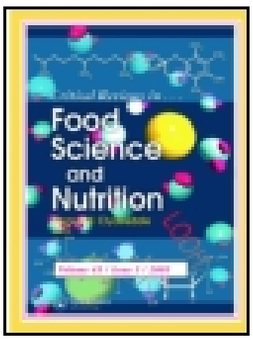

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Critical Reviews in Food Science and Nutrition

ISSN: 1040-8398 (Print) 1549-7852 (Online) Journal homepage: http://www.tandfonline.com/loi/bfsn20

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To cite this article: Telmo J. R. Fernandes, Joana Costa, Isabel Carrapatoso, Maria Beatriz P.P. Oliveira & Isabel Mafra (2015): Advances on the Molecular Characterisation, Clinical Relevance and Detection Methods of Gadiform Parvalbumin Sllergens, Critical Reviews in Food Science and Nutrition, DOI: 10.1080/10408398.2015.1113157

To link to this article: http://dx.doi.org/10.1080/10408398.2015.1113157

Advances on the molecular characterisation, clinical relevance and detection methods of Gadiform parvalbumin allergens

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Abstract

Gadiform order includes several fish families, from which Gadidae and Merlucciidae are part of, comprising the most commercially important and highly appreciated fish species, such as cod, pollock, haddock and hake. Parvalbumins, classified as calcium-binding proteins, are considered the main components involved in the majority of fish allergies. Nine and thirteen parvalbumins were identified in different fish species from Gadidae and Merlucciidae families, respectively. This review intends to describe their molecular characterisation and the clinical relevance, as well as the prevalence of fish allergy. Additionally, the main protein- and DNA-based methods to detect fish allergens are fully reviewed owing to their importance in the safeguard of sensitised/allergic individuals.

Keywords: food allergen; fish allergy; Gadidae; Merlucciidae; molecular characterisation; prevalence; parvalbumin detection; protein-based methods; DNA-based methods.

INTRODUCTION

The taxonomic order of Gadiformes is ubiquitous in every ocean of the world and includes several fish families of great commercial interest. In this order, the most commercially important and highly appreciated species are included in two main families: the Gadidae and the Merlucciidae. The Gadidae family (Gadoids) contains a well-known group of fishes such as Atlantic cod (*Gadus morhua*), pollock (*Pollachius pollachius*) and haddock (*Melanogrammus aeglefinus*), while Merlucciidae family encompasses species such as hakes (*Merluccius merluccius*) (Cohen et al., 1990; Di Finizio et al., 2007). Approximately 18% of the global marine catch regards Gadiform species, corresponding to almost 6.5 million tons in 2011 (FAO, 2014). Like other commonly consumed fish families, the species belonging to Gadidae and Merlucciidae are traditional dietary components in several worldwide populations, being used in many of the existing manufactured fish products (fresh or frozen fillets, smoked, salted, among others) (Calo-Mata et al., 2003).

Beyond the incontestable nutritional value of their flesh as a source of highly assimilated proteins, fish plays a vital role in human diet. Several health benefits have been related to fish consumption, such as prevention of cardiovascular diseases and cancer or glycaemic control, mainly associated with the ingestion of omega-3 polyunsaturated fatty acids (PUFA) (Larsen et al., 2011; Mozaffarian, et al., 2005; Sirot et al., 2008). Along with the increased awareness of the benefits of fish intake and consequent consumption, the number of fish allergies has also increased (Van Do et al., 2005a).

Fish is one of the eight groups responsible for almost 90% of food allergies reported at global scale (CODEX, 2010). For the sensitised/allergic patients, the prevention of an allergic reaction

relies mostly on the total avoidance of the offending food. Therefore, accurate and reliable food labelling systems have become imperative to improve consumer's protection and life quality (Costa et al., 2014; Fæste et al., 2011; Monaci, and Visconti, 2010; Prado et al., 2015; Rencova et al., 2013; Taylor, and Baumert, 2015). According to the recent European Union (EU) regulations, food producers are obligated to declare and highlight from the rest of the list of ingredients, the presence of fourteen groups of potentially allergenic foods, which include fish, crustaceans, molluscs, celery, mustard, sesame seed, gluten, tree nuts, peanuts, milk, eggs, soybeans, lupine and sulphites (Directive 2006/142/EC, Directive 2007/68/EC, EFSA, 2014; Regulation (EU) No 1169/2011).

In the present work, a concise and updated overview on fish allergens, focusing on Gadiform order will be described. Issues such as the prevalence of fish allergy, the molecular characterisation of Gadiform parvalbumins and their clinical relevance will be especially addressed. The analytical techniques used for the detection and quantification of fish allergens will be also particularly reviewed.

PREVALENCE OF FISH ALLERGY

Despite the general perception of the increasing frequency of food allergies, estimates of their actual prevalence and incidence are still uncertain (Nwaru et al., 2014). Most of the diagnoses is based on self-reporting symptoms, specific immunoglobulin E (IgE) sensitisation assays or skin prick tests (SPT) to common food allergens, so the real frequency of food allergies could be probably overestimated (Burks et al., 2012; Nwaru et al., 2014). Although objective assessments such as SPT are considered reliable evaluations (Zuidmeer et al., 2012), relatively few epidemiological studies have been performed using open food challenges (OFC) or double-blind

placebo-controlled food challenge (DBPCFC) as the gold standard diagnosis in defining food allergy (Nwaru et al., 2014).

Presently, overall data suggest that 1-10% of the general population suffers from food allergies (Chafen, et al., 2010), with a higher frequency among children than in adults (Sicherer, and Sampson, 2009; Sicherer, and Sampson, 2010). During the first years of life, the prevalence of food allergy reaches 6-8%, mainly due to the consumption of milk, egg, peanut, fish, and shellfish (Berin, and Sampson, 2013; Wang, and Sampson, 2011). Children often acquire natural tolerance to some foods such as egg, milk, wheat or soybean during childhood (mainly in the first decade), in opposition to fish, shellfish, peanut and tree nuts allergies that are more likely to be life-persisting (Priftis et al., 2008; Wang, and Sampson, 2011).

Fish allergy is estimated to affect 0.1-0.4% of the general population (Codex, 2010; Kuehn et al., 2013), although these numbers can vary among different countries/regions. In a population-based study performed by random call survey in Canada, the prevalence of perceived and further confirmed fish allergy was 0.51% and 0.10%, respectively. This discrepancy suggests that among Canadian population, the prevalence of fish allergy is probably overestimated, highlighting the importance of using confirmatory tests for food allergy diagnosis (Ben-Shoshan et al., 2010). In the USA, another random call survey estimates that 0.4% of the population suffers from fish allergy and 0.2% from both shellfish and fish allergies. In the same study, the prevalence of fish allergy was found to be higher among adults and among women, respectively, in terms of age and gender (Sicherer et al., 2004). In a different report from the USA targeting adult population, the incidence of self-reported and clinically diagnosed fish allergy was 0.7% and 0.6%, respectively (Vierk et al., 2007). Contrarily to the previous report (Sicherer et al.,

2004), no significant differences were found in the prevalence of fish allergy between gender or race/ethnic groups (Vierk et al., 2007). In Europe, the latest reported overall incidence of fish allergy was approximately 0.2%, with Germany presenting the highest prevalence (0.9%), followed by Spain with 0.5% (Burney et al., 2010; Burney et al., 2014). Although the percentage in Europe was estimated on the basis of a study that included eleven countries and other partners (Australia and USA), only six of them reported data regarding fish allergy. Similarly to other food allergies, there is a proportional relationship between the prevalence of fish allergy and the total consumption of fish in each country. In fact, fish is one of the most common food allergens in the European coastal countries like Norway, Finland, Portugal or Spain (Kamath et al., 2013; Perez-Gordo et al., 2011; Sharp, and Lopata, 2013; Tsabouri et al., 2012). Considering that countries such as Portugal or Finland were not included in the study described by Burney et al. (2010), the prevalence of fish allergy in Europe is most likely underestimated. The scarce data from Asia and South Africa suggest a high incidence of fish allergy among the allergic population. In Singapore, fish allergy presented an estimated prevalence of 4.1% (Thong et al., 2007).

It is also important to highlight that although food intolerance does not result from a specific immune response because it is mainly due to digestion, absorption or metabolic disorders, the clinical symptoms are extremely similar to the ones presented by allergic individuals. This fact may also contribute to frequent errors on the estimation of the prevalence of the perceived and confirmed food allergies (Prado et al., 2015).

MOLECULAR CHARACTERISATION OF GADIFORM PARVALBUMINS

Over the last years, the number of identified allergen sequences has been exponentially increasing, which emphasises the need for a systematic classification. Together with the recent advances of bioinformatics, researchers are now more capable of establishing evolutionary and structural relationships among allergens from distinct origins (Radauer, and Breiteneder, 2007; Radauer et al., 2008).

As a result, allergen platforms such as the ALLERGOME, the Resource Program Allergen, the Official List of Allergens issued by the International Union of Immunological Societies (IUIS) Allergen Nomenclature Sub-committee and the InFormAll databases that report molecular, biochemical and clinical data about allergenic proteins have become excellent tools for allergen classification. Particularly, ALLERGOME database has been designed to provide relevant information on allergenic molecules selected from international renowned scientific journals, reporting significant IgE-mediated reactions. Even though most of the allergenic molecules are officially named and recognised by the IUIS Allergen Nomenclature Sub-committee, ALLERGOME database expands data available from the scientific literature, following careful immunological and allergological criteria based on structural relationships with known allergens and IgE-binding capacity. Thereby, a wide number of allergenic molecule entries that are not listed in the IUIS allergen nomenclature website are included in the ALLERGOME database (Mari et al., 2009).

Although the number of food allergens has been continuously rising, literature seems to suggest that they are limited to some protein groups, corresponding to about 5% of all structural protein families (Radauer et al., 2008). So far, plant food allergens have been considered a diversified

group of proteins that belong to few specific families, evidencing their distinct biological and biochemical roles (Breiteneder, and Radauer, 2004; Breiteneder, and Mills, 2005a; Breiteneder, and Mills, 2005b). In the case of animal food allergens, the number of families is even smaller. Contrasting with plant food or pollen allergens, practically all animal food allergens have homologs in the human proteome, which is thought to affect the recognition of such proteins by the human immune system (Jenkins et al., 2007).

Parvalbumins belong to the second largest family of animal food allergens, the calcium binding proteins (Jenkins et al., 2007; Breiteneder, and Radauer, 2005a). They are vertebrate-specific, primarily cytosolic, small in length (composed by 106-113 aa), with a globular tri-dimensional structure and acidic isoelectric points (3.9-5.5), being expressed in fast-twitch muscles, specific neurons, certain kidney and endocrine gland cells, Corti's cells and others (Permyakov et al., 2008). Biochemically, parvalbumins are characterised by the high proportions of glutamic and aspartic acids and phenylalanine, with little or no histidine, proline, cysteine, methionine, tyrosine, or tryptophan (Heizmann, 1984). Owing to their unbalanced amino acid composition (high content in phenylalanine), parvalbumins exhibit a characteristic ultraviolet absorption spectrum with low absorbance at 280 nm (Arif, 2009). They are well-known for possessing one or more EF-hand motifs that correspond to a 12-residue loop flanked on both sides by a 12-residue alpha-helical domain (Grabarek, 2006). Parvalbumins contain, specifically, three EF-hand motifs, in which two domains bind a calcium ion each and a third silent domain forms a cap covering the hydrophobic surface of the two functional domains, stabilising the two EF-hand motifs (Grabarek, 2006; Ikura, 1996). They are abundant proteins (up to 5 mg per g of fresh weight) in the white muscle of many fish species, with estimated biological functions related to

the relaxation of muscle fibres through the binding of free intracellular calcium. The binding of calcium ion to the protein is thought to be determinant for the conformational integrity of the IgE epitopes, affecting their allergenic capacity. Parvalbumins have remarkable resistance to high temperatures, denaturing agents and proteolytic activity (Arif et al., 2007; Bugajska-Schretter et al., 1998; Bugajska-Schretter et al., 2000; Capony, and Pechère, 1973).

Parvalbumins can be divided into two lineages – alpha-parvalbumins and beta-parvalbumins – that present very similar overall folding. These two groups can be distinguished by their isoelectric points (pI) (alpha: pI $\geq$5; beta: pI $\leq$4.5), sequence characteristics, affinities for calcium and magnesium, cell-type specific expression and physiologic functions (Wopfner et al., 2007). The alpha-parvalbumins are abundantly present in the muscle of fish and amphibians, but in general they are not allergenic. The beta-parvalbumins seem to present allergenic properties, which are evidenced by the great number of beta-parvalbumins identified as allergens in fish species (Jenkins et al., 2007). Beta-parvalbumins are considered important allergenic proteins with well conserved sequences, exhibiting at least 53% of sequence identity among homologues from distantly related or even unrelated fish species (Radauer et al., 2008). Beta-parvalbumins of fish represent one of the largest animal families of food allergens, reaching the second place just behind tropomyosin family in crustaceans and molluscs (Jenkins et al., 2007). Cross-reactions between beta-parvalbumins from cod and other species are frequently the main cause of observed polysensitisation to multiple fish species in allergic individuals (Griesmeier et al., 2010).

So far, the Gadidae and the Merlucciidae families of fish allergens are considered the most important in terms of clinical relevance. Few members of other families of Gadiform order,

namely Phycidae and Muraenolepididae (Cohen et al., 1990), have been reported as allergenic fishes, though with lower consumption rate and, subsequently, with lower exposure. Among them, the Gadidae is, by far, the most representative family of allergens. The Merlucciidae is the family of cod-like fish with several of its members having commercial interest, which include 5 different genera of fish. The most well-known species of this family belong to the *Macruronus* and *Merluccius* genera (Lloris et al., 2005).

At the time, only Gad c 1 and Gad m 1 parvalbumins have been included in the WHO/IUIS official nomenclature list of allergens. Due to the high number of reported immunoreactivity of other fish parvalbumins, several of them have been proposed as allergens in the ALLERGOME database, which are expected that after careful evaluation by the WHO/IUIS Executive Committee are also included in the official list of allergens, justifying their inclusion in the present review.

Gadidae parvalbumins

Gad c 1 (former Allergen M)

This protein corresponds to the parvalbumin allergen present in Baltic cod (*Gadus callarias*) and it was formerly designated as allergen M. Its identification and characterisation was first reported almost four decades ago (Elsayedet al., 1974; Elsayed, and Bennich, 1975) and since then, Gad c 1 has been the source of extensive study.

Gad c 1 has a primary structure composed of 113 aa with an estimated molecular mass of 12.1 kDa and a pI of 4.75 (Table 1). Its sequence includes a monomer of glucose and residues of tyrosine, tryptophan and arginine (one of each), with arginine being thought to play an essential

role in the conformational structure of the mature protein (Elsayed, et al., 1974). The trypic cleavage of the arginyl peptide bond of allergen M allowed the identification of two allergenically active fragments (TM1 and TM2), evidencing that their linear sequences are most directly related to cod allergy than conformational structure (Elsayed et al., 1974; Elsayed, and Bennich, 1975). Gad c 1 was the first allergenic protein to undergo epitope mapping, thus peptides have been generated by tryptic hydrolysis and analysed for immunoreactivity (Elsayed et al., 1974; Elsayed et al., 1981; Elsayed, and Apold, 1983). Based on Gad c 1.0101 isoform, four hexapeptides, namely DEDGFY, IADEDK, KGILSN and SNADIK were *in silico* analysed by Minkiewicz et al. (2012), revealing a wide distribution of Gad c 1 epitopic hexapeptides in the universal proteome. In the reported study, the four hexapeptides were found, not only across plant and animal kingdoms, but also in several microorganisms, which might explain potential cross-reactivity with unrelated food species (Minkiewicz et al., 2012).

In general, parvalbumins are thought to be well conserved among different fish species (Radauer et al., 2008), indicating a high potential for cross-reactivity with distinct parvalbumins in fish-allergic patients. The analysis of purified extracts of fish allergens by SDS-PAGE and immunoblotting, using sera of fish-allergic individuals, allowed Van Do et al. (2005a) to compare the IgE-binding proteins of nine fish species. In the reported study, an intensive coloured band at 12 kDa could be observed in cod, salmon, pollock and wolfish, confirming the presence of parvalbumins in these fish species. The use of rabbit polyclonal IgG raised against recombinant Gad c 1 by means of immunoblotting assay, also enabled the identification of strong reactivity of cod with salmon, pollock, herring, wolfish and flounder, while reactivity for other fish species (tuna, halibut and mackerel) was considered weak, confirming the immunoblotting

results with IgE from sera of fish-allergic patients (Van Do et al., 2005a). Primary protein sequence of Gad c 1 showed high identity indices with the parvalbumins Sal s 1 (68%) from salmon and The c 1 (62%) from pollock, supporting the strong cross-reactivity among cod and these species. This cross-reactivity is of clinical relevance since individuals with a positive DBPCFC to cod also reacted to other fish, such as herring, plaice and mackerel.

Gad m 1

From *Gadus morhua* species, the Gad m 1 is another allergenic protein of major importance. So far, two isoallergens – Gad m 1.01 and Gad m 1.02 – and four respective isoforms – Gad m 1.0101/Gad m 1.0102 and Gad m 1.0201/Gad m 1.0202 – have been identified in Atlantic cod (ALLERGEN, 2014). The four isoforms of Gad m 1 present a primary structure of 109 aa (NCBI, 2014; UniProt, 2014) containing the residue alanine N-acetylated, although their encoded nucleotide sequences vary between 546 base pair (bp) and 797 bp (Table 1) (NCBI, 2014). The amino acid sequence and 3-D representation of Gad m 1 molecule is presented in Figure 1.

Gad m 1 was first reported by Das Dores et al. (2002) as an allergen from Atlantic cod, presenting a sequence identity of 62.3% with Gad c 1 (*Gadus callarias*) and 75% with Sal s 1 (*Salmo salar*) allergens. This finding suggested that, in cod and salmon species, the Gad m 1 and the Sal s 1 are probably encoded by the same gene, while Gad m 1 and Gad c 1 are most likely to be coded by two distinct genes (Das Dores et al., 2002).

Van Do et al., (2003) reported the identification and characterisation of two distinct parvalbumin transcripts (T1 and T2) in cod by means of reverse transcriptase-polymerase chain reaction (PCR) using the RNA of cod muscle. Both parvalbumin mRNA sequences encoded similar polypeptide chains of 108 aa (including the Methionine start codon), and show unambiguously

strong relationship to the beta-parvalbumins. The amino acid sequence of T1 had identity indices with beta-type parvalbumins of teleost fish spanning from 73.1% (Northern pike) to 92.6% (whiting). In relation to T2, the identity indices were from 71.3% (whiting) to 86.0% (European hake) and with alpha-type parvalbumins, identity ranged from 53.9% (human) to 59.8% (Northern pike). The identification of two distinct cDNA in Atlantic cod seems to suggest that isotypic variants are normally present in fish. The T1 isotype evidenced high similarity with beta-parvalbumin of whiting (92.6%), whereas the T2 isotype presented less identity (71.3%) with the same sequence (Van Do et al., 2003).

More recently, Ma et al. (2008) cloned and expressed two full-length nucleotide sequences (Gad m 1.01 and Gad m 1.02). The coding regions of each of the two parvalbumin cDNA encompass approximately 330 bp for proteins with 109 aa, both with a theoretical pI of 4.58. The deduced sequences of the two cod beta-parvalbumin isoforms were 71% identical, justifying their classification as isoallergens of Gad m 1. Both isoallergens also evidenced 80-81% sequence identity with Cyp c 1.01 from *Cyprinus carpio* (common carp). After purifying native Gad m 1 from muscle protein extract of cod, nGad m 1 and rGad m 1.02 were evaluated for their immunoreactivity to sera obtained from fish-allergic patients (*n*=26). All sera were reactive to both nGad m 1 and rGad m 1.02, and 25 out of 26 tested sera were also reactive to Cyp c 1.01, confirming that cod and carp parvalbumins share at least some B-cell epitopes. In the same study, supported by mass spectrometry (MS), authors speculate that the majority of nGad m 1 molecules undergo post-translational modification by acetylation. Therefore, sequences of Gad m 1 isoforms that were included in the available databases (ALLERGEN, 2014; NCBI, 2014; UniProt, 2014) present the acetylated N-terminal alanine (Ma et al., 2008).

Gad m 1 was the first beta-parvalbumin studied by NMR spectroscopy (Ma et al., 2008), although no structure is yet available in the Protein Data Bank (PDB). The protein revealed a secondary structure composed of six alpha-helices (7-10 aa each) and two small beta-sheets with residues 56-57 and 97-99. These regions are thought to correspond to the putative calcium-binding sites that are present in parvalbumin family members as an EF-hand motif (Moraes et al., 2013). Since Gad m 1 is a calcium-binding protein, in the absence of calcium, its structure becomes less ordered, losing integrity at a pressure of 200 MPa. According to infrared results, above 50ºC the polarity in protein core increases, indicating a loss of the tertiary structure that leaded to the appearance of a molten globule conformation (Somkuti et al., 2012). In the same study, the authors established that the complete unfolding of Gad m 1 was possible combining high temperatures and pressures (e.g. 40ºC at 1.14 GPa or 50ºC at 890 MPa). Besides the normally observable native, unfolded and aggregated states, the authors also reported a molten globule and a partially unfolded state possessing different calcium-binding capacities. However, the immunoreactivity of those proteins seemed unaffected by any of the tested treatments since IgE-binding capacity was neither reduced nor enhanced in the presence of these proteins (Somkuti et al., 2012).

Mic po 1

Mic po 1 allergen was identified in the *Micromesistius poutassou* species, frequently known as blue whiting. Piñeiro et al. (1998) have described the identification of several proteins from *Micromesistius poutassou* species ranging from high molecular weight (~67 kDa) down to approximately 14 kDa and with high isoelectric points (pI>5.5), suggesting their classification as parvalbumins. However, the set of proteins with molecular mass below 14 kDa and pI in the

interval of 3.9-4.6 were defined as potential allergenic parvalbumins in blue whiting. More recently, Sletten et al. (2010) reported the identification of a 12 kDa parvalbumin in blue whiting using two antibodies: a monoclonal anti-frog parvalbumin antibody PARV-19 and a polyclonal rabbit anti-cod parvalbumin antibody K991. In the referred study, data suggested that blue whiting resembles cod and haddock, with a prominent 12 kDa parvalbumin band and other additional bands between 17 and 62 kDa.

Mel ae 1

The *Melanogrammus aeglefinus* (haddock) has the parvalbumin Mel ae 1 identified as an allergen. Like other parvalbumins, it is present in the white muscle tissue of haddock and its route of sensitisation is ingestion (ALLERGOME, 2014). Mel ae 1 was identified by means of ELISA with the monoclonal PARV-19 mouse anti-frog parvalbumin antibody. In the same research, parvalbumins with molecular masses ranging from 8-11 kDa were detected in different species, namely cod, pollock, hake, haddock, salmon, sturgeon, and tilapia (Weber et al., 2009). Using polyclonal antibodies raised against Atlantic cod, Koppelman et al. (2012) were also able to identify a haddock parvalbumin that was 100% reactive with the developed antibodies, suggesting high homology between parvalbumins from cod and haddock. Supporting this fact, Sun et al. (2009) were able to amplify parvalbumin sequences from twenty-eight fish species using universal real-time PCR.

Sera from a total of nineteen fish-allergic patients were incubated with haddock parvalbumin, presenting 100% immunoreactivity with fresh haddock. With respect to processed haddock, the immunoreactivity seems to be increased in most of the sera tested with parvalbumin from the smoked fish (Sletten et al., 2010). The identification of this parvalbumin in haddock

(*Melanogrammus aeglefinus*) was also confirmed using a monoclonal anti-frog parvalbumin antibody PARV-19 and a polyclonal rabbit anti-cod parvalbumin antibody K991 (Sletten et al., 2010).

The ch 1

This protein has been identified as the allergenic parvalbumin in *Theragra chalcogramma* species, which is commonly known as Alaska pollock or Walleye pollock (ALLERGOME, 2014). The ch 1 presents a primary sequence of 109 aa, with an estimated molecular mass of 11.5 kDa that is encoded by the *Theragra chalcogramma* clone 2 parvalbumin mRNA with 679 bp (Table 1). It was first described by Van Do et al. (2005b) as the allergenic parvalbumin from pollock, exhibiting similar patterns of reactivity with polyclonal rabbit anti-cod and anti-pollock antibodies. The immunoreactivity of The ch 1 was also confirmed by immunoblotting using a panel of six sera of fish-allergic patients, which exhibited 100% reactivity to The ch 1 (Alaska pollock) as well as to Gad c 1 (Baltic cod).

Two isoforms (P1 and P2) were obtained by means of a reverse transcriptase-PCR, evidencing properties from the beta-lineage of parvalbumins. Both cDNA encoded proteins with 109 aa had theoretical molecular weights of approximately 11.5 kDa and pI of 4.39 and 4.60 for P1 and P2 isoforms, respectively. The alignment of the referred isoforms with cod and salmon parvalbumins revealed different sequence identities. Pollock P1 showed a 67% of sequence identity with P2, which suggested their classification as isoallergens. Regarding the other parvalbumins, isoform P1 presented a sequence identity that ranged from 59% with Baltic cod up to 75% with salmon. With respect to isoform P2, identity indices varied between 62% with Baltic cod and 77% with cod T2 (Van Do et al., 2003; Van Do et al., 2005b). Recombinant The

ch 1 (P1 and P2 isoforms) were expressed in transformed *Escherichia coli*, but small IgE-reactivity was verified for each isoform. Contrarily to the expressed recombinant isoforms, native proteins may undergo post-translational modifications in calcium binding sites, increasing their capacity for IgE recognition (Van Do et al., 2005b). The differences observed by Sun et al. (2009) in the real-time PCR amplification of parvalbumin genes from Atlantic cod and Alaska pollock suggest a more distant genetic relationship between these two species (Sun et al., 2009). Van Do et al. (2005a), using SPT in twelve fish-allergic patients and respective sera, revealed high reactivity to nGad c 1 (cod), nThe ch 1 (Alaska pollock) and nSal s 1 (salmon).

Mer me 1

The species *Merlangius merlangus*, which was previously named *Gadus merlangus*, is popularly known as whiting. In this species, a major parvalbumin was first described by Joassin, and Gerday (1977) with a primary sequence containing 108 aa and an acetylated terminal aa group (Table 1). This protein has no disulphide bridges and the alignment of the hydrophobic amino acids suggests that the structures of the two calcium-binding sites, as well as its hydrophobic core are well preserved. The comparison of the amino acid composition of Mer me 1 with other parvalbumins is indicative of belonging to the beta-lineage (Joassin, and Gerday 1977). Additionally, data suggest a strong interaction and a positive cooperation between the two calcium-binding sites. The structure of whiting parvalbumin appears to be very stable at temperatures up to 70ºC, demonstrating some conformational alterations when heated between 70-90ºC, with expected complete denaturation at temperatures above 100ºC. Whiting parvalbumin is also stable in a wide range of pH (5.5-9.5), but out of this interval seems to lose integrity, lowering its affinity to calcium (Permyakov et al., 1980). Its tertiary structure was

already determined by X-ray crystallography and is publically available in PDB (accession no. 1A75).

Gad ma 1, Bor sa 1 and Pol vi 1

Gad ma 1 and Bor sa 1 parvalbumins have been recently identified in *Gadus macrocephalus* and *Boreogadus saida*, also commonly known as Pacific cod and Polar cod/Artic cod, respectively (Table 1). Pol vi 1 is the allergenic protein of *Pollachius virens*, known as Pollock, which was described as presenting the same parvalbumin profile as cod and haddock, with molecular mass <14 kDa determined by immunoblotting with the monoclonal anti-frog parvalbumin antibody PARV-19 (Weber et al., 2009). Koppelman et al. (2012) identified pollock parvalbumins with polyclonal antibodies raised against Atlantic cod, revealing a reactivity of at least 92%. Like in the case of haddock, cod and pollock seem to share high sequence homology with allergenic parvalbumins. Gad ma 1, Bor sa 1 and Pol vi 1are present in the white muscle tissue and their route of exposure seems to be ingestion (ALLERGOME, 2014). In terms of molecular characterisation, no information is yet available.

Merlucciidae parvalbumins

Mer ap 1

Belonging to the species *Merluccius australis*, the allergen Mer ap 1 was identified in the subspecies *Merluccius australis polylepis*, more commonly known as Southern hake or Patagonian hake (UniProt, 2014). So far, three sequences defined as parvalbumins of beta-lineage have been identified in this species (Table 1) (Carrera et al., 2010). The parvalbumin beta 1 has a primary structure of 109 aa, with a pI of 4.30 and a molecular mass of 11.30 kDa that was

determined by two-dimensional (2D) gel electrophoresis and confirmed by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) (Carrera et al., 2006; Carrera et al., 2010). With the same number of residues, the parvalbumin beta 2 has a pI of 4.14 and an estimated molecular mass of 11.33 kDa (MALDI-TOF-MS determination). The third sequence defined as parvalbumin beta 3 presents identical molecular mass (~11.33 kDa) and a pI of 3.98. According to its molecular mass, the primary structure should present 109 aa, though only about 76 aa have been experimentally identified (Carrera et al., 2010). Parvalbumins beta 1 and beta 2 exhibited a sequence identity of 74%, suggesting their classification as isoallergens. Regarding the isoallergens beta 1 and beta 2, high sequence homology (>90%) was verified with different members of the *Merluccius* genus, being designated as isoform sequences (Carrera et al., 2010). Like other parvalbumins, the isoallergens from *Merluccius australis polylepis* present characteristic residues such as cysteine and arginine in positions 18 and 75, respectively.

Mer au 1

Mer au 1 was classified as an allergen of the subspecies *Merluccius australis australis*, frequently denominated as Austral hake or Southern hake (UniProt, 2014). In this subspecies, four protein sequences have been identified, exhibiting primary structures with 108 aa, with the exception of the parvalbumin beta 4 that revealed a structure with 69 aa. All four peptides are suggested to present pI ranges of 3.98-4.51 and molecular mass of approximately 11.3 kDa (2D-gel and MALDI-TOF-MS), even parvalbumin beta 4 with residues partially unidentified (Carrera et al., 2010). In Blastp analysis, parvalbumin beta 1 showed a high sequence homology with parvalbumin beta 2 (~99%), indicating that they are probably variants (isoforms) of the same isoallergen (Chapman et al., 2007; Chapman, 2008). Regarding parvalbumin beta 3, its sequence

identity with parvalbumin beta 1 was of 74%, suggesting their forthcoming classification of isoallergens. Since parvalbumin beta 4 was not yet fully sequenced, its classification as an isoallergen of Mer ap 1 is still not yet possible, according to current nomenclature criteria. Like the isoallergens from Mer ap 1, high sequence homology (>90%) was verified among Mer au 1 and other parvalbumins from the *Merluccius* genus (Carrera et al., 2010). In addition to high homology, similar molecular masses and isoelectric points, the isoallergens from *Merluccius australis australis* also possess other characteristic traits of beta-parvalbumins such as a cysteine in position 18 and an arginine in position 75.

Mer bi 1

The *Merluccius bilinearis* species is frequently nominated as silver hake and is also identified as an allergenic fish. In this species, four proteins sequentially designated as parvalbumin beta 1 to parvalbumin beta 4 have been characterised and classified as isoallergens or isoforms of Mer bi 1 (Table 1). The Mer bi 1 includes three sequences that exhibit a primary structure composed of 108 aa and a fourth sequence with 94 aa. All sequences presented an estimated molecular size of approximately 11.3 kDa and pI ranging from 3.98-4.23 (UniProt, 2014). From Blastp search, the sequence parvalbumin beta revealed 95% of sequence identity with parvalbumin beta 3, suggesting that they are possibly two variants of the same isoallergen (NCBI, 2014; UniProt, 2014). In addition, parvalbumin beta 2 and parvalbumin beta 4 presented sequence identity of 76% and 68%, respectively with parvalbumin beta, confirming that they are isoallergens (NCBI, 2014; UniProt, 2014). When compared to other species from the same or even different families, sequence homology with Mer bi 1 is, in general, very high (Carrera et al., 2010).

The isoallergen parvalbumin beta, also referred as parvalbumin isoform B, was firstly described by Revett et al. (1997) and its tri-dimensional structure was determined by X-ray diffraction (PDB accession no. 1BU3) (PDB, 2014; Richardson et al., 2000). According to its conformation, parvalbumin isoform B exhibits the same structural features as other parvalbumins, consisting of six alpha-helices (A-F) arranged in a globular shape. Chains A and B are folded into antiparallel conformation, while helices C/D and E/F pair up and each adopt a perpendicular arrangement. Like in other parvalbumins, the loops between helices CD and EF form the two predominant metal ion (calcium) binding sites. Other invariant features of parvalbumins such as the Ile58-Ile97 beta-sheet, the Arg75-Glu81 salt bridge and the characteristic hydrophobic core composed by Phe, Leu, Ile and Val, are also present in this protein (Richardson et al., 2000).

Mer ca 1

In the *Merluccius capensis* (stockfish or cape hake as common names) species, three beta-parvalbumins have been identified, from which two presented 108 aa and the third one 58 aa. Exhibiting molecular masses of approximately 11.3 kDa and pI of 3.95-4.55, parvalbumin beta 1 presents 75% of sequence identity with parvalbumin beta 2, suggesting that both sequences are isoallergens (NCBI, 2014; UniProt, 2014). Regarding the parvalbumin beta 3, further information about its primary sequence is needed (Chapman et al., 2007; Chapman, 2008).

In a study described by Weber et al. (2009), the parvalbumin from *M. capensis* exhibited a molecular size of approximately 11 kDa as determined by SDS-PAGE with silver staining and by immunostaining with monoclonal PARV-19 mouse anti-frog parvalbumin antibody. Using a competitive indirect ELISA with the referred antibody, the same authors reported 80% of cross-reactivity with cod (*Gadus* spp.).

Mer ga 1

The allergen identified in the *Merluccius gayi* species (known as South Pacific hake or English hake) is Mer ga 1. It was reported for the first time by Piñeiro et al. (1998), presenting four parvalbumins with molecular weights of 12-13 kDa and pI of 3.6-4.5 (Table 1). More recent data on the molecular size/pI and the primary structures of each parvalbumin were completely/partially identified, confirming previous findings (Carrera et al., 2010). The two sequences with 108 aa in length revealed a 74% identity, making them isoallergens of Mer ga 1 (Table 1). Parvalbumins beta 3 and 4 evidenced lower sequence identity (61-62%) with parvalbumin beta 1, probably due to their incomplete sequence (Uniprot, 2014).

Mer hu 1

Mer hu 1 is a parvalbumin from *Merluccius hubbsi* species, commonly known as Argentine hake. Like in *Merluccius gayi*, four parvalbumins were identified in *M. hubbsi* with pI of 3.9-4.5 (Piñeiro et al., 1998). MALDI-TOF-MS revealed two sequences with a primary structure composed of 108 aa and a third one with 86 aa, all presenting molecular masses of approximately 11.3 kDa and pI of 4.09-4.57 (Carrera et al., 2010), which confirmed the previous data (Piñeiro et al., 1998). However, the fourth parvalbumin identified by Piñeiro et al. (1998) with a pI of 3.9, common to other three *Merluccius* species (*M. capensis*, *M. australis* and *M. merluccius*), was not reported in the study of Carrera et al. (2010) or in ALLERGOME database.

Mer mr 1

The isolation of a major parvalbumin with 108 aa (11.47 kDa) from the white muscle of *Merluccius merluccius* species was firstly described by Pechère et al. (1971) and further

confirmed by Capony, and Pechère (1973). Thereafter, other authors identified a set of three aa sequences containing 108 residues in length, molecular masses around 11.3 kDa and pI ranging from 3.9-4.5 (Carrera et al., 2006; Carrera et al., 2010; Piñeiro et al., 1998; UniProt, 2014). Together with these three sequences, a fourth structure displaying 69 aa was also classified as a parvalbumin from *M. merluccius*, sharing similar biochemical properties to other parvalbumins (UniProt, 2014). A Blast search on the available sequences of parvalbumins evidenced high homology (>99%) between parvalbumin beta and beta 2, suggesting their classification as isoforms or variants of Mer mr 1 (Table 1). The primary structure of parvalbumin beta 4 was not yet fully sequenced, presenting only 61% of sequence identity with the remaining three parvalbumins. In spite of this fact, the four parvalbumins are considered isoallergens of Mer mr 1 (Carrera et al., 2010).

Mer pr 1

Mer pr 1 belongs to *Merluccius productus*, commonly named as North Pacific hake. It comprises two sequences with 108 aa and one with 88 aa, exhibiting more than 57% of homology among sequences. The characteristic traits of beta-parvalbumins such as a molecular weight of approximately 11.3 kDa and pI below 5 are also shared by Mer pr 1 (Wopfner et al., 2007).

So far, no nucleotide sequences encoding these proteins have become available in the GenBank database, although in the study of Sun et al. (2009), the amplification of an universal nucleotide sequence evidenced a more close genetic relation among *M. productus* and other species such as *Hymenocephalus striatissimus* (hoki), *Sciaenops ocellatus* (red fish) or *Theragra chalcogramma* (Alaska pollock) than with *Gadus macrocephalus* (Pacific cod) or *Aristichthys nobilis* (Bighead carp).

Mer se 1, Mer po 1 and Mer pa 1

Mer se 1, Mer po 1 and Mer pa 1 belong to *Merluccius senegalensis*, *M. polli* and *M. paradoxus* species, frequently known as Senegalese hake or Black hake, Benguela hake and deepwater hake/deepwater Cape hake, respectively. Each allergen is composed of three isoallergens and besides presenting the same properties of the beta-parvalbumins already described (Table 1), limited information is yet available regarding these molecules.

Mac ma 1 and Mac n 1

The *Macruronus magellanicus* or *Macruronus novaezelandiae magellanicus*, frequently designated as Patagonian grenadier, is also considered an allergenic fish. Recently, the isoallergens Mac ma 1 have been completely/partially sequenced (Table 1). Presenting primary structures with 98 aa, 108 aa and 74 aa for parvalbumins beta 1, beta 2 and beta 3, respectively, all isoallergens evidenced similar molecular weights (~11.3 kDa) and pI (<5). Besides the common features associated with beta-parvalbumins, the isoallergens Mac ma 1 also exhibit two EF-hand repeats and conserved amino acid regions, such as an aspartic acid in position 61, which is common to several parvalbumin sequences (Mer ap 1, Mer au 1, Mer bi 1, Mer ca 1; Mer ga 1, Mer hu 1, Mer mr 1, Mer pa 1, Mer po 1, Mer pr 1 and Mer se 1) (Carrera et al., 2006; Carrera et al., 2010). Additionally, for all the referred sequences, as well as for Mac ma 1, a cysteine in position 18 and an arginine residue in position 75 were always conserved, which are considered characteristic traits of parvalbumins of beta-lineage. In relation to the parvalbumins that were completely sequenced, homology among different species from *Merluccius* or *Macruronus*

genera is frequently higher than 80%, explaining the high incidence of cross-reactivity among them.

The *Macruronus novaezelandiae* or *Macruronus novaezelandiae novaezelandiae*, commonly known as Blue grenadier, has been described as an allergenic fish. Like the Mac ma 1, the Mac n 1 is composed by three sequences with 98 aa, 108 aa and 83 aa, which were firstly reported by Carrera et al. (2006). Mac n 1 isoallergens present all the biochemical traits of beta-parvalbumins, namely molecular weight of approximately 11.3 kDa, pI<5 and a set of well-conserved amino acid regions (a cysteine in position 18, an aspartic acid in position 61 and an arginine in position 75) (Carrera et al., 2006; Carrera et al., 2010).

Other Gadiform parvalbumins

The Mur mi 1 is the allergenic parvalbumin identified in the *Muraenolepis microps* species, commonly known as Smalleye Moray Cod and included in the Muraenolepididae family. It was firstly reported by Sun et al. (2009), presenting close sequence amplification with other members of Gadidae family (e.g. *Micromesistius poutassou*, *Gadus macrocephalus*), suggesting their high sequence homology.

The Uro te 1 allergen was identified in *Urophycis tenuis* species, commonly known as Boston ling or White hake, which is a member of Phycidae family. It was detected using the monoclonal anticarp antibody, suggesting high structural homology between respective sequences (Koppelman et al., 2012; Lee et al., 2011). In the same sense, parvalbumin Uro te 1 also evidenced 100% of cross-reactivity with the polyclonal anticod antibody described by Koppleman et al. (2012), which reinforces that most parvalbumins share similar traits. Regarding this allergen, no relevant biochemical information could be found in literature.

CLINICAL RELEVANCE OF GADIFORM PARVALBUMINS

From a clinical point of view, parvalbumins are major allergens that are present in a broad spectrum of fish species (Beale et al., 2009; Lindstrom et al., 1996; Perez-Gordo et al., 2011; Van Do et al., 2005a). During an allergic episode, fish-allergic patients can exhibit individual symptoms or combined manifestations, depending on the target organ/systems affected. The observable clinical presentations of fish allergy can be classified as mild, such as oral allergy syndrome (OAS) and general erythema, moderate (urticaria, vomiting) or severe (angioedema, asthma, anaphylaxis) (Bock et al., 2001; Helbling et al., 1999; Sicherer et al., 2000). In the specific case of fish allergy, patients with severe and systemic symptoms are often common. A great part of the described symptoms occur after ingestion, though urticaria and eczema may also appear upon skin contact with fish/fish products, as well as upper and lower airway syndromes as consequence of the inhalation of odours during fish preparing/processing (Jeebhay et al., 2008). Most fish sensitised/allergic patients are positively reactive to multiple species from taxonomically distinct classes (genetically close or distantly related species). Moreover, serological studies and skin testing seem to suggest high cross-sensitisation (70-90%) (Bernhisel-Broadbent et al., 1992), though clinical cross-reactivity is only confirmed in 50% to 70% of the cases (De Martino et al., 1990; Helbling et al., 1999; Sicherer, and Sampson, 2010). Reported differences in the allergenicity among different fish species are mainly related to variable allergen content and to the effects of food processing (Kuehn et al., 2010).

Several parvalbumins have been identified in members of the Gadidae family and comprised in the ALLERGOME database, but only few molecules have been included in the IUIS official list of allergens. In spite of their relevance, clinical information could only be found for Gad c 1,

Gad m 1, Mic po 1 and The ch 1 parvalbumins, which belong to the most consumed fish species (Bugajska-Schretter et al., 1998; Griesmeier et al., 2010; Ma et al., 2008; Rancé et al., 1999; Sletten et al., 2010; Van Do et al., 2005a). With respect to Gad c 1, it has been classified as a major allergen in cod (Bugajska-Schretter et al., 1998), considering that more than 50% of the sera of fish-allergic patients were reactive to this allergen (Chapman, 2008). In a study described by Bugajska-Schretter et al. (1998), using a test population of 30 patients with clear history of at least one clinical symptom (dermatitis, urticaria, angioedema, diarrhoea, asthma and/or anaphylactic reaction) after ingestion, inhalation or skin contact with fish proteins, the sera of all individuals were IgE-reactive to Gad c 1. In the same study, Gad c 1 was submitted to different chemical treatments to evaluate its immunoreactivity. Similarly to Gad c 1, high immunoreactivity to Gad m 1 was also observed in patients' sera with clear history of well-defined symptoms that range from mild (skin rash or OAS) to potentially life-threatening (anaphylaxis) (Griesmeier et al., 2010; Ma et al., 2008). From a total of 19 fish-allergic patients presenting a wide variety of clinical symptoms (atopic dermatitis, asthma, urticaria, throat swelling, vomiting, breathlessness, abdominal pain and/or anaphylaxis), the sera of 18 individuals were IgE-reactive to blue whiting parvalbumin (Mic po 1) (Sletten et al., 2010).

For members of Merlucciidae family, only two studies reporting clinical symptoms were found in the literature (Beale et al., 2009; González-de-Olano et al., 2012), both regarding the Mer mr 1. Beale et al. (2009) reported that, using sera of ten patients with clinical and serological history of self-reported allergy after fish ingestion, they were able to identify four individuals IgE-reactive to *Merluccius merluccius*. The reported allergic reactions varied from moderate to severe, in general with systemic clinical symptoms of urticaria, dermatitis, vomiting, asthma,

rhinoconjunctivitis and/or angioedema. Additionally, a rare clinical case evidencing the cross-reactivity between chicken and fish parvalbumins was recently described by González-de-Olano et al. (2012). In the referred study, a 23-year-old patient with a documented severe fish-allergic episode at age of nine (chest tightness, wheezing and facial angioedema) presented a very similar clinical condition (chest tightness and wheezing), within minutes upon the ingestion of chicken. Using this patient's serum, strong IgE-reactivity towards hake and chicken extracts could be observed.

DETECTION OF GADIFORM PARVALBUMINS

The risk of allergic individuals of suffering from an abnormal immune episode remains a reality, even when following a daily diet with total avoidance of the offending foods. Despite the EU regulations requesting the compulsory labelling of food products that may contain food allergens (Directive 2007/68/EC; EFSA, 2014; Regulation (EU) No. 1169/2011), incorrect labelling or cross-contamination cases may occur upon food processing, resulting in the inadvertent presence of hidden allergens. This fact emphasises the need for accurate, fast and highly sensitive methods in order to detect trace levels of allergens in foods and protect fish-allergic consumers, while ensuring the accurate labelling of products (Carrera et al., 2012; Costa et al., 2014; Herrero et al., 2014; Lee, and Taylor, 2011; Rencova et al., 2013).

Despite the lack of available testing/reference materials and the absence of official methods, there are currently several reports describing molecular tools to detect food allergens. However, within the sphere of fish allergen detection, the number of available methods is still limited.

In recent years, methods targeting proteins and DNA have been proposed for the detection of fish allergens (mostly parvalbumins). Protein-based methods are the most widely used and they

include two main groups of techniques: immunoassays and mass spectrometry methods. DNA-based analysis has allowed the development of indirect allergen detection methods, predominantly by means of PCR amplification (Carrera et al., 2012; Rencova et al., 2013; Zheng et al., 2012).

Protein-based methods

Protein-based methods are particularly helpful in the food industry since they offer high specific and sensitive tools for fast screening of allergens within a complex food matrix, without needing an extensive sample preparation step (Lee, & Taylor, 2011). Considering the detection of Gadiform allergens, the available commercial kits and literature reports mostly rely on immunochemical techniques based on allergen/antibody interaction (Tables 2 and 3).

Lateral flow devices

Lateral flow devices (LFD) are simple and rapid tests that provide qualitative or semi-quantitative information without the need for specialised equipment. Presently, only one LFD is commercially available for the analysis of general fish proteins (Table 2), with a sensitivity of 2 mg/kg of fish in foodstuffs and a test performance time of about 10 minutes.

To detect *Gadus macrocephalus* parvalbumin (Table 2), a superparamagnetic nanoparticle-based lateral flow immunoassay (SPMN-LFIA) was recently developed by means of monoclonal antibodies raised against fish parvalbumin, allowing its rapid detection in foods (~20 min) and a limit of detection (LOD) of 0.046 mg/kg (Zheng et al., 2012).

The simplicity of LFD with the possibility of visually reading the result makes these assays suitable for wide applications in the control of allergens at industrial level, though they are

highly prone to false negative results in part caused by the complexity of the matrix (Diaz-Amigo, 2010).

ELISA

As noticed in Tables 2 and 3, the enzyme-linked immunosorbent assay (ELISA) is by far the most used method to trace Gadiform allergens, as in the case of other allergenic foods (e.g. tree nuts, peanut and soybean). ELISA allows the direct detection and quantification of allergens or other marker proteins, usually providing a rapid and low-cost method with no special requirements for expertise knowledge (Costa et al., 2014). Currently, there are two ELISA kits commercially available to enable the detection of fish in foods: the "Fish Protein ELISA kit" targeting parvalbumins with a limit of quantification (LOQ) of 1 mg/kg; and the "AgraQuant® Fish" with a LOQ of 4-100 mg/kg, though without clear information regarding the target class of fish proteins (Table 2). Due to the structural homology of parvalbumins, the possibility of cross-reactivity is referred in the Fish Protein ELISA kit namely for cod, haddock or mackerel species (Table 2).

Three ELISA, each one in the formats of sandwich-type, competitive indirect and quantitative, have been proposed in literature for the detection and quantification of parvalbumins in fish (e.g. *Gadus morhua* or *Pollachius virens*) and in derived-fish foodstuffs and gelatines (Table 3). Recently, Fæste and Plassen (2008) also developed a sandwich ELISA targeting a fish muscle protein (34 kDa) rather than parvalbumin. In general, these assays present high sensitivity and no cross-reactivity with plant or animal-derived food (fish not included), excluding the positive result for European squid (Fæste, and Plassen, 2008).

Despite the predominance of ELISA, both as commercial kits or as reported methods, their results should be carefully analysed. These assays are likely to present false positive results since the target proteins might be highly affected by conformational changes upon processing (thermal treatments, pH, fermentation and partial hydrolysis). However, the capacity of multiple epitope recognition by polyclonal antibodies leads to a better detection of modified proteins (Costa et al., 2014; Lee, & Taylor, 2011).

MS platforms

In recent years, the enormous progress in MS technology has given a boost to the field of proteomics, including the identification. characterisation and determination of food allergens (Fæste et al., 2011; Monaci, and Visconti, 2010). The application of proteomic methods for the analysis of allergenic proteins has been termed allergenomics. The many different allergenic proteins are included in the allergenome, whose information is continuously being accumulated in ALLERGOME database (Mari et al., 2009). For further reading, Fæste et al. (2011) provide a compendium of studies on proteomic analysis, marker peptides, liquid chromatography (LC) methods and quantitative assays for the 14 main food allergens.

The protein analysis by LC and MS has greatly advanced in recent years, allowing allergen identification, characterisation and quantification (Fæste et al., 2011). There are several advantages that make this technique suitable for allergen detection, such as high accuracy, sensitivity, specificity and reproducibility, while presenting less problems regarding cross-reactivity, frequently associated with immunoassays (Costa et al., 2014).

Carrera et al. (2012) proposed a MS-driven detection, as an alternative method for the direct identification of parvalbumin in any food product, in less than 2 h (Table 3). This method was

tested in 16 fish species (including *Gadus morhua)* and involved reverse phase LC for the separation of peptides and selected MS/MS ion monitoring for multi-target analysis of beta-parvalbumin biomarkers.

MS strategies have been suggested as having high potential for unequivocal identification of food allergens. Nevertheless, the need for specialised personnel and the high cost of the equipment are some disadvantages that hamper the wide application of this technology.

DNA-based methods

Lately, methodologies based on DNA analysis have been referred as adequate alternatives to proteins and considered as methods of choice for the differentiation and identification of distinct components in foods (Mafra et al., 2008). DNA molecules can be found in most biological tissues, presenting higher stability/resistance to adverse conditions than proteins. Therefore, DNA is less prone to be affected by thermal treatments, pH alterations or partial hydrolysis than proteins, which normally suffer structural changes that, consequently, affect their detection. As a result, DNA-based methods are especially useful to analyse highly processed foodstuffs, emphasising their important role for the management of allergens in food industry (Eischeid et al., 2013; Herrero et al., 2014). In spite of the growing number of DNA-based methods for the detection and quantification of food allergens, only few studies report fish allergens, particularly considering the Gadiformes.

PCR

PCR provides a fast, simple and sensitive tool for the specific detection of sequences encoding allergenic proteins or species-specific markers. Since it does not target directly the offending

proteins, it is considered an indirect method of detecting food allergens (Costa et al., 2014; Hildebrandt, 2010; Mafra et al., 2008).

Up to date, the majority of the reports describing the application of PCR-based methods for fish analysis have mainly been focused on species-specific identification for authentication purposes. Concerning commercial applications, there is one kit available for fish DNA detection (Table 2). The five reported fish-specific PCR methods are resumed in Table 4. The specific detection of the parvalbumin gene of fish (including species from the Gadiform order) by a real-time PCR assay was solely proposed by Sun et al. (2009), allowing a sensitivity of 5 pg.

Generally, these methods provide high specificity by minimising or avoiding cross-reactivity with other non-target food species. Therefore, the application of DNA-based methods has high potential for the detection and quantification of parvalbumin sequences.

Biosensors

Biosensors represent probably one of the most promising ways of simple, fast, reproducible, and cheap multi-analyte detection alternatives. A biosensor is an integrated receptor-transducer device that converts the biological-recognition event into a measurable chemical physical signal, which is proportional to the target concentration. The receptor can be an antibody raised against an allergen, a single-stranded DNA molecule capable of hybridising with an allergen-specific DNA fragment, or an aptamer selected to recognise the target allergen directly (Pilolli et al., 2013). In the case of food allergens (Alves et al., 2015; Pilolli et al., 2013; Prado et al., 2015), as in the case of other food analysis, such as the detection of genetically modified organisms (Plácido et al., 2016), three major groups of biosensors, depending of the type of transducer can be found: electrochemical, optical (surface plasmon resonance, SPR) and piezoelectric (quartz

crystal microbalance, QCM). Concerning the type of recognition element, immunosensors are the most commonly applied to food allergen analysis, followed by genosensors (DNA-sensors) and, more recently, aptasensors (Alves et al., 2015).

In the specific case of biosensing approaches applied to Gadiform parvalbumin detection, to our knowledge, no reports have become available. To detect fish parvalbumins, Lu et al. (2004) developed a SPR immunosensor based on a monoclonal antibody against tuna and carp parvalbumins that was applied to processed seafoods (Table 3). More recently, Wang et al. (2011) developed a highly efficient assay to simultaneously identify eight food allergens (soybean, wheat, peanut, cashew, shrimp, fish, beef, and chicken) consisting of a silicon-based optical thin-film biosensor chip. The assay was based on two tetraplex PCR systems that were developed and validated, followed by an enzyme-labelled indirect detection. The enzymatic reaction products precipitated on the thin-film surface and modified the interference pattern of light on the biosensor surface, producing a significant colour change on the surface. The main advantage of this approach was the possibility to perceive the colour change with the naked eye, without any extra analysis/equipment. Fish detection was achieved after PCR amplification of a sequence of the mitrocondrial 16S rRNA gene of *Plecoglossus altivelis*, only reporting sensitivity for cashew nut (Wang et al., 2011).

FINAL REMARKS

The consumption of fish is growing as a result of the continuous recommendations for its inclusion in the context of a healthy diet. Nevertheless, the number of reported cases of fish allergy has increased in recent years, representing nowadays an important issue of food safety.

Recently, relevant information on the biochemical classification of fish allergens has become available, which has improved its correlation with the elicited clinical symptoms. Currently, the parvalbumins are considered the most important class of fish-allergens, being responsible for inducing moderate to severe and systemic adverse immunological reactions in fish-sensitised/allergic individuals. So far, cod has been frequently described as the model case regarding the study of fish allergy, being also the most well studied. Although the patterns of sensitisation to fish parvalbumins have been related to ingestion, direct contact and/or inhalation, they are yet not fully understood, suggesting the need for additional studies. From a clinical point of view, it is expected that the increasing consumption of fish could lead to a rising number of fish-allergic individuals, which emphasises the importance of this subject. Although there are reports stating the relevance of parvalbumins, more clinical data should be made available regarding other less studied allergens. Furthermore, it becomes clear that more reliable and extensive epidemiological studies are essential to support the existing data on the prevalence of fish allergy.

Until now, the avoidance of offending foods is the only effective means of preventing adverse immunological reactions. Therefore, regardless of being protein- or DNA-based techniques, the development of new methodologies for the detection and quantification of parvalbumins in fish is markedly significant for the better management of allergens by the food industry. Food technologies can induce changes due to processes, such as mechanical stress, high temperature, pH variations, enzymatic activities or fermentations may result in significant degradation of both proteins and DNA, which can affect the sensitivity of the analysis, particularly the limits of detection and quantification (Fernandes et al., 2015; Prado et al., 2015).

Regarding Gadiform allergens, immunochemical assays, namely ELISA, are the most used methods for parvalbumin testing, in terms of both commercial and reported assays. Recently, PCR technologies have emerged as excellent alternatives to identify parvalbumin or other gene sequences and assess the effect of food processing on its detection/allergenicity, although much effort is also required for the establishment of novel assays as effective tools. New methodologies based on biosensor devices have also been developed for the detection of allergenic foods, as well as the use of nanomaterials for analytical methods improvement (Pilolli et al. 2013; Prado et al., 2015). However, in the particular case of fish allergen detection, the biosensing developments are still very scarce, highlighting the need for future research in such promising approaches. Moreover, forthcoming advances should become available upon the development of testing/reference materials, as well as further improvements to establish threshold levels.

CONFLICT OF INTERESTS

The authors declare no conflict of interests.

ACKNOWLEDGEMENTS

This work has been supported by Fundação para a Ciência e a Tecnologia (FCT) through grant no. UID/QUI/50006/2013 and NORTE-07-0124-FEDER-000069-Food Science. Telmo J. R. Fernandes is grateful to FCT PhD grant (SFRH/BD/93711/2013) and Joana Costa to FCT Post-doc grant (SFRH/BPD/102404/2014), both financed by POPH-QREN (subsidised by FSE and MCTES).

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Table 1. General data on fish parvalbumin isoallergens from the Gadiform order.

Allergen	Species	Common name	MW (kDa)	Isoallergens	Protein (UniProt)
Gad c 1	*Gadus callaris*	Baltic cod	12.1 (113 aa)	Gad c 1.01	P02622
Gad m 1	*Gadus morhua*	Atlantic cod	11.45 (109 aa)	Gad m 1.01	Q90YL0
			11.47 (109 aa)		A5I873
			11.55 (109 aa)	Gad m 1.02	Q90YK9
			11.54 (109 aa)		A5I874
Gad ma 1	*Gadus macrocephalus*	Pacific cod	No data available	No data available	No data available
Bor sa 1	*Boreogadus saida*	Arctic cod	11.57 (109 aa)	No data available	C0LEL4
Mic po 1	*Micromesistius poutassou*	Blue whiting	12	No data available	No data available
Mel ea 1	*Melanogrammus*	Haddock	12	No data available	No data

	aeglefinus				available
Pol vi 1	*Pollachius virens*	Pollock	10	No data available	No data available
The ch 1	*Theragra chalcogramma*	Alaska pollock	11.50 (109 aa)	Parvalbumin beta 1 (predicted classification The ch 1.01)	Q90YK8
			11.51 (109 aa)	Parvalbumin beta 2 (predicted classification The ch 1.02)	Q90YK7
Mer me 1	*Merlangius merlangus*	Whiting	11.33 (108 aa)	Parvalbumin beta	P02621
Mer ap 1	*Merluccius australis polylepis*	Patagonian hake	11.27 (108 aa)	Parvalbumin beta 1 (predicted classification Mer ap 1.01)	P86749
			11.32 (108 aa)	Parvalbumin beta 2 (predicted classification Mer ap	P86750

				1.02)	
			8.22 (76 aa)	Parvalbumin beta 3 (predicted classification Mer ap 1.03)	P86751
Mer au 1	*Merluccius australis australis*	Southern hake	11.32 (108 aa)	Parvalbumin beta 1 (predicted classification Mer au 1.01)	P86745
			11.53 (108 aa)	Parvalbumin beta 2 (predicted classification Mer au 1.01)	P86747
			11.30 (108 aa)	Parvalbumin beta 3 (predicted classification Mer au 1.02)	P86748
			11.33 (69 aa)	Parvalbumin beta 4 (predicted classification Mer au 1.03)	P86746

Mer bi 1	*Merluccius bilinearis*	Silver hake	11.27 (108 aa)	Parvalbumin beta 1 (predicted classification Mer bi 1.01)	P56503
			11.24 (108 aa)	Parvalbumin beta 3 (predicted classification Mer bi 1.01)	P86753
			11.32 (108 aa)	Parvalbumin beta 2 (predicted classification Mer bi 1.02)	P86752
			11.27 (94 aa)	Parvalbumin beta 4 (predicted classification Mer bi 1.03)	P86754
Mer ca 1	*Merluccius capensis*	Stockfish	11.30 (108 aa)	Parvalbumin beta 1 (predicted classification Mer ca 1.01)	P86756
			11.38 (108	Parvalbumin beta 2	P86757

			aa)	(predicted classification Mer ca 1.02)	
			11.39 (58 aa)	Parvalbumin beta 3	P86755
Mer ga 1	*Merluccius gayi*	English hake	11.33 (108 aa)	Parvalbumin beta 1 (predicted classification Mer ga 1.01)	P86761
			11.35 (108 aa)	Parvalbumin beta 2 (predicted classification Mer ga 1.02)	P86759
			11.20 (75 aa)	Parvalbumin beta 3	P86758
			11.33 (91 aa)	Parvalbumin beta 4	P86760
Allergen	Species	Common name	MW (kDa)	Isoallergens	Protein (UniProt)

Mer hu 1	*Merluccius hubbsi*	Argentine hake	11.33 (108 aa)	Parvalbumin beta 1 (predicted classification Mer hu 1.01)	P86764
			11.35 (108 aa)	Parvalbumin beta 2 (predicted classification Mer hu 1.02)	P86762
			11.35 (86 aa)	Parvalbumin beta 3	P86763
Mer mr 1	*Merluccius merluccius*	European hake	11.33 (108 aa)	Parvalbumin beta 1 (predicted classification Mer mr 1.01)	P02620
			11.30 (108 aa)	Parvalbumin beta 2(predicted classification Mer mr 1.01)	P86765
			11.38 (108 aa)	Parvalbumin beta 3(predicted classification Mer	P86766

				mr 1.02)	
			11.39 (69 aa)	Parvalbumin beta 4(predicted classification Mer mr 1.03)	P86767
Mer pa 1	*Merluccius paradoxus*	Deepwater hake	11.36 (108 aa)	Parvalbumin beta 1 (predicted classification Mer pa 1.01)	P86768
			11.37 (108 aa)	Parvalbumin beta 2(predicted classification Mer pa 1.02)	P86769
			11.35 (95 aa)	Parvalbumin beta 3 (predicted classification Mer pa 1.03)	P86770
Mer po 1	*Merluccius polli*	Benguela hake	11.33 (108 aa)	Parvalbumin beta 1(predicted classification Mer po 1.01)	P86773

			11.35 (108 aa)	Parvalbumin beta 2 (predicted classification Mer po 1.02)	P86771
			11.35 (69 aa)	Parvalbumin beta 3 (predicted classification Mer po 1.03)	P86772
Mer pr 1	*Merluccius productus*	North Pacific hake	11.37 (108 aa)	Parvalbumin beta 1 (predicted classification Mer pr 1.01)	P86774
			11.31 (108 aa)	Parvalbumin beta 2 (predicted classification Mer pr 1.02)	P86775
			11.35 (88 aa)	Parvalbumin beta 3 (predicted classification Mer pr 1.03)	P86776
Mer se 1	*Merluccius*	Black hake	11.33 (108	Parvalbumin beta 1	P86778

	senegalensis		aa)	(predicted classification Mer se 1.01)	
			11.38 (108 aa)	Parvalbumin beta 2 (predicted classification Mer se 1.02)	P86779
			11.37 (58 aa)	Parvalbumin beta 3(predicted classification Mer se 1.03)	P86777
Mac ma 1	*Macruronus magellanicus*	Patagonian grenadier	11.25 (98 aa)	Parvalbumin beta 1 (predicted classification Mac ma 1.01)	P86739
			11.34 (108 aa)	Parvalbumin beta 2 (predicted classification Mac ma 1.02)	P86741
			11.35 (74 aa)	Parvalbumin beta 3 (predicted	P86740

				classification Mac ma 1.03)	
Mac n 1	*Macruronus novaezelandiae*	Blue grenadier	11.25 (98 aa)	Parvalbumin beta 1 (predicted classification Mac n 1.01)	P86739
			11.35 (108 aa)	Parvalbumin beta 2 (predicted classification Mac n 1.02)	P86741
			11.35 (83 aa)	Parvalbumin beta 3 (predicted classification Mac n 1.03)	P86740
Mur mi 1	*Muraenolepis microps*	Smalleye Moray Cod	No data available	No data available	No data available
Uro te 1	*Urophycis tenuis*	Boston ling	No data available	No data available	No data available

Table 2. Commercial LFD, ELISA and real-time PCR kits available for the detection and quantification of fish allergens.

Commercial kit/brand	Assay type	Cross-reactivity	LOD	LOQ	Estimated time to perform assay
Fish Protein Rapid Test (Elution Technologies, Vermont, USA)	LFD	No cross-reactivity observed	2 mg/kg	No information available	~10 min (sample preparation)
Fish Protein ELISA Kit (Elution Technologies, Vermont, USA)	Sandwich ELISA	Salmon, Cod, Trout, Sole, Tilapia, Haddock, Bass, Sardine, Mackerel, Halibut.	No information available	1 mg/kg	~50 min
AgraQuant® Fish (Romer Labs	Quantitative – Sandwich	No information	1.4 mg/kg	4-100 mg/kg	~40 min (sample extraction) + ~60

Division Holding GmbH, Austria)	ELISA	available			min (incubation time)
SureFood Allergen Fish (R-Biopharm AG Darmstadt, Germany)	Real-time PCR (qualitative)	No cross-reactivity observed	≤ 5 DNA copies, ≤ 0.4 mg/kg	No information available	~60 min (Real-time reaction)

Table 3. Most relevant protein-based methods for the detection of Gadiform fish allergens available in the literature.

Method	Antibody	Cross-reactivity	Sensitivity level	Food matrices	References
Sandwich ELISA	Rabbit antibodies (raised against cod parvalbumin, *Gadus morhua*)	1/24 plant and animal-derived food (fish not included). Cross-reactivity with European squid (0.6 mg/kg parvalbumin)	LOD = 0.01 mg/kg LOQ = 0.02 mg/kg	Fish (32 species, including *Gadus morhua* and *Pollachius virens*) and food/ingredient (24) samples	Faeste, and Plassen (2008)
Competitive indirect ELISA	Rabbit antibodies (raised against frog muscle	4/5 fish species. Strong cross-reactivity related to	LOD = 18.7 mg/kg fish gelatin	Fish (8 species, including *Gadus spp*., *Pollachius virens*, *Merluccius capensis*, *Melanogrammus*	Weber et al. (2009)

	parvalbumin)	cod (80-179%)		*aeglefinus*) Fish gelatins Isinglass	
Sandwich ELISA	Polyclonal rabbit antibodies (raised against a fish muscle protein, 36 kDa)	0/16 animal and plant-derived food (fish not included)	LOD = 0.1 mg/kg	Fish (63 species, including *Gadus morhua* and *Pollachius pollachius*) Fish in crab meat mixtures	Chen, and Hsieh (2014)
Quantitative ELISA	Polyclonal rabbit antibodies (raised against purified parvalbumin from fish muscle)	Not verified	LOD = 2-6 ng parvalbumin/ml	Fish (8 species, including *Gadus morhua*)	Kuehn et al. (2010)

SPMNP-LFIA	Monoclonal antibody (raised against fish parvalbumin)	Not verified	LOD = 0.046 mg/kg	Fish (17 species, including *Gadus macrocephalus*) Plant and animal food matrices	Zheng et al. (2012)
LC-MS/MS (multi-target approach)	Not applicable (peptide biomarkers for parvalbumin detection)	Not verified	No information available	Fish (16 species, including *Gadus morhua*) Non-fish (6 species) Commercial sea-foodstuffs	Carrera et al. (2012)

Table 4. Most relevant DNA-based methods for the detection of Gadiform fish allergens available in the literature.

Method	Target gene	Fragment size (bp)	Cross-reactivity	Sensitivity level	Food matrices	References
Conventional PCR	Atlantic herring parvalbumin partial sequence	189	1/26 fish species (Strong cross-reactivity with *Clupea Pallasii* (Pacific herring)	10 pg/µL	Fish (26 species, including *Gadus morhua*, *Gadus merlangus*, *Merluccius merluccius*, *Theragra chalcogramma*, *Pollachius virens*)	Rencova et al. (2013)
Real-time PCR for fish allergen detection	18S RNA gene sequence	Not available	0/47 plant and animal-derived food (fish not included)	50 pg	Fish (54 species, including *Gadus macrocephalus*, *Gadus morhua*, *Merluccius australis* and *Merluccius*	Herrero et al. (2014)

					merluccius)	
Real-time PCR for fish parvalbumin detection	Parvalbumin gene	Not available	0/13 plant and animal-derived food (fish not included)	5 pg	Fish (30 species, including 8 Gadiform varieties)	Sun et al. (2009)
Real-time PCR for multiple fish species detection	Rhodopsin gene partial sequence	Not available	Not available	0.05 ng	Animal feeds with fish as an ingredient (targeting 22 fish species, including 12 Gadiform species)	Prado et al. (2012)
Real-time PCR for multiple fish species detection	12S rRNA gene sequence	87 bp	Not available	0.1 pg	Animal feeds with fish as an ingredient (targeting 39 fish species, including 3 Gadiform	Pegels et al. (2013)

					species)	

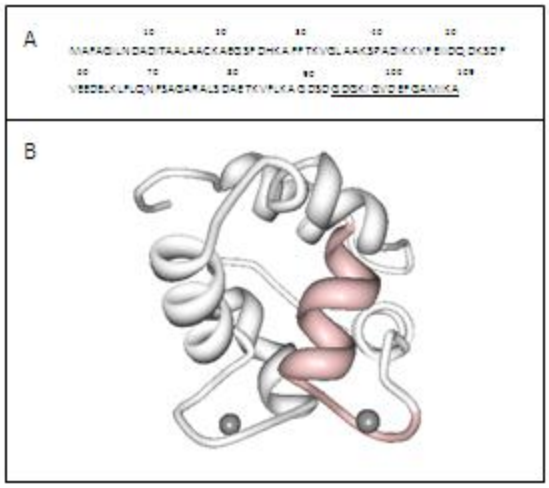

Figure 1
(to be reproduced colour on the web)

Fig. 1. (A) Aminoacid sequence of Gadus morhua (Gad m 1) parvalbumin – 2MBX (RCSB-PDB, http://www.rcsb.org). The main epitopes are underlined. (B) Ribbon representation of Gad m 1 (main epitopes highlighted) and calcium cations represented by spheres.