some fish species show a higher potential to elicit allergic reactions. In Spain, mainly fat-poor white fish like whiff, sole or hake can elicit fish allergy in young infants from 6 months to 1 year [22]. At this age fish will be introduced in the diet of Spanish children [22]. As fish allergy is persistent, approximately 80% of patients are still allergic even after 10 years after initial diagnosis [22]. Polysensitization to multiple fish species like cod and whiff is very common, but patients may tolerate some fish like tuna or swordfish [10, 22].

Thus, in the second study we examined the IgE binding capacity of 16 sera from Spanish patients to cod, whiff, and swordfish protein extract. IgE of 15 sera recognized the parvalbumins in cod and whiff extracts, however only 12 sera showed a weak IgE binding to swordfish parvalbumin (III, Fig. 2). Additionally, the extractable amount of swordfish parvalbumin was considerably less in comparison to the abundance of parvalbumins present in cod and whiff tissues (III, Fig. 3). We confirmed the cross-reactivity of all purified parvalbumins with a sequence identity of 62 to 74% (III, Fig.

1B). Further, we could attribute the low allergenicity of swordfish to the low expression level of its parvalbumin. These results are in line with the study of Pascual et al. [10, 22] who describes the members of the Tunidae (e.g. tuna) and Xiphiidae (e.g. swordfish) families as least common to elicit allergic reactions. The high cross-reactivity was explained by the presence of the highly conserved EF-hand motifs of parvalbumins. Beta-parvalbumins from fish have at least 53% sequence identity between homologues from unrelated fish species [26].

IgE mediated allergic reactions may result from ingestion, but also from inhaling the allergen [10, 22]. Since some patients have IgE to parvalbumins from fish species which were clinically tolerated [27], “food challenges should represent the most reliable way to establish or rule out an adverse reaction to a food in children and adults [28].” Pascual et al. [22], for example, started fish challenges with swordfish, the species with the lowest IgE response. If the patients could not tolerate this fish, they were recommended to exclude all fish species from their diet [22].

Knowing that whiff parvalbumin is a common allergen in Spain [10], we finally studied the resistance of whiff parvalbumin to heat treatment and digestion. The calcium-bound beta-parvalbumin showed a remarkable thermal stability up to 65°C as observed by CD spectroscopy (IV, Fig. 2B). Structural changes at acidic pH (IV, Fig. 2A) did not impair the IgE binding ability of this major fish allergen tested by IgE ELISA inhibition assay (IV, Tab. 1). In contrast to calcium-depleted Gad m 1 [4], the calcium-unbound Lep w 1 lost its natural conformation (IV, Fig. 2C).

The whiff parvalbumin with or without bound calcium was easily degraded by pepsin (IV, Fig. 3). In contrast to proteins in raw fish extract, that are digested *in vitro* within

15 minutes (IV, Fig. 5A), cooked fish proteins were detected for more than 120 minutes by IgE immunoblotting after the start of the digestion process (IV, Fig. 6A). Additionally, a different IgE binding capacity of raw or cooked whiff protein extracts was observed (IV, Fig. 4). We concluded that the high molecular weight proteins, only present in the cooked fish extract, were parvalbumin aggregates. This conclusion was supported by detecting a parvalbumin dimer of 24 kDa using a polyclonal anti-Gad m 1 antibody (IV, Fig. 6B). Dimerization of parvalbumins has already been described for Gad c 1, as detected by a monoclonal anti-parvalbumin antibody [29]. In our study, protein aggregates were formed by cooking, which could explain the high allergenicity of whiff.

Thermal treatment during food processing can influence the IgE binding ability to epitopes of food allergens by inducing protein aggregation or chemical modification [30]. The enhancement of allergenicity by protein aggregation due to processing has been observed in soya, where glycinins form heat set gels [31]. Another common protein modification by thermal treatment is the *Maillard's reaction*. The interaction of free amino groups on proteins and the aldehyde or ketone groups of sugars and further rearrangements during roasting may lead to the formation of adducts (Amadori products) [32, 33]. These high molecular weight aggregates are more resistant to gastric digestion and may affect the allergenicity of food proteins like Ara h 1 from peanut [33, 34]. Further observations of food allergen aggregation have been shown for milk alpha- and beta-lactoglobulin after pasteurization [35]. Systemic administration of aggregated beta-lactoglobulin and alpha-lactalbumin induced anaphylactic reactions in mice [35]. Besides food processing, the influence of the food matrix on the stimulation of the immune system should be considered. The susceptibility of some allergens to degradation and interactions between allergens and other food ingredients, particularly

lipids or polysaccharides has been reported [36]. Digestion of beta-lactoglobulin by duodenal enzymes was retarded by polysaccharides such as pectins or gum Arabic [36, 37]. This observation was explained by the existence of non-specific interactions between allergen and polysaccharides [30, 37]. Additonally, Moreno et al. [38] observed a retarded gastric digestion of alpha-lactalbumin in the presence of phosphatidylcholine.

The physico-chemical and immunological characterization of animal food allergens are essential for the understanding of the structural and biological features which together result in their allergenicity. The observed stability of the parvalbumins to high temperatures and low pH values contributes to the understanding of the sensitizing potential of these proteins. In addition, the results described in this thesis are also useful for explaining the high cross-reactivity of different fish species and the existence of low allergenic fish.

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# Summary

Food allergy is increasing worldwide. One of the most frequent causes of IgE-mediated food allergy in coastal countries is fish. Following ingestion, in severe cases fatal anaphylaxis can be induced. Thus, fish is of high interest in allergy research. Parvalbumins were identified as the major allergens in various fish species and are subdivided into two distinct phylogenetic lineages, alpha and beta. Most allergic reactions are caused by beta-parvalbumins. In this study, we cloned and expressed cDNAs encoding cod (*Gadus morhua*) and carp (*Cyprinus carpio*) beta-parvalbumins and purified natural cod beta-parvalbumin. Biochemical characterizations revealed a similarity of their overall secondary structure. The IgE reactivity of fish allergic patients sera was similar to the natural and to the recombinant proteins. Additionally, a high cross-reactivity among cod and carp parvalbumins was observed. Natural and recombinant parvalbumins displayed comparable biochemical properties and allergenic activity.

Furthermore, polysensitization to various fish species is frequently reported possibly due to the cross-reactivity of their parvalbumins. Nevertheless, some studies indicate the existence of low allergenic fish such as tuna and swordfish. These findings prompted us to compare the cross-reactivity and allergenicity of the purified natural parvalbumins from cod, whiff (*Lepidorhombus whiffiagonis*) and swordfish (*Xiphias gladius*), all eaten frequently in Spain. We tested total protein extracts for their IgE binding capacity and found fewer patients' IgE reactive to swordfish as to cod and whiff. The extractable amounts of parvalbumins from cod and whiff were considerably higher than from swordfish. We observed a high cross-reactivity and comparable sequence identities of the three parvalbumins. The low allergenicity of swordfish is due to the lower expression levels of its parvalbumins.

A further report describes the stability of Lep w 1, the major allergen of whiff. Purified Lep w 1 was thermally stable at neutral pH. Calcium depletion and acidic conditions resulted in structural changes, however the allergen retained its full IgE binding ability. Further, the allergenic activity of proteins extracted from cooked fish, and their stability to proteolysis, were analysed. A higher number of IgE reactive bands was observed in the cooked in contrast to the uncooked fish extract. IgE binding to these proteins could

not be inhibited by an extract from uncooked fish. Furthermore, the cooked extract showed higher resistance to pepsinolysis. It is likely that the observation of stability of Lep w 1 to thermal denaturation and the formation of protein aggregates in cooked fish, partially resistant to *in vitro* gastric digestion, explain the high allergenicity of whiff.

In conclusion, identification and characterization of fish parvalbumins, their cross-reactivity and physiochemical properties will contribute to a better understanding why these proteins are able to elicit allergic reactions.

# Zusammenfassung

Allergie auf Nahrungsmittel ist ein weltweites Problem. In Küstenländern ist die, durch IgE Antikörper vermittelte, allergische Reaktion gegen Fisch sehr häufig. Die Aufnahme von Fisch kann schwere Symptome in Fischallergikern auslösen. Im schlimmsten Fall kann es zu einem tödlichen, anaphylaktische Schock kommen. Aus diesem Grund beschäftigt sich die Allergieforschung mit Fischallergie.
Parvalbumine sind die Hauptallergene in vielen Fischarten. Sie werden phylogenetisch in zwei Typen aufgeteilt, die Alpha- und Beta-Parvalbumine. Allergische Reaktionen werden aber meist von Beta-Parvalbuminen ausgelöst. In unserer Studie haben wir die Beta-Parvalbumine von Kabeljau (*Gadus morhua*) und Karpfen (*Cyprinus carpio*) kloniert und exprimiert. Außerdem haben wir das natürliche Beta-Parvalbumin von Kabeljau gereinigt. Biochemische Charakterisierungen zeigte eine Ähnlichkeit der Sekundärstruktur der Parvalbumine. Das natürliche Parvalbumin von Kabeljau war sowohl mit als auch ohne gebunden Kalzium thermisch stabil. Patientenseren von Fischallergikern aus Spanien, den Niederlanden und aus Griechenland zeigten eine vergleichbare IgE-Reaktivität auf natürliche und rekombinante Proteine. Zusätzlich konnten wir eine hohe Kreuzreaktivität zwischen Kabeljau- und Karpfen-Parvalbumin feststellen. Das Ergebnis dieser Studie ergab vergleichbare Resultate bei der Biochemischen und allergenen Charakterisierung von rekombinanten und natürlichen Parvalbuminen.

Es wird oft über Polysensibilisierung von Fischallergikern auf mehrere Fischarten berichtet, die wahrscheinlich durch die Kreuzreaktivität ihrer Parvalbumine erklärt werden kann. Trotzdem zeigen einige Studien Fische auf, die geringere allergische Reaktion auslösen. Zu diesen Fischen gehören der Thunfisch und der Schwertfisch. Um herauszufinden, warum Fische verschieden starke allergische Reaktionen auslösen können, haben wir die Kreuzreaktivität und die allergene Wirkung von Kabeljau, Flügelbutt (*Lepidorhombus whiffiagonis*) und Schwertfisch (*Xiphias gladius*) untersucht. Alle erwähnten Fische werden in Spanien häufig gegessen. Wir haben die IgE-Reaktivität von spanischen Patientenseren auf die Proteinextrakte aller drei Fische getestet, und fanden heraus, dass auf Schwertfischextrakt weniger Seren reagierten als auf Kabeljau- und Flügelbuttextrakt. Anschließend wurden die Parvalbumine dieser Fische gereinigt und die cDNA von Flügelbutt und Schwertfisch bestimmt. Aus

Kabeljau und Flügelbutt konnte beträchtlich mehr Protein extrahiert werden als aus Schwertfisch. Wir stellten eine hohe Kreuzreaktivität und eine vergleichbare Sequenzidentität der Parvalbumine fest. Die geringere Allergenität von Schwertfisch erklärten wir durch die niedrigere Expressionsrate von Parvalbumin in diesem Fisch.

Ein weiterer Themenbereich beschäftigt sich mit der Stabilität von Lep w 1, den Hauptallergen von Flügelbutt. Wir untersuchten die Stabilität des Proteins gegen Hitze und während des Verdauvorganges. Das Allergen war thermisch stabil unter neutralen Bedingungen, aber durch die Entfernung von Kalzium oder durch sauren pH änderte es seine Struktur. Es wies aber trotzdem eine vollständige IgE-bindende Aktivität auf. Weiters analysierten wir die Allergenität und die proteolytische Stabilität von extrahierten Proteinen aus gekochten Fisch. Es wurden mehr IgE-reaktive Banden im Proteinextrakt aus gekochtem als aus ungekochtem Fisch gefunden. Die IgE-Bindung an diese Proteine konnte nicht durch den Extrakt aus ungekochtem Fisch inhibiert werden. Zusätzlich war der Extrakt aus gekochten Fisch, im Gegensatz zum rohen Fischextrakt, stabiler im Verdau durch Pepsin. Die Stabilität gegenüber der thermischen Denaturierung und die Bildung von Proteinaggregaten in gekochten Fisch, die teilweise im *in vitro* gastrischen Verdau stabil waren, kann die hohe Allergenität von Flügelbutt begründen.

Die Identifizierung und Charakterisierung von Fischallergenen, Studien deren Kreuzreaktivität und dessen physiochemische Eigenschaften können einen Beitrag zum besseren Verständnis leisten, warum diese Proteine Allergie auslösen können.

# Curriculum Vitae


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# Publication list

## Papers as first author

- Ma, Y[*], **Griesmeier, U[*]**, Susani, M, Radauer, C, Briza, P, Erler, A, Bublin, M, Alessandri, S, Himly, M, Vàzquez-Cortés, S, Reig Rincon de Arellano, I, Vassilopoulou, E, Saxoni-Papageorgiou, P, Knulst, AC, Fernández-Rivas, M, Hoffmann-Sommergruber, K, Breiteneder, H (2007): **Comparison of natural and recombinant forms of the major fish allergen parvalbumin from cod and carp.** (Mol Nutr Food Res.) (* contributed equally)

- **Griesmeier, U[*]**, Vázquez-Cortés, S[*], Bublin, M, Radauer, C, Ma, Y, Briza, P, Fernández-Rivas, M, Breiteneder, H (2009): **Expression levels of parvalbumins determine allergenicity of fish species.** reviewed and minor revisions required (Allergy) (* contributed equally)

- **Griesmeier, U**, Bublin, M, Radauer, C, Vázques-Cortés, S, Ma, Y, Fernández-Rivas, M, Breiteneder, H (2009): **Physicochemical properties and thermal stability of**
  **Lep w 1, the major allergen of whiff.** reviewed and major revisions required (Mol Nutr Food Res.)

## Congress presentations as presenting author

- **Ulrike Griesmeier**, Sonia Vázquez-Cortés, Merima Bublin, Otto Scheiner, Monserrat Fernánder-Rivas, Heimo Breiteneder (2006): **IgE reactivity to fish allergens in a Spanish study population.** Poster presentation at the congress of the European Academy of Allergology and Clinical Immunology, June 10 - 14, 2006, Vienna, Austria

- **Ulrike Griesmeier**, Stefan Wagner, Bettina Zwölfer, Birgit Wagner, Marianne Gerstmayr, Christian Radauer, Barbara Bohle, Otto Scheiner, Heimo Breiteneder (2005): **Production of a Biologically active recombinant human interleukin 10 in a bacterial expression system for immunomodulation of**

**type I allergy.** Poster presentation at the Annual Meeting of the Austrian Society for Allergology and Immunology, December 1-3, 2005, Graz, Austria

- **Ulrike Griesmeier**, Birgit Wagner, Marianne Gerstmayr, Barbara Bohle, Otto Scheiner, Heimo Breiteneder (2004): **Production of recombinant human interleukin 10 in a bacterial expression system for immunomodulation of type I allergy**. Poster Presentation at the Annual Meeting of the Austrian Society for Allergology and Immunology, December 2-4, 2004, Vienna, Austria

- **Ulrike Griesmeier,** Sonia Vázquez-Cortés, Merima Bublin, Otto Scheiner, Monserrat Fernánder-Rivas, Heimo Breiteneder (2006): **IgE reactivity to fish allergens in a Spanish study population.** Poster presentation at the EAACI, June 10-14, 2007, Vienna, Austria

- **Ulrike Griesmeier**, Sonia Vázquez-Cortés, Merima Bublin, Otto Scheiner, Montserrat Fernández-Rivas, Heimo Breiteneder (2006**): Identification of Parvalbumin in Tuna and Swordfish and Analysis of their Isoforms.** Poster presentation at the Pirquet Symposium, December 7-9, 2006, Vienna, Austria

- **Ulrike Griesmeier,** Sonia Vázquez-Cortés, Merima Bublin, Otto Scheiner, Monserrat Fernánder-Rivas, Heimo Breiteneder (2007): **Cross-reactivity among three different fish parvalbumins.** Poster presentation at the 2nd ISMA, April 22-24, 2007, Rome, Italy

- **Ulrike Griesmeier**, Sonia Vázquez-Cortés, Merima Bublin, Otto Scheiner, Montserrat Fernández-Rivas, Heimo Breiteneder (2007): **Cross-reactivity among cod and whiff beta-parvalbumins.** Poster presentation at the Annual Meeting of the Austrian Society for Allergology and Immunology, December 13-15, 2007, Vienna, Austria

- **Ulrike Griesmeier**, Sonia Vázquez-Cortés, Merima Bublin, Montserrat Fernández-Rivas, Heimo Breiteneder (2008): **Effect of pH and calcium depletion on thermal stability and IgE binding ability of whiff parvalbumin**.

Poster presentation at the congress of the European Academy of Allergology and Clinical Immunology, June 7-11, 2008, Barcelona, Spain

## Other Congress Presentations

- Maria Kostadinova**,** Merima Bublin, **Ulrike Griesmeier**, Nina Balazs, Stefan Wagner, Christian Radauer, Heimo Breiteneder (2007): **Selection of monoclonal single chain variable fragment antibodies against the major fish allergen, parvalbumin, using a phage display library.** Poster presentation at the Annual Meeting of the Austrian Society for Allergology and Immunology, December 13-15, 2007, Vienna, Austria

- Sonia Vázquez-Cortés, Sara Cano Escudero, Beatriz Nuñez Acevedo, Christian Radauer, L. Zayas Romero, **Ulrike Griesmeier**, Heimo Breiteneder, Montserrat Fernández-Rivas (2009): **Diagnostic performance of ImmunoCAP to rGad c 1 and rCyp c 1.** accepted for the Poster presentation at the EAACI, June 6-10, 2009, Warsaw, Poland